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UNIVERSITY OF CALIFORNIA SAN DIEGO

SAN DIEGO STATE UNIVERSITY

Mechanistic Studies of two tRNA Modification Enzymes and Utilization in Drug
Discovery

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Chemistry

by

Naduni Nipunika Paranagama

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Professor Tom Huxford
Professor Marina Kalyuzhnaya

2021

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Chair

University of California San Diego

San Diego State University

2021

Dedication

This thesis work is dedicated to my husband, who has been a continuous source of encouragement during the ups and downs of research and graduate school. I would also like to dedicate this thesis to my parents and siblings, who made many sacrifices that allowed me to pursue a career in scientific research and for teaching me to work hard to achieve my ambitions and goals.

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List of Abbreviations

ADP	Adenosine diphosphate
AFRPT	2-amino-5-formylamino-(6 β)-ribosylamino-4(3H)-pyrimidone 5'-triphosphate
AK	Arginine Kinase
AMP	Adenosine monophosphate
aPK	Atypical protein kinase
aSK	Atypical serine kinases
ASL	Anticodon Stem Loop
ATP	Adenosine triphosphate
AUC	Analytical Ultracentrifugation
AWI	Air water interface
BCR-ABL	Breakpoint cluster region protein-Abelson tyrosine kinase
BH4	Tetrahydrobiopterin
BME	β -betamercaptoethanol
Bs	Bacillus subtilis
BYK	Bacterial Tyrosine Kinase
CDC	Center for Disease Control and Prevention
CMC	Critical Micellar Concentration
Cryo-EM	Cryo-Electron Microscopy
CTF	Contrast transfer function
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase
DNA	Deoxyribonucleic acid
DRPT	2,5-diamino-6 β -ribosylamino-4(3H)-pyrimidone 5'-triphosphate
DTT	DL-dithiothreitol
Ec	Escherichia coli
EcAP	Escherichia coli Alkaline Phosphatase
EDTA	Ethylenediaminetetraacetic acid
EMSA	Electrophoretic mobility shift assay
ePK	Eukaryotic protein kinase
eSTK	Eukaryotic-like S/T kinases
FSC	Fourier Shell Correlation
GCYH	GTP Cyclohydrolase
GSH	Glutathione
GTP	Thre Guanosine triphosphate
H ₂ NTP	7,8-dihydroneopterin triphosphate

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hi	Haemophilus influenzae
His ₆	Hexa Histidine Tag
HK	Histidine Kinase
Hs	Homo sapien
ITC	Isothermal Calorimetry
MERRF	Myoclonic epilepsy with ragged red fibers
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MIC	Minimal inhibitory concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
Ng	Neisseria gonorrhoeae
NO	Nitric Oxide
NP40	Nonidet P-40
NSCLC	Non-small cell lung cancer
NTP	Nucleoside triphosphate
PAGE	Polyacrylamide gel electrophoresis
PDB	Protein Database Bank
PEG	Polyethylene glycol
PES	Polyethersulfone
PSMF	Phenylmethylsulfonyl fluoride
SAXS	Small Angle X-ray Scattering
SDS	Sodium Dodecyl sulfate
t ⁶ A	N ⁶ -Threonylcarbamoyl adenosine
TC	Threonylcarbamoyl
TC-AMP	Threonylcarbamoyl adenosine monophosphate
TCT	Threonylcarbamoyl transfer
TEM	Transmission Electron Microscopy
THF	Tetrahydrofolate
TLC	Thin layer chromatography
Tm	Thermotoga maritima
TMP	Trimethoprim
TMT	Tandem Mass Tag TM
TRIS	2-Amino-2-(hydroxymethyl)-1,3-propanediol
tRNA	transfer ribonucleic acid
Tt	Thermus Thermophilus
UbK	Ubiquitous bacterial kinase

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Publications

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Fields of Study

Major Field: Biochemistry and Biophysics

Abstract of the Dissertation

Mechanistic Studies of two tRNA Modification Enzymes and Utilization in Drug
Discovery

by

Naduni Nipunika Paranagama

Doctor of Philosophy in Chemistry

University of California San Diego

San Diego State University

2021

Professor Manal A. Swairjo, Chair

Threonylcarbamoyl adenosine (t^6A) and Queuosine (Q) are two structurally complex modified nucleosides found at position 37 and 34, respectively, in the anticodon stem-loop of tRNA, and are required for accurate and efficient ribosomal translation. The goal of this dissertation is to understand the molecular mechanisms underlying t^6A and Q biosynthesis and explore the potential of the biosynthesis enzymes for drug target development. t^6A biosynthesis involves the transfer of a threonylcarbamoyl (TC) moiety from a pathway intermediate to tRNA by a multisubunit enzyme complex possessing an ATPase activity required for multi-turnover of the t^6A cycle. In chapter 2, I used structure-

guided mutagenesis and kinetic assays to investigate the mechanism of ATP hydrolysis-driven turnover of the t^6A cycle in *T. maritima*, and show that residues in conserved “switch” loops of the TsaE subunit of the complex mediate this process. In chapter 3, I show that a stable trimeric form of TsaE possesses a kinase activity, autophosphorylating at conserved switch residues, suggesting a role for phosphorylation in cycle reset. In chapter 4, I present efforts toward structure determination of the tRNA bound biosynthesis complex, resulting in a 10.1 Å cryo-EM map showing the mode of tRNA binding to a pre-TC-transfer state of the complex. Q biosynthesis starts with the GTP cyclohydrolase-catalyzed conversion of GTP to H_2NTP , a step shared with the folate pathway in bacteria and the biopterin pathway in humans. Many pathogen rely on a form of the enzyme (GCYH-IB) that is structurally distinct from the human GCYH-IA enzyme, positioning GCYH-IB as an attractive antimicrobial target. In chapter 5, I investigate the catalytic mechanism of *Neisseria gonorrhoeae* GCYH-IB and the role of active-site S-nitrosylation in enzyme function; and test enzyme inhibition by 8-oxo-guanine analogs. The results provide a broad map of the catalytic site and suggest a novel role of S-nitrosothiol in catalysis. The outcome of these studies is a proposal of the detailed catalytic mechanism of GCYH-IB, and identification of a selective inhibitor of GCYH-IB. Together, these studies contribute to a better mechanistic understanding of t^6A and Q biosyntheses, and the utilization of enzymes in these pathways for drug discovery.

Chapter 1 : Introduction

1.1 tRNA modifications

As key molecules of translation, transfer RNAs (tRNAs) decode information in messenger RNA (mRNA) during protein synthesis. A mature tRNA adopts a clover-leaf secondary structure with five characteristic regions, the acceptor stem, D-arm, T-arm, variable loop and the anticodon (Figure 1-1A). Additional tertiary interaction, particularly between D-arm and T-arm, create the elbow of the characteristic L-shaped tertiary structure of cytosolic tRNAs (Figure 1-1B). Mitochondrial tRNAs may be deficient in a D-arm or a T-arm or both (1, 2). tRNAs are extensively modified with diverse chemical groups, and these modifications account for nearly 80% of a total of ~160 known RNA modifications, as reported to date in the MODOMICS database (3). Modified nucleotides constitute ~10% of the total nucleotides in an average tRNA, and they are widely distributed across phylogenetic domains (4). Modifications occur throughout the tRNA molecule, but particularly cluster in regions of functional significance such as the anticodon stem-loop (ASL) and the elbow/core region of the tRNA. The tendency to be modified in areas of functional importance is conserved in all domains of life, but the identities of those modified nucleosides can be domain-specific (4). The ASL modifications are usually bulky and can be species specific and anticodon specific. These modifications define codons, and fine-tune ribosomal function by promoting translational accuracy and preventing ribosomal frameshifting (2). They are also often required for recognition by aminoacyl-tRNA synthetases. In contrast, most of the modifications in the tRNA elbow are small, generally common among tRNA species, and play important roles in the structural stability of tRNA. Alterations to tRNA modifications and to their dynamic

abundances have detrimental and pleiotropic effects on the cell (5–7). Due to the essentiality of some of these tRNA modifications in bacteria, the related biosynthetic enzymes have become interesting candidates for the development of new antibiotics. For example, TrmD, the methyl transferase responsible for the ASL modification m¹G37, is an essential enzyme in *Streptococcus pneumoniae*, *Bacillus subtilis*, *E. coli* and *Mycobacterium Tuberculosis* (8, 9) and is currently being investigated for the development of inhibitors using fragment-based approaches (8). Bacterial tRNA modification genes that are essential are suitable targets for broad spectrum antibiotic development. However, to preserve commensal bacteria and only target the pathogenic bacteria, tRNA modification genes related only to virulence can be targeted. An example of this is the 5-methylaminomethyl-2-thiouridine (mnm⁵s²U34) tRNA modification. It is synthesized by two enzymes, GidA and MnmE, which are required for virulence in pathogens like *Salmonella enterica serovar Typhimurium* and *Pseudomonas aeruginosa* (10, 11).

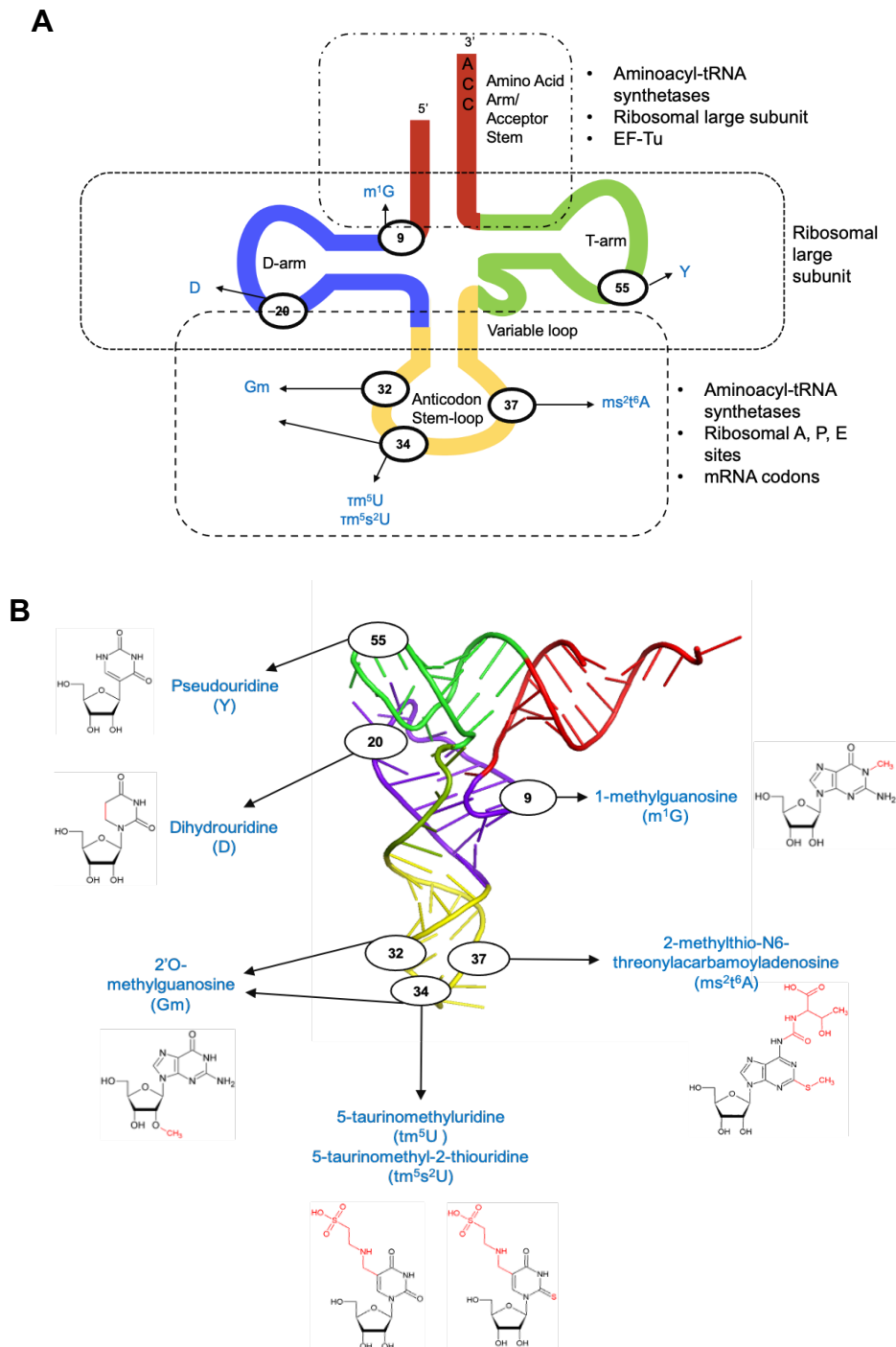


Figure 1-1: tRNA structure and modification.

A: Clover-leaf secondary structure of tRNA and examples of its modified nucleotides, their locations in tRNA and the translational components with which they interact.

B: Structures of some ASL and elbow modifications in tRNA, figure made using yeast tRNA^{Phe}, PDB ID: 1EHZ.

1.2 Threonylcarbamoyl adenosine (t⁶A)

N⁶-threonylcarbamoyl adenosine (t⁶A) is a universal tRNA modification that occurs at position 37 in the anticodon stem-loops of tRNAs that decode ANN codons (N is any nucleotide) (12) (Figure 1-2). t⁶A plays a critical role in maintaining the local structure of the ASL and its interactions with the ribosome, by providing additional base stacking interactions (13, 14). Cells deficient in t⁶A exhibit an increase in translational frameshifting at discrete codons, an increase in inaccurate selection of start codons, and deregulation of codon-specific translation elongation rates (15, 16). t⁶A-unmodified tRNAs show a decrease in aminoacylation by aminoacyl-tRNA synthetases (17). The cumulative outcomes of these effects are global growth defects as was shown in bacteria, drosophila and yeast (17–19). t⁶A is essential in most bacteria including pathogens such as *E. coli*, *Staphylococcus aureus* (20) and *Pseudomonas aeruginosa* (17). In bacteria where t⁶A is not essential, its deficiency causes deleterious phenotypes, such as altered homeostasis, increased sensitivity to antibiotics, compromised biofilm formation and defective cell wall synthesis (17, 20–23). In humans, defects in cytoplasmic t⁶A production has been linked to the rare renal-neurological disease Galloway Mowat syndrome (24–26) and low levels of mitochondrial t⁶A have been linked to the mitochondrial disease MERRF (myoclonic epilepsy with ragged red fibers) (27). Elevated levels of t⁶A have been detected in the urine of patients with metastatic breast cancer, positioning t⁶A as a potential breast cancer biomarker (28). Likewise, a recent study revealed the upregulation of the t⁶A biosynthetic enzyme TsaC2 in non-small cell lung cancer (NSCLC) cells, pointing to the potential of TsaC2 as a biomarker of NSCLC (29).

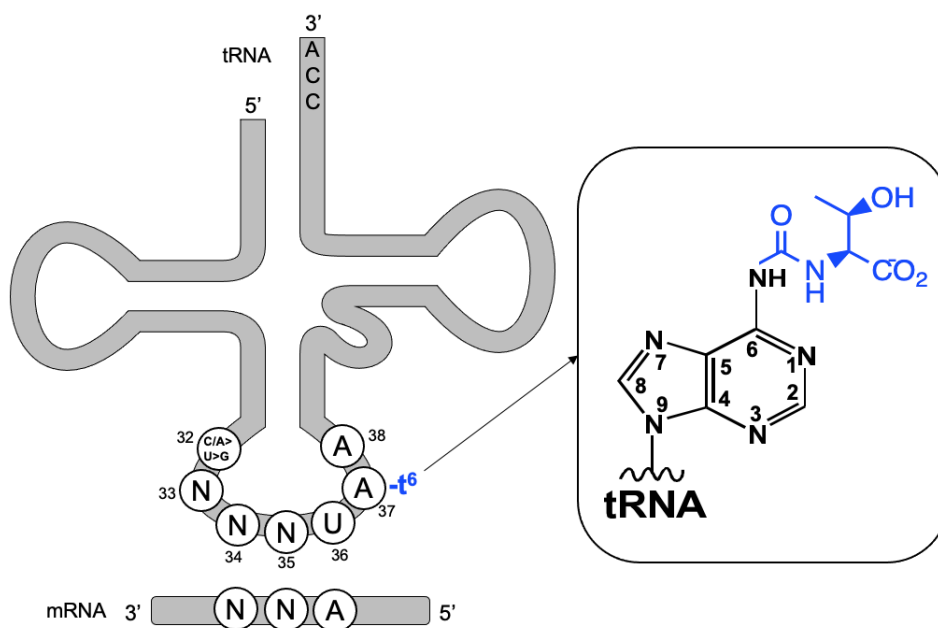


Figure 1-2: Structure of t^6A and its location in tRNA.

t^6A biosynthesis occurs in two steps (Figure 1-3): Members of the TsaC/TsaC2 (formerly YrdC/Sua5) family, present in all three domains of life (30, 31), utilize L-threonine, bicarbonate, and ATP to synthesize the pathway intermediate threonylcarbamoyl adenylate (TC-AMP) (32). The threonylcarbamoyl (TC) moiety of TC-AMP is then transferred to N⁶ of adenosine-37 of the substrate tRNA by the threonylcarbamoyl transfer (TCT) complex, composed of TsaB, TsaD and TsaE in bacteria (12, 32, 33) (Tsa proteins); by the KEOPS complex composed of Pcc1, Kae1, Bud32 and Cgi121 in archaea (34) and the cytosols of eukaryotes (35, 36); and by the TsaD ortholog Qri7 in mitochondria (37). The TsaD/Kae1/Qri7 protein harbors the catalytic site for the transfer reaction. The structures of these proteins and their mechanistic roles in the t^6A cycle have been elucidated to various degrees in different organisms. In bacteria, TsaB and TsaD associate to form a TsaB/TsaD hetero complex with a 1:1 stoichiometry in the case of the *S. typhimurium* and *E. coli* systems (38, 39), or

a 2:2 stoichiometry where TsaB acts as a dimerization module in the *Thermotoga maritima* system ($TmTsaB_2D_2$) (33, 40). This complex binds the tRNA substrate and carries out the TC transfer reaction, while TsaE provides an ATPase activity required for turnover of the catalytic cycle (33, 40). Using the *T. maritima* and *E. coli* enzyme systems, it has been shown that in the absence of tRNA, TsaE binds to the TsaB/TsaD complex in an ATP-dependent manner (33, 38–40) and occupies the same or an overlapping site with that of tRNA, forming a 1:1:1 complex (e.g. in the *E. coli* system), or a 2:2:2 complex in the case of the *T. maritima* system ($TmTsaB_2D_2E_2$) (33, 40).

TsaE is an atypical P-loop ATPase that harbors the Walker A (GX4GKT/S) and Walker B motifs (h4D/E, h is a hydrophobic residue) characteristic of P-loop ATPases and responsible for nucleotide binding and hydrolysis, respectively. It also harbors the Switch I and Switch II loops known in P-loop ATPases to be responsible for signal transduction of ATP hydrolysis to remote sites in the enzyme or in an adjacent subunit (41, 42). The ATPase function of TsaE is activated in the presence of TsaB and TsaD by ~10 fold (12, 40), and this activity is required for cell viability (43). It has been recently shown that the $TmTsaB_2D_2$ complex catalyzes TC transfer in the absence of TsaE, however ATP hydrolysis in the presence of TsaE is required for multiple turnover of the t^6A synthesis reaction (40). Furthermore, product tRNA is released before the binding of TsaE and before ATP hydrolysis occurs (40). This led to a proposal of the t^6A catalytic cycle in which $TmTsaB_2D_2$ is inactivated after TC transfer, requiring ATP hydrolysis in the ATPase site to “reset” the system to the active state for the next cycle (Figure 1-3). Despite this understanding, the mechanism of specific selection of ANN-decoding tRNAs by the TC-transfer complex, and of its ATP hydrolysis-driven “reset” remain unclear.

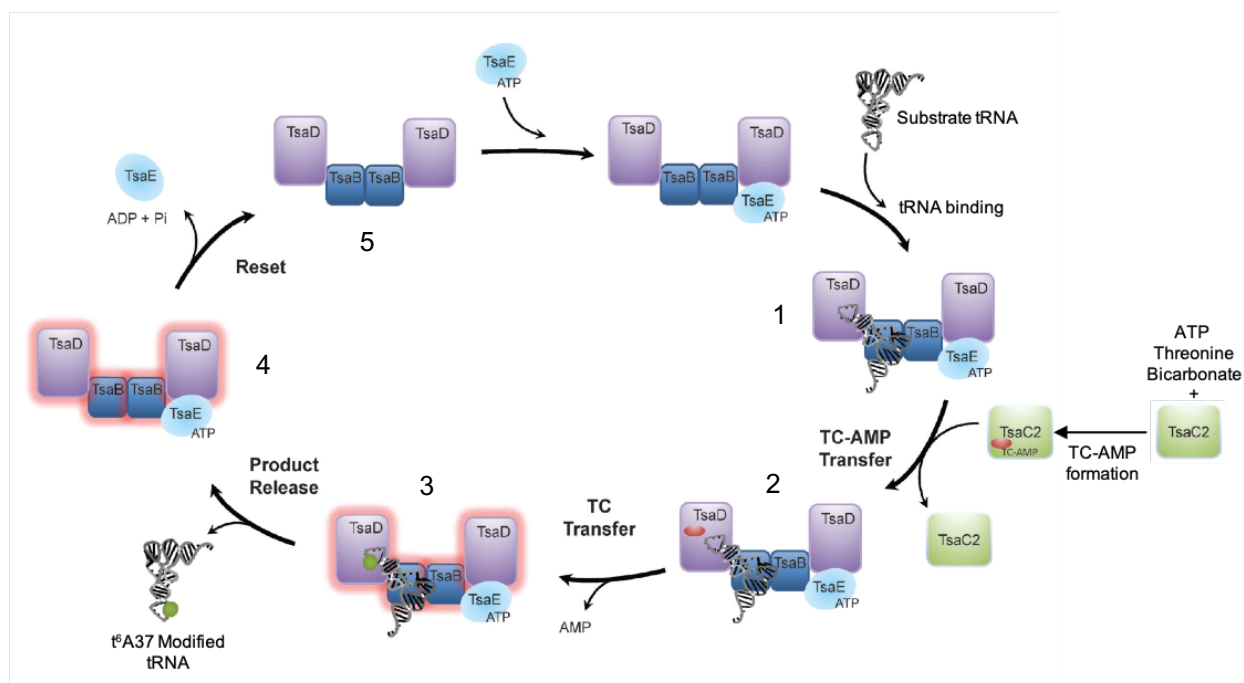


Figure 1-3: Current proposal of the bacterial t⁶A catalytic cycle, shown for the *T. maritima* enzyme system.

tRNA and ATP-bound TsaE bind to separate sites on the *Tm*TsaB₂D₂ heterocomplex (1). TC-AMP, synthesized by TsaC2, is then delivered or putatively channeled to the active site of the tRNA-bound TsaD subunit (2), and the threonylcarbamoyl (TC) moiety is transferred to A37 of tRNA, forming t⁶A (3). Following dissociation of the t⁶A-modified tRNA from the complex (4), ATP hydrolysis resets the system to the initial state and TsaE is released (5), allowing the next substrate tRNA to bind for the next cycle. The two halves of the complex are reset and utilized in alternating cycles. Halos indicate a pre-reset state for a given half.

1.3 Antibiotic resistance and the folate pathway

Antibiotics have revolutionized modern medicine. They are not only used to treat bacterial infections but also made modern medical procedures, such as surgeries and cancer therapy that would otherwise involve a high risk of bacterial infections, possible (44). However, due to decades of misuse, many antibiotics have become ineffective due to acquired resistance by their bacterial targets, resulting in untreatable multi-drug resistant infections. Resistance can emerge against a new antibiotic as early as 2 years after its initial clinical use (45). In 2019, the Centers for Disease Control and Prevention (CDC) reported that more than 35,000 people in the USA die annually as a result of multi-drug resistant bacterial infections, and listed 14 resistant bacteria as urgent and serious threats (46). One of these pathogens is *Neisseria gonorrhoeae* (*Ng*), the causative agent of the sexually transmitted disease gonorrhea (infection of the genitalia and urinary tract) and major cause of pelvic inflammatory disease and infertility in women (47). *Ng* has adeptly developed resistance to all first-line antibiotics such as penicillin (inhibitor of cell wall synthesis), tetracycline (inhibitor of ribosomal translation) and fluoroquinolones (DNA gyrase and topoisomerase inhibitor); and multiple strains of *Ng* are developing resistance to the last class of therapy – third generation antibiotics cephalosporin and ceftriaxone (both inhibitors of cell wall synthesis) (48). With more than 580,000 gonorrhea cases reported to the CDC in 2018 (49), the fear of widespread untreatable gonorrhoeal infections calls for urgent development of new therapeutics.

One of the important and clinically validated antibiotic targets is the bacterial folate biosynthesis pathway (50). Folate (folic acid, tetrahydrofolate, THF) is an essential vitamin that donates a one-carbon unit during the synthesis of purines, formylmethionyl-

tRNA, thymidylate, pantothenate, glycine, serine, and methionine in most life forms. Bacteria, fungi and plants synthesize THF *de novo*, while animals do not make folate and depend on a dietary source of it (51). The bacterial folate pathway has been targeted at two steps for antibiotic intervention: Sulfonamides, the first synthetic drugs developed with broad-spectrum antibacterial activity (52), block folate biosynthesis at dihydropteroate synthase (DHPS), and lethality is due to the subsequent loss of thymidine production (53). Later, trimethoprim (TMP) was introduced as a dihydrofolate reductase (DHFR) inhibitor blocking a second step in the pathway (52). However, resistance emerged due to alterations in both enzymes and the spread of plasmids carrying the altered genes (52, 54). Because of the clinical success of antifolates and the emerging resistance to these drugs, a new class of antibiotics targeting an unexploited enzyme of folate biosynthesis, GTP cyclohydrolase I (GCH-I), would present a prime opportunity to target resistant pathogens that rely on this enzyme.

1.4 GTP cyclohydrolase I

GCH-I catalyzes the conversion of GTP to 7,8-dihydroneopterin triphosphate (H_2NTP), the first step in three major metabolic pathways (Figure 1-5): 1) the folate pathway in bacteria and plants; 2) the tetrahydrobiopterin (BH_4) pathway in higher eukaryotes; 3) the biosynthesis pathway to the 7-deazaguanine modified nucleosides queuosine (in bacteria) and archaeosine (in archaea and phage), and the 7-deazapurine natural antibiotics (in some bacteria) (55). In mammals, tetrahydrobiopterin functions as an important cofactor for enzymes involved in aromatic amino acid synthesis and neurotransmitter synthesis (56).

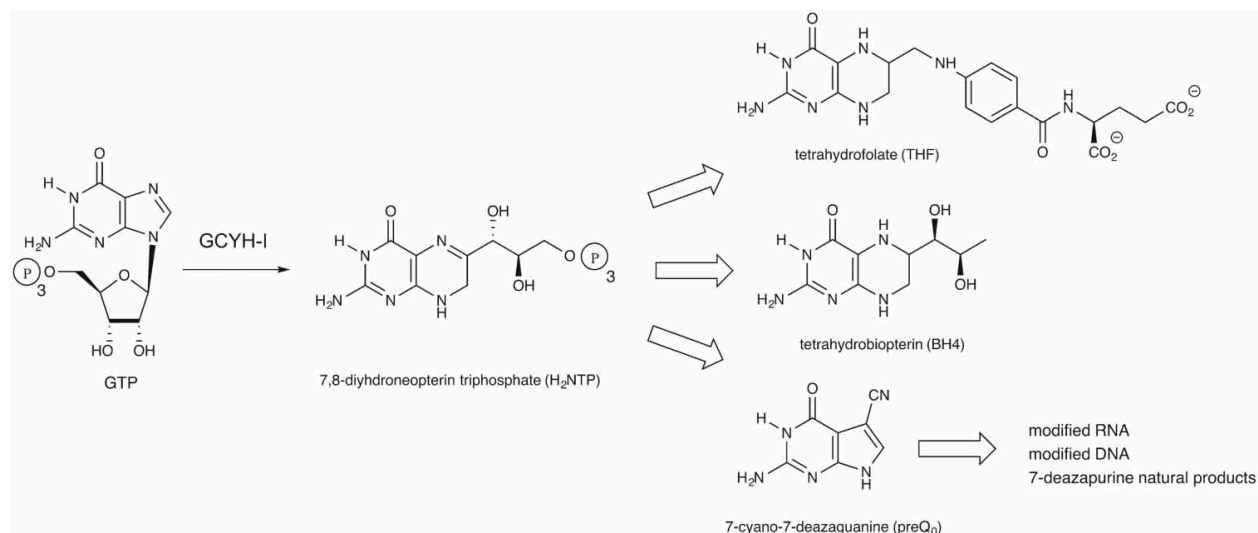


Figure 1-4: The reaction catalyzed by GCYH-I and the metabolic pathways in which it is found.

GCYH-I represents one of four classes of GTP cyclohydrolase enzymes, all of which require divalent cations for activity and use GTP as the substrate to initiate the biosynthesis of important vitamins/cofactors. GCYH-II converts GTP to 2,5-diamino-6- β -ribosyl-4(3*H*)-pyrimidinone 5'-phosphate and is the first step in riboflavin (vitamin B₂) and toxoflavin (toxic antibiotic) biosynthesis. GCYH-III converts GTP to 2-amino-5-formylamino-6-ribosylamino-4(3*H*)-pyrimidinone 5'-phosphate and is the first step in deazaflavin biosynthesis, a flavin derived cofactor involved in redox reactions. GCYH-IV (also known also MptA) is an archaeal specific cyclohydrolase that converts GTP to dihydroneopterin-2',3'-cyclophosphate, the first step in tetrahydromethanopterin biosynthesis, a cofactor involved in methanogenesis (55).

Despite the essential role of GCYH-I as the initiating step in the bacterial folate pathway, its equally essential role in the tetrahydrobiopterin pathway in humans, coupled with the high homology between the bacterial and human GCYH-I enzymes, precluded

GCYH-I as a viable target for new antibacterials. Recently, a second family of GCYH-I enzymes (named GTP cyclohydrolase type IB or GCYH-IB) was discovered in a significant number of bacteria and found to be structurally distinct from the eukaryotic enzyme (renamed GTP cyclohydrolase type IA or GCYH-IA) (57). Crystal structures have been determined for GCYH-IA from *E. coli* (58), human (59), rat (60) and *Thermus thermophilus* (*Tt*) (61); and for GCYH-IB from *Neisseria gonorrhoeae* (62). Although both families belong to the Tunnel fold (T-fold) structural superfamily and catalyze the same reaction, they show structural differences in tertiary and quaternary structure and in the active-site architecture (62, 63). GCYH-IA is a unimodular, homodecameric, Zn^{2+} -dependent enzyme while GCYH-IB is a bimodular, homotetrameric enzyme activated by a variety of divalent cations, with maximal activity observed with Mn^{2+} . In both enzymes, as in all T-fold enzymes, catalysis occurs at the interfaces between the T-fold subunits (63, 64). However, several helical insertions and deletions near the interface render the active sites of the two enzymes significantly different. These differences, coupled with the occurrence of the latter enzyme in clinically important human pathogens that lack GCYH-IA, such as *Ng* (57, 63), and thus depend solely on GCYH-IB for folate biosynthesis, have positioned GCYH-IB as a potential new antibacterial target in the folate pathway (63, 65). These differences also led to the proposal that the two GCYH-I families may employ different catalytic strategies (62, 63). While details of the mechanism have been proposed for the type IA enzyme (59, 61, 66), the mechanism of the type IB enzyme has not been explicitly addressed.

1.5 Protein kinases

Protein kinases are enzymes that use ATP as a phosphate donor to attach one or more phosphate groups to a substrate protein to give a phosphorylated form of that protein. Protein kinases can autophosphorylate themselves or transphosphorylate another protein, and a kinase may have more than one substrate protein, and in some cases may be activated by another kinase upstream in a pathway. This post-translational modification, known to occur on various amino acids, can act as a molecular switch to turn on or off a substrate protein or trigger temporary protein-protein interactions to achieve a cellular function (67). Protein kinases usually act alongside protein phosphatases, enzymes that dephosphorylate proteins, in order to maintain a balance between phosphorylated and dephosphorylated forms of proteins. This balance is important as kinases and phosphatases work in opposite directions in a signaling pathway (67). Protein kinases are involved in numerous, complex networks of signal transduction cascades in order to sense and communicate both intra- and extracellular stimuli in regulatory pathways.

The field of protein kinases was established in the 1950s by Krebs and Fischer who studied eukaryotic kinases. The canonical eukaryotic protein kinases (ePKs) fall into 9 families (68) and share a core structure consisting of two lobes; a small, largely β -stranded N-terminal (N-) lobe, and a large, mostly helical C-terminal (C-) lobe. ATP binds in a cleft between the N- and C- lobes. The N-lobe hosts the ATP binding glycine-rich loop (P-loop). The C-lobe harbors a catalytic loop (C-loop), a metal binding loop (M-loop), an activation loop that harbors the site of autophosphorylation and is responsible for modulation of the kinase activity (A-loop, also called the F-loop/ flexible loop in some kinases), and a P+1 loop which is an extension of the A-loop and provides the docking

site for the peptide substrate (69). These motifs are conserved in eukaryotic kinases both in sequence and structure, except for the A/F-loop which tends to be the most structurally diverse part of the protein, hence the name flexible loop (70). Another class of eukaryotic protein kinases are the atypical protein kinases (aPKs), which fall into 13 families that are defined by structure including the RIO kinase family (71). All aPK families are small and some of these families are restricted to metazoans while others are widely distributed in eukaryotes and are found even in prokaryotes (72). aPKs are remarkably similar in structure to the ePKs but show low sequence similarity with the glycine-rich P-loop completely replaced by another motif (HRD motif) (68) (73). Both ePKs and aPKs target a variety of phosphorylation sites, with serine/threonine, tyrosine or dual specific serine/threonine/tyrosine being the most common, but phosphorylation of histidine, arginines, lysines and aspartates have also been observed (74, 75). ePKs serve in regulating a variety of cellular processes such as cell metabolism, cycle, proliferation, differentiation, motility, adhesion, invasion, inflammation, angiogenesis and vessel maturation via multiple protein kinase pathways (76). While aPKs are clear homologs of ePKs and have demonstrated *in vitro* protein kinase activity, not much research has been conducted on the aPKs and their physiological functions (72). Two well studied aPKs, atypical MAPK and atypical protein kinase C, were found to play a role in the same pathways as their conventional counterpart (ePKs) (77, 78) .

ePKs are critical players in many cancers and degenerative diseases, and numerous anticancer drugs have been developed based on inhibiting some of these kinases (79). Imatinib, a very successful chemotherapy drug used for chronic myelogenous leukemias, works by inhibiting the protein breakpoint cluster region protein-

Abelson tyrosine kinase (BCR-ABL). BCR-ABL is involved in the Ras/MAPK and JAK/STAT cancer proliferation pathways (79).

1.6 Bacterial protein kinases

The study of protein kinases in bacteria is an emerging field. As of 2017, there were five families of protein kinases identified in bacteria: eukaryotic-like S/T kinases (eSTK), bacterial tyrosine (BY) kinases, histidine kinases (HK), arginine kinases (AK), and atypical serine kinases (aSK) (80). Recently, a new eukaryotic-like S/T/Y Hanks-type kinase was described in bacteria and proposed as a sixth family of bacterial kinases named “Ubiquitous bacterial kinase” (Ubk) (21).

The bacterial eukaryotic-like S/T kinases (eSTKs) are either cytoplasmic or inserted in the plasma membrane with an extracellular domain and, as the name indicates, they resemble eukaryotic protein kinases in that they contain ePK motifs and undergo autophosphorylation in the activation loop. eSTKs phosphorylate a wide array of regulatory proteins involved in virulence, cell division and metabolism (80, 81).

BY kinases bear no resemblance to the ePK counterpart. BY kinases are found in the form of a single membrane protein or two separate subunits (one membrane bound and one cytoplasmic), and the kinase core harbors Walker A, Walker B, Walker A' and tyrosine-cluster motifs where Walker B and Walker A' are very similar in sequence and the tyrosine-cluster contains the phosphorylation sites (82). The Walker motifs are usually found in NTPases and therefore BY kinases share structural similarities to some ATPases and GTPases (83). BY kinases autophosphorylate and transphosphorylate a large array

of proteins involved in numerous regulatory pathways in bacteria, e.g. exopolysaccharide production, biofilm formation, virulence, stress response and DNA metabolism (80).

HKs, the first bacterial protein kinases identified and characterized, are a hallmark of the bacterial two-component signal transduction systems (TCSs) (81). TCSs typically consist of a sensor HK and a corresponding response regulator. The sensor HK comprises an extracellular ligand-binding sensory domain and a cytoplasmic kinase domain that interacts with the response regulator. When stimulated, the kinase domain autophosphorylates at a conserved histidine and then transfers the high energy phosphoryl group to an aspartate residue on the response regulator which ultimately leads to changes in gene expression through specific signal transduction cascades (84). The kinase domain of a histidine kinase is characterized by five motifs (also called boxes): an H motif which harbors the conserved phosphoacceptor histidine, and N, G1, G2 and G3 motifs which contain conserved residues that form the ATP binding cleft (85).

Arginine phosphorylation in bacteria is a relatively new discovery found in *B. subtilis* with the protein McsB. McsB was shown to play a role in heat shock regulation and protein quality control via chaperones (GroEL, DnaK) and protease systems (Hsp100/Clp) (86). According to the structure of McsB, the kinase core consists of a conserved cysteine, two glutamates and a series of arginine residues which together position the substrate arginine and ATP for phospho transfer (86).

Atypical serine kinases (bacterial aSK) also employ Walker motifs like BY kinases (except for Walker A') and are unusual in that they use the same active site to catalyze both serine phosphorylation and dephosphorylation. There are only two known proteins of this type, Hpr kinase/phosphorylase and isocitrate dehydrogenase

kinase/phosphorylase. aSKs act on substrate proteins only and have been shown to be specifically involved in the control of metabolism (80, 81).

The eukaryotic-like S/T/Y Hanks-type Ubk bacterial kinases have been reported in two studies by the same research group thus far (21, 87). The Ubk family of proteins is essentially the TsaE family involved in the biosynthesis of the tRNA modified nucleotide t⁶A. TsaE has been described as a P-loop ATPase, but this activity is stimulated by its binding to other t⁶A biosynthesis proteins (see chapter 2). The Ubk/TsaE family, like the BY kinases and aSKs, possess Walker A and Walker B motifs, but are unique among bacterial kinases in that they also contain the Switch I and Switch II motifs characteristic of P-loop NTPases. To date, the only established biological function of this family is its role in t⁶A biosynthesis.

Chapter 2 : Conformational communication mediates the reset step in threonylcarbamoyl adenosine biosynthesis.

Abstract

The universally conserved *N*⁶-threonylcarbamoyl adenosine tRNA modification is essential for translational efficiency and survival of most bacteria. Differences between the eukaryotic and bacterial t⁶A biosynthesis machineries have positioned the bacterial t⁶A pathway an attractive target for antimicrobial development. Bacterial t⁶A biosynthesis starts with the TsaC/TsaC2-catalyzed synthesis of the intermediate threonylcarbamoyl adenylate (TC–AMP), followed by transfer of the threonylcarbamoyl (TC) moiety to adenine-37 of tRNA by the TC-transfer complex comprised of TsaB, TsaD and TsaE subunits and possessing an ATPase activity required for multi-turnover of the t⁶A cycle. Previous structural and biochemical studies of the *Thermotoga maritima* enzyme system led to a model of the t⁶A catalytic cycle in which TC transfer is catalyzed by the active site of the TsaD subunit (the TC-transfer site) of the tRNA-bound TsaB/TsaD heterocomplex, rendering it inactive. ATP hydrolysis in an interfacial ATPase site between the TsaD and TsaE subunits then ‘resets’ the system to the active state for the next cycle. In this study, I investigated the mechanism of reset using structure-guided mutagenesis of the TsaE subunit and quantitative *in vitro* evaluation of mutants for their ability to support formation of the TC-transfer complex, ATP hydrolysis, and multi-turnover t⁶A synthesis. The results show that residues in the conserved Switch I and Switch II motifs of TsaE conformationally mediate the ATP hydrolysis-driven reactivation/reset step of the t⁶A cycle.

2.1 Introduction

The crystal structure of the TC-transfer complex TsaB₂D₂E₂ from *Thermotoga maritima* (Tm) was determined at 3.1 Å (PDB ID 6FPE (88)) and at 2.50 Å (PDB ID 6N9A, Figure 2-1A (33)). The structure showed the TsaE subunit positioned at the entrance of the TC-transfer site in TsaD, and blocking access of tRNA to the site, consistent with previous biochemical and SAXS data (38, 40). The structure also revealed an ATP molecule bound in the ATPase site at the interface between the TsaD and TsaE subunits, and a carboxy-AMP molecule bound in the TC-transfer site of the TsaD subunit and mimicking the TC-AMP substrate (Figure 2-1B). The distance between the two sites is 18 Å.

The observation that a nonhydrolyzed nucleotide is bound in the ATPase site identifies this structure as a pre-“reset” structure in the proposed course of the t⁶A cycle, i.e. representing the inactivated half of the complex in proposed states 1, 2, 3 and 4 in Figure 1-3.

In this chapter, I used the TmTsaB₂D₂E₂ crystal structure to guide mutagenesis experiments to elucidate the mechanism by which ATP hydrolysis in the ATPase site is communicated to the TC-transfer site during the reset step of the t⁶A catalytic cycle.

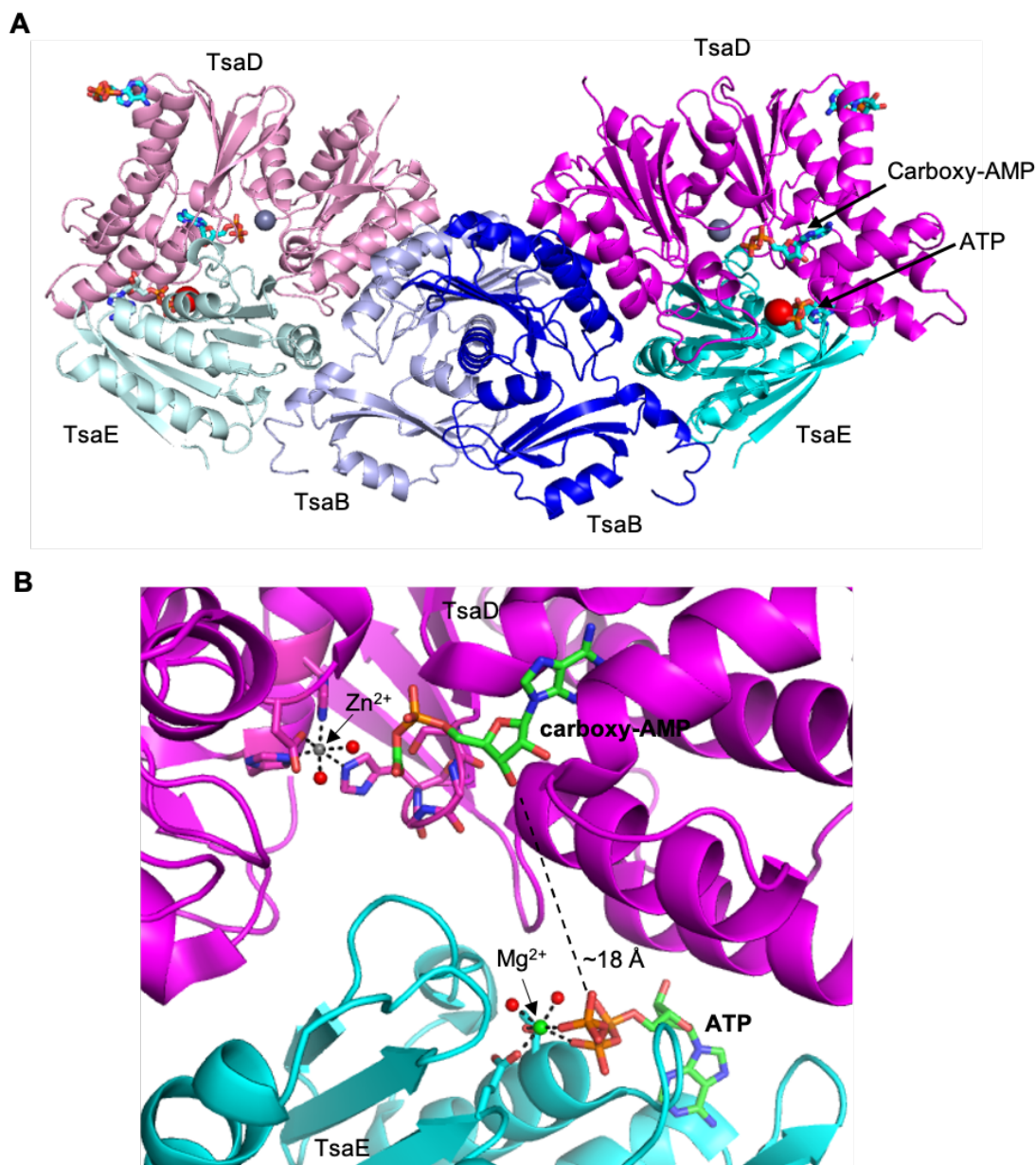


Figure 2-1: Crystal structure of *TmTsaB₂D₂E₂* in complex with ATP and carboxy-AMP.

A: Overall structure showing locations of the ATPase site and TC-transfer site. Subunits TsaB, TsaD and TsaE are colored in two shades of blue, magenta and cyan, respectively. Bound Zn²⁺ and Mg²⁺ ions are shown as gray and green spheres, respectively, and bound nucleotides as stick model and labeled. Bound water molecules shown as red spheres.

B: Close up view of ATP and Mg²⁺ bound in the interfacial ATPase site between TsaD and TsaE, and of carboxy-AMP and Zn²⁺ bound in the TC-transfer site in TsaD. Metal coordination spheres are indicated with dashed lines.

2.2 Materials and Methods

Analysis of TsaB₂D₂E₂, TsaBD and TsaE crystal structures

The *Tm*TsaE subunit from the *Tm*TsaB₂D₂E₂ crystal structure with ATP bound in the ATPase site (representing the pre-reset or pre ATP hydrolysis conformation) was compared with *Haemophilus infleunzae* (*Hi*) TsaE bound with ADP (representing the post ATP hydrolysis conformation) (41). Likewise, the *Tm*TsaB₂D₂ subcomplex from the *Tm*TsaB₂D₂E₂ crystal structure (representing the conformation of the tRNA binding platform in the presence of bound TsaE) was compared with the *E. coli* TsaBD structure (representing the conformation in the absence of bound TsaE) (38). In both cases, the two crystal structures were superimposed and visually analyzed for conformational changes using Pymol (89). To understand the significance of observed conformational changes, a sequence-based alignment of ~1000 non-redundant sequences and a structure-based alignment of available crystal structures were generated using the NCBI Protein Blast, Clustal Omega and EsPript servers (90–92) to inspect the conservation of residues in TsaE and TsaD (including the eukaryotic TsaD orthologs - Kae1 and Qri7).

Mutagenesis

Point mutagenesis was carried out on pET28a-*Tm*TsaE (provided by Dr. Amit Luthra of Prof. Manal Swairjo Lab) using the Q5® Site-Directed Mutagenesis Kit (New England Biolabs Inc.) and the primers listed in Table 2-1. All constructs were verified by sequencing.

Table 2-1: Primers used for the preparation of *TmTsaE* mutants.

Mutant	Sense Primer	Antisense Primer
Ser61Ala	5'-AATGGTGAAAGCACCTACTTTACCCC-3'	5'-TTTTCATCGAGACCTATC-3'
Thr63Ala	5'- GAAAAGTCCTGCATTACCCCTCATG-3'	5'-ACCATTTTTTTCATCGAGAC-3'
Glu80Ala	5'-ATACCACCTCGCACTTTACAGAC-3'	5'- ATTGTTTTCAAACCTGGATAAAC- 3'
Phe64Ala	5'-AAGTCCTACTGCAACCCTCATGAATG-3'	5'-TTCACCATTTTTTTCATCGAG-3'
Tyr82Ala	5'-CCTCGATCTTGCAAGACTTCAAGACACG-3'	5'-GGTATATTGTTTTCAAACC-3'
Arg83Ala	5'-CGATCTTTACGCACTTCAAGACACG-3'	5'- AGGTGGTATATTGTTTTCAAAC- 3'

Protein purification

All recombinant wild-type and mutant *TmTsaE* proteins were purified as described previously (40). Briefly, His₆-tagged *TmTsaE* was overexpressed in *E. coli*, purified by Ni-NTA and the His₆ tag was removed by thrombin digestion followed by gel filtration chromatography. Purity was verified by SDS-PAGE to be > 95%.

Assessment of formation of *TmTsaB*₂*D*₂*E*₂ complexes with mutant proteins using analytical gel filtration

Analytical gel filtration experiments were performed on a calibrated Enrich SEC650 10 mm × 300 mm column (Bio-Rad, Hercules, CA, USA) using a Bio-Rad NGC chromatography system, as described previously (40). Briefly, 500 µl of sample containing 10 µM each *TmTsaB*^{WT}, *TmTsaD*^{WT} and *TmTsaE*^{WT/Mut} in buffer containing 50 mM Tris–HCl pH 7.5, 50 mM KCl, 1 mM DTT, 1 mM ATP and 1 mM MgCl₂ were loaded on the column and run using buffer containing 50 mM Tris (pH 7.5) and 50 mM KCl, at a flow rate of 0.3 ml/min, with detection at 280 nm.

ATPase activity of the TC-transfer complex

The ATP hydrolysis was carried out at 37°C for 60 min in 40 µl reactions containing 5 µM wild-type *TmTsaB₂D₂* and mutant *TmTsaE* (or *TmTsaB₂D₂^{mutant}* and wild-type *TmTsaE*), 50 µM [γ -³²P] ATP (22×10^9 DPM/µmol, PerkinElmer), 25 mM Tris–HCl (pH 7.5), 50 mM KCl and 2 mM MgCl₂. Two microliters of the reaction mix were spotted on a PEI-cellulose thin layer chromatography (TLC) plate. A reaction mixture lacking enzyme was likewise analyzed as a control. The radioactive nucleotide and product [γ -³²P]P_i were separated on the plate using 0.5 M KH₂PO₄ (pH 3.5) as solvent, visualized by phosphorimaging and quantified by densitometry. The percentage of ATP hydrolyzed was calculated using equation (1).

$$\text{Fraction of ATP hydrolyzed} = \frac{\text{quantity of } [\gamma\text{-}^{32}\text{P}]\text{P}_i}{\text{quantities of } ([\gamma\text{-}^{32}\text{P}]\text{P}_i + \text{unhydrolyzed } [\gamma\text{-}^{32}\text{P}]\text{ATP})} \quad (1)$$

t⁶A biosynthesis assay

Time-course enzyme assays to measure the activity of *TmTsaD* and *TmTsaE* mutants in t⁶A synthesis were executed in 275 µl-reactions containing 100 mM Tris–HCl (pH 8.0), 300 mM KCl, 5.0 mM DTT, 2 mM ATP, 20 mM MgCl₂, 50 mM NaHCO₃, 250 µM L-[U-¹⁴C]-threonine (36 µCi/µmol), 20 µM *E. coli* tRNA^{Thr}_{CGU}, 2 µM each *TmTsaB*, *TmTsaC2* and *TmTsaD^{WT/Mut}* and 1 µM *TmTsaE^{WT/Mut}*. Assays were incubated at 37°C and 50 µl aliquots were taken at 15, 30, 60, 90, 120, 180 min. tRNA was precipitated by the addition of 500 µl 10% TCA and cooling on ice for 15 min. tRNA was then collected by filtration through Whatman GF/C filters, and the filters were subsequently washed with 100% cold ethanol. The dried filters were combined with Econo-Safe scintillation cocktail (RPI™) and counted in an LS 6500 Multi-purpose

scintillation counter (Beckman Coulter, Inc.). The initial velocity for each reaction was estimated from the initial slope (first derivative) of the concentration of modified tRNA product as a function of time. Note that because the goal of the experiments was to discriminate between single- and multiple-turnover regimes of the enzyme complex, full steady-state kinetic analysis was unnecessary and the assays with mutants were designed as time-courses at a single concentration of substrates.

2.3 Results

TsaE-induced conformational changes in the tRNA binding platform TsaB₂D₂

Comparison of the crystal structures of *Tm*TsaB₂D₂E₂ and *Ec*TsaBD (PDB ID 4YDU (38), r.m.s.d. = 2.0 Å over 408 C_α atoms) highlights the TsaE-induced conformational changes in the tRNA binding platform *Tm*TsaB₂D₂ (Figure 2-2). The main changes are the concerted movements of the C-terminal tail (Leu191-Glu206) of the TsaB subunit and the helical hairpin α 1- α 2 (Ser31-His50) in the TsaD subunit, allowing the Switch I loop of TsaE to lodge near the TC-transfer site. In the absence of TsaE, as seen in the *Ec*TsaBD structure (38), these two parts of the complex are pinned together. The movement of the C-terminal tail of TsaB, and its refolding from its extended conformation seen in *Ec*TsaBD to a 3-turn helix. The N-terminal 2.5 turns of the 6-turn helix α 2 of TsaD unfolds, and the resulting loop and preceding helix α 1 (altogether named “the lever arm”) rotate by 67° away from the TsaB subunit (Figure 2-2). The hinge residues around which the lever arm rotates are Ser31 and Arg49, both invariant in TsaD proteins (Figure 2-3), and the two residues are stabilized by specific interactions with TsaE.

Although these conformational changes result in 21% reduction in the protein surface area buried in the TsaB/TsaD intersubunit interface upon TsaE binding (2170 Å² in *Tm*TsaB₂D₂E₂ versus 2758 Å² in *Ec*TsaBD), the additional surface area buried by TsaE increases the total area buried within the complex by two-fold (5942 Å² in *Tm*TsaB₂D₂E₂ versus 2758 Å² in *Ec*TsaBD). This explains the stabilizing effect, previously observed by SAXS, of TsaE on the structure of the tRNA binding platform (40). Lastly, TsaE pushes apart the two domains of TsaD through a 15° rotation in the C-terminal domain, resulting in expansion of the cavity available for TsaE binding by another 4 Å, relative to its size in

EcTsaBD. Collectively, the conformational changes seen in the tRNA binding platform are consistent with an induced-fit mechanism for binding of the TsaE subunit.

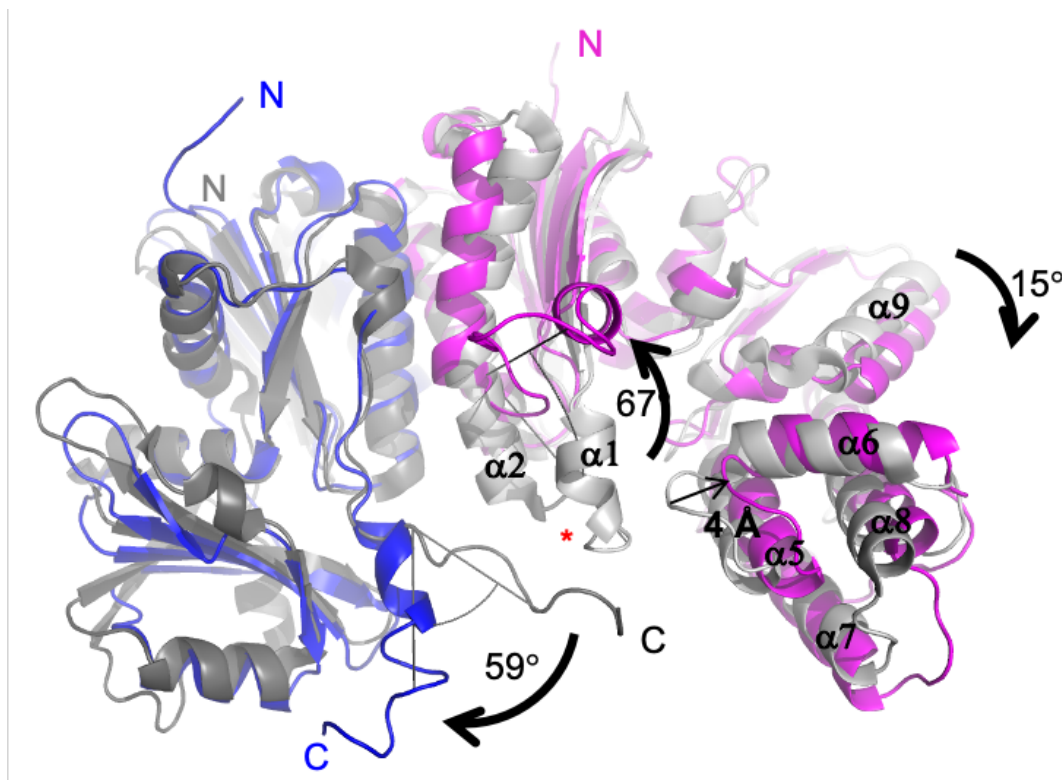


Figure 2-2: TsaE-induced conformational changes in the TsaB₂D₂ platform.

Superposition of the crystal structures of *TmTsaB₂D₂E₂* (colored) and *EcTsaBD* (grey) showing TsaE-induced separation of the TsaB C-terminal tail and “lever arm” of TsaD. The TsaB and TsaD subunits of *TmTsaB₂D₂E₂* are in blue and magenta, respectively. The corresponding subunits of *EcTsaBD* are in dark grey and light grey, respectively. For clarity, only half the *TmTsaB₂D₂E₂* complex is shown, and the TsaE subunit is not shown. The red asterisk marks the position of the center of mass of *TmTsaE* in *TmTsaB₂D₂E₂*. The angles of rotation of the C-terminal tail of TsaB and the lever arm of TsaD ($\alpha 1$ - $\alpha 2$), and the shift in the 5-helical bundle of the C-terminal domain of TsaD ($\alpha 5$ - $\alpha 9$) are indicated.

ATP hydrolysis induced conformational changes in TsaE

In *TmTsaE*, Switch I and Switch II loops contain of the sequences SPT/SF/Y (S⁶¹PTF⁶⁴) and HXDXY/FR (H⁷⁸LDLYR⁸³), respectively, and are characteristically located adjacent to the ATP binding site (Figure 2-4A). Superimposing the crystal structure of *HtTsaE* (post ATP hydrolysis conformation) with *TmTsaE* (pre ATP hydrolysis conformation) (RMSD = 1.482, 93 aligned Ca atoms) revealed two residues, Phe64 and Arg83 located in Switch I and Switch II loops respectively, to have undergone a conformational change (Figure 2-4B). This observation was used to formulate the following hypothesis: In the course of a t⁶A cycle, ATP hydrolysis in the ATPase site is transmitted to the TC-transfer site via residues in Switch I and Switch II loops, via Phe64 and Arg83 in particular.

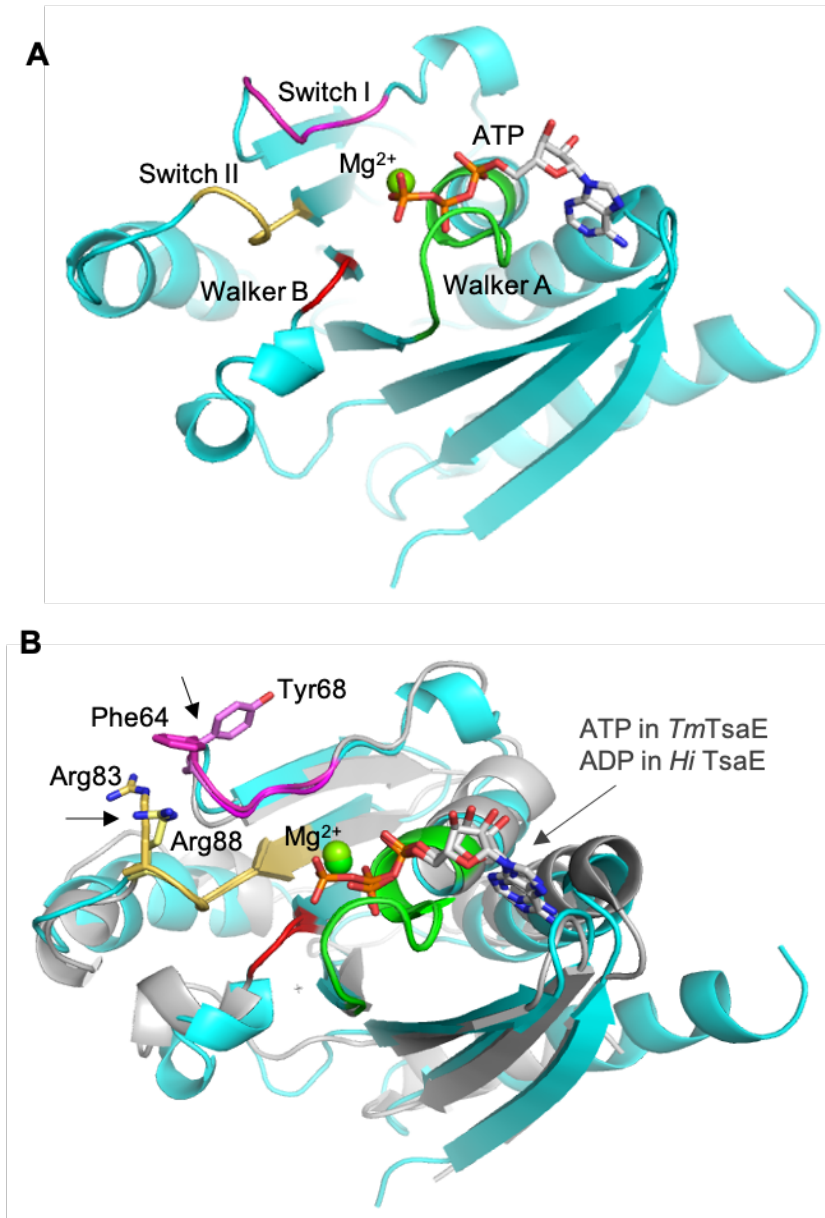


Figure 2-4: TsaE active site and ATP hydrolysis-induced conformational changes.
A: The active site of *TmTsaE* from the *TmTsaB₂D₂E₂* structure. Walker A (P-loop), Walker B, Switch I and Switch II loops are shown in green, red, magenta and yellow, respectively.
B: Superposition of the crystal structures of *TmTsaE* (colored) and *HiTsaE* (grey) showing pre and post ATP hydrolysis conformations, respectively. The black arrows point to the conformational changes by Switch I residue Phe64 (in *TmTsaE*) or Tyr68 (in *HiTsaE*) and Switch II residue Arg83 (in *TmTsaE*) or Arg88 (in *HiTsaE*).

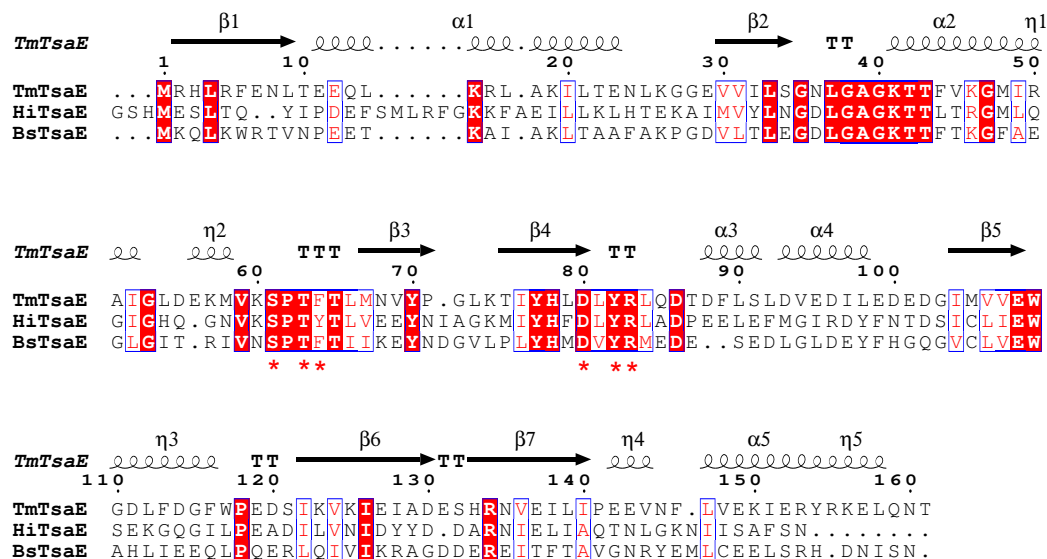


Figure 2-5: Representative structure-based multisequence alignment of TsaE subunits from all known crystal structures of Tsa protein complexes.
Tm: *Thermotoga maritima* (this study), Hi: *Haemophilus influenza* (PDB ID 1FL9), Bs: *Bacillus subtilis* (PDB ID 5NP9). Residues mutated to test the main hypothesis in this chapter are indicated with red asterisks.

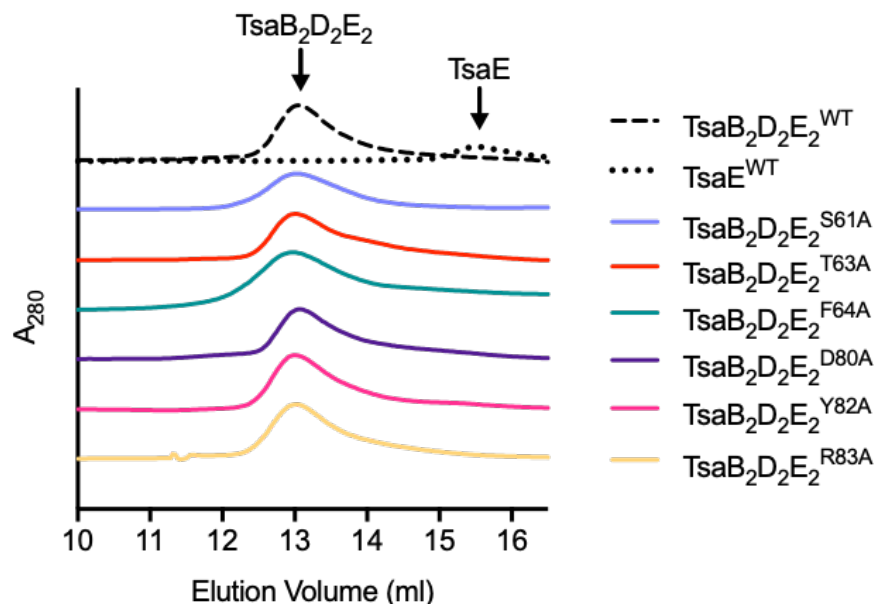


Figure 2-6: Assessment of complex formation by *TmTsaE* mutants by analytical size exclusion chromatography (SEC).
SEC profiles of *TmTsaB₂D₂E₂* formed by mixing 10 μ M each wild-type *TmTsaB*, *TmTsaD* and mutant *TmTsaE* in the presence of 1mM ATP and 1 mM $MgCl_2$.

Interactions and mutagenesis of the ATPase site

The crystal structure reveals a Mg^{2+} ion in the ATPase site that is coordinated in an octahedral geometry in which the equatorial ligands are the TsaE Walker B motif residues Thr42 and Glu108, the ATP γ -phosphate O3 atom, and a water molecule, while the axial ligands are the ATP β -phosphate O2 atom and a second water molecule that in turn is coordinated by Ser61 and Asp80 from the Switch I and Switch II motifs, respectively (Figure 2-5 and 2-7). Mutating any of these invariant side chains (Thr42 and Glu108 in previously published work by Swairjo Lab (40), and Ser61 and Asp80 in this study) to alanine results in mutants that support only a single turnover of the t^6A cycle (Figure 2-8C) with initial velocities similar to that of the wild-type *TmTsaB₂D₂* (Table 2-2). The loss of multiple-turnover t^6A synthesis activity appears to be due to loss of ATP hydrolysis as reflected by their extremely low ATPase activities (even lower than that of wild-type *TmTsaE* alone, Figure 2-8A and B), even though the mutant proteins still make stable complexes with *TmTsaB₂D₂* (Figure 2-6).

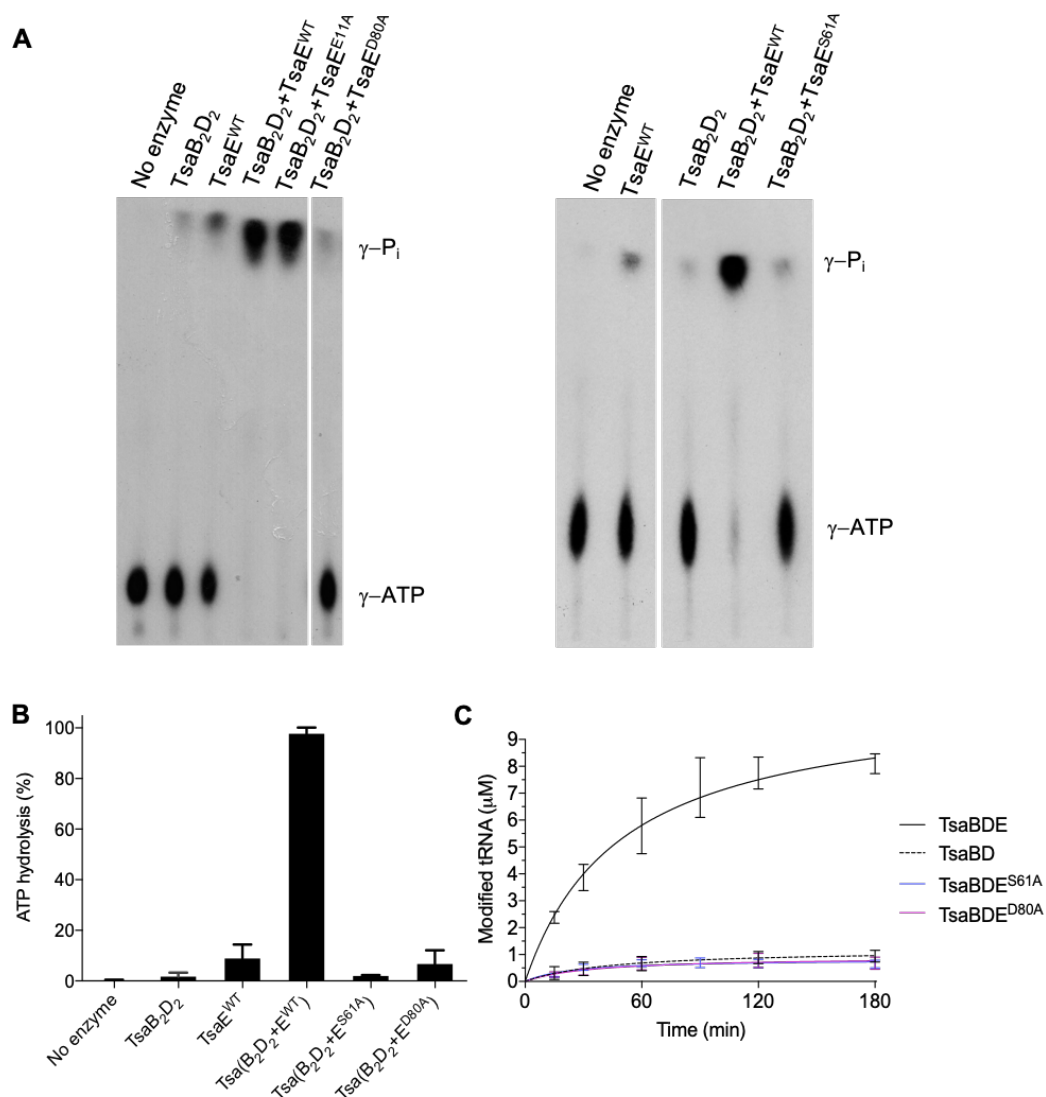


Figure 2-8: Mutagenesis of the ATPase site of *TmTsaB₂D₂E₂*.

A: TLC radiograms of ATP hydrolysis reactions by ATP binding *TmTsaE* mutants catalyzed by equimolar mixtures of pre-isolated wild-type *TmTsaB₂D₂* and *TmTsaE^{wild-type/mutant}*. Reactions conducted with 5.0 μ M enzyme for 60 minutes. B: Effect of mutations in the ATPase site on the ATP hydrolysis activity of the TC-transfer complex. Shown are the % hydrolysis of ATP by equimolar mixtures of pre-isolated *TmTsaB₂D₂* and *TmTsaE^{wild-type/mutant}*. Data represent averages of duplicate measurements from three independent reactions. C: Effect of mutations in the ATPase site on the turnover of the t⁶A biosynthesis reaction, assessed using the time-course radiochemical t⁶A assay measuring the incorporation of [¹⁴C]-L-threonine into tRNA. Assays contained mutant *TmTsaE* harboring mutations in the ATPase site, and the other three wild-type Tsa proteins. Multiple-turnover activity in the presence of all wild-type proteins (solid black line) and single-turnover activity in the absence of *TmTsaE* (dashed black line) are also shown. Data represent duplicate measurements from at least three independent reactions.

Residues in the conserved Switch I and Switch II motifs of TsaE mediate communication between the ATPase site and the TC-transfer site

To address the potential role of Switch I and Switch II loops in conformational changes associated with the t^6A catalytic cycle, we targeted Thr63 and Phe64 in Switch I, and Tyr82 and Arg83 in Switch II with mutagenesis. These residues lie outside the ATP binding pocket, i.e. they do not interact directly or via water with the metal or nucleotide in the ATPase site, and two of the residues (Phe64 and Arg83) were observed to undergo conformational changes upon ATP hydrolysis according to the analysis carried out on the TsaE crystal structures (Figure 2-4).

Mutating Thr63 or Tyr82 of *TmTsaE* to alanine, results in mutants that can bind to *TmTsaB₂D₂* and form a stable TC-transfer complex (Figure 2-6) but complete loss of ATP hydrolysis activity (Figure 2-9A, 2-9B and 2-10B). As expected, the loss of ATPase activity is accompanied by the loss of multi-turnover t^6A synthesis (Figure 2-10C and Table 2-2) by these mutants. Significantly, mutating Phe64 or Arg83 to alanine maintains formation of a stable TC-transfer complex (Figure 2-6) with a robust ATPase activity (Figure 2-9A, 2-9B and 2-10B), but compromises t^6A synthesis, with the $TsaE^{Phe64Ala}$ mutant exhibiting only single-turnover activity, and $TsaE^{Arg83Ala}$ exhibiting multiple turnovers but at a very slow rate (Figure 2-10C and Table 2-2). These results are consistent with a role for Phe64 and Arg83 in post ATP-hydrolysis signal transduction that leads to ‘resetting’ the *TsaB₂D₂* platform for the next catalytic cycle.

In the structure, the phenyl ring of Phe64 stacks against (within 4 Å) and stabilizes a rigid conformation of the glycine-rich β -turn $S_{134}GGH_{137}$ in TsaD (Figure 2-10A), which appears to stabilize the binding pocket for the carbamoyl moiety of TC-AMP. Given that TsaE is not bound during TC-transfer, this interaction may recapitulate an interaction with

a tRNA base during the reaction. In contrast, Arg83 of TsaE makes a salt bridge with Asp11 of TsaD at the N-terminal hinge of the lever arm, thus stabilizing it in the up position (Figure 2-10A). The observation that Arg83 is required for efficient multi-turnover t⁶A synthesis is consistent with movement of the lever arm of TsaD comprising at least part of the reset mechanism.

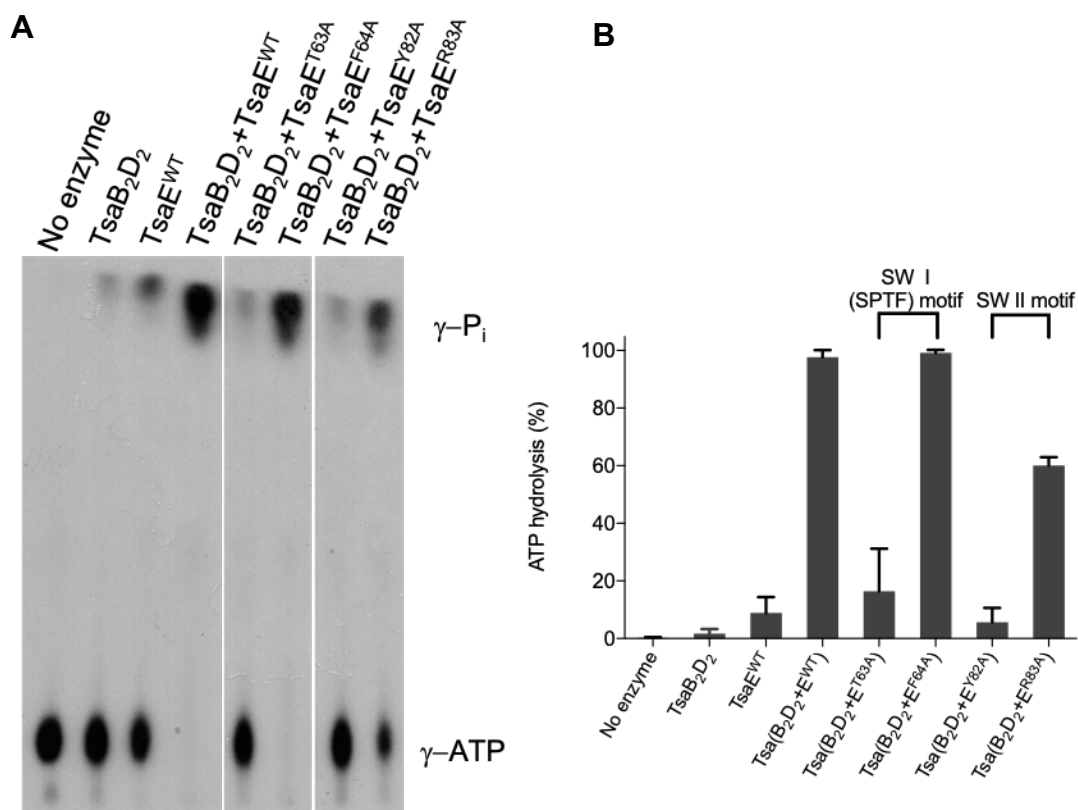


Figure 2-9: ATP hydrolysis reactions by Switch I and Switch II *TmTsaE* mutants.

A: TLC radiograms of ATP hydrolysis catalyzed by wild-type or mutant *TmTsaE* in the presence of pre-isolated wild-type *TmTsaB₂D₂*. Reactions conducted with 5.0 μ M enzyme for 60 minutes. B: Percent ATP hydrolysis as quantified by densitometry to calculate % ATP hydrolysis.

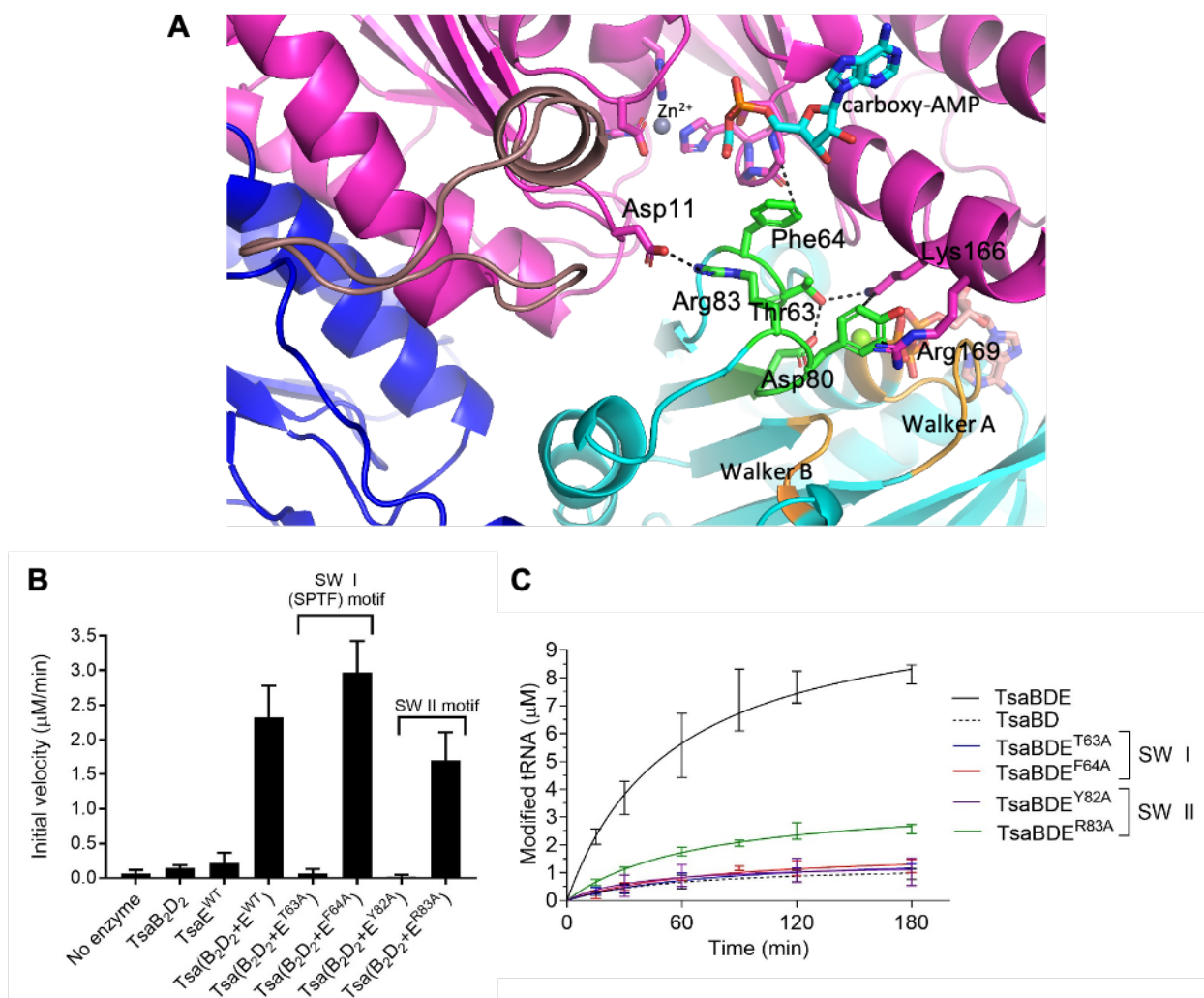


Figure 2-10: Role of Switch I and Switch II residues of *TmTsaE* in signal transduction between the ATPase site and TC-transfer site.

A: Interactions of Thr63 and Phe64 in Switch I loop, and Tyr82 and Arg83 in Switch II loop of TsaE and their locations relative to the bound nucleotides in the ATPase and TC-transfer sites. B: Effect of mutations in Switch I and Switch II motifs on the ATPase activity of the TC-transfer complex. Shown are initial velocities of ATP hydrolysis reactions catalyzed by an equimolar mixture of pre-isolated wild-type *TmTsaB₂D₂* and mutant *TmTsaE* proteins harboring mutations in Switch I or Switch II. Data represent averages of duplicate measurements from three independent reactions. C: Effect of mutations in Switch I and Switch II motifs on the turnover of the t⁶A biosynthesis reaction, assessed using the time-course radiochemical t⁶A assay measuring the incorporation of [¹⁴C]-threonine into tRNA^{Thr}_{CGU}. Assays contained mutant *TmTsaE* and the other three wild-type Tsa proteins (colored lines). Multiple-turnover activity catalyzed by a mixture of all wild-type proteins (solid black line), and single-turnover activity catalyzed in the absence of *TmTsaE* (dashed black line) are shown. Data represent duplicate measurements from three independent reactions.

Table 2-2: Initial velocities of t⁶A synthesis reactions.

The reactions contained *Tm*TsaE carrying mutations in the ATPase site, Switch I, or Switch II; and the other two wild-type Tsa proteins. *: standard error of the mean.

Functional site of mutation	Enzyme	Initial velocity of t ⁶ A synthesis (μM/min)
	Wild-type TsaBDE	0.15 ± 0.013*
	Wild-type TsaBD	0.013 ± 0.0025
ATP binding site	TsaBDE ^{S61A}	0.016 ± 0.002
	TsaBDE ^{D80A}	0.012 ± 0.0025
SW I	TsaBDE ^{T63A}	0.018 ± 0.0063
	TsaBDE ^{F64A}	0.016 ± 0.0020
SW II	TsaBDE ^{Y82A}	0.017 ± 0.007
	TsaBDE ^{R83A}	0.039 ± 0.0020

2.4 Discussion

We had proposed that the full catalytic cycle involves an ATP-dependent structural change in the *TmTsaB₂D₂* complex during the ‘reset’ step that occurs after the release of product tRNA (40). The results in this chapter are consistent with this proposal (Figure 1-3) and provide additional insight into the nature and timing of these changes.

Mutagenesis of the ATP binding site of TsaE, and demonstration that loss of ATPase activity is always accompanied by a loss of multi-turnover t⁶A activity, further establishes the coupling between ATPase activity and multi-turnover synthesis of t⁶A-modified tRNA. The data from mutagenesis of the Switch I/Switch II residues Phe64 and Arg83 demonstrate uncoupling of the ATPase activity from the t⁶A activity when the structural changes generated by ATP hydrolysis cannot be communicated throughout the complex. Given the large conformational changes associated with the movement of the lever arm, it is not unreasonable to propose that ATP hydrolysis might power that structural change, although these data do not directly address that question.

2.5 Future Directions

The next step of this study is to investigate the structural characteristics of the inactivated TsaB₂D₂ complex. This is necessary in order to understand how ATP hydrolysis-mediated reset is brought about. One can prepare the inactivated TsaB₂D₂ complex by performing the t⁶A synthesis reaction to completion in the presence of all reaction components except for TsaE. The ‘inactivated’ TsaB₂D₂ complex can then be purified and subjected to structural studies using either x-ray crystallography or cryo-EM. While the crystal structure by Luthra *et al.* represents the pre-reset conformation in the presence of TsaE, a structure of the inactivated TsaB₂D₂ complex in the absence of TsaE

will be necessary to properly delineate the structural features that are specifically associated with inactivation and not with TsaE binding.

2.6 Acknowledgment

Chapter 2, in part, is a reprint of the material as it appears in Luthra, A., Paranagama, N., Swinehart, W., Bayooz, S., Phan, P., Quach, V., Schiffer, J. M., Stec, B., Iwata-Reuyl, D., & Swairjo, M. A. (2019). Conformational communication mediates the reset step in t6A biosynthesis. *Nucleic acids research*, 47(12), 6551–6567. The dissertation author was the second author of this paper.

Chapter 3 : Quantitative assessment of the kinase activity of the threonylcarbamoyl adenosine biosynthesis protein TsaE

Abstract

Recently, TsaE proteins from various bacterial sources have been reported to possess S/T/Y kinase activities *in vitro*. In this chapter, I describe *in vitro* characterization of the kinase activity of *T. maritima* TsaE using a combination of quantitative kinase assays, phosphate release assays, and site directed mutagenesis. The results show that a stable trimeric form of *Tm*TsaE possesses a kinase activity *in vitro*, autophosphorylating at the conserved residues Thr65 and Tyr82, located in the Switch I and II loops that are responsible for communicating the ATP hydrolysis signal to the TC-transfer site in the course of the t⁶A cycle, suggesting that phosphorylation of these sites may play a role in the communication. Site-directed mutagenesis of the ATP binding site and the Switch I and II loops revealed residues that are required for either the ATPase activity or the kinase activity but not both. These results suggest that the kinase function of TsaE requires different conformations of its Switch I and II loops than those seen in the TCT complex or in the free form, and support the notion that TsaE proteins carry kinase activities that may be integral to their function in t⁶A biosynthesis and/or regulation. Further, monomeric TsaE is heterologously phosphorylated in *E. coli* and this observed phosphorylation is independent of the autokinase activity, suggesting that it is a substrate of a yet unidentified bacterial kinase.

3.1 Introduction

Aside from the ATPase function in t⁶A biosynthesis, TsaE from *B. subtilis* and *E. coli* were recently reported to autophosphorylate at Ser/Thr residues in the Switch I loop *in vitro* (21). In the *B. subtilis* system, TsaE was also reported to transphosphorylate TsaD at unidentified residues (21). Similarly, a recent report showed that *S. pneumoniae* TsaE autophosphorylates at a single Tyr residue of Switch I loop *in vivo* (87). TsaE contains another conserved motif HxD, just upstream of Switch II loop. The same motif is conserved in some eukaryotic protein kinases (ePKs). When crystal structures of TsaE were compared with ERK2 (an ePK), the HxD motifs were observed at similar spatial positions with respect to the nucleotide (21). Mutation of the HxD motif abolished *B. subtilis* TsaE autophosphorylation activity. Depletion of TsaE in *E. coli*, *B. subtilis* and *S. pneumoniae* cells under aerobic conditions resulted in abnormal cell morphologies, with peeling of cell walls and mispositioning of septa in *S. pneumoniae* cells (20, 21, 87). However, TsaE is not essential for cell growth under anaerobic conditions and the expression of TsaE is upregulated in the presence of oxidative stressors (94). Treatment of *B. subtilis* wild-type and TsaE-depleted cells with an oxidative compound showed that the abnormal cell morphologies were even greater in the TsaE-depleted cells (21). Taken together, these observations led to the proposal that TsaE is a novel family of Hanks-type S/T/Y bacterial kinases involved in stress response (21).

The functional ortholog of TsaE in archaeal and eukaryotic t⁶A biosynthetic systems is Bud32, a member of the eukaryotic Ser/Thr Rio kinase family that is closely related to the atypical Rio kinases. Like TsaE, Bud32 also displays kinase and ATPase activities. Bud32 kinase activity is stimulated in the presence of Cgi121 and inhibited in the presence of the Kae1-Pcc1 complex. It was proposed that Kae1 induces Bud32 to

“switch” from a kinase to an ATPase (34, 95). Bud32 was also reported to transphosphorylate Kae1 (95). Bud32 is involved in the PI3K-Akt/TOR signaling pathway in yeast, drosophila and human (96–98).

In this chapter, I investigate the kinase activity of *TmTsaE*, a Tsa family member for which the ATPase activity and role in the t⁶A cycle are well characterized. I demonstrate that a trimeric form of *TmTsaE* autophosphorylates weakly *in vitro*. In addition, heterologously expressed *TmTsaE* is substantially phosphorylated *in vivo* and this phosphorylation is specific to the monomeric form. Using mutagenesis experiments I show that different active site residues are required for the *in vitro* autokinase activity versus the *in vivo* phosphorylation. Likewise, some residues are required for the ATPase or the autokinase activity but not both. I also show that *TmTsaE* rather behaves as an ATPase than an autokinase under the *in vitro* conditions tested in this study.

3.2 Materials and Methods

Purification of recombinant wild-type and mutant *TmTsaE* proteins

Some single site mutants of *TmTsaE* were prepared as described in Chapter 2 while the other mutants of *TmTsaE* were provided by Dr. Amit Luthra from Prof. Swairjo's Lab. His₆-tagged *TmTsaE* wild-type and mutant proteins were overexpressed in *E. coli*, purified by Ni-NTA affinity chromatography, their His₆-tags removed by thrombin digestion followed by gel filtration as previously described in Chapter 2 (33). For all proteins, purity of >95% was verified by SDS-PAGE.

Separation of trimeric and monomeric forms of *TmTsaE*

500 μ L of *TmTsaE* at 400-430 μ M were injected into an ENRICH SEC70 column (Biorad), pre-equilibrated in buffer containing 50 mM Tris pH 7.5, 100 mM KCl and 1 mM DTT, operated on a NGC liquid chromatography system (Biorad). Trimeric and monomeric *TmTsaE* eluted at ~9.8 mL and ~11.8 mL, respectively, using the same buffer. The fractions were collected and freshly used in further steps.

Assessment of *TmTsaE* trimer stability using SEC

Following initial separation of monomeric and trimeric *TmTsaE* by gel filtration chromatography, the trimeric fraction was diluted to 2.5 μ M (8-fold dilution) and 5.0 μ M (4-fold dilution) *TmTsaE* using the same running buffer. These dilutions were then incubated at room temperature or 60 °C for 11 minutes to 2 hours, before re-injecting into ENRICH SEC70 column (Biorad). The monomeric fraction was also diluted to 5.0 μ M (4-fold dilution) *TmTsaE* and incubated at room temperature or 60 C for 2 to 7 hours before re-injection. The areas under the peaks in the chromatograms were calculated using ChromeLab software (Biorad).

SDS-PAGE analysis of *TmTsaE* trimer under non-reducing conditions

Isolated *TmTsaE* trimer (40 μ L of 4 μ M) was either treated with 360 mM β -betamercaptoethanol (BME) or not before subjecting it to SDS-PAGE analysis using a 12% polyacrylamide gel and a running buffer composed of 25 mM Tris pH 8.3, 192 mM glycine and 0.1% SDS. The gel was run at 200 V for 30 minutes at room temperature before staining with Coomassie Blue.

³²P radiolabel assay for measurement of *in vitro* *TmTsaE* autokinase activity

End-point experiments. *TmTsaE* trimer and monomer were isolated as mentioned above. Final reactions contained 10 μ M wild-type *TmTsaE* trimer or monomer, 50 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT, 2 mM MgCl₂ buffer, 100 μ M ATP (spiked with 0.016 μ M ³²P- γ -ATP, Perkin Elmer) in a total volume of 40 μ L at 60 °C for 30 minutes. Each reaction was split into two, one half was treated with 5 μ L of 1% SDS and 5 μ L of 10 mM EDTA solution before boiling and subjecting to SDS PAGE (200 V for 30 minutes at room temperature with a running buffer composed of 25 mM Tris pH 8.3, 192 mM glycine and 0.1% SDS), and the other half was treated with 5 μ L of 10 mM EDTA solution and 5 μ L of 50% glycerol before subjecting to native PAGE analysis (100 V for 2.5 hours at room temperature with a running buffer composed of 25 mM Tris pH 8.3 and 192 mM glycine). The same procedure was followed to probe the effect of single-site mutations on *TmTsaE* autokinase activity using unfractionated wild-type and mutant *TmTsaE* proteins and analyzed by SDS-PAGE. The resulting gels were stained using Coomassie Blue to visualize the protein followed by autoradiography to visualize phosphorylation and subjected to densitometry analysis using Image Studio Lite software. Radiogram intensities were divided by the Coomassie intensities and the resulting values were then

divided by that of the wild-type protein to relative *in vitro* kinase activities. The same procedure was followed to probe the effect of substrate concentration on *TmTsaE* autokinase activity, using 5 μM wild-type *TmTsaE* trimer or monomer and 0 – 1000 μM ATP (spiked with 0.016 μM ^{32}P - γ -ATP, Perkin Elmer) in a total volume of 20 μL at 60 $^{\circ}\text{C}$ for 2 hours. The amount of *TmTsaE* trimer used for the calculation of total phosphate incorporation per *TmTsaE* were accounted for by the real amount of trimer as evident in the SEC profile under that condition (Figure 3-3B, Trimer 2 hours at 60 $^{\circ}\text{C}$).

Time-course experiments. *TmTsaE* trimer and monomer was subjected to autokinase activity assay as mentioned above (100 mM KCl and 60 $^{\circ}\text{C}$), except that parallel reactions were set up containing 5 μM enzyme and 50 μM ATP (spiked with 0.008 μM ^{32}P - γ -ATP, Perkin Elmer) in a total volume of 20 μL . All the reactions were started by adding ATP and stopped at 1, 6, 11, 16, 21, 26, 41 and 56 minutes, by adding 5 μL of 0.1% SDS and 1 mM EDTA solution, boiled and subjected to SDS PAGE analysis as described above. For ^{32}P standards, the same ^{32}P - γ -ATP spiked ATP stock was serially diluted to final concentrations of 10, 5, 2.5, 1.25, 0.625, 0.3125 μM , and 1 μL of was spotted on a PEI-cellulose thin layer chromatography plate (TLC, Sigma-Aldrich) and air dried at room temperature. The TLC plate and SDS-PAGE gel were subjected to autoradiography simultaneously, followed by densitometry analysis using Image Studio Lite software. For the ^{32}P standards, the slope of the linear plot of intensity versus total ATP (unlabeled + radiolabeled) was calculated and the measured intensities from the reaction samples were converted to covalent phosphates in nanomoles using equation 1.

$$\text{Covalent phosphates (nmol)} = \frac{\text{Intensity of sample radiogram (a.u.)}}{\text{Slope of linear plot of } ^{32}\text{P} \text{ standards (a.u. nM}^{-1}\text{)}} \quad (1)$$

Nanomoles were converted to molarity using equation 2.

$$\text{Covalent phosphates } (\mu\text{M}) = \frac{\text{Covalent phosphates (nmol)}}{\text{Reaction volume (nL)}} \times 10^6 \quad (2)$$

Molarity of covalent phosphates versus time were plotted using non-linear least squares method. All calculations and graphing were done using GraphPad Prism version 8. The amount of *TmTsaE* trimer used for the calculation of initial rates were accounted for by the real amount of trimer as evident in the SEC profile under that condition (Figure 3-3B, Trimer 11 mins at 60 °C).

Effect of temperature on TmTsaE autokinase activity. *TmTsaE* was subjected to autokinase activity as mentioned above, except that parallel reactions were set up containing 2.5 μM unfractionated *TmTsaE* and 50 μM ATP (spiked with 0.008 μM ^{32}P - γ -ATP, Perkin Elmer) in a total volume of 40 μL . The reactions were started by adding ATP and incubated at 25 °C, 37 °C or 60 °C for 30, 60 or 120 minutes before analyzing by SDS-PAGE and autoradiography.

Verification of E.coli alkaline phosphatase activity. *TmTsaE* was subjected to autokinase activity as mentioned above, except the reaction conditions contained 500 μM unfractionated *TmTsaE* and 1 mM ATP (spiked 0.008 μM ^{32}P - γ -ATP, Perkin Elmer) in a total volume of 100 μL and an incubation period of 60 minutes. Excess ATPs were removed from the sample using a G-50 Sephadex spin column (Roche) pre-equilibrated in buffer containing 50 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT and 2 mM MgCl_2 . The resulting ^{32}P - γ -phosphate incorporated *TmTsaE* eluant was either treated with 2 Units (20

μL of 0.1 U/μL) of *E. coli* alkaline phosphatase (*EcAP*, Sigma-Aldrich) or 20 μL of water at 37 °C for 60 minutes. The samples were then subjected to SDS-PAGE and visualized via Coomassie blue staining and autoradiography.

Malachite green phosphate assay for measurement of *in vivo* phosphates on purified *TmTsaE*

Final reactions contained 6.25 μM wild-type or mutant *TmTsaE* (fractionated or unfractionated) and 0.1 units of *EcAP* in 50 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT, 2 mM MgCl₂ buffer. Control reactions consisting of buffer, buffer and *EcAP* or buffer and the respective protein (*TmTsaE* wild-type or mutant) were also set up. Phosphate standards were prepared by serial dilution using the manufacturer provided stock of 1 mM phosphate (Malachite green phosphate assay kit, Sigma-Aldrich MAK307-1KT). Experimental, control and phosphate-standard reactions were made to a total volume of 240 μL and incubated at 37 °C for 60 minutes before stopping the reaction by adding 60 μL of malachite green dye (Malachite green phosphate assay kit, Sigma-Aldrich). All samples were incubated at room temperature for 30 minutes before reading the absorbance at 620 nm in arbitrary units (a. u.) using a spectrophotometer (Tecan infinite M200). For each experimental sample, the absorbance due to released phosphates was calculated using equation 4, where Abs_{Exp} is sample absorbance, Abs_{Protein} is protein control absorbance, Abs_{EcAP} is *EcAP* control absorbance and Abs_{Buffer} is buffer control absorbance.

$$\text{Abs}_{\text{released phosphates}} = \text{Abs}_{\text{Exp}} - [\text{Abs}_{\text{Protein}} + (\text{Abs}_{\text{EcAP}} - (\text{Abs}_{\text{Buffer}}))] \quad (4)$$

The slope of the linear plot of absorbance at 620 nm in arbitrary units versus phosphates in picomoles was calculated and the $Abs_{\text{released phosphates}}$ were converted to released phosphates in picomoles using equation 5.

$$\text{Released phosphates (pmol)} = \frac{Abs_{\text{released phosphates (a.u.)}}}{\text{Slope of linear plot of phosphate standards (a.u. pmol}^{-1})} \quad (5)$$

Picomoles were converted to molarity using equation 6.

$$\text{Released phosphates (}\mu\text{M)} = \frac{\text{Released phosphates (pmol)}}{\text{Reaction volume (pL)}} \times 10^6 \quad (6)$$

Where relevant, relative activities were calculated by dividing released phosphates in molarity, by that of wild-type *TmTsaE*. All calculations and graphing were done using GraphPad Prism version 8.

Denaturation of *TmTsaE* to remove any bound ATPs

A sample containing 10 μM unfractionated *TmTsaE*, 50 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT and 0.1% SDS in a total volume of 4 mL, was incubated at 60 °C for 60 minutes. The resulting denatured protein sample was applied to a protein concentrator (PES 5 kDa, Sartorius) and washed three times using the same buffer, before exchanging the buffer to 50 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT. Control samples containing either water in place of *TmTsaE* or *TmTsaE* in the absence of 0.1% SDS was subjected to the same procedure as mentioned above.

Dephosphorylation of *TmTsaE*

TmTsaE monomeric fraction was dephosphorylated by treatment with *EcAP* following the manufacturer's instructions. Briefly, 500 μ L reactions containing 80 μ M *TmTsaE* monomer and 2 Units of *EcAP* in 50 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT buffer was incubated at 37 °C for 60 minutes. The dephosphorylation was verified by subjecting the same reaction to the malachite green phosphate assay as described before.

In vitro ATPase activity

TmTsaE trimer and monomer were isolated as mentioned above. Final reaction contained 2.5 μ M wild-type *TmTsaE* trimer or monomer, 50 mM Tris pH 7.5, 100 mM KCl, 1 mM DTT, 2 mM $MgCl_2$ buffer, 50 μ M ATP (spiked with 0.008 μ M ^{32}P - γ -ATP, Perkin Elmer) in a total volume of 30 μ L at 60 °C. The reaction was started by adding ATP. At various time points (2.5 - 300 minutes), 2 μ L of the reaction mix was taken out and mixed with 1 μ L of 30 mM EDTA solution before spotting a total of 2.5 μ L on a PEI-cellulose thin layer chromatography (TLC) plate. $[\gamma\text{-}^{32}P]\text{ATP}$ and the product $[\gamma\text{-}^{32}P]P_i$ were separated on the plate using 0.5 M KH_2PO_4 pH 3.5 as solvent. A reaction mixture lacking enzyme was monitored as a control. The TLC was visualized by autoradiography and quantified by densitometry using Image Studio Lite software. The percentage of ATP hydrolyzed was calculated using the equation 1 of page 21.

3.3 Results

***TmTsaE* exists in monomeric and oligomeric forms *in vivo*.**

It was known from previous *TmTsaE* protein preps, that *TmTsaE* exists in an equilibrium between oligomers and monomers. Therefore, the oligomerizations at different salt concentrations were characterized using analytical ultracentrifugation (AUC) in collaboration with Dr. Andrey Bobkov at Sanford Burnham Institute (Figure 3-1). At low salt concentrations of 50 mM KCl, majority of oligomeric species were calculated to be composed of trimers and dimers, while a minority of the oligomeric species were composed of higher order species (6X, 14X and 20X monomer). Upon increasing salt concentrations to physiological levels of 100 mM and all the way to 400 mM KCl, the higher order species completely disappear indicating that these higher order species are non-specific. Even at 400 mM KCl, the trimeric-dimeric species were maintained albeit to a lesser extent indicating a remarkably stable oligomer. Here onwards, the trimer-dimeric species will be referred to as oligomer. A general trend of increasing monomeric species and decreasing oligomeric species were observed with increasing salt concentrations (Figure 3-2A). The salt dependency of *TmTsaE* oligomerization is consistent with that previously reported for *Bacillus subtilis* TsaE (*BsTsaE*) (43). The stability of the *TmTsaE* oligomer was assessed by isolating *TmTsaE* oligomeric (primarily trimeric) fraction using size exclusion chromatography (SEC) and subsequently injecting dilutions of that fraction to the same column following increasing incubation time at room temperature or 60 °C. At room temperature, both the oligomeric and monomeric species of *TmTsaE* were observed to be remarkably stable for several hours. At 60 °C, the trimer dissociated to about ~35% trimer, ~25% dimer and ~39% monomer in 30 mins and maintained that

population of species for 2 hours. On the other hand, the monomer did not considerably re-equilibrate to a mixture of trimer, dimer and monomer even at 60 °C (Figure 3-3). This indicated a very slow equilibrium between monomer and oligomer of *TmTsaE* at room temperature while a reasonably faster equilibrium was observed at 60 °C, consistent with thermophilic proteins. A fast equilibrium was observed with *BsTsaE* (43). SDS-PAGE was used to probe if the high stability displayed by the *TmTsaE* trimer was due to disulfide linkages, however no disulfide linkages were observed after treatment with reducing agent and analysis by SDS PAGE (Figure 3-2B).

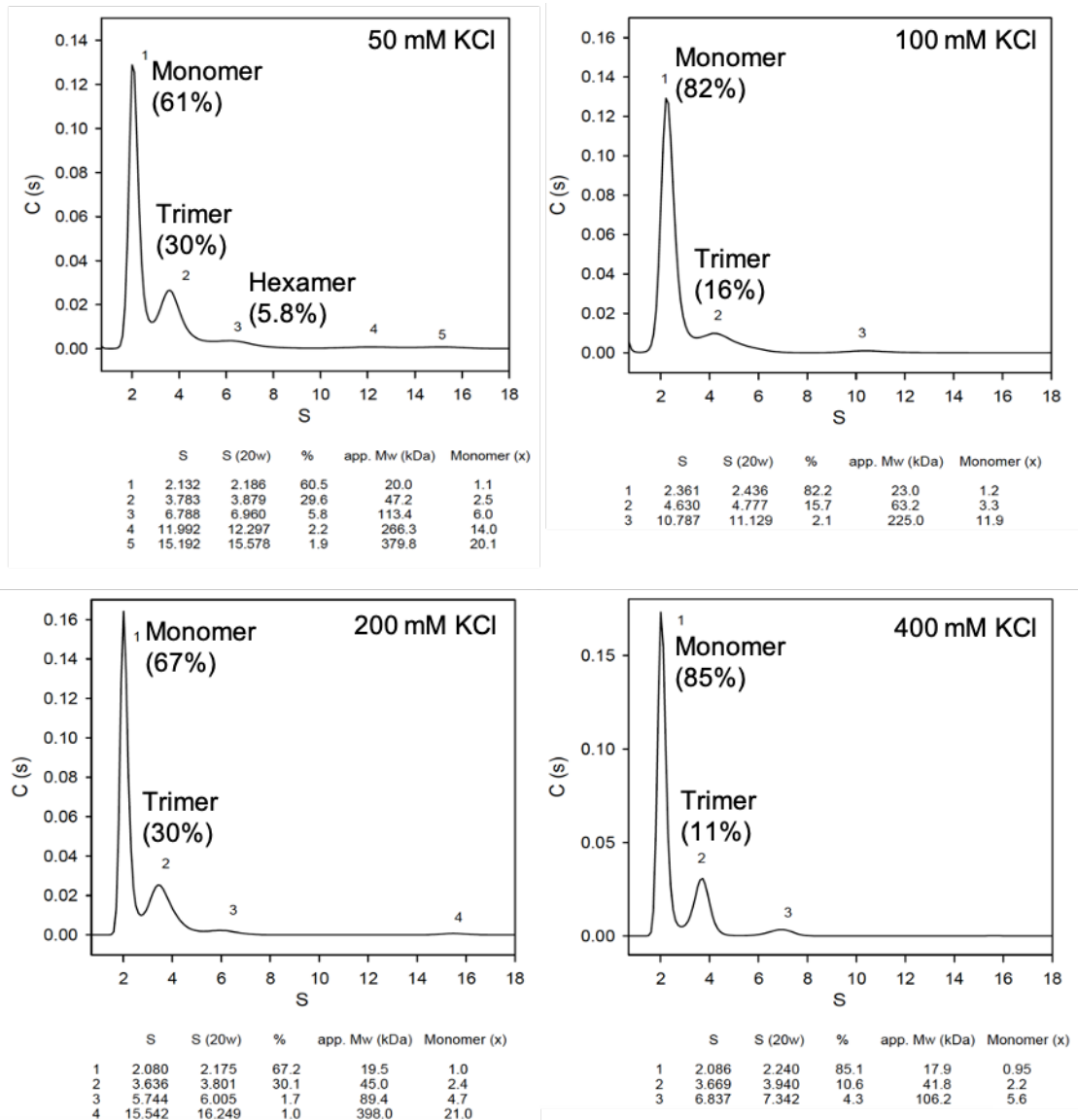


Figure 3-1: *TmTsaE* exists in monomeric and oligomeric forms *in vivo*.

AUC analysis of *TmTsaE* at low, physiological and high salt concentrations reporting the sedimentation coefficients (S), sedimentation coefficients normalized to water at 20 °C (S 20w), population of each species as a percentage (%), apparent molecular weights (app. MW) and monomer multiples (monomer X).

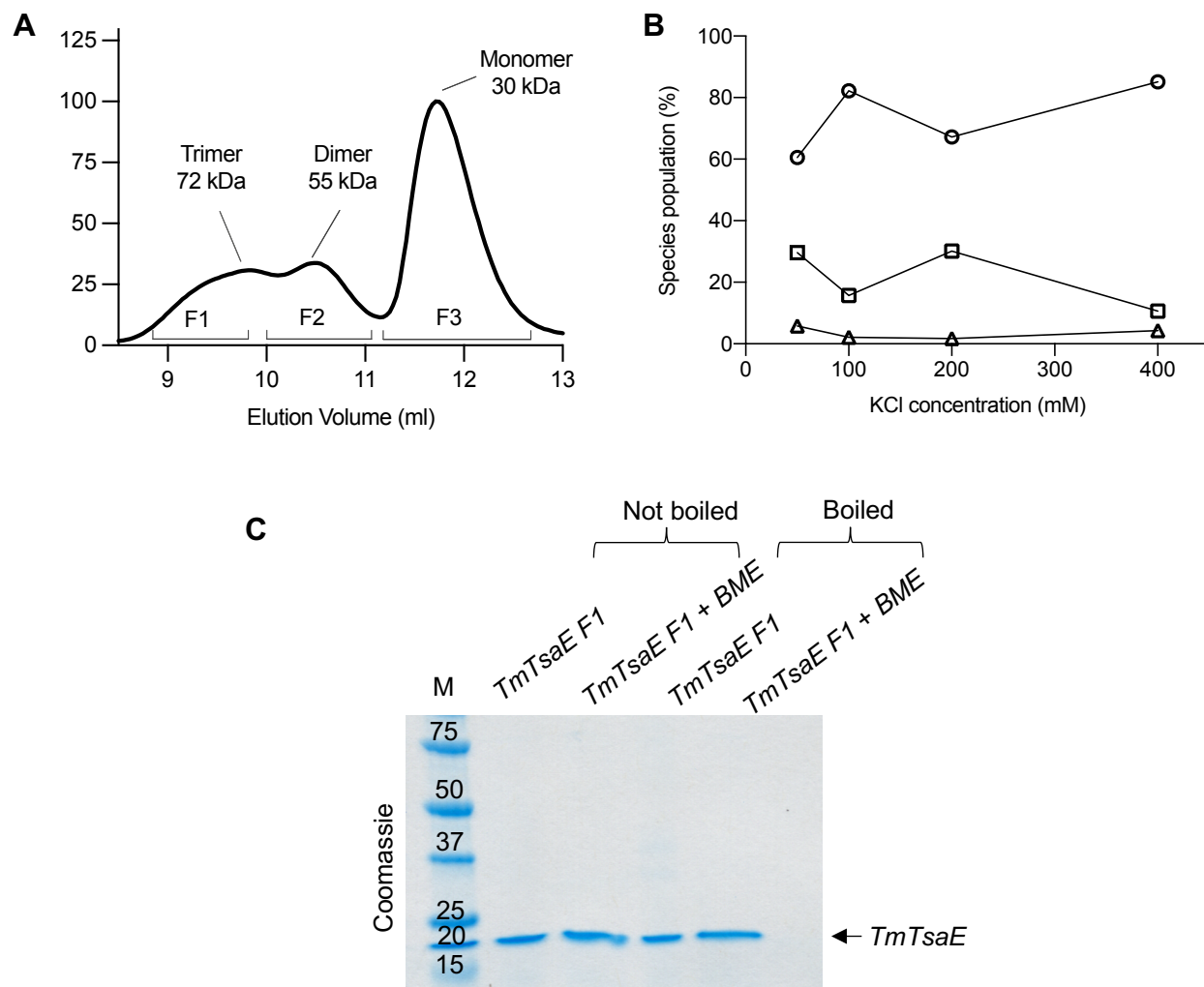


Figure 3-2: *TmTsaE* oligomerization decreases with increasing salt concentration and the oligomer is non-covalent.

A: SEC chromatogram of *TmTsaE*. B: AUC analysis of *TmTsaE* oligomerization with increasing salt concentration. Monomer shown in circles, dimer/trimer shown in squares and pentamer/hexamer shown in triangles. C: SDS-PAGE analysis of SEC purified *TmTsaE* Trimer (fraction F1) in non-reducing and reducing conditions. M: molecular weight marker.

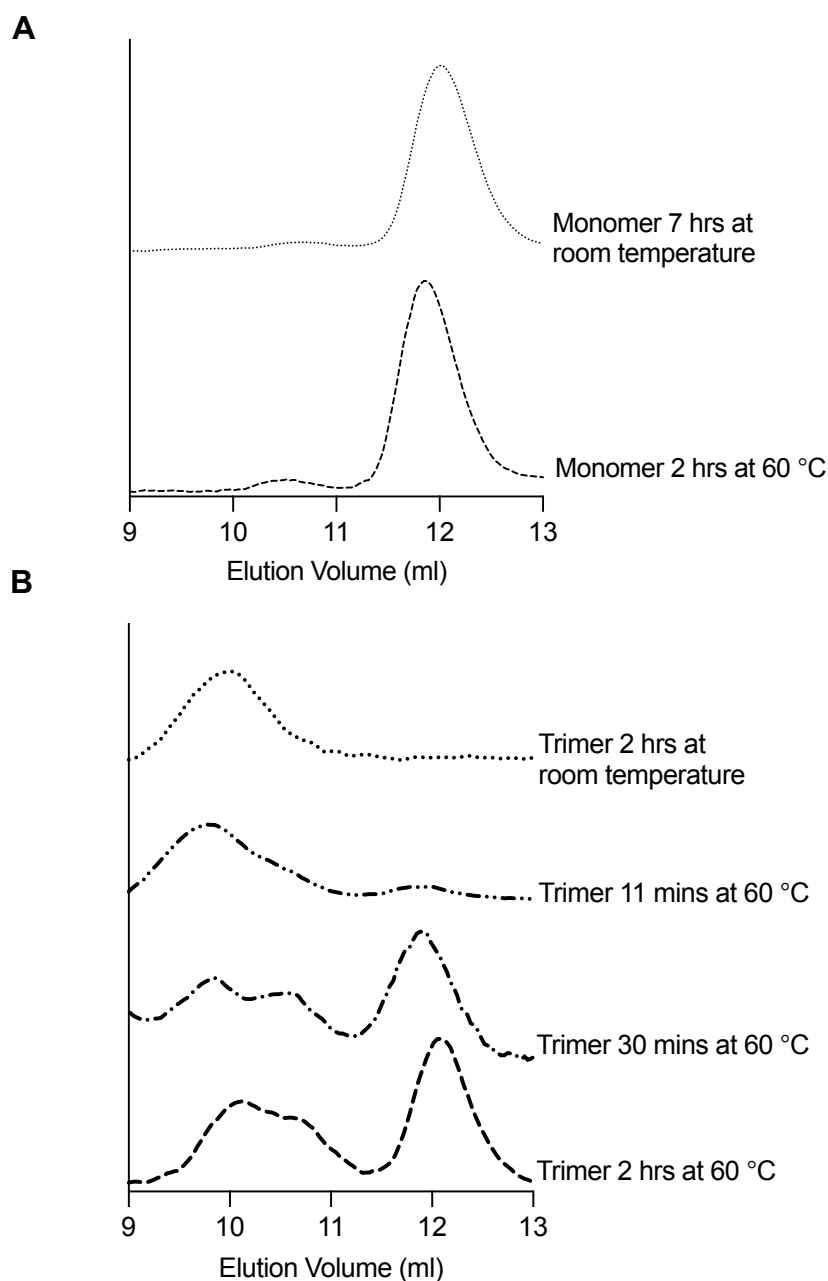


Figure 3-3: Effect of dilution on *TmTsaE* oligomer-monomer equilibrium assessed using SEC.

A: SEC chromatograms of monomeric *TmTsaE*, prepared by diluting a previously isolated *TmTsaE* monomer, after incubation for several hours at room temperature or at 60 °C. B: SEC chromatograms of trimeric *TmTsaE*, prepared by diluting a previously isolated *TmTsaE* trimer, after incubation for 11 mins to 2 hours at room temperature or 60 °C. In each case, the samples incubated for 30 mins at 60 °C were of 8-fold dilution and that incubated for 2 hours at 60 °C were of 4-fold dilution.

***TmTsaE* trimer possesses a moderate autokinase activity *in vitro*.**

To test if the autokinase activity displayed by *TmTsaE* was dependent on the oligomerization, *TmTsaE* was fractionated by SEC and then subjected to *in vitro* phosphorylation assay. As shown in Figure 3-4A, the autokinase activity was seen to be specific to the oligomeric species, and it is the trimeric species to be precise as evident on the Native PAGE gel.

The autokinase activity of *TmTsaE*, measured as concentration of phospho-*TmTsaE* formed, was evaluated over increasing ATP concentration as detected on the SDS-PAGE gel (Figures 3-4B, 3-5 and 3-6). The percentage of phosphate incorporation by the trimeric and monomeric species of *TmTsaE* was calculated by dividing the maximum concentration of covalent phosphates, as shown in Figure 3-4B (0.435 μ M for trimer and 0.087 μ M for monomer), by the concentration of trimeric or monomeric *TmTsaE*. The concentration of trimeric and monomeric *TmTsaE* present after 2 hours at 60 °C was evaluated by SEC (Figure 3-3) and integration of the areas under the SEC profiles. 33.5% of trimer and 96% of monomer from a total protein concentration of 5 μ M was present after those conditions. The autokinase activity for the trimer was moderate with ~26% phosphate incorporation and that of the monomer was weak with 2% phosphate incorporation. The trimer is 13-fold more active than the monomer. The activity level calculations are the result of two independent experiments.

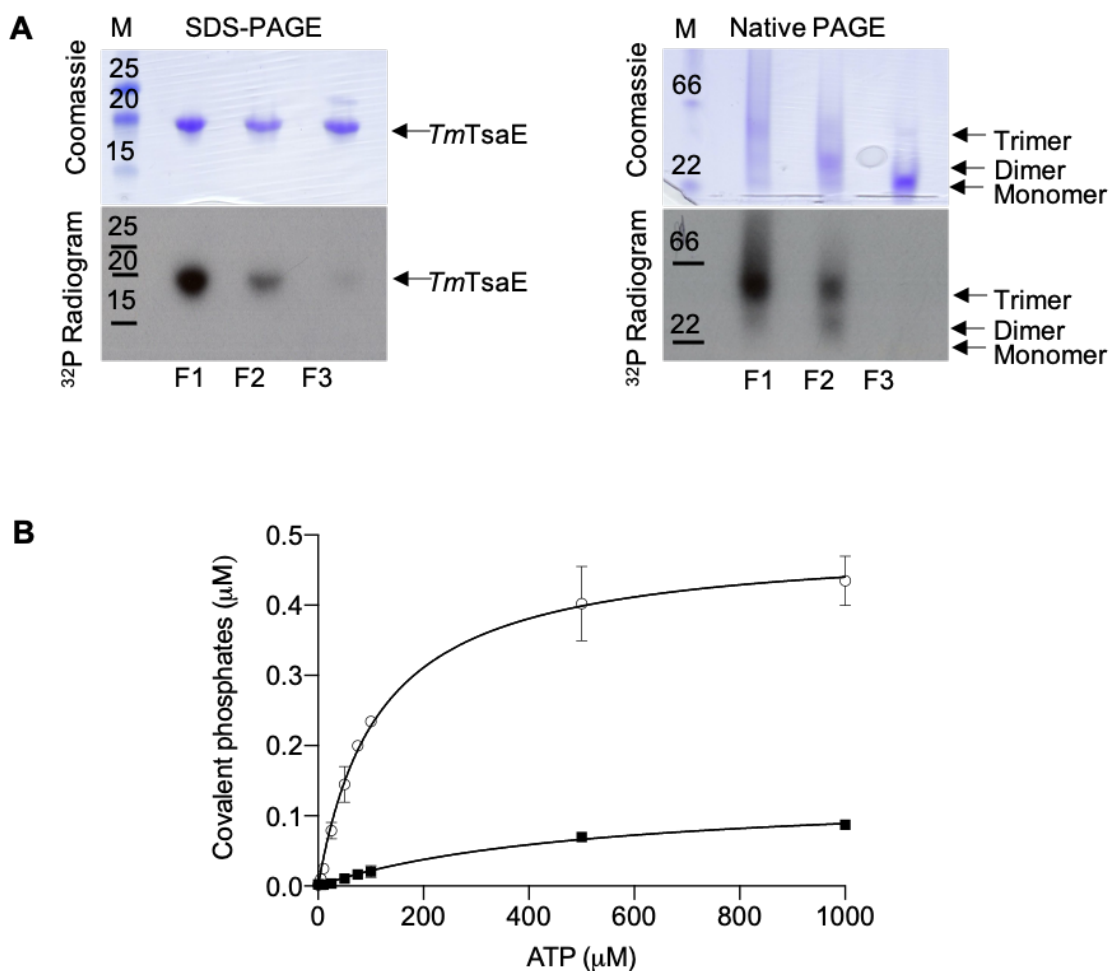


Figure 3-4: *TmTsaE* oligomer possesses a weak autokinase *in vitro*.

A: SDS-PAGE and Native PAGE analysis of trimeric (F1), dimeric (F2) and monomeric (F3) *TmTsaE* following incubation with 100 μM ^{32}P - γ -ATP, 10 μM protein and 2 mM MgCl_2 . M: molecular weight marker. B: Quantification using ATP standard of trimeric (open circles) and monomeric (closed squares) *TmTsaE* autokinase activities against increasing ATP concentrations, $n=2$. Error bars in the lower plot (monomer) is too small to display, the average error is 0.00194 μM .

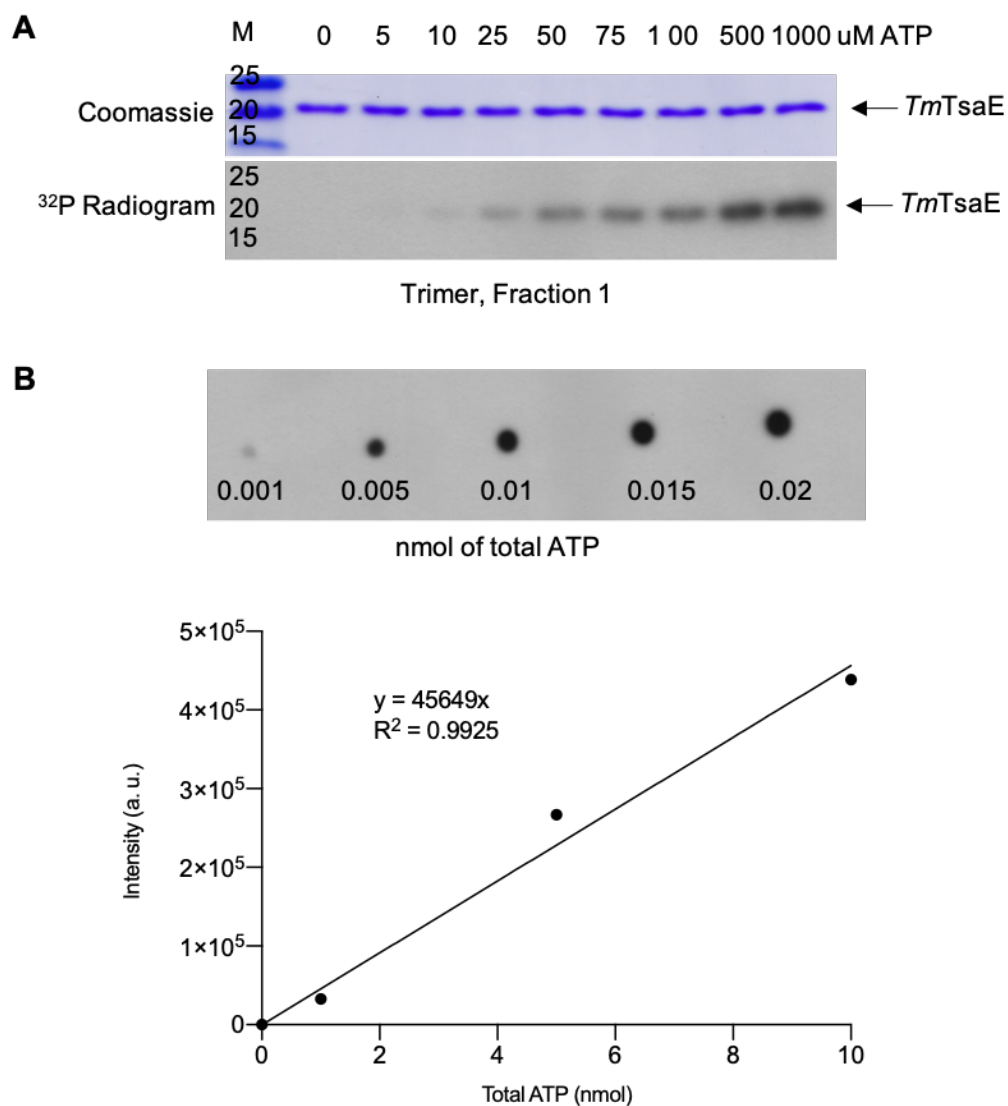


Figure 3-5: Quantification of *TmTsaE* oligomer *in vitro* autokinase activity.

A: SDS-PAGE analysis of SEC purified *TmTsaE* Trimer (F1) following incubation with ³²P-γ-ATP and MgCl₂ (5 μM protein, 0 – 1000 μM ATP, 2 mM MgCl₂). M is the protein marker.

B: Known amounts of total ATP spotted on a TLC and autoradiographed simultaneously with the sample and subjected to densitometric quantification using Image Studio Lite software.

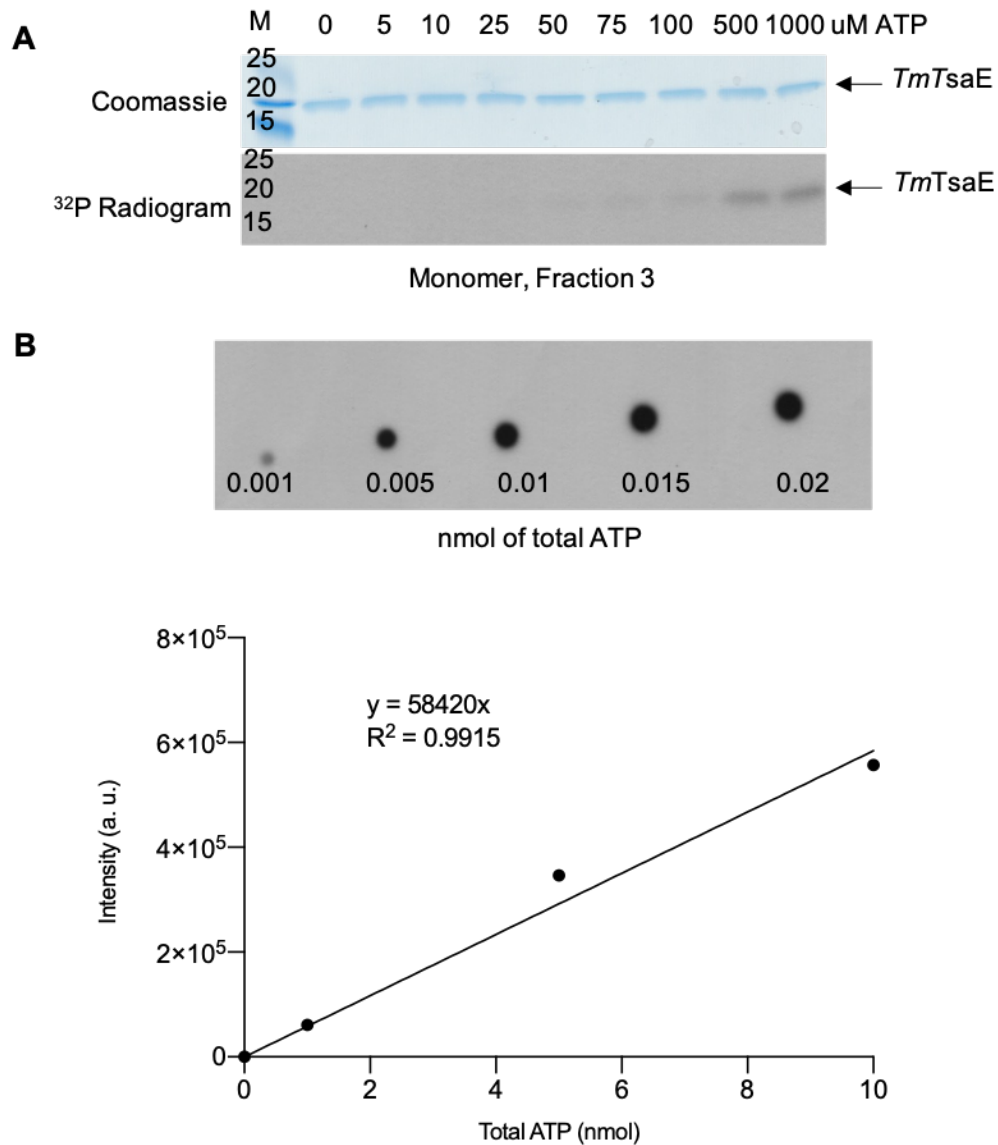


Figure 3-6: Quantification of *TmTsaE* monomer *in vitro* autokinase activity.

A-B: SDS-PAGE analysis of SEC-purified *TmTsaE* monomer (fraction F3) following incubation with ^{32}P - γ -ATP (5 μM protein, 0 – 1000 μM ATP, 2 mM MgCl_2). M is the protein marker. Autoradiogram of known amounts of total ATP spotted on a TLC plate (top) (autoradiography was done simultaneously with the gel) and subjected to densitometric quantification (bottom) using Image Studio Lite software.

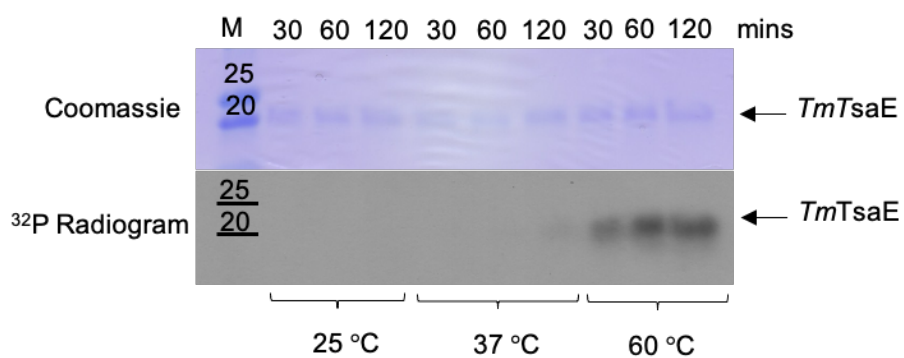


Figure 3-7: Temperature dependence of *TmTsaE* *in vitro* autokinase activity. SDS-PAGE analysis of the effect of temperature on *TmTsaE* autokinase activity using the ^{32}P incorporation assay.

***TmTsaE* monomer is phosphorylated heterologously *in vivo*.**

In order to test if the weak autokinase activity of trimeric *TmTsaE* was a result of already present covalent cold phosphates in the as-purified protein, I adapted a malachite green assay kit to quantify released phosphates from *TmTsaE* using a phosphatase. I tested multiple phosphatases to assess their ability to remove covalent phosphates from *TmTsaE* and found *E. coli* alkaline phosphatase (*EcAP*) to successfully do so. (Figure 3-9A).

Upon analysis of as-purified, fractionated *TmTsaE* phosphate levels, it was evident that the trimeric *TmTsaE* was minimally phosphorylated *in vivo*, while monomeric *TmTsaE* was observed to be substantially more phosphorylated *in vivo* than *in vitro* (Figure 3-8, 3-9B and C). Since *EcAP* has the ability to hydrolyze free AMP, ADP and ATPs, the possibility that the detected *in vivo* phosphates from *TmTsaE* may be originating from bound nucleotides was considered. To address this concern, the amount of phosphates in water-treated and 0.1% SDS-treated *TmTsaE* was measured using *EcAP* and malachite green assay. As shown in Figure 3-10, the amount of phosphates on both water-treated and 0.1% SDS-treated *TmTsaE* is nearly equal. This result demonstrated two points; (1) the apparent weakness of the autokinase activity of *TmTsaE* is not caused by dephosphorylation due to hydrolysis under denaturing conditions of SDS-PAGE. (2) the released phosphates detected from as-purified *TmTsaE* using *EcAP* and malachite green assay do not originate from bound AMP, ADP or ATP but are covalently bound to *TmTsaE*.

Further, despite detection of phosphorylation in the as-purified *TmTsaE* trimer, it was never detected to be ~85% phosphorylated in all independent experiments carried out even using *TmTsaE* trimeric fractions from different protein preparations. Therefore,

the *in vivo* phosphate levels of as-purified *TmTsaE* trimer do not explain its weak autokinase activity *in vitro*.

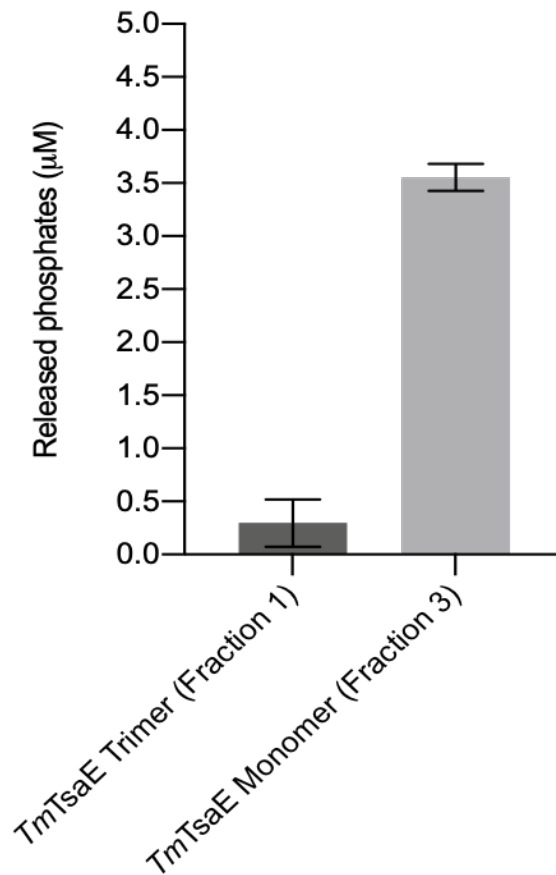


Figure 3-8: *TmTsaE* monomer is phosphorylated heterologously *in vivo*. Quantification of *in vivo* phosphates detected on SEC separated as purified *TmTsaE* trimer and monomer using malachite green phosphate assay, n=3.

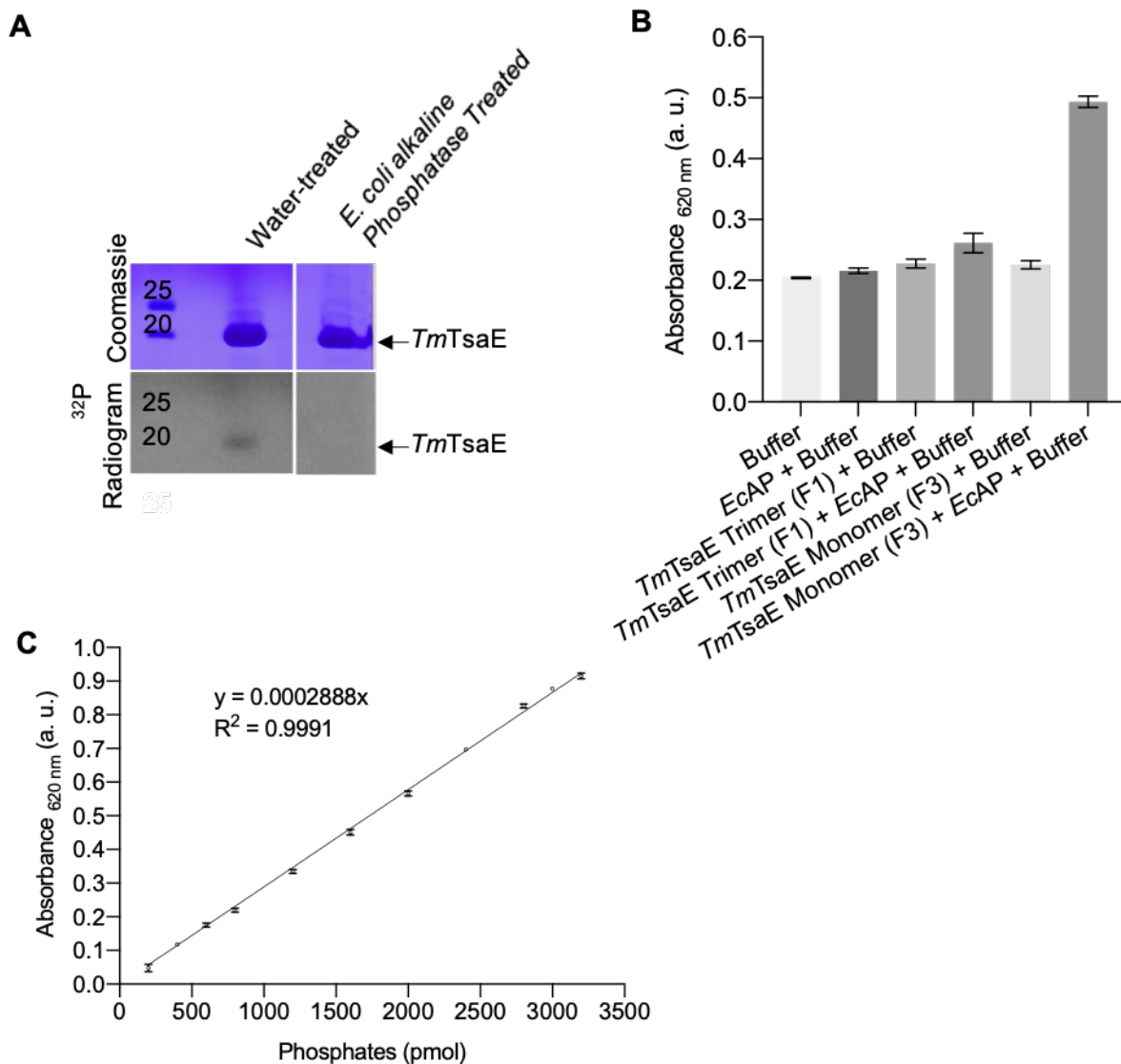


Figure 3-9: Dephosphorylation of *TmTsaE* by *E.coli* Alkaline dephosphorylation and quantification of released phosphates.

A: *E. coli* Alkaline phosphatase was tested for its ability to dephosphorylate *TmTsaE*. *TmTsaE* was phosphorylated using ^{32}P incorporation assay and excess ATP was removed using a spin column, before phosphatase treatment followed by SDS-PAGE and autoradiography. B: Raw absorbances of samples and controls used for experiment in Figure 3-8 using the malachite green phosphate assay, $n=3$. C: Standard curve of absorbances at 620 nm against known amounts of phosphates for malachite green phosphate assay, $n=3$.

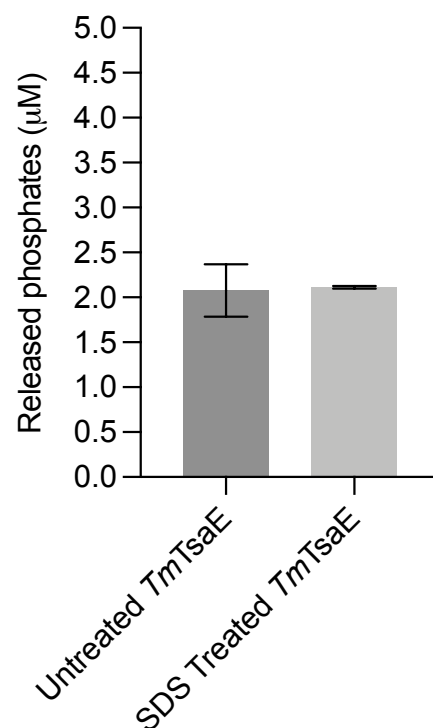


Figure 3-10: Assessment of heterologous *in vivo* phosphate levels of as purified *TmTsaE* with and without denaturation.

TmTsaE was subjected to denaturing (0.1% SDS) conditions and washed with 50 mM Tris pH 7.5, 50 mM KCl and 1 mM DTT buffer, before quantification of phosphates using the malachite green phosphate assay, n=3.

***TmTsaE* oligomerization is independent of its phosphorylation.**

Inspired by the trimer-specific autokinase activity *in vitro* and the monomer-specific heterologous phosphorylation of *TmTsaE* *in vivo*, I hypothesized that phosphorylation modulates oligomerization. SEC was used to assess whether, before and after dephosphorylation of *TmTsaE* monomer (previously fractionated), caused a change to the oligomer-monomer distribution. As shown in Figure 3-11A, dephosphorylation of *TmTsaE* monomer did not cause a significant change in the oligomer-monomer distribution, at least not to an extent based on the number of phosphates the same *TmTsaE* monomer was verified to have, i.e. ~45% (Figure 3-11B). This finding is consistent with the fact that *TmTsaE* monomeric fractions isolated and quantified for the amount of phosphate using malachite green assay, were rarely detected to be 100%. In other words, *TmTsaE* monomer exists as phosphorylated and unphosphorylated monomers. If phosphorylation does not modulate oligomerization, then *TmTsaE* trimers must also exist in phosphorylated and unphosphorylated forms. And this has been the case for all *TmTsaE* trimeric fractions analyzed by malachite green assay albeit, the levels have been either none or less than 10%. Next, the ability of ATP binding to impact the oligomer-monomer equilibrium of *TmTsaE* was tested. This was done by performing SEC in the presence of 1 mM ATP and then comparing with the absence of ATP (Figure 3-11C). The K_d of ATP to *TmTsaE* is 9 μ M at room temperature as reported previously (40). The SEC profile did not show a noticeable change in distribution, it would seem *TmTsaE* is not modulated by ATP interaction either. The only factors that were able to modulate *TmTsaE* oligomerization *in vitro*, thus far are salt concentration and temperature.

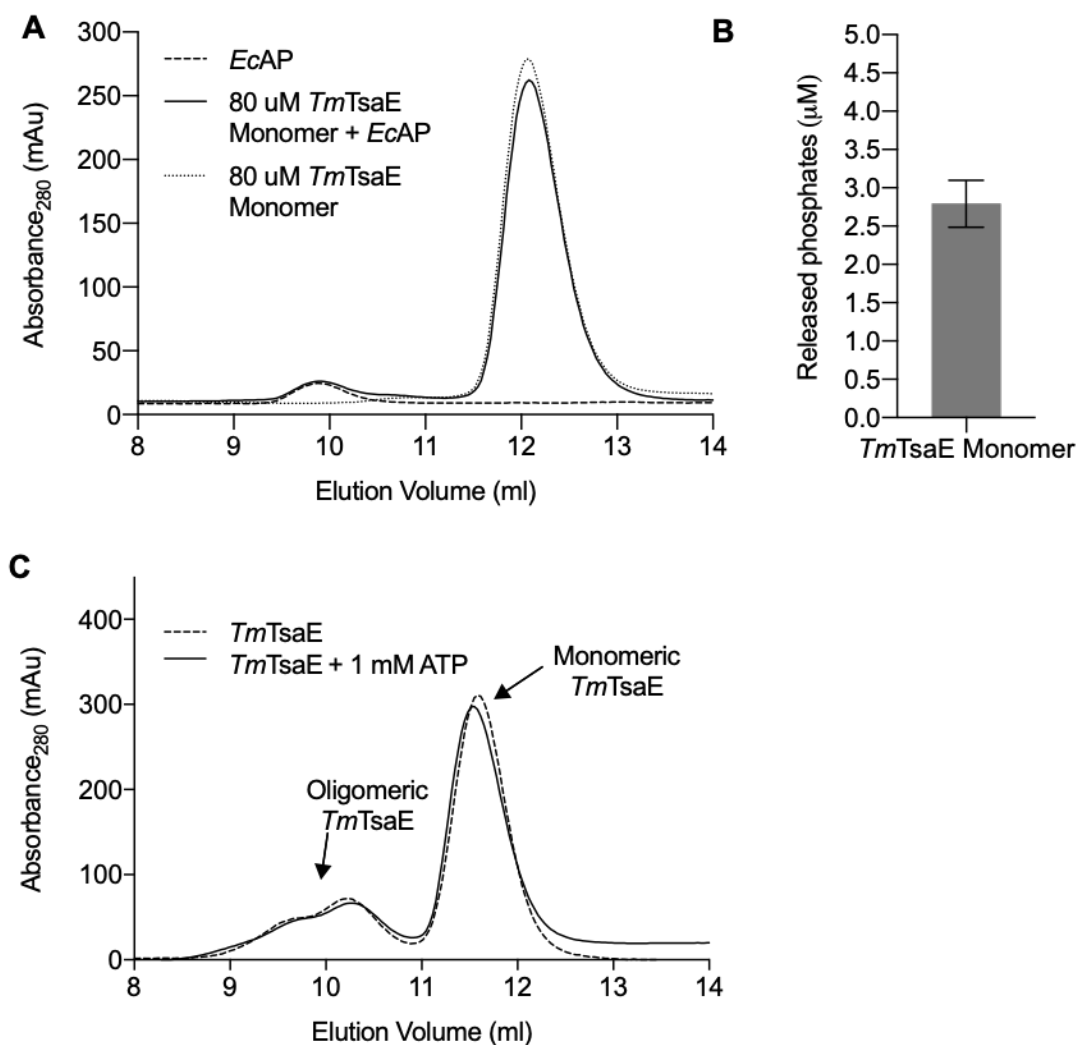


Figure 3-11: *TmTsaE* oligomerization is independent of phosphorylation.

A: *TmTsaE* monomer was dephosphorylated using *EcAP* and analyzed for oligomer-monomer distribution using SEC. *EcAP* control shown in dashed line, as-purified *TmTsaE* monomer control shown in dotted line and the *EcAP*-treated *TmTsaE* monomer shown in solid line. B: Amount of phosphates on the same *TmTsaE* monomer quantified using malachite green phosphate assay, n=3. C: Effect of ATP on *TmTsaE* oligomer-monomer equilibrium in the presence and absence of 1 mM ATP using SEC.

Mutational analysis of *TmTsaE* *in vitro* kinase activity and comparison with the observed *in vivo* phosphorylation

Upon investigation of *TmTsaE* mutants for *in vitro* kinase activity and the heterologous phosphorylation *in vivo*, it was clear that two activities show prominent differences (Table 1). To analyze kinase activities of *TmTsaE* mutants, some structural insight from former crystal structures (*BsTsaE*-ADP, *HtTsaE*-ADP and *TmTsa* B₂D₂E₂-ATP-carboxy-AMP (21, 33, 41)) were used. Since *TmTsaE* exists as a complex between TsaB₂ and TsaD₂ in the *TmTsa*B₂D₂E₂ crystal structure, *BsTsaE* and *HtTsaE* were superimposed onto *TmTsa*B₂D₂E₂ and found that there were no major changes to the ATP binding pocket of *TmTsaE* in the presence of *TmTsa*B₂D₂. Thus, the ATP binding pocket architecture of *TmTsaE* in *TmTsa*B₂D₂E₂ crystal structure was considered to be akin to that of free *TmTsaE* (Figure 3-12).

Residues that are absolutely necessary for *in vitro* kinase activity are Lys41, Thr42, Phe64, Tyr82, Glu108 and Arg134. All of these residues, with the exception of Phe64 and Tyr82, are well known directly interacting ATP-Mg²⁺ residues in P-loop ATPases (99). Tyr82 is a Switch II residue that is located very close to the γ -phosphate according to *TmTsaE* crystal structure (bound with ATP and *TmTsa*B₂D₂). In the absence of *TmTsa*B₂D₂, where *TmTsaE* ATP site is bare open as seen with free TsaE crystal structures (*BsTsaE* and *HtTsaE* bound with ADP (21, 41)), it is likely that Tyr82 might make a direct and indispensable stabilizing interaction with γ -phosphate of ATP. Phe64, located in Switch I a highly flexible loop, was demonstrated to be a key communication residue in the case of t⁶A activity (33). Thus, like in t⁶A activity, Phe64 could play the role of propagating a conformational change that might be necessary for *in vitro* kinase

activity. Residues that are not absolutely essential but cause a decrease in activity when mutated to an Alanine are Trp109, Arg83 and Glu11. Glu11 despite holding a direct interaction with the 2' OH of ATP (33), shows the least decrease in activity when mutated. Trp109, making an indirect interaction with the γ -phosphate of ATP via a water molecule (33), exhibits a stark requirement for *in vitro* kinase activity, albeit not crucial. And this might be due to the positioning of Trp109 right next to Tyr82 allowing aromatic stacking interactions to stabilize each other. Arg83, a Switch II residue without direct or indirect interaction to ATP-Mg²⁺ (33) show a similar trend as Trp109 but to a lesser extent. Arg83 of Switch II might also play a conformational role like Phe64 of Switch I as suggested before (33). Other residues interacting indirectly with ATP-Mg²⁺, such as Ser61, Thr63 and Asp80 are all dispensable for the *in vitro* kinase activity. The residues equivalent to Ser61 and Thr63 in *BsTsaE* were also dispensable for *in vitro* kinase activity but they were essential for the trans-kinase activity (using myelin basic protein) which was not tested in the present study (21).

Strikingly, for the observed heterologous phosphorylation of *TmTsaE* *in vivo*, only Lys41, Thr42 and Arg134 are required among the residues tested, while substitution of Thr43 with alanine resulted in partial reduction in phosphorylation. Further, despite Glu108 being a crucial Mg²⁺ coordinating residue, it appears to be completely expendable for the observed *in vivo* phosphorylation. Likewise, Phe64 and Tyr82, both required for the *in vitro* kinase activity, are dispensable for the *in vivo* heterologous phosphorylation. Given that both phosphorylations (*in vitro* kinase activity and *in vivo* heterologous phosphorylation) rely on the core ATP binding residues Lys41, Thr42, Thr43 and Arg134, it can be postulated that both activities use the same ATP binding site.

Table 3-1: The *in vitro* autophosphorylation of *TmTsaE* and the observed *in vivo* phosphorylation are two distinct activities.

<i>TmTsaE</i>	<i>In vitro</i> kinase activity relative to Wild-type	<i>In vivo</i> phosphates detected relative to Wild-type
Wild-Type	100%	100%
Glu11Ala	41%	95%
Lys41Ala	1.1%	7.3%
Thr42Ala	1.5%	0.0%
Thr43Ala	62%	60%
Ser61Ala	82%	118%
Thr63Ala	130%	130%
Phe64Ala	0.7%	94%
Asp80Ala	180%	122%
Tyr82Ala	0.5%	121%
Arg83Ala	35%	121.8%
Glu108Ala	0.7%	224%
Trp109Ala	17%	202%
Arg134Ala	4%	0.0%

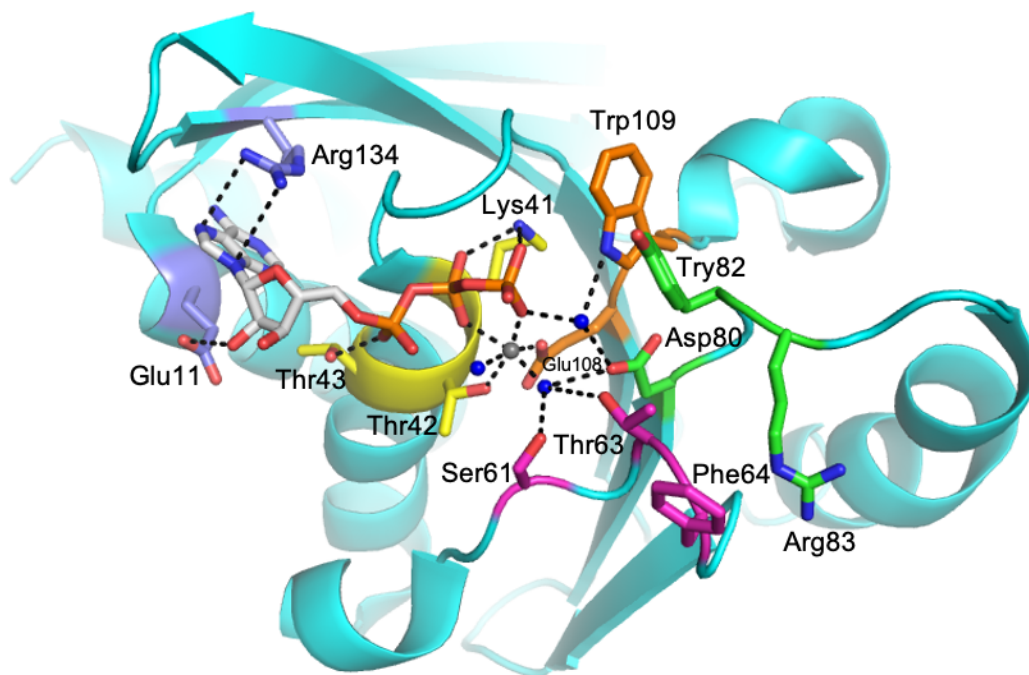


Figure 3-12: *TmTsaE* active site.

Ribbon diagram of *TmTsaE* (extracted from PDB ID 6N9A) showing ATP bound in the active site (33). Conserved TsaE motifs are colored as follows: Walker A or P-loop motif shown in yellow, Walker B motif shown in orange, Switch I shown in green and Switch II shown in magenta. Residues mutated in this study are displayed in stick. Residues interacting with the adenine ring of ATP are in blue. Mg^{2+} shown as a gray sphere while water molecules shown as blue spheres.

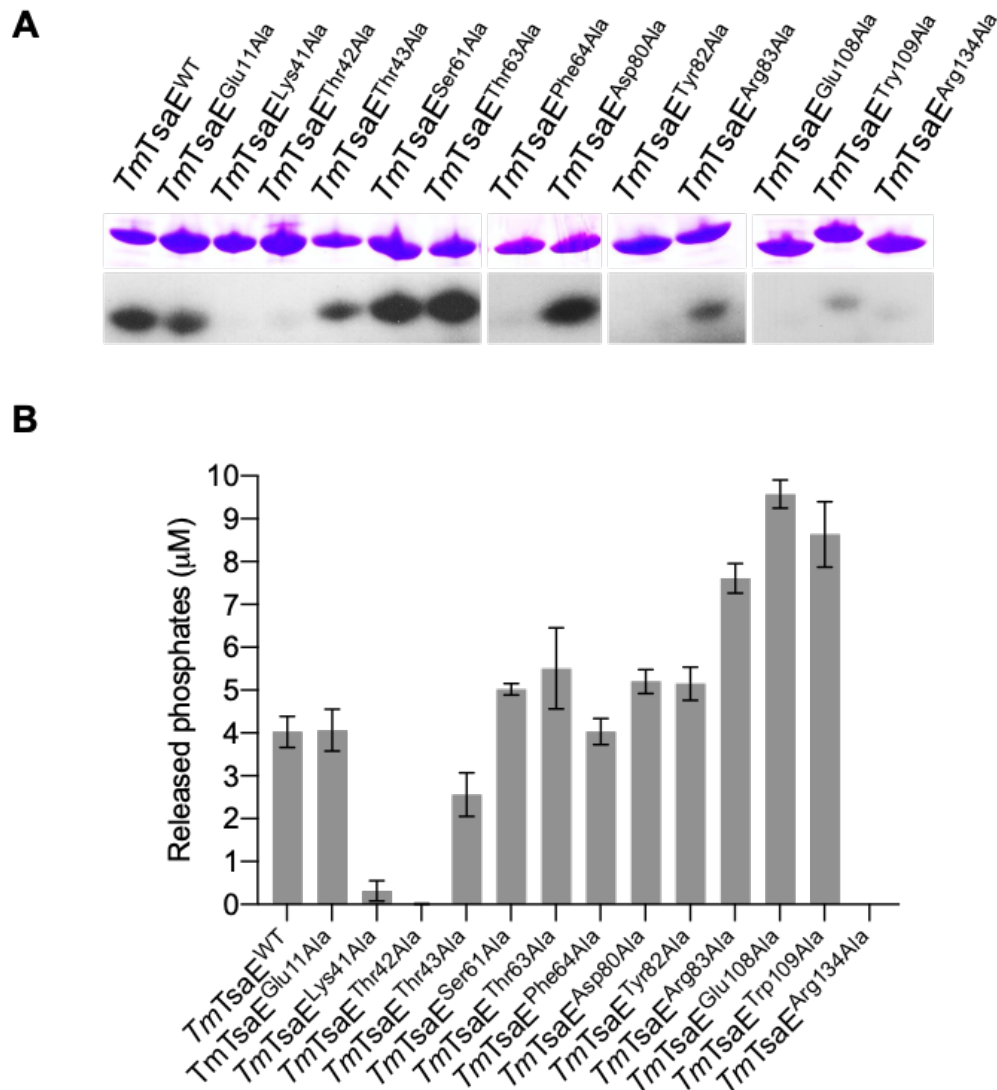


Figure 3-13: *In vitro* autokinase activity and observed *in vivo* phosphates of *TmTsaE* mutants.

A: SDS-PAGE analysis of wild-type and mutant *TmTsaE* proteins after incubation with 100 μ M 32 P- γ -ATP, 10 μ M protein and 2 mM MgCl₂. B: Quantification of *in vivo* phosphates released from unfractionated wild-type and mutant *TmTsaE* proteins as determined by the malachite green phosphate assay, n=3. Note: Data in panel A was provided by Dr. Amit Luthra from Prof. Manal Swairjo Lab.

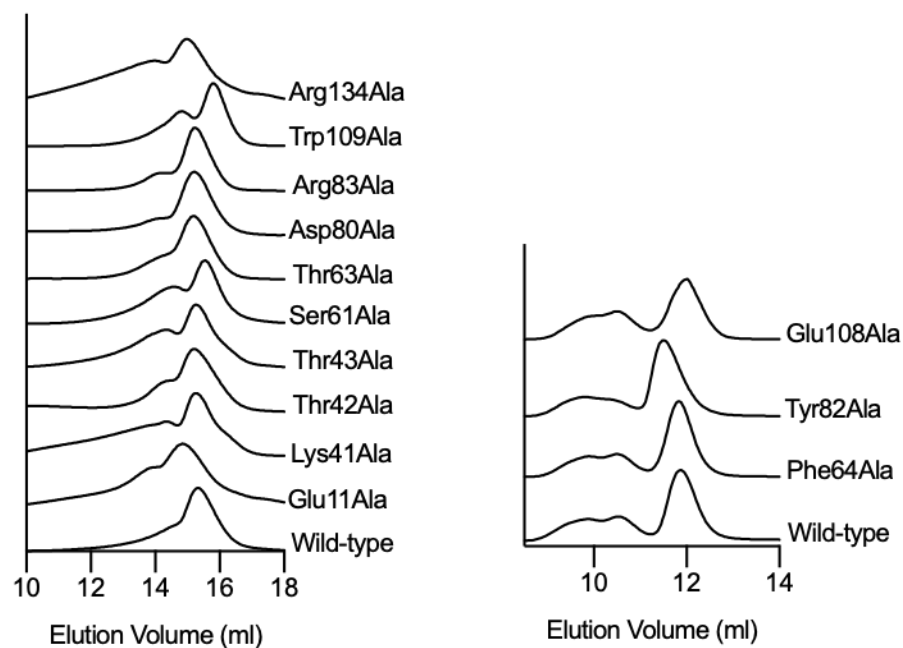


Figure 3-14: Distribution of oligomer and monomer of *TmTsaE* mutants.

Size exclusion chromatography profiles of *TmTsaE* mutants. On the left, profiles were obtained using a Biorad SEC70 column while the profiles on the right were obtained by using a Biorad SEC650 column.

Phosphorylation sites of *TmTsaE*

To find the sites of phosphorylation, LC-MS/MS was performed at the Stanford Burnham Medical Research Institute's Proteomics facility on an unfractionated *TmTsaE* (as purified) *sample* in the presence and absence of ATP. The results show phosphorylation of Ser61, Thr63, Thr65, Tyr70, Tyr77 and Tyr82.

Ser61, Thr63 and Thr65 are located in the Switch I loop of *TmTsaE* and Tyr82 is located in Switch II loop. Tyr77 is located on a β -strand (β 4) leading up to Switch II loop and Tyr70 is located on a β -strand (β 3) preceding Switch I loop. For each sample, there was only one major phosphorylation site among those listed above, and that is Tyr82 for as-purified *TmTsaE* and Thr65 for *TmTsaE* in the presence of ATP (Table 3-2). The other phospho sites were either one or two orders of magnitude less abundant than the major site detected in each sample. This result is consistent with the malachite green phosphate assay which showed that mutating Tyr82 did not abolish the observed *in vivo* phosphorylation levels, consistent with multiple phosphorylation sites in the enzyme. These data are consistent with previous reports of a serine and three threonine residues, all in Switch loops, as phosphorylation sites in *B. subtilis* TsaE (21), and a single tyrosine residue in the Switch I loop in *S. pneumoniae* TsaE (87).

Notably, in the presence of ATP, the major phosphorylation site changes by two orders of magnitude from Tyr82 to Thr65 (Table 3-2), suggesting a potential relaying of phosphates between the Switch I and II loops of *TmTsaE*.

Table 3-2: Major phosphorylation sites of *TmTsaE* in the presence and absence of ATP detected by phosphoproteomic LC/MS analysis.

Sample	Residue	Phosphopeptide and probability of phosphosite localization	Intensity
<i>TmTsaE</i> as purified	Thr65 (SW I)	SPT(0.007)FT(0.993)LMNVYPGLK	4.0E+06
	Tyr82 (SW II)	TIYHLDLY(1)R	5.9E+09
<i>TmTsaE</i> + ATP	Thr65 (SW I)	SPT(0.002)FT(0.998)LMNVYPGLK	8.8E+07
	Tyr82 (SW II)	TIYHLDLY(1)R	9.3E+05

***In vitro* ATPase activity of *TmTsaE* trimer and monomer.**

Intrigued by the co-existence of two activities in the presence of ATP, i.e. autokinase activity and ATPase activity, I quantified and compared the two activities for monomeric and trimeric *TmTsaE* under the same conditions (Table 3-3, Figures 3-15 and 3-16). The results show that the initial velocity of the ATPase activity of trimeric *TmTsaE* is approximately 1.5X higher than that of monomeric *TmTsaE*. This apparent difference in the ATPase activities of monomeric and trimeric *TmTsaE* is still much smaller than the difference in their autokinase activities. Further, the ATPase activity of the trimer and the monomer are ~200 fold and ~45,700 fold higher than the autokinase activity under the same conditions, respectively.

Table 3-3: Autokinase activity vs. ATPase activity of *TmTsaE* *in vitro*.

Wild-type <i>TmTsaE</i>	Initial velocity of <i>in vitro</i> autokinase activity ($\mu\text{M min}^{-1} \text{mg}^{-1}$)	Initial velocity of <i>in vitro</i> ATPase activity ($\mu\text{M min}^{-1} \text{mg}^{-1}$)
Trimer, 60 °C	3.035 ± 0.035	591 ± 18.738
Monomer, 60 °C	0.00865 ± 0.004	396 ± 19.102

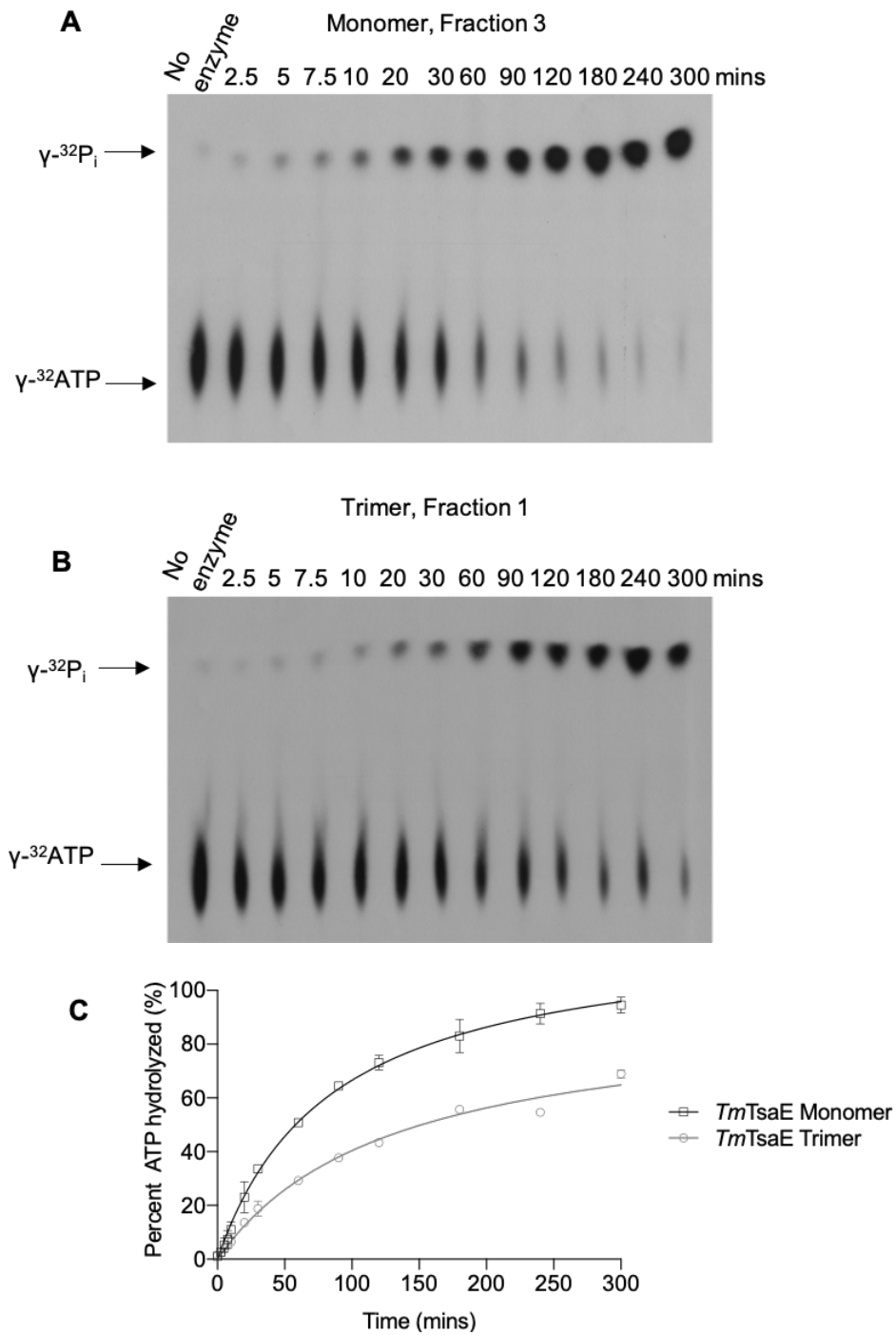


Figure 3-15: ATP hydrolysis of *TmTsaE* over time.

A, B: ATPase activity of monomeric and trimeric *TmTsaE* respectively. TLC of wild-type *TmTsaE* proteins after incubation with 50 μM ^{32}P - γ -ATP, 2.5 μM protein and 2 mM MgCl_2 . C: Percent ATP hydrolyzed by trimeric and monomeric *TmTsaE* plotted against time, n=2.

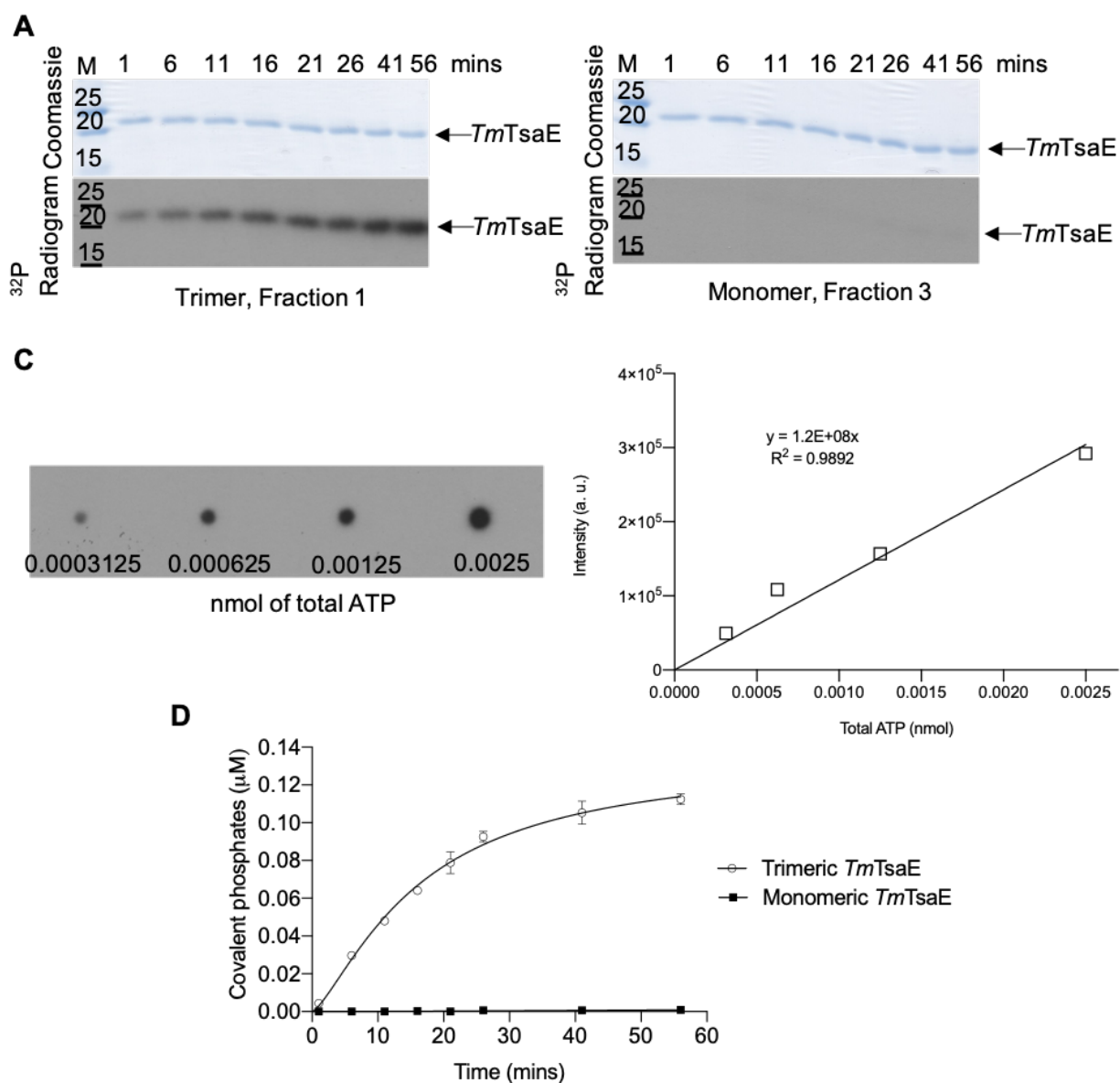


Figure 3-16: Time-course of the autokinase activity of monomeric and trimeric *TmTsaE*.

A-B: SDS-PAGE analysis of SEC-purified *TmTsaE* trimer (F1) and monomer (F3) following incubation with ^{32}P - γ -ATP and MgCl_2 (5 μM enzyme, 50 μM total ATP and 2 mM MgCl_2) for increasing periods of time. M denotes molecular weight markers. C: Known amounts of total ATP spotted on a TLC plate and autoradiographed (left) simultaneously with the gels shown in A - B and subjected to densitometric quantification using Image Studio Lite software (right). D: phospho-*TmTsaE* for trimeric and monomeric fractions plotted against time, $n=2$. Error bars in the lower plot (monomer) are too small to display, the average error is 0.000132 μM .

TmTsaE* trimer forms *TmTsaB₂D₂E₁* complex in the presence of *TmTsaB₂D₂

After finding that *TmTsaE* trimer can behave as both a robust ATPase and a weak autokinase, the ability of *TmTsaE* trimer to form complex with *TmTsaB₂D₂* was tested by running a Native PAGE (Figure 3-17). This result demonstrates that *TmTsaB₂D₂* has the ability to disrupt the very stable *TmTsaE* trimer in order to form *TmTsaB₂D₂E₁* complex.

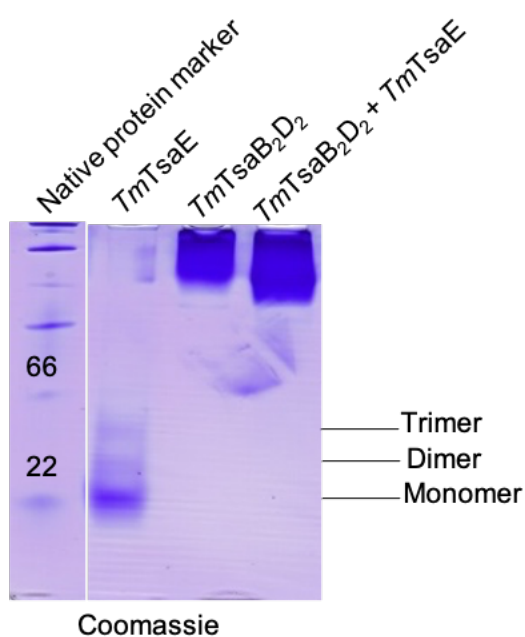


Figure 3-17: Native PAGE analysis of unfractionated *TmTsaE* in the presence and absence of *TmTsaB₂D₂* complex.

3.4 Discussion

Is *TmTsaE* an autokinase or an ATPase?

As-purified *TmTsaE* exists as both phosphorylated and unphosphorylated monomers and trimers. It is clear that ATP binds to *TmTsaE* given a modest K_d of 9 μM as previously reported (40) under the reaction conditions used in this study (50 μM ATP). Despite binding of ATP to either trimer or monomer or both, the weak autokinase activity of trimeric *TmTsaE* implies that the active site architecture of the trimer weakly imitates that of a real autokinase, while the active site architecture of the monomer does not. Given the weak activity of the trimer, phosphorylation independent oligomerization and a phosphorylated *TmTsaE* monomer *in vivo*, it is very unlikely that *TmTsaE* trimer is the source of *in vivo* phosphates on *TmTsaE* monomer.

Taken together, it seems very likely that *TmTsaE* alone under the conditions of this study is not a bona-fide autokinase. And the *in vivo* phosphates on *TmTsaE* suggests that one of the following cases could be true: *TmTsaE* monomer is a substrate of an unknown protein kinase or *TmTsaE* recruits an unknown effector protein and that together becomes a bona-fide autokinase. The latter is case for Bud32, the eukaryotic ortholog of TsaE. Bud32 has an extremely weak intrinsic autokinase activity that is greatly stimulated by Cgi121 (34, 95). Perhaps in bacterial cells, the $t^6\text{A}$ system also utilizes an unidentified accessory protein such as Cgi121, that is not crucial for $t^6\text{A}$ activity but greatly enhances it as in the case of the eukaryotic $t^6\text{A}$ system.

Comparing ATPase activity and autokinase activity of trimeric and monomeric *TmTsaE in vitro*, indicated that both species of *TmTsaE* have a much greater ATPase activity than their respective autokinase activities. Under the *in vitro* conditions that this

study was carried out, it can be concluded that *TmTsaE* is an ATPase rather than an autokinase. However, the same cannot be said for *in vivo* conditions as *TmTsaE* may still function as a robust autokinase in the presence of other unidentified, cellular factors.

Is *TmTsaB₂D₂* the *in vivo* protein kinase of *TmTsaE*?

Analyzing the mutational data of *TmTsaE* *in vivo* kinase activity and previously reported *TmTsaB₂D₂E* ATP binding data by ITC (40), one mutation in particular stands out, i.e. Glu108Ala of *TmTsaE*. It is clear that Glu108 is needed for ATP binding and ATP hydrolysis in the *TsaB₂D₂E* complex but not needed for *in vivo* heterologous phosphorylation of *TmTsaE*. The Glu108Ala mutant effectively decouples the ATPase activity of *TsaB₂D₂E₁* complex and *in vivo* phosphorylation of *TmTsaE*, indicating that the *TsaB/TsaD* complex of *E. coli* is not the *in vivo* protein kinase of *TmTsaE*, and suggesting that this observed *in vivo* phosphorylation is unlikely occurring in *T. maritima*.

Does phosphorylation modulate ATP hydrolysis?

In 2019, Pelletier *et al.* identified that *SpTsaE* was singularly phosphorylated at Tyr58 of SWI using mass spectrometry. They generated Tyr58Phe and Tyr58Glu mutants which are “phosphor-abrasive” and “phosphor-mimetic” mutants of *SpTsaE* respectively. They then measured the ATP hydrolysis activity of these mutants and compared with that of Wild-type *SpTsaE*. They found the phosphor-abrasive mutant had a lower ATPase activity than Wild-type and phosphor-mimetic mutant had a higher ATPase activity than Wild-type. And they argue that phosphorylation modulates ATP hydrolysis of *SpTsaE*. However, when you consider the error within the measurements, neither of the two mutations dramatically changed the ATP hydrolysis activity. Considering that ATPase activity of *TsaE* is $\sim 1/10^{\text{th}}$ that of *TsaB₂D₂E₁* complex, all the activities reported by Pelletier

et al. which are all in the same order of magnitude, is hardly an increase or decrease in ATP hydrolysis activity. If one compares the ATPase activities of the trimeric and monomeric forms of *TmTsaE* (Table 3-3), the ATPase activities are of the same order of magnitude for both species. However, the level of phosphorylation detected for the trimeric species was about 26X that of the monomeric species. This data suggests that there is no phosphorylation mediated modulation of ATP hydrolysis.

TsaE and Rio kinases/ATPases

A protein family related to Bud32, called Rio kinases/ATPases (Rio1 and Rio2) also share similar biochemical traits to Bud32 and TsaE. A study done by Kiburu *et al.* (2012) demonstrated Rio1 kinases/ATPases to oligomerize in the presence of ATP and undergo substrate inhibition at high concentrations of ATP (100). *TmTsaE* did not display substrate inhibition at high concentrations of ATP nor an ATP driven oligomerization (Figure 3-4B and 3-11C). Kiburu *et al.* also performed inhibition studies using Toyocamycin, crystallographic analysis of Rio1-Toyocamycin complex and SEC analysis of Rio1 in the presence of Toyocamycin. The results of which led to the discovery that Rio1 undergoes autokinase activity and monomerizes to give the most active form of the enzyme, indicating modulation of Rio1 autokinase activity by oligomerization (100). Although *TmTsaE* monomer was detected to be the most phosphorylated form *in vivo*, it was not the most active form of autokinase. Both Rio1 and Rio2 kinases/ATPases were shown to possess weak autokinase activity (less than 0.1% of total Rio1 or Rio2 ATP hydrolysis events) in contrast to the ATPase activity (101, 102), a behavior consistent with that of *TmTsaE*.

Interestingly, both Rio1 and Rio2 ATPase activities were also shown to mediate via an ADP-phospho-aspartate intermediate, characteristic of P-type ATPases. The aspartate that gets phosphorylated before the phosphate is released is an invariant residue involved in Mg^{2+} metal binding in Rio1 and Rio2 kinases/ATPases (Asp257 in *ctRio2* and Asp298 in *ctRio1*) (101, 102). *TmTsaE* also possess such an invariant residue involved in metal binding, but it is a glutamate residue (Glu108). *TmTsaE*^{Glu108Ala} lacks any autokinase activity as well as ATPases activity, and it also lacks ATP binding unlike in the case of Rio2 where ATPase activity was independent of ATP binding in *ctRio2*^{Asp257Ala} mutant (40, 102). However, *TmTsaE* contains another invariant acidic residue positioned at the ATP site (Asp80). *TmTsaE*^{Asp80Ala} mutant was observed to have wild-type like autokinase activity and no ATPase activity (33) opening the possibility of the TsaB₂D₂E₁ complex to behave as a P-type ATPase.

Is the kinase activity of *TmTsaE* needed for multi-turnover t⁶A synthesis?

It was known that t⁶A synthesis requires the ATPase activity of the TCT complex for turnover of the cycle, however the role of the autokinase activity of TsaE in the 'reset' of the TCT complex has not been previously investigated. The mutagenesis data from Chapter 2 (33) and this chapter combined (summarized in Table 3-4 below) show that some residues are required for individual activities but not both. For example, Phe64 in the Switch I loop (highlighted in light grey in Table 3-4) is not required for ATP binding nor hydrolysis, yet it is required for the kinase activity. Similarly, Ser61 in Switch I and Asp80 in Switch II (highlighted in dark grey in Table 3-4), which are involved indirectly in coordinating the Mg^{2+} ion in the ATP binding site and are required for ATPase activity, are dispensable for the kinase activity. Further, Tyr82, a primary phosphorylation site (red

text in Table 3-4), is a Switch II residue that is not involved in ATP binding, yet it is necessary for both activities. The addition of ATP to *TmTsaE* resulted in re-localization of the phosphate from Tyr82 to Thr65. Taken together, these results suggest that the kinase function of TsaE requires different conformations of the Switch I and II loops than what is seen in the TCT complex (33) or in the free forms (21, 41). This supports the idea that the TsaB₂D₂E₁ complex may carry a P-type ATPase-like kinase and/or phosphatase/phosphotransfer activities that may be integral to its function in t⁶A biosynthesis and/or regulation.

Table 3-4: Summary of autokinase, ATPase and t⁶A synthesis activities of *TmTsaE* mutants.

<i>TmTsaE</i>	Relative autokinase activity (%)	Relative ATPase activity (%)	t ⁶ A synthesis turnover
Wild-Type	100	100	multiple
Ser61Ala	82	2	single
Thr63Ala	130	4	single
Phe64Ala	0.7	117	single
Asp80Ala	180	2	single
Tyr82Ala	0.5	0	single
Arg83Ala	35	74	Weak multiple

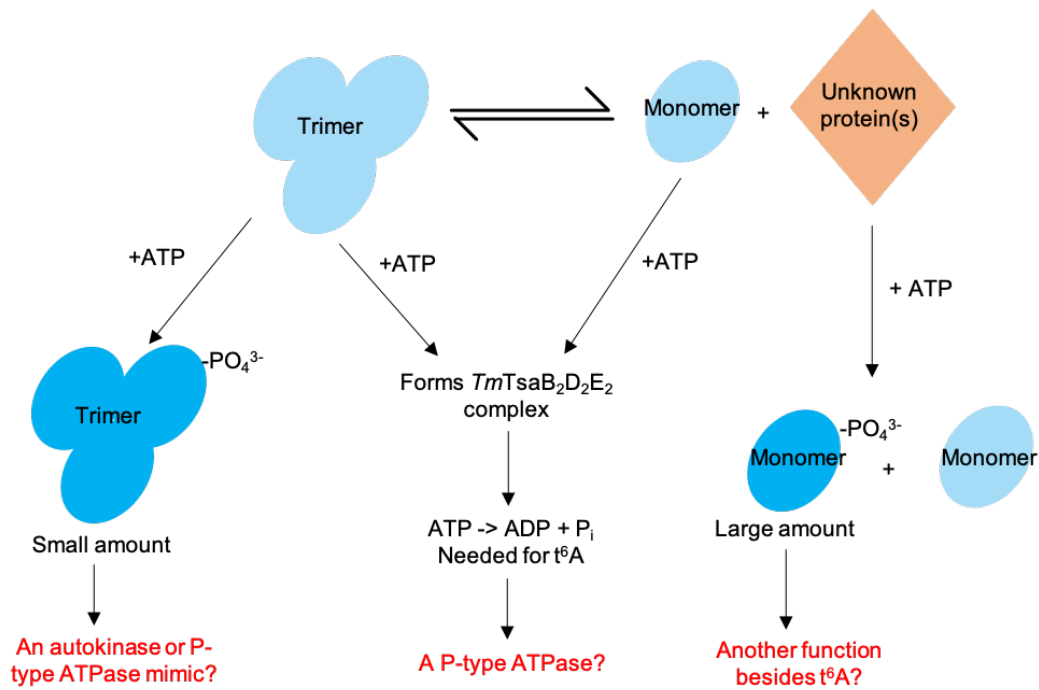


Figure 3-18: Model of *TmTsaE* autokinase and ATPase activities.

Unphosphorylated *TmTsaE* shown as light blue circles while phosphorylated *TmTsaE* shown as dark blue circles.

3.5 Future Directions

The next step of this study is to test the hypothesis that the TCT complex is a P-type ATPase that requires both kinase and phosphatase activities during the reset step. One can test this hypothesis by testing for autokinase activity using *TmTsaE* mutants that are active in autokinase activity but inactive in ATPase activity (Asp80Ala, Ser61Ala and Thr63Ala) in the presence of wild-type *TmTsaB₂D₂*. One can also use ATPase inactive mutants of *TmTsaD* (Lys166Ala and Thr213Ala (33)) with wild-type *TmTsaE* and *TmTsaB*. One can then assess if the autokinase activity observed with *TmTsaB₂D₂E₁*^{mutant} and *TmTsaB₂D₂*^{mutant}*E₁* is higher than observed with wild-type *TmTsaE* or wild-type *TmTsaD* (which would have wild-type ATPase function and thereby release the phosphate). If there is a retention of phosphates within the TCT complex when assayed using mutants that are kinase active but ATPase inactive, then this could be an indication of a P-type ATPase in action.

Another future aspect of this study is the identification of potential other *in vivo* protein partners of *TmTsaE*, especially if *TmTsaE* is a substrate of an unknown protein kinase. One can use pull down experiments coupled with mass spectrometry to probe potential non-Tsa binding partners.

3.6 Acknowledgment

Chapter 3, in full, is currently being prepared for submission for publication of the material. Paranagama, N., Luthra, A., Bayooz, S., Swairjo, M. A. Quantitative assessment of the kinase activity of the tRNA modification enzyme TsaE. The dissertation author is the first author of this manuscript.

Chapter 4 : Structural studies of the threonylcarbamoyl transfer complex bound to substrate tRNA

Abstract

An important prerequisite to developing an enzyme as an antimicrobial target is a clear understanding of its catalytic mechanism, including the intermediates involved in the enzymatic reaction. One of the gaps in our understanding of the t⁶A cycle is how the TC-transfer complex specifically recognizes ANN-decoding tRNAs for modification, and the intermediates involved in the TsaD-catalyzed TC-transfer reaction. To answer these questions, I pursued the structure of *Tm*TsaB₂D₂ in complex with tRNA^{Thr} by cryo-EM and X-ray crystallography as alternative approaches. Using the CryoSparrc software suite to process a dataset of ~2000 micrographs, a 10.1 Å cryo-EM map of *Tm*TsaB₂D₂-EctRNA^{Thr} complex was reconstructed. This map revealed that the tRNA binds to the back surface of the TsaD subunit and interfaces with the TsaB subunit. The structure also shows the lever arm of TsaD in the downward conformation, consistent with a pre-TC-transfer state of the complex. Although the map is of low resolution and does not address the mechanistic question, it represents the first structure of the tRNA-bound TC-transfer complex to date, and provides a more accurate view of the mode of tRNA binding than previously provided by SAXS. Further, *Tm*B₂D₂E₁ in complex with ASL^{Thr/Lys} RNA was subjected to manual optimization of initial crystallization conditions, yielding larger and less clustered crystals, and enabling assessment of X-ray diffraction. The improved crystals belonged to space group P21 and diffracted to 7.5 Å.

4.1 Introduction

One of the gaps in our understanding of the reaction mechanism of the t⁶A cycle is how the TCT complex recognizes ANN decoding tRNAs and the intermediates involved in the TsaD-catalyzed TC-transfer reaction. The position of TC-AMP within TsaBD active site was just recently discovered by a crystal structure of *Ec*TsaBD in complex with a stable phosphonate mimic of TC-AMP (103). A small angle x-ray scattering (SAXS) model of the *Tm*TsaB₂D₂-*Ect*RNA^{Thr} suggested that the tRNA (anticodon) bound at the interface of TsaD and TsaB with specific interaction with the TsaB C-terminal end (Figure 4-1) (33). According to this model, the tRNA did not make any other contacts with TsaD or TsaB. Electrophoretic mobility shift assays that probed for tRNA binding performed in the presence of delta C-term TsaB confirmed that the TsaB C-terminal tail is required for tRNA binding, and that in its absence all tRNA binding was lost (33). Electrophoretic mobility shift assays (EMSAs) conducted on the *Tm*TsaB₂D₂E₁ complex have shown that only one molecule of tRNA binds at a time, despite the presence of two TsaBD interfaces in the complex, and tRNA binds competitively at the same site as TsaE (40). Based on the crystal structure of *Tm*TsaB₂D₂E₂ (33), TsaE binds at a positively charged cavity formed by TsaB and TsaD (33). This observation is consistent for tRNA binding in general, as tRNAs are highly negatively charged molecules. TsaE approaches the TsaBD heterocomplex from the back side, and the TsaD lever arm adopts an upward conformation, while the C-terminal tail of TsaB adopts a helical conformation induced by TsaE binding (33). Thus far, there is no high-resolution structure of the TCT-tRNA complex. This structure would reveal the exact positioning of A37 of tRNA in the active site, and therefore enable elucidation of the mechanism of the TC-transfer reaction, and

will provide a structural understanding of specificity of t⁶A modification to ANN-decoding tRNAs.

Several attempts to determine the structure of the TCT-tRNA complex by crystallography have been made by Prof. Manal Swairjo Lab. However, none of those attempts yielded well diffracting crystals or a high-resolution structure thus far. Two approaches in particular have been tried, one approach is to co-crystallize *TmTsaB₂D₂E₁-EctRNA^{Thr}* and second approach is to co-crystallize *TmTsaB₂D₂E₁* with anticodon stem-loop RNAs (ASL). For the second approach, initial crystallization conditions were identified as the G3 reagent of the Morpheus I screen (Molecular Dimensions) by Eric Hellie and Albert Susanto of Prof. Manal Swairjo Lab. The initial crystals were grown at 20 °C in vapor-diffusion sitting drops made of 100 nl *TmTsaB₂D₂E₁-ASL* (43 µM) and 100 nl reservoir. This initial hit yielded highly clustered, small crystals (Figure 4-12A).

In this chapter, I used Cryo-EM and x-ray crystallography methods to probe the structures of tRNA-TsaB₂D₂ and TsaB₂D₂E₁-ASL complexes. For Cryo-EM I prepared the *TmTsaB₂D₂-EctRNA^{Thr}* complex and determined a structure resolved to ~10 Å. For crystallography, I used an existing initial hit established for *TmTsaB₂D₂E₁* in complex with three anticodon stem-loops (*TmASL^{Thr}*, *TmASL^{Lys}* and *TmASL^{Ser}*) to improve the size, reduce the clustering and increase diffraction quality of the crystals.

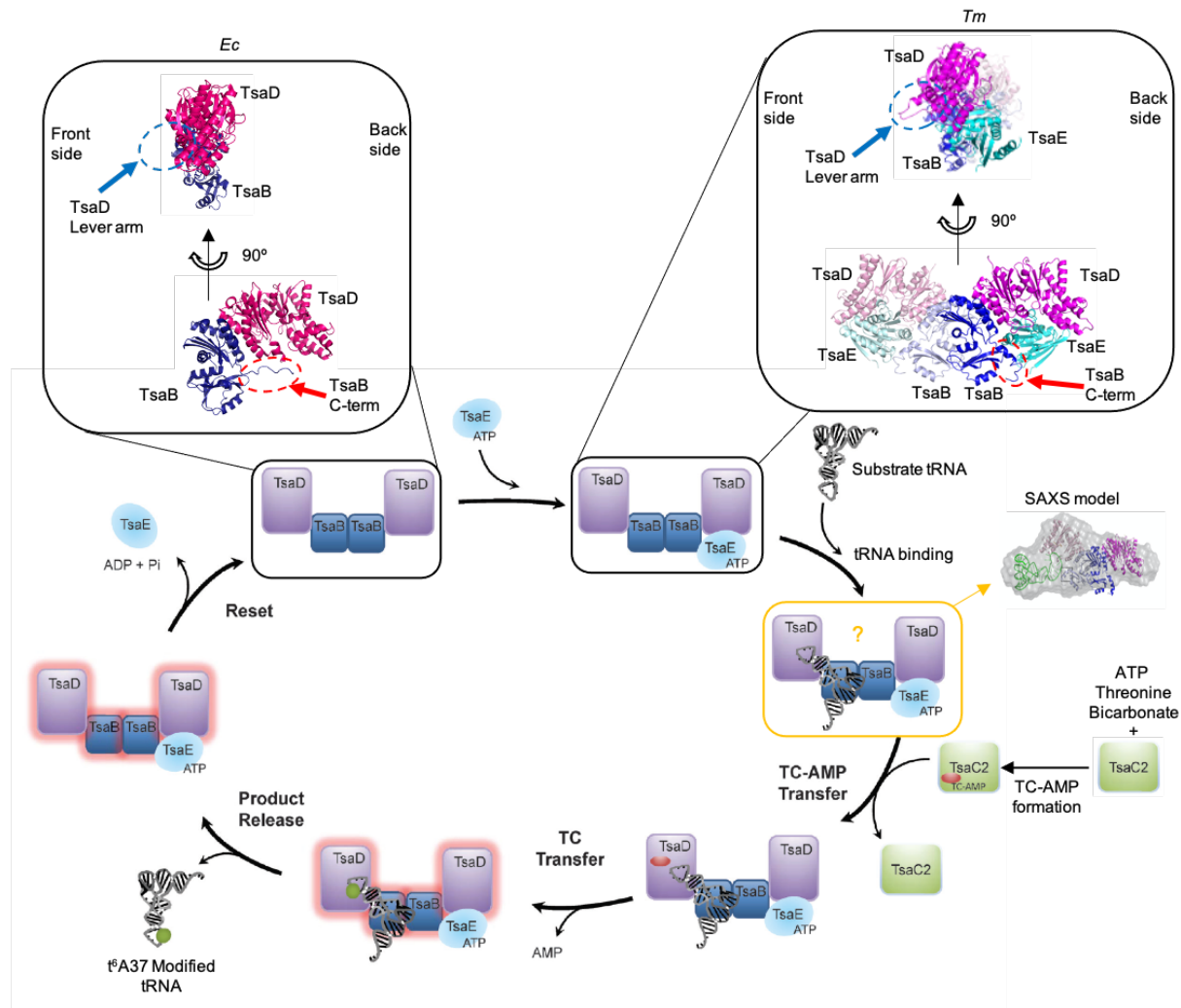


Figure 4-1: Experimentally determined TsaB/D and TsaB/D/E structures or molecular models for the bacterial t^6A catalytic cycle.

Crystal structures of *Tm*TsaB₂D₂E₂ (PDB ID: 6N9A) and *Ec*TsaBD (PDB ID: 4YDU). SAXS model of *Tm*TsaB₂D₂-EctRNA^{Thr} (33)

4.2 Materials and Methods

Purification of proteins

His₆-tagged *TmTsaB* and His₆-tagged *TmTsaD* were overexpressed in *E. coli* and purified by Ni-NTA and gel filtration chromatography as described previously (40). Purity was verified by SDS-PAGE to be > 95%.

Preparation of tRNA

E. coli tRNA^{Thr} transcript was synthesized by *in vitro* transcription from a BstN1 linearized DNA template containing the *E. coli* tRNA^{Thr}_{CGU} gene *thrW* (provided by Dr. Manal Swairjo) using T7 RNA polymerase as described previously with minor modifications and an additional step (31, 40). Briefly, preparative *in vitro* transcription was conducted in 3 mL reactions at 37 °C for 12h, and the DNA template was subsequently digested with RQ1 RNase-Free DNase (Promega Corporation). Transcribed RNA was analyzed by electrophoresis on a 10% TBE-Urea gel (Thermo Fisher Scientific). Transcripts were purified by anion exchange chromatography using a HiPrep Q Fast Flow 16/10 column (GE life sciences). The column was equilibrated with buffer containing 0.1 M Tris-HCl (pH 6.9), 0.4 M sodium acetate, and 0.2 mM EDTA. RNA was eluted with a linear gradient of elution buffer containing 0.1 M Tris-HCl (pH 7.3), 1.5 M sodium acetate, and 0.2 mM EDTA. Fractions of the elution peak were pooled, ethanol precipitated, re-dissolved in sterile water, and stored at -20 °C until further use. Final RNA purity of >95% was verified by 10% urea-PAGE and methylene blue staining. RNA was annealed by incubation at 95 °C for 5 minutes, followed by cooling at room temperature in 50 mM Tris-HCl pH 7.5, 50 mM KCl, 1 mM DTT and 2 mM MgCl₂. The annealed RNA was then purified by size exclusion chromatography in the same buffer using an ENRICH SEC 650

column and NGC chromatography system (Biorad). The elution peak was collected and used for subsequent experiments.

Isolation of *TmTsaB₂D₂* by size exclusion chromatography

Gel filtration experiments were performed on a calibrated Enrich SEC 650 column (Bio-Rad) using a Bio-Rad NGC chromatography system, as described previously (40). Briefly, 500 μ l of sample containing 15 μ M each *TmTsaB* and *TmTsaD* in buffer containing 50 mM Tris–HCl pH 7.5, 50 mM KCl, 1 mM DTT and 2 mM MgCl₂ were loaded on the column and eluted in the same buffer. Elution fractions containing *TmTsaB₂D₂* were verified by SDS-PAGE and before pooling for further steps.

Detection of *TmTsaB₂D₂*/tRNA complex formation by Native PAGE

tRNA fractions from the SEC run were collected and mixed with *TmTsaB₂D₂* to a final volume and concentration of 10 μ L and 2 μ M respectively with a 1:1 stoichiometry. The reactions were incubated at room temperature for 30 mins before mixing with 5 μ L of 50% glycerol and loading onto an anykD Tris Glycine gel (Biorad). The gel was run using 25 mM Tris 8.0, 192 mM glycine and 1 mM EDTA running buffer at 80V for 60 mins before staining for RNA visualization using Syber gold (Invitrogen) and protein visualization by Coomassie Blue staining.

Complex preparation for Cryo-EM analysis

Sample for grid preparation was made by mixing equimolar amounts of *TmTsaB₂D₂* and *EctRNA^{Thr}_{CGU}* to a final concentration of 1.5 μ M in 10 μ l. The sample was incubated at room temperature for 30 mins.

Cryo-EM Image processing

Gold grids (R1.2/1.3 UltrAuFoil) were plasma cleaned for 7 seconds using a Solarus plasma cleaner (Gatan Inc.). 2.5 μl of the sample was applied to the grid and incubated for 30 s before blotting with filter paper for 3 seconds and manually plunge freezing in liquid ethane at liquid nitrogen temperature ($-180\text{ }^{\circ}\text{C}$). A total of 2124 raw micrographs were collected in one session using a 200 kV Talos Arctica electron microscope (ThermoFisher) and a K2 Summit direct electron detector (Gatan Inc.) operated in counting mode controlled by the Legion software (104). A magnification of 57,000X with a calibrated pixel size of $0.73\text{ \AA}$ and a defocus range from $-1.0\text{ }\mu\text{m}$ to $-3\text{ }\mu\text{m}$ was used. Micrographs were collected with a total dose of $35.79\text{ e}^{-}/\text{\AA}^2$ that was applied across 80 frames at a dose rate of $\sim 6.1\text{ e}^{-}/\text{pix/s}$. Of the 2124 micrographs collected half was collected at a tilt angle of 30° and the other half was collected without a tilt. This is a common practice if the sample behavior on the grid with respect to the air-water interface is not well known (Refer to page 98-99 in Results and Discussion section). The grid preparation and data collection were done by Youngmin Jeon of Prof. Dmitry Lyumkis Lab.

Image processing was performed by combining both tilted and untilted images. Frames were aligned and dose weighted using MotionCor2 (105). The aligned sums were imported to CryoSparc and all subsequent image processing procedures were performed in CryoSparc (106). Local CTFs were calculated using CryoSparc patch-based CTF estimation for each micrograph (107). Particle picking was performed using CryoSparc template picker. Initial templates were generated by 2D classification of 158 manually picked particles. Automated particle selection resulted in 130,001 particles, which were

subjected to several cycles of 2D classifications. At the end of which 82,719 particles were deemed suitable for further data processing steps and the rest was rejected. This selection was based on the criteria that particles need to provide clean 2D class averages with a clean background and characterized by a good resolution, as measured by the Fourier ring correlation. Particles that did not yield good averages, according to the criteria above, were rejected. The 82,719 particles were used to build an ab-initio model using the Ab-initio Reconstruction module available within CryoSparc. Using the ab-initio model, the 82,719 particles were subjected to several cycles of 3D classifications using C1 symmetry. A class containing 8216 particles was used to generate a 10.4 Å map according to the Fourier shell correlation cut off at 0.143 (gold standard).

Crystallization and assessment of X-ray diffraction

The G3 condition of Morpheus I screen (Molecular Dimensions) consisted of 0.1 M Buffer system I (0.1 M Imidazole/MES pH 6.5), 30% Precipitant mix 3 (20% v/v Glycerol, 10% PEG4000) and 0.1 M Carboxylic acid mix (0.02 M Sodium formate, 0.02 M Ammonium acetate, 0.02 M Sodium citrate tribasic dihydrate, 0.02 M Potassium sodium tartrate tetrahydrate, 0.02 M Sodium oxamate). For the optimization experiments, Buffer system I and Precipitant mix 3 were prepared in the lab while the carboxylic acid mix was purchased from Molecular Dimensions. Using these reagents, sitting-drop crystallization screens were set up for each *TmTsaB₂D₂E₁*-ASL using sample containing 43 µM each protein and ASL in buffer containing 50 mM Tris pH 7.5, 50 mM KCl and 1 mM DTT. The volume of the reservoir was 80 µl and the drop was prepared by mixing 100 nl of sample with 100 nl of reservoir in 96-well MRC2 plates (Hampton Research) using a Mosquito crystallization robot (SPT Lab Tech). The precipitant concentration was

screened between 19% and 46% with increments of 2%, pH was screened between 6.0 and 7.0 with increments of 0.1 and the salt concentration (Carboxylic acid mix) was screened between 0.02 M and 0.2 M with increments of 0.01 M.

The best condition from each screen for each *TmTsaB₂D₂E₁*-ASL sample was selected for an additive screen (Hampton Research) that was done using the same set-up as described above.

Several screens were set up using the optimized conditions excluding Precipitant mix 3 which was replaced by either PEG1500 (at 5, 10 or 15%), PEG 2000 (at 5, 10 or 15%), PEG3350 (at 5 or 10%) and PEG 4000 (at 5 or 10%), to assess the dispensability of glycerol from the optimized condition.

Using the optimized condition, crystals with size 0.3–0.4 mm appeared in 10 days. The crystals were flash frozen in liquid nitrogen without cryoprotectant and X-ray diffraction was assessed on beamline 14-1 at the Stanford Synchrotron Radiation Lightsource.

Assessment of crystals for presence of bound ASL

Crystals from the optimized condition were picked up and washed in three consecutive drops of buffer containing 50 mM Tris pH 7.5, 50 mM KCl and 1 mM DTT. Next, the 3X washed crystals were placed in a drop containing 50 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 7M urea to dissolve. The resulting solution was analyzed on a 10% TBE-Urea gel (ThermoFisher) with controls for the protein and ASL and using 1X TBE as running buffer. SYBR gold staining followed by Coomassie staining was used to visualize the RNA and protein, respectively.

4.3 Results and Discussion

Sample preparation for Cryo-EM study

TmTsaB₂D₂ was reconstituted *in vitro* by SEC using recombinant His₆ tagged *TmTsaD* and His₆ tagged *TmTsaB* (Figure 4-2). *EctRNA^{Thr}* was prepared by using *in vitro* transcription followed by anion exchange chromatography (Figure 4-3). tRNA was purified using the protocol that was in use in Prof. Manal Swairjo Lab at the time this project was initiated.

To prepare sample for Cryo-EM, the purified tRNA was annealed and mixed with *TmTsaB₂D₂*, this sample did not yield good particle distribution on the grid. The particles displayed aggregates mixed with string-like particles (Figure 4-4A). Upon inspecting what *EctRNA^{Thr}* alone looked like on the micrograph, the string-like particles were observed again, suggesting the string-like particles were contributed from the *EctRNA^{Thr}* sample and not the protein sample (Figure 4-4B). Based on this observation, I hypothesized that the string-like particles were misfolded or oligomerized tRNAs that led to the aggregations seen on the micrographs. Misfolded tRNAs is a common phenomenon that occurs with *in vitro* transcribed tRNAs due to the absence of tRNA modifications. And as a result, the free energies of the correctly folded and misfolded tRNAs are quite similar (2, 108).

Incorporating an additional purification step, a SEC step that was designed to isolate the differently folded or oligomeric forms of tRNA, to the existing protocol greatly reduced the tRNA mediated aggregation on the grid. As observed in Figure 4-5A and B, the SEC profile of *EctRNA^{Thr}* after annealing revealed a broad peak, suggesting multiple forms of tRNA. Fractions collected from the SEC were analyzed by Native PAGE in the presence and absence of *TmTsaB₂D₂* to check for sequestration of a specific tRNA

species in the complex (Figure 4-5B). The Native PAGE analysis shows multiple bands of tRNA in each fraction. When comparing each tRNA fraction in the absence and presence of *TmTsaB₂D₂*, Fraction 8 and 9 revealed the presence of a faint band that disappeared in the presence of *TmTsaB₂D₂*. Based on this observation, which was checked multiple times, Fraction 9 was collected, concentrated and mixed in equimolar ratio with *TmTsaB₂D₂* to give a final complex concentration of 1.5 μ M (Figure 4-6A). This sample yielded a good particle distribution on the grid (Figure 4-6B).

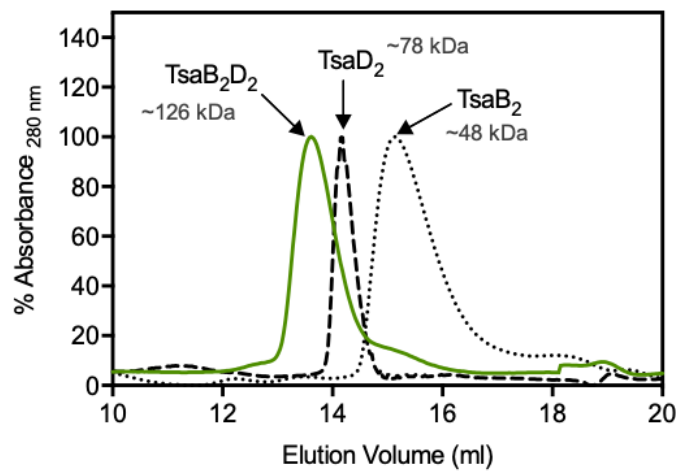


Figure 4-2: *In vitro* reconstitution of *TmTsaB₂D₂* complex using size exclusion chromatography (SEC).

SEC profiles of *TmTsaB₂D₂* formed by mixing 15 μ M of *TmTsaB₂* and *TmTsaD₂*.

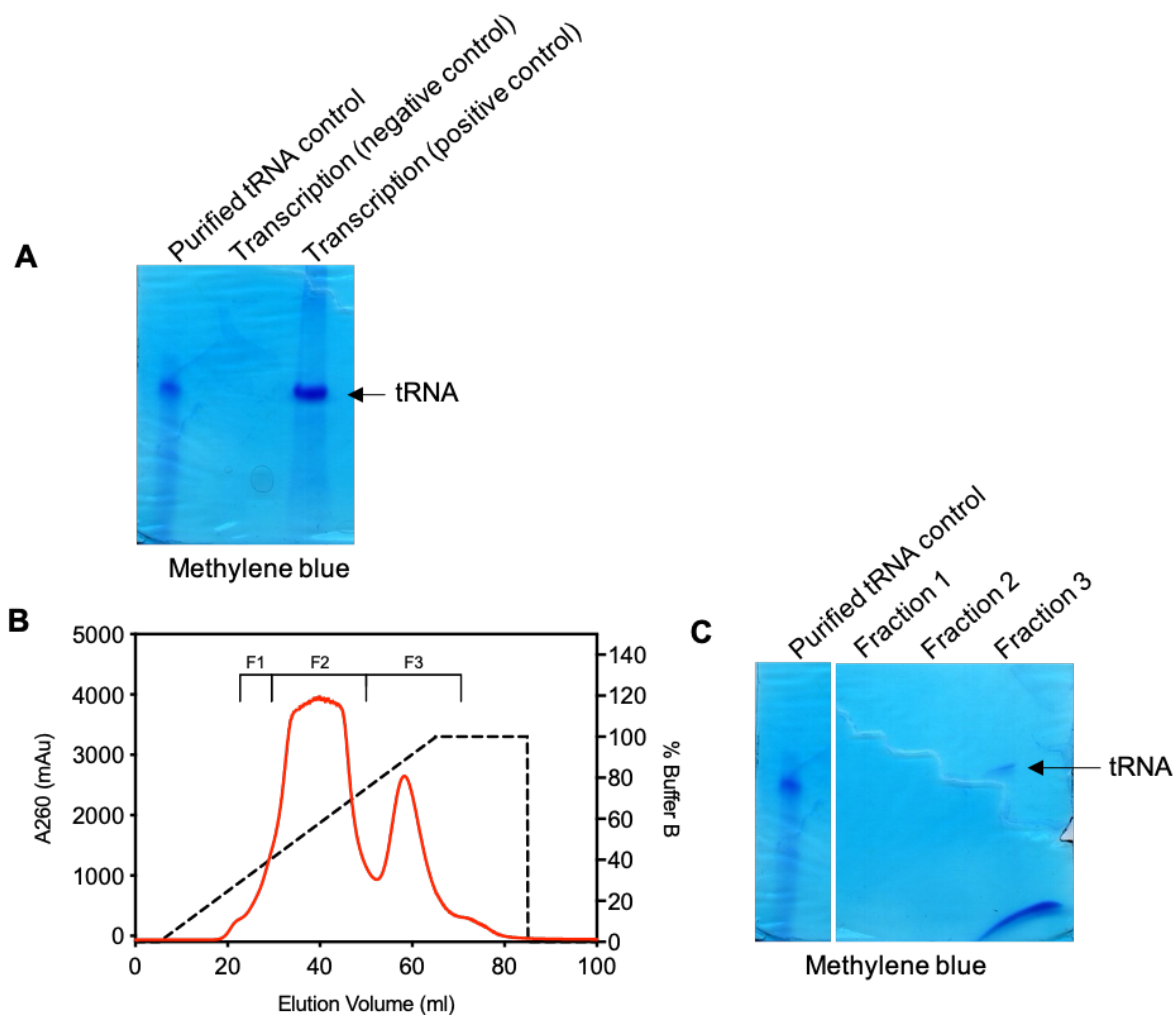


Figure 4-3: Purification of *EctRNA*^{Thr}.

A: *In vitro* transcription products run on a denaturing TBE urea gel indicating successful transcription by T7 RNA polymerase. B: Anion exchange chromatography purification of the *EctRNA*^{Thr}. The dotted line indicates the % Buffer B gradient applied. F1, F2 and F3 corresponds to Fraction 1, Fraction 2 and Fraction 3 respectively C: Anion exchange chromatography fractions run on a denaturing TBE urea gel indicating the presence of tRNA in Fraction 3.

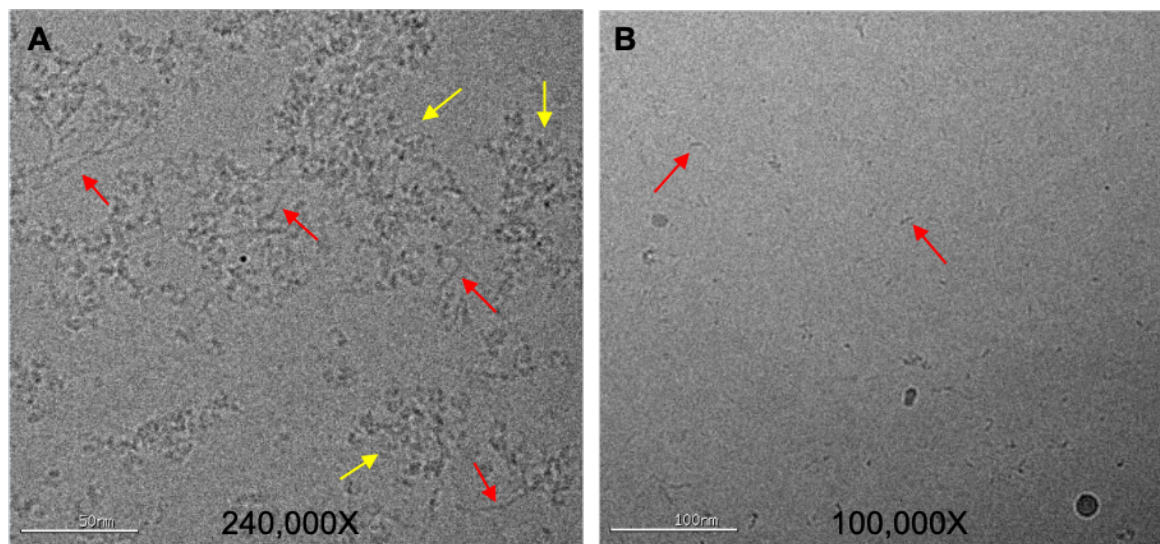


Figure 4-4: Sample micrographs of *TmTsaB₂D₂*-*EctRNA^{Thr}* sample (A) and *EctRNA^{Thr}* only sample (B) before SEC purification of *EctRNA^{Thr}*.

Yellow arrow points to aggregations of *TmTsaB₂D₂*-*EctRNA^{Thr}* complex. Red arrow points to string-like particles that were observed in *TmTsaB₂D₂*-*EctRNA^{Thr}* sample as well as *EctRNA^{Thr}* only sample. Only one concentration of *EctRNA^{Thr}* only sample was tested for this control.

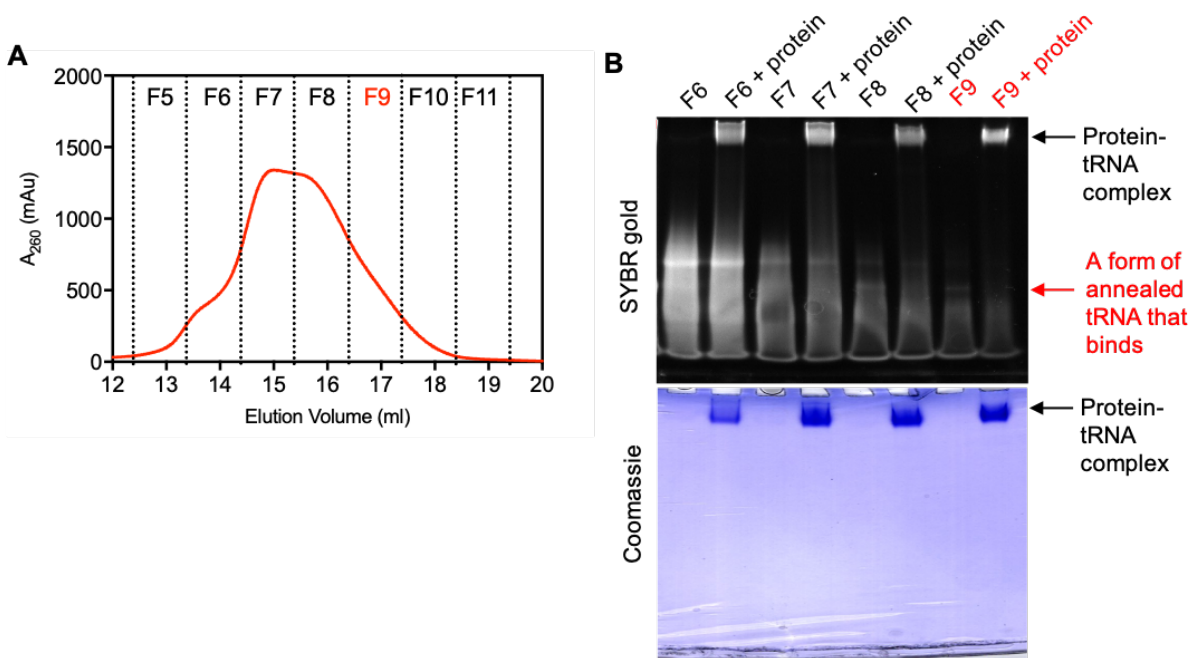


Figure 4-5: SEC of annealed of *EctRNA*^{Thr}.

A: Size exclusion chromatography (SEC) purification of annealed Fraction 3. B: Native Page analysis of each fraction with and without 2 μ M *TmTsaB*₂D₂ protein complex. The red arrow indicates a particular folded tRNA species that binds to the protein. Fraction 9 of the SEC run was used for Cryo-EM sample preparation.

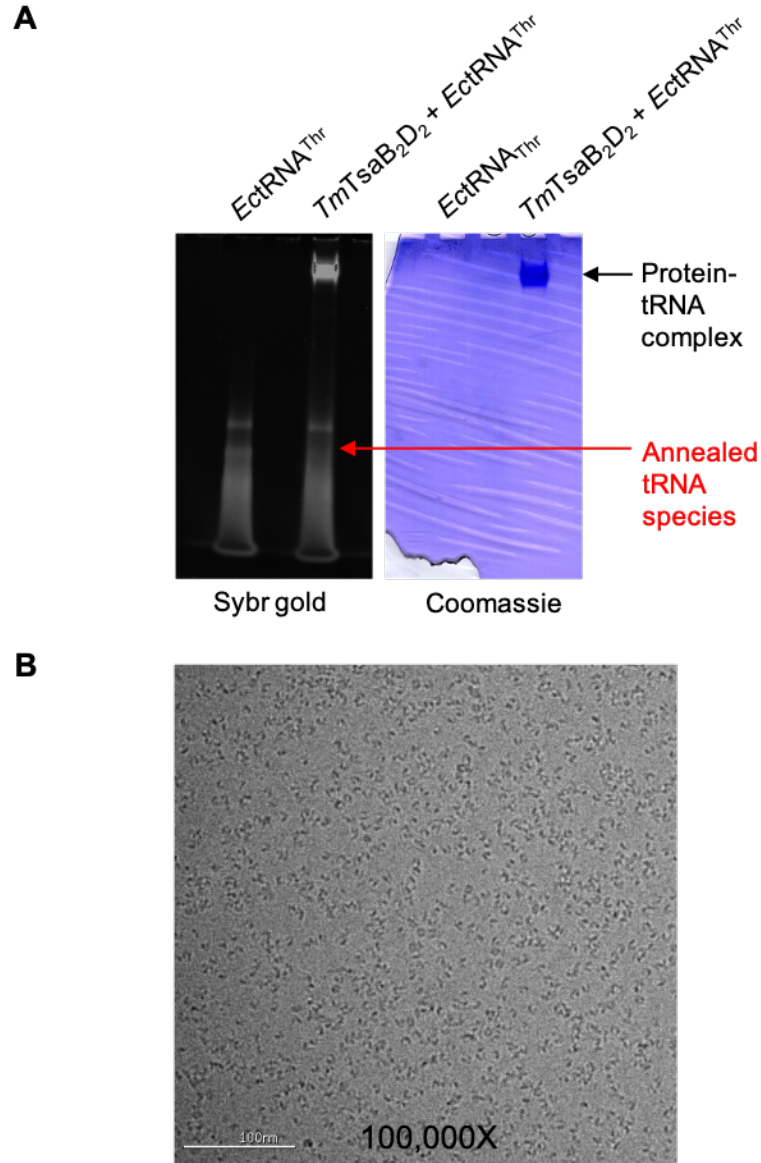


Figure 4-6: Assessment of SEC purified *EctRNA^{Thr}* binding to *TmTsaB₂D₂* in sample prepared for Cryo-EM grid preparation.

A: Native Page analysis of SEC isolated Fraction 9 *EctRNA^{Thr}* with and without *TmTsaB₂D₂* protein complex. The samples were prepared with a 1:1 stoichiometry at 1.5 μ M. B: Sample micrograph of the same complex (1.5 μ M) obtained on the Tecnai F20 electron microscope at 100,000X magnification.

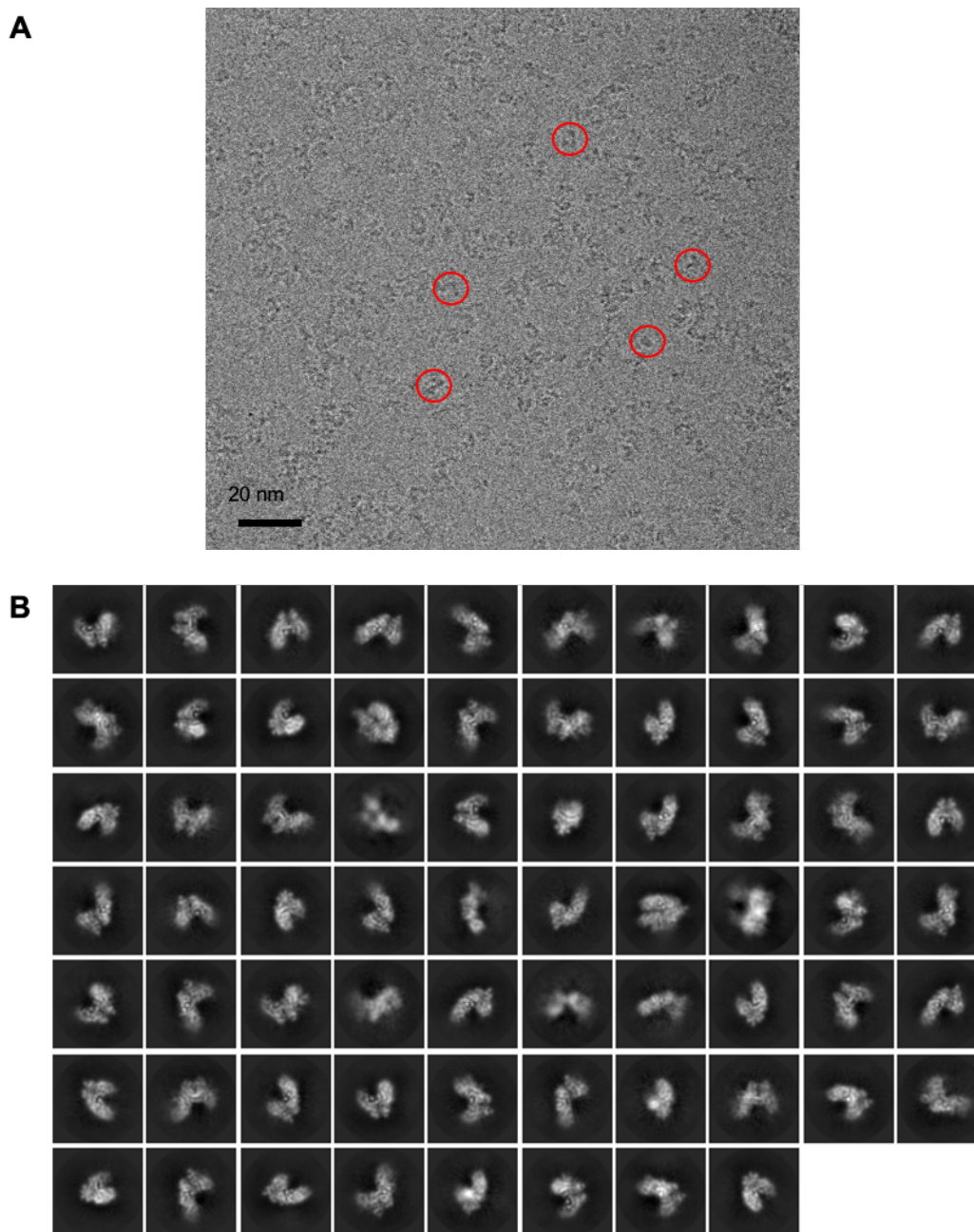


Figure 4-7: Data collection.

A: Representative micrograph for the *TmTsaB₂D₂*-EctRNA^{Thr} complex. Images were collected on a Talos Arctica equipped with a Gatan K2 direct detector operating in counting mode; the magnification was set to 57,000X, with a calibrated pixel size of 0.73 Å. B: Representative 2D class averages as calculated with Cryosparc (106).

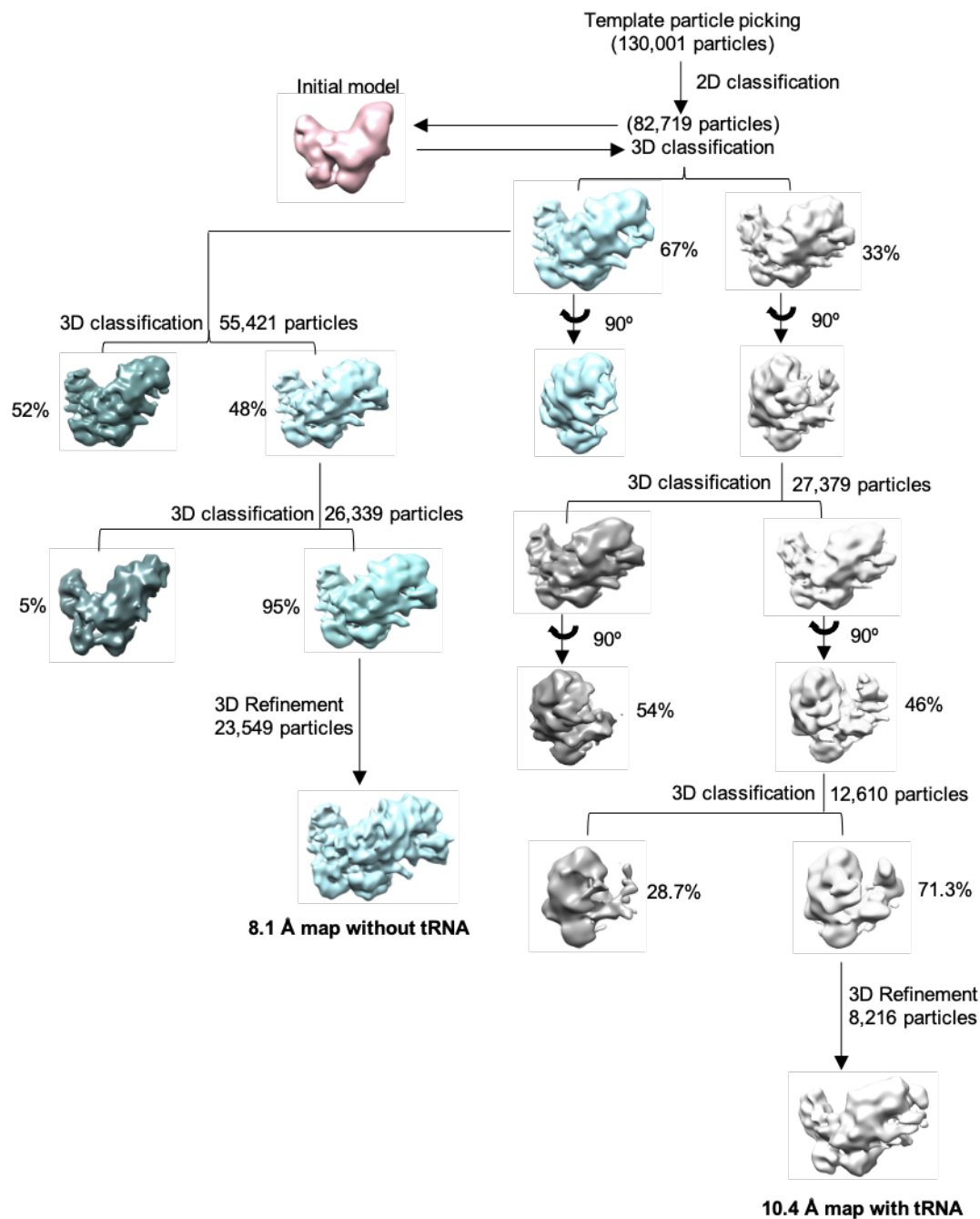


Figure 4-8: Schematic diagram of the data processing.

Two classes, shown in grey and blue were pursued. Rejected particles at each 3D classification are shown in the darker version of each color (dark grey and dark blue). Detailed description of this processing pipeline is mentioned in the Results and Discussion section.

Cryo-EM map of *TmTsaB₂D₂*-*EctRNA^{Thr}*

To analyze the dataset, I performed image analysis using the Cryosparc software suite. The goal was to achieve a Cryo-EM map of *TmTsaB₂D₂*-*EctRNA^{Thr}* complex. From the initial 3D classifications, it was apparent that there are two major classes, one was protein with an L-shaped extra density (shown in grey in Figure 4-8), and one was just protein (shown in blue in Figure 4-8). The extra density although, fragmented and low resolution, was interpreted as the bound tRNA. Based on this observation, further 3D classifications were done with the goal of finding the best set of particles that would give a good protein-tRNA map. At each 3D classification stage, the maximum number of particles that showed a better tRNA density was retained and refined. After refinement, if a good resolution of the tRNA, i.e. helical structure of tRNA with major and minor grooves visible, could not be achieved then it was interpreted as the particle stack needing more pruning to remove the low signal to noise ratio particles within the stack. As a result, more 3D classifications were performed with the same goal as before. At each stage the tRNA density was improved in terms of an L-shaped blob but the resolution of this L-shaped blob did not converge with the pruning of the particle stack. Ultimately, the best map that was achievable was a 10.4 Å map of 8216 particles (Figure 4-9A).

This map was the product of only ~10% of a total of 82,719 particles. This is in part due to the dissociation of protein-RNA complex on the grid at the concentrations used (1.5 µM). This is consistent with the knowledge that the K_d of the *EctRNA^{Thr}* to *TmTsaB₂D₂* complex is ~ 1.3 µM (40). Another reason for the low protein-tRNA particle number is evident in the Euler plot of this map, which shows that the map was reconstructed from particles that had only a few orientations (Figure 4-9C). A sample may lack sufficient

orientations when the sample has a tendency to orient in a few limited orientations versus a multitude of random orientations. This 'preferred specimen orientation' is generated by diffusion mediated adherence of the particle to the air-water interface (AWI) on either sides of the grid (109). Preferred orientation can cause multiple artifacts: AWI adherence leads to an artificial concentrating of the particles on the grid, creating a protein network film, and this protein network may undergo full or partial denaturation at AWI (110, 111). An insufficient number of orientations may result in an oversampling in a few directions and an undersampling (or complete loss of sampling) in other directions of Fourier space during 3D reconstruction, which would lead to an anisotropic electron potential map that lacks features and is characterized by lower resolution along the direction of imaging (112). The complex formed by *EctRNA*^{Thr} and *TmTsaB*₂D₂, like many other samples, adheres to the AWI and is characterized by poor directional resolution. Due to this adherence, and the fact that particles are artefactually concentrated at the AWI, using a sample concentration above 1.5 μ M – a strategy that could help overcome dissociation of the tRNA from the protein complex on the grid – did not yield a good particle distribution. Consistent with AWI adherence-mediated effects, the cryo-EM density map of *TmTsaB*₂D₂-*EctRNA*^{Thr} shows denaturation of one of the *TmTsaB*₂D₂ complex subunits, *TmTsaD* (Front view, Figure 4-9A). Denaturation of one of the *TmTsaD* subunits is consistent with the predominant view observed in the 2D class averages (Figure 4-7), this is the direction of imaging. And along this direction is also where the tRNA density is observed (red arrow in Figure 4-9A, Top view).

A combination of high K_d between *EctRNA*^{Thr} and *TmTsaB*₂D₂, and potential AWI mediated denaturation led to the low *TmTsaB*₂D₂-*EctRNA*^{Thr} particle number of the final map. AWI mediated preferred specimen orientation and thereby lack of sampling along

the direction of imaging, led to loss of directional resolution which in turn led to loss of global resolution. This is displayed well by the histogram of per angle Fourier Shell Correlation (FSC) percentage. The histogram refers to FSC (at 0.143) derived resolutions in 100 evenly spaced directions in 3D space. According to this histogram, the final map shows that ~60% of the directions (out of the 100) has a resolution of ~10 Å while ~40% of the direction has a resolution much lower than 10 Å (Figure 4-9B).

Tilting the sample cannot address denaturation of the sample at the AWI or artificial concentrating of the sample at AWI but it can address non-uniform particle distributions by increasing the sampling in Fourier space. While the 30° tilt images resulted in 2D class averages that had slightly more information than the no tilt images, the 30° tilt was not sufficient to completely overcome the anisotropy as indicated by the Euler plot and the per angle Fourier Shell Correlation (FSC) percentage (Figure 4-9B and C). The Euler plot of this map shows two major views, and this is consistent with the views seen in the 2D class averages of this map. However, the ‘sprinkling’ of views observed in other areas of the Euler plot are indicative of some degree of orientation misalignment during 3D reconstruction. Orientation misalignment is essentially overfitting spatial information in directions spatial information does not exist (or not captured due to preferred orientation) during the 3D reconstruction. A combination of low particle number and severe lack of orientations, as seen in the Euler plot, can lead to orientation misalignment.

The *TmTsaB*₂D₂-EctRNA^{Thr} map shows an extra density that cannot be accounted for by the protein complex. The extra density is L-shaped, as expected for a folded tRNA. However, at a resolution of ~10 Å and possible denaturation, the secondary structural features of the tRNA cannot be resolved, and therefore this density appears as a blob

(Side, Back and Top views, Figure 4-9A). Due to this reason, one cannot model the correct orientation of the tRNA (acceptor stem vs. anticodon) based on experimental evidence. However, given the knowledge that A37 needs to be positioned close to the TC transfer active site in TsaD, and the overall orientation observed in the map, one can logically model that the anticodon approaches the active site from below, while the acceptor stem interacts with the top back surface of TsaD. If the anticodon was modelled to the back surface of TsaD, A37 would be too far away and out of reach of TC transfer site (Figure 4-10A). tRNA binds at the interface of TsaB and TsaD and approaches the TsaBD heterocomplex from the backside (Figure 4-10B) (33, 40). The tRNA makes contact with the back surface of TsaD and binds with the lever arm of TsaD in downward conformation which is consistent with the pre-TCT state of the complex (Front and Side view, Figure 4-9A). The lever arm of TsaD would need to be positioned downwards in order to create the binding area where tRNA is observed to bind with TsaBD heterodimer.

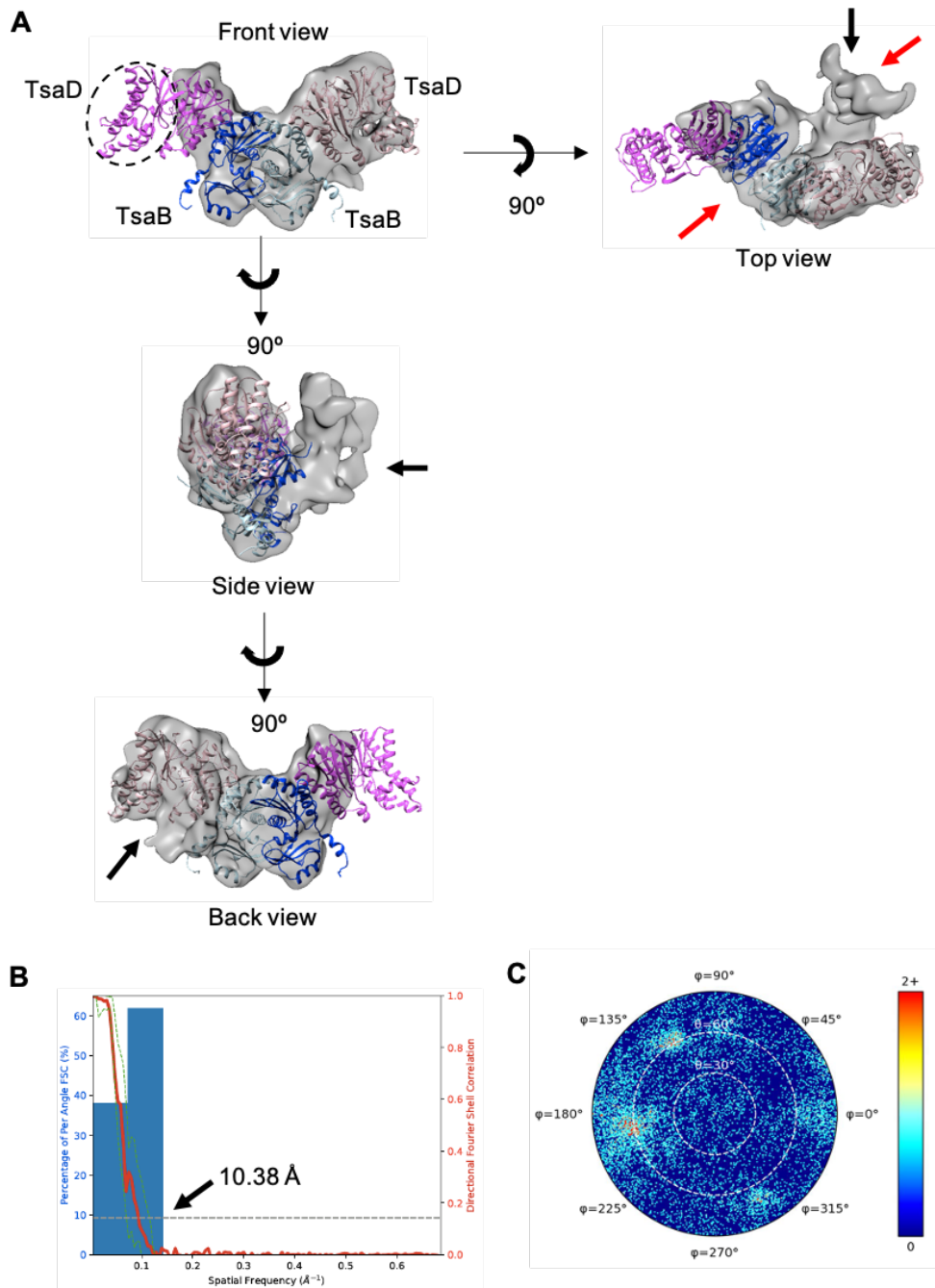


Figure 4-9: Final reconstruction of *TmTsaB₂D₂-EctRNA^{Thr}* resolved to 10.4 Å.

A: Final map of 10.4 Å superimposed with *TmTsaB₂D₂* crystal structure (PDB ID 6N9A) modelled to have the TsaD lever arm in down position. *TmTsaB₂* is shown in blue and cyan and *TmTsaD₂* is shown in magenta and pink. The dotted circle indicates missing cryo-EM density on one of the subunits of the *TmTsaB₂D₂* complex (*TmTsaD*) and the black arrow points to an extra density that represents the *EctRNA^{Thr}*. B: Plot of the global half-map FSC (solid red line) and spread of directional resolution values ($\pm 1\sigma$ from mean is shown in green dotted lines; the blue bars are a histogram of 100 such values evenly sampled over the 3D FSC) generated by 3DFSC (112). C: Angular distributions plot generated by cisTEM (113).

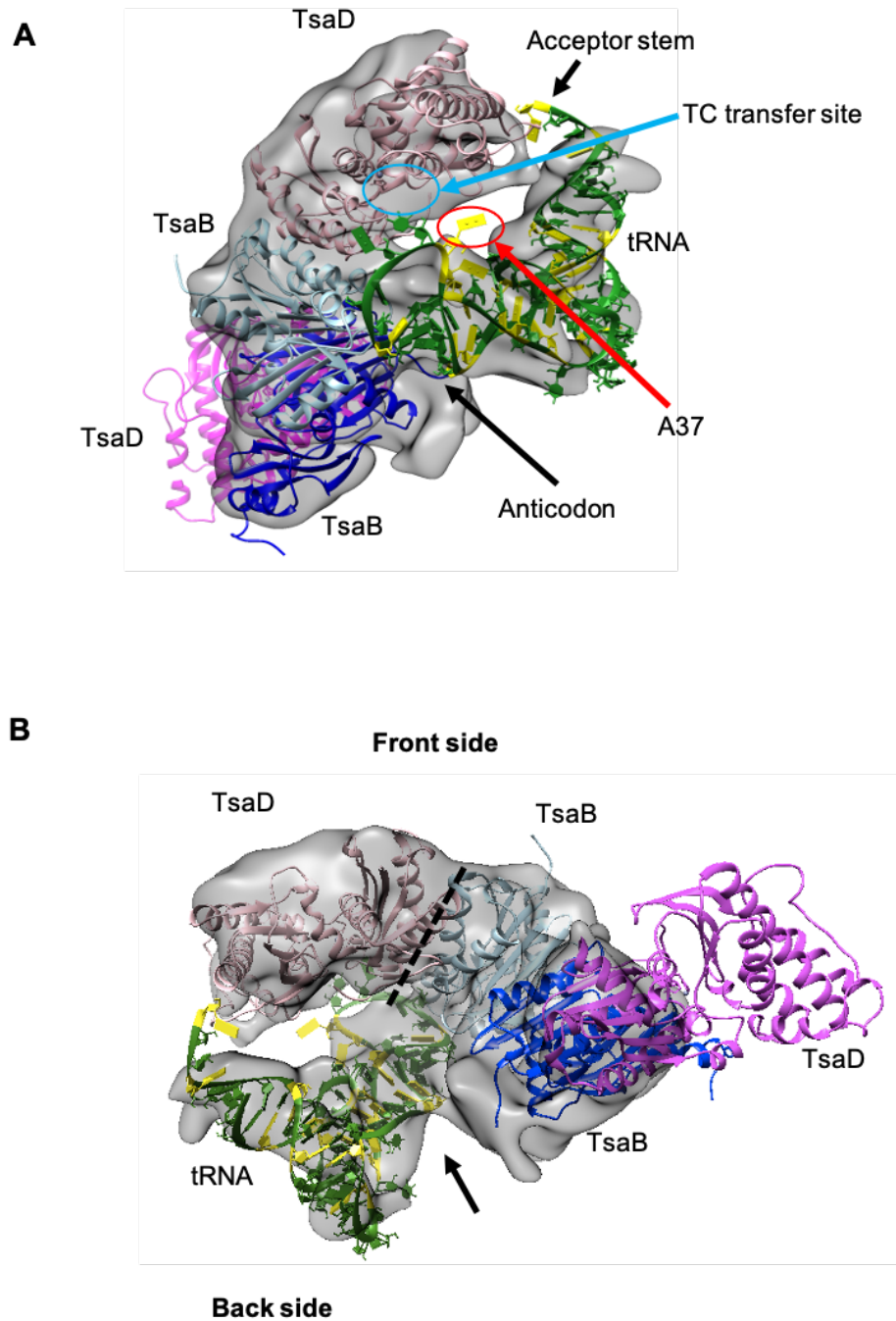


Figure 4-10: *TmTsaB₂D₂*-*EctRNA^{Thr}* map with tRNA model.

A: Side view of the EM map superimposed with *TmTsaB₂D₂* crystal structure (PDB ID 6N9A, modelled to have the TsaD lever arm in down position) and manually docked with *EctRNA^{Thr}* (PDB ID: 1QF6) into the tRNA density. B: Top view of same as in (A). Black dotted line indicates the binding interface between *TmTsaB* and *TmTsaD*. Black arrow points to the tRNA density emerging from the interface of *TmTsaBD* heterodimer.

Cryo-EM map of *TmTsaB₂D₂*

To compare maps in the presence and absence of a L-shaped tRNA density, the particles from the initial 3D classification that yielded density for just the protein was pursued. During each subsequent 3D classification step the maximum number of particles, that yielded the most conformationally homogenous, the least denatured and the best resolved electron potential map, were retained and refined. The final map is 8.1 Å comprised of 23,549 particles (Figure 4-11A). Histogram of per angle FSC percentage shows that ~40% of the directions has a resolution of ~10 Å, ~30% of the directions has a resolution of ~6 Å and another ~30% of the directions has a resolution lower than ~10 Å (Figure 4-11B). The Euler plot shows that the map was reconstructed from particles that had a better spread of orientations (Figure 4-11C). Unlike the previous map, the ‘sprinkling’ effect in the Euler plot of this map is a lot less, and this is likely due to the larger number of particles and particle orientations used for the 3D reconstruction. Between the higher number of particles and a slightly better number of orientations, the reconstruction of this *TmTsaB₂D₂* map has a slightly higher resolution than that of *TmTsaB₂D₂-EctRNA^{Thr}* map (Figure 4-9). Like observed in the *TmTsaB₂D₂-EctRNA^{Thr}* map, this map too shows denaturation of one *TmTsaD* (Figure 4-11A) as both reconstructions were from the same grid.

With the exception of the one *TmTsaD* subunit that is consistently denatured in this dataset, the *TmTsaB₂D₂* map shows a near complete density for the protein. *TmTsaB₂D₂* of this sample appears to be in the same overall conformation as observed for *EcTsaBD* (Figure 4-1). The lever arm of *TmTsaD* is adopting a downward conformation as shown in Figure 4-11C when superimposed with *TmTsaB₂D₂* model (adapted from PDB ID: 6N9A with lever arm modelled down and *TmTsaE* removed). At

this resolution, it is difficult to properly delineate the exact conformation of the C-terminal tail of *TmTsaB* (Front, Side and Top views, Figure 4-11C). The *TmTsaB₂D₂* map does not show density for the helical conformation represented by the *TmTsaB₂D₂* model but rather some density closer to the lever arm of *TmTsaD* (Front view, Figure 4-11C), potentially suggesting the same conformation as that observed in the TsaB subunit of the *EcTsaBD* crystal structure (Figure 4-1)

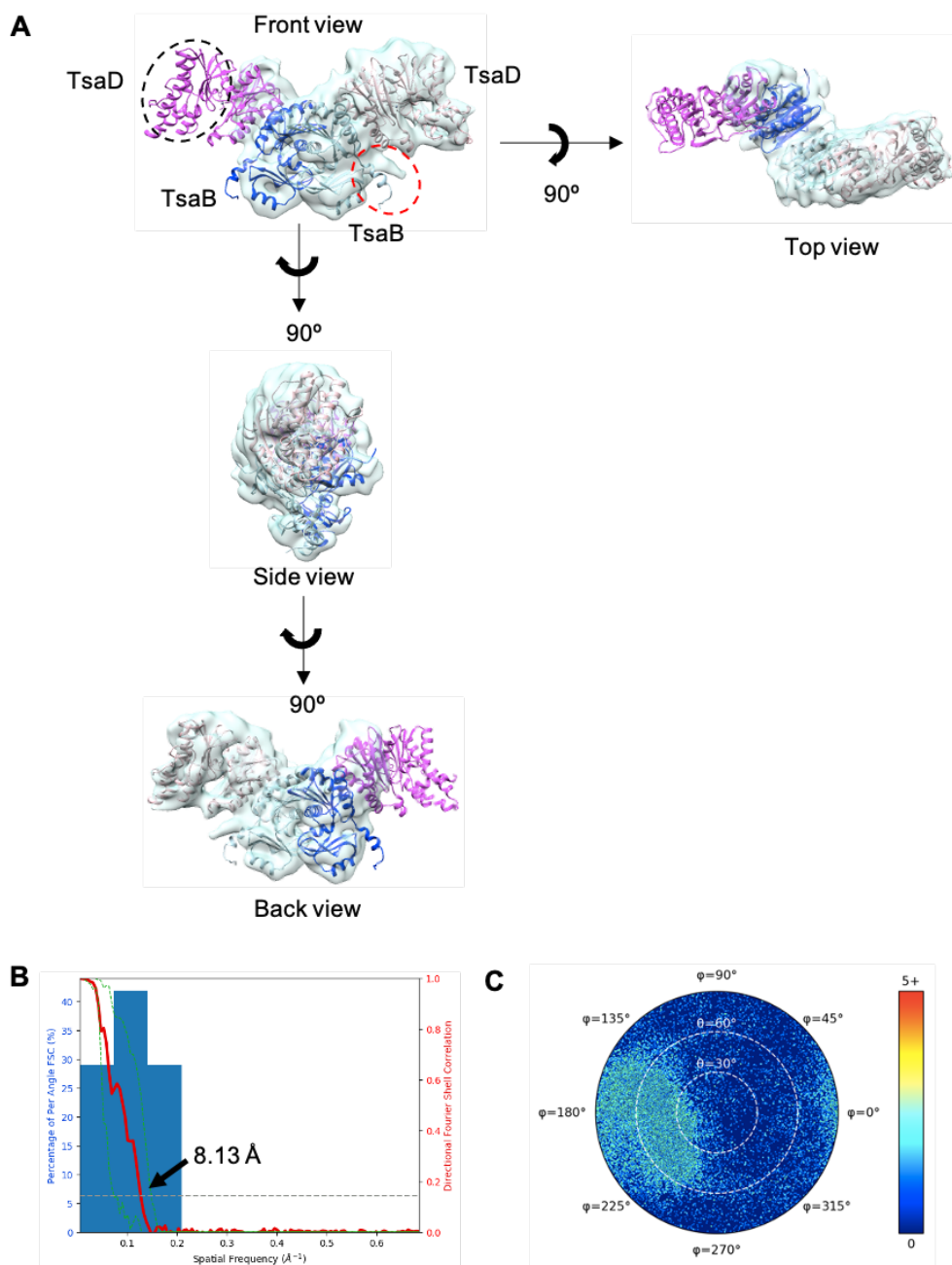


Figure 4-11: Final reconstruction of *TmTsaB₂D₂* using C1 symmetry to a resolution of 8.13 Å.

A: Final map of 8.1 Å superimposed with *TmTsaB₂D₂* crystal structure (PDB ID 6N9A) modelled to have the TsaD lever arm in downward position. *TmTsaB₂* is shown in blue and cyan and *TmTsaD₂* is shown in magenta and pink. The dotted circle indicates missing EM density on one of the subunits of the *TmTsaB₂D₂* complex (*TmTsaD*) and the red dotted circle shows the C-terminal tail of *TmTsaB*. B: Plot of the global half-map FSC (solid red line) and spread of directional resolution values ($\pm 1\sigma$ from mean is shown in green dotted lines; the blue bars are a histogram of 100 such values evenly sampled over the 3D FSC) generated by the program 3DFSC (112). C: Angular distributions plot generated by the program cisTEM (113).

Other attempted experiments for Cryo-EM study

The low resolution of the cryo-EM maps reported in this chapter is due to the low number of particles containing bound tRNA and the AWI-mediated preferred specimen orientation and denaturation of the particles on the grid. AWI-mediated preferred orientation is very common, and most samples exhibit amount of preferred orientation on grids (114). However, the extent of the detrimental effects caused by AWI are sample-dependent. There are cases where substantial denaturation is observed (115), but in other instances, the effects may be negligible.

Other factors that can affect resolution of a reconstruction is the ice thickness and beam induced motion. If ice is too thick, this can decrease the signal-to-noise ratio of the final images. While technological advances have almost completely overcome beam induced motion, there may still be some motions that are too large especially when the sample is tilted at high angles.

In order to overcome some of the potential causes contributing to the low resolution of the maps, several approaches were attempted and had no success. These attempts are tabulated in Table 4-1.

Table 4-1: Attempted experiments for Cryo-EM study

Potential cause	Approach	Protein complex	Conditions screened	Negative staining control	Vitrification outcome
Insufficient number of protein-RNA particles	Crosslink using glutaraldehyde	<i>TmTsaB₂D₂-EctRNA^{Thr}</i> in 50 mM HEPES pH 7.5, 50 mM KCl, 1 mM DTT, 2 mM MgCl ₂	Glutaraldehyde concentration, incubation time, temperature and complex concentration	Could not detect a protein-RNA crosslinked species	
Anisotropy and AWI mediated denaturation	Apply surfactants to cover the AWI	SEC isolated <i>TmTsaB₂D₂E₁-TmtRNA^{Thr}</i> in 50 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 2 mM MgCl ₂	1X CMC*, 2X CMC and 4X CMC of NP40 against 100 μ M complex	N/A	Vitrification did not yield good particle distribution. Thick ice. Data collected and processed did not yield a resolution better than 10 Å.
Sample specific	Try different protein-tRNA complexes	SEC isolated <i>TmTsaB₂D₂E₁-TmtRNA^{Thr}</i> in 50 mM Tris pH 7.5, 50 mM KCl, 1 mM DTT, 2 mM MgCl ₂	Changed protein complex and tRNA species	N/A	Vitrification gave ok particle distribution. Thick ice. Data collected and processed did not yield a resolution better than 10 Å.
Buffer	Optimize buffer and pH based on a study that correlated optimal buffer conditions as that of crystallization conditions (116).	SEC isolated <i>TmTsaB₂D₂-TmtRNA^{Thr}</i>	One condition that is close to crystallization pH of <i>TmTsaB₂D₂E₂</i> tested: 100 mM Na Citrate pH 5.6, 100 mM KCl, 1 mM DTT, 18 mM MgCl ₂ , 500 mM NaCl.	Particles are intact and stable in this buffer and resulted in a good negative stained EM map of 14 Å.	Vitrification gave ok particle distribution. Thick ice. Data collected and processed did not yield a resolution better than 10 Å.

*CMC is critical micellar concentration

Crystallization of *TmTsaB*₂D₂E₁ in complex with ASL RNA.

Attempts to co-crystallize *TmTsaB*₂D₂E₁ in complex with *TmASL*^{Lys/Thr/Ser} yielded improved crystals only for the complex with *TmASL*^{Lys} or *TmASL*^{Thr}, and the improvement was primarily in the overall intensity and quality of diffraction, but not in resolution which stayed at 7.5 Å (Figure 4-12). The new crystallization conditions were 24% Precipitant mix 3, 0.1 M Buffer system 1 pH 6.5, and 0.13 M Carboxylic acid mix. These conditions more than doubled crystal size and greatly reduced clustering of the crystals (although did not fully eliminate it) which grew mostly as thin plates with a few satellite plates in the center. Analysis of dissolved crystals by native PAGE confirmed the presence of the RNA in the crystal as shown in Figure 4-12C. The crystals were anisotropically disordered, as reflected by large variations in diffraction quality along different crystal axes. It is possible that the presence of the chaotropic agent glycerol in the Precipitant mix 3 contributed to crystal disorder, as glycerol breaks hydrogen bond networks between protein molecules, potentially interfering with the crystallization process. The additive screen gave a single crystal which grew in the presence of added 10 mM ZnCl₂, but could not be reproduced, nor did it diffract.

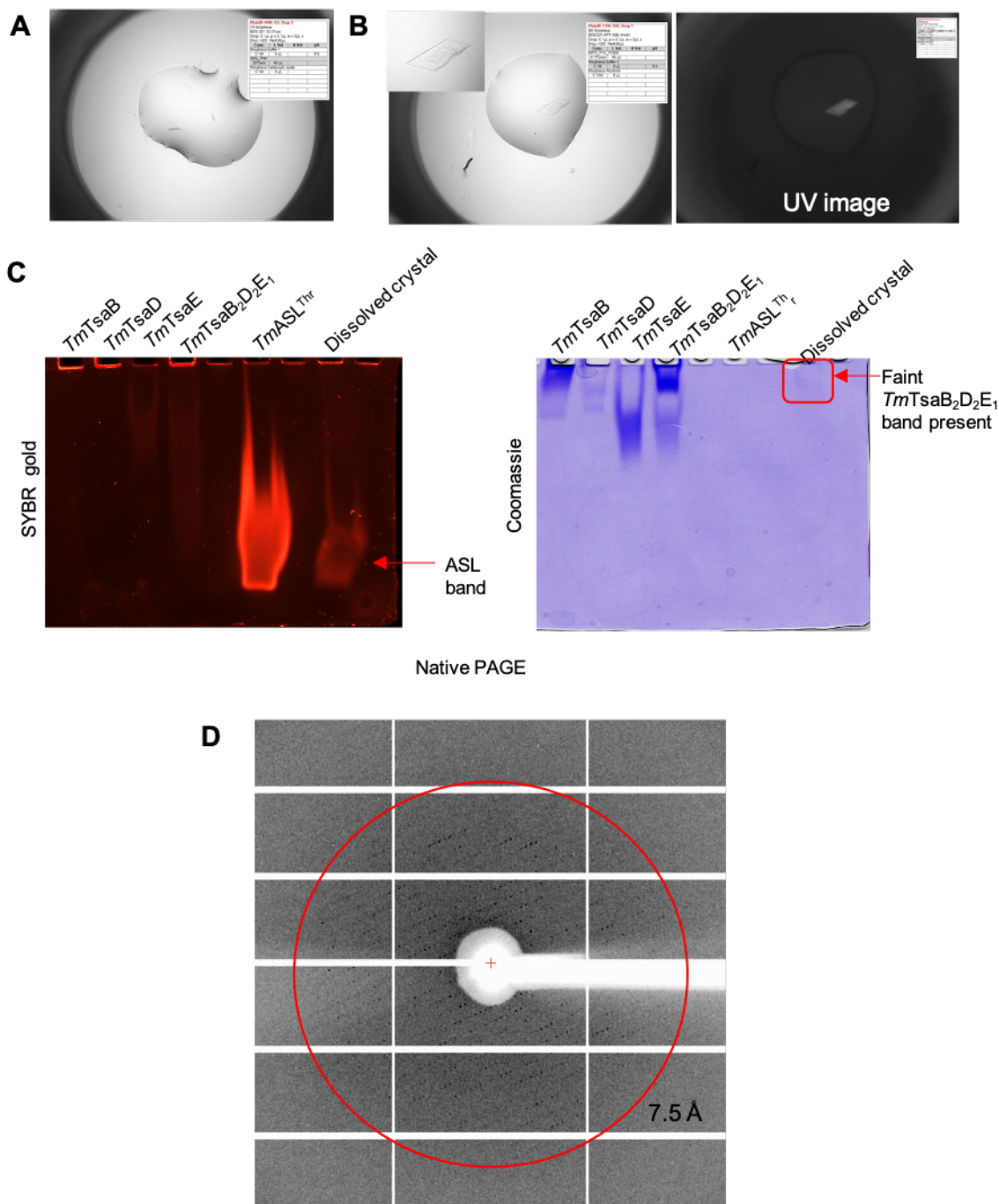


Figure 4-12: Crystallization of *TmTsaB₂D₂E₁* and *TmASL^{Thr}*

A: Initial hit from Morpheus I screen, reagent G3. B: Crystal after improving buffer pH, salt and precipitant concentrations. C: Native PAGE analysis of washed crystals for presence of bound ASL. D: Synchrotron X-ray diffraction by crystals grown from optimized conditions thus far. Space group is $P2_1$ and unit cell dimensions are 182.25, 108.29, 251.32 Å.

4.5 Future Directions

Within each approach mentioned in Table 4-1, there are still several conditions one can test. For example, the crosslinking of protein to tRNA using glutaraldehyde did not yield a crosslinked product. However, glutaraldehyde is a $\sim 5 \text{ \AA}$ crosslinker and may not have been too short to crosslink the complex. One can test other free amine crosslinkers with a longer spacer such as DSP or BS3, both of which have $\sim 12 \text{ \AA}$ spacer. Free amine mediated crosslinking of tRNA to protein relies on the amines available on either macromolecule to react with the crosslinker. While both tRNA and protein have free amines, free amine crosslinkers have traditionally favored protein-protein crosslinking over other types of crosslinking. Therefore, even if this method successfully crosslinked the protein and tRNA, it may not provide a high yield. However, an advantage of cryo-EM is that it only requires small amount of sample ($\sim 1 \text{ mg/ml}$, $\sim 10 \text{ \mu l}$). Crosslinking will not deter AWI adherence or its detrimental effects, but it can increase the number of protein-RNA particles.

Another approach that can be revisited is the application of surfactants to cover the AWI. NP40, a non-ionic surfactant did not result in a good particle distribution on the grid, as not every surfactant will. One can test other non-ionic surfactants as well as zwitterionic and ionic surfactants. Since zwitterionic and ionic surfactants may interfere with complex formation which in this case is largely mediated by electrostatic interaction, with tRNA being a negatively charged species and the *TmTsaB₂D₂* complex hosting a positively charged cavity, one can test for complex formation in the presence of CMC levels of a particular surfactant using SEC. Alternatively one can also test amphipathic polymers such as Amphipol. The concentration of the protein-RNA complex needed to give a good distribution at CMC is different for every sample and surfactant, therefore one

would need to titrate the sample to find a good particle distribution. It is important to keep surfactants at CMC or higher, as this will enable the complete coverage of the AWI and thus prevention of AWI mediated preferred orientation.

Another approach to avoid AWI, is to outrun the diffusion mediated AWI adherence by decreasing the time between spot to plunge. An instrument named Spotiton that can perform very short spot-to-plunge times was first designed by Prof. Bridget Carragher Lab. Use of Spotiton on some samples with a history of preferred orientation, have demonstrated an increase in the particle orientations and an improvement in the resulting reconstruction (117). Higher concentration of sample will be needed to execute this approach as with the minimizing of AWI adherence, the artefact of artificial concentration is also minimized. However, this method may not work on all samples, as every sample behaves differently.

For crystallographic study of *Tm*TsaB₂D₂E₁-ASL^{Thr/Lys} complex, one can try two approaches to decrease the disorder in axis perpendicular to the thin plate. The additive screen was conducted at a single concentration. At this concentration, an additive might have resulted in a clear or precipitated drop, but that does not mean that those conditions have no productive effect at all, as a clear drop could be an indication of too little additive and a precipitated drop could be an indication of too much additive. Therefore, one can screen a range of concentrations for each of the 96 additives. Another approach in decreasing the disorder is to lower the temperature at which the crystal grows.

4.6 Acknowledgment

I am extremely thankful for Prof. Dmitry Lyumkis for the very generous opportunity to learn cryo-EM. His kindness, scholarly guidance and persistent interest in this project was pivotal for the completion of this task. I would like to thank Youngmin Jeon of Lyumkis Lab for preparing the grid and collecting the dataset that yielded the two Cryo-EM maps presented in this chapter. I would also like to thank the following Lyumkis Lab members for training me and being a constant support through data processing, grid preparation and microscope use: Dr. Jessica Bruhn, Dr. Jessica Rabuck-Gibbons, Dr. Sriram Aiyer, Dr. Zelin Shan, Dr. Cheng Zhang, Dr. Dario Passos, and Dr. Phil Baldwin.

Chapter 5 : Catalytic mechanism and inhibition of the prokaryotic-specific GTP cyclohydrolase IB

Abstract

GTP cyclohydrolase I (GCYH-I) catalyzes the first step in folate biosynthesis in bacteria and plants, biopterin biosynthesis in mammals, and the biosynthesis of 7-deazaguanosine-modified nucleosides of tRNA and DNA in various microbes. Many pathogen, including *Neisseria gonorrhoeae*, rely on a form of the enzyme (GCYH-IB) that is structurally distinct from the human GCYH-IA enzyme involved in biopterin biosynthesis, positioning GCYH-IB as an attractive antimicrobial target. In this study, we investigated the catalytic mechanism of GCYH-IB and potential for selective targeting by structure-based inhibitors. Crystal structures of *N. gonorrhoeae* GYCH-IB with bound reaction-intermediate mimics have revealed an S-nitrosylated active-site Cys149 in contact with or in proximity of the ligands. Using an iodo-TMT/Western blot assay and single-site mutagenesis, I confirmed the site-specific nitrosylation of Cys149 and showed that S-nitrosylation maintains enzyme activity, suggesting a potential role of the S-nitrosothiol in catalysis. Further, structure-guided-mutagenesis data show that, together with Cys149, conserved active site residues His246 and Glu243 are required for H₂NTP production. Based on these results and the crystal structures, we proposed a catalytic mechanism for the GCYH-IB enzyme that is consistent with all experimental data, and in which His246 and Glu243, respectively, coordinate and deprotonate several catalytic water molecules in the early steps of the reaction, while Cys149 participates in opening the GTP ribose ring and subsequent amadori rearrangement in the late stage of the reaction. Finally, four 8-oxo-G derivative compounds, designed using a structure-based approach with ether linkages at the guanine O⁶ and O⁸ and synthesized by the Purse lab,

were tested for selective inhibition of *N. gonorrhoeae* GCYH-IB and human GCYH-IA *in vitro*. The most potent of these compounds, G3, is three-fold more potent against GCYH-IB than GCYH-IA, and 31-fold more selective against the –IB enzyme than the reaction intermediate analog 8-oxo-GTP, supporting the promise of GCYH-IB as a target for a new class of small molecule antibiotics.

5.1 Introduction

Proposed reaction mechanism for GCYH-IA

In 2005, *Tanaka et al.* proposed a fairly detailed novel reaction mechanism for the *Thermus thermophilus* (*Tt*) GYCH-IA enzyme (61). They found 8-oxo-GTP to have a binding affinity that was three orders of magnitude greater than that of GTP and thereby proposed 8-oxo-GTP to be a transition state analogue of GCYH-IA. They solved, interpreted a high-resolution crystal structure of *Tt*GCYH-IA bound with 8-oxo-GTP and factored in any experimentally validated reaction intermediates from prior studies (118, 119) to propose a reaction mechanism. In their proposal, an activated water molecule nucleophilically attacks the C8 atom of GTP to give a tetrahedral transition state. N7 atom gets protonated from a water molecule and this breaks the C8-N9 bond to produce the experimentally validated intermediate AFRPT (2-amino-5-formylamino-(6 β)-ribosylamino-4(3H)-pyrimidone 5'-triphosphate). A hydroxyl ion resulting from the deprotonation of a water molecule attacks the C8 atom that leads to the cleavage of C8-N7 bond to form another tetrahedral intermediate, DRPT (2,5-diamino-6 β)-ribosylamino-4(3H)-pyrimidone 5'-triphosphate) and the bi-product, formate. DRPT is a proposed reaction intermediate (120). DRPT forms a Schiff base at N9 atom, which initiates an Amadori rearrangement, where the Schiff base is converted an enol form and then immediately isomerized to the keto form. N7 atom of the keto form then nucleophilically attacks C2' atom resulting in the closing and dehydration of the pteridine ring to give the final product dihydroneopterin (61).

Crystal structures of *N. gonorrhea* GYCH-IB bound with ligands

Two *Ng*GYCH-IB enzyme structures have been determined, enzyme bound with the molecule TRIS and enzyme bound with the inhibitor nucleotide 8-oxo-GTP in the active sites (62). The structures reveal a homotetramer with each subunit composed of two T-fold modules, characteristic of bimodular T-fold enzymes. The two β -barrels join together head-to-head to form a central tunnel (Figure 5-1). The enzyme active sites are located at the interfaces between three subunits, two from the same β -barrel and one from the opposite β -barrel. The active site consists of a Zn^{2+} ion that binds at the interface between opposite β -barrels and is coordinated by Cys147, His159 and Glu201 (all invariant residues). While the complex makes a total of four active sites, two of the active sites observed in the crystal structures are more solvent exposed and a little disordered (62). Interestingly, an invariant active site residue, Cys149 was observed to be nitrosylated on the Sy atom.

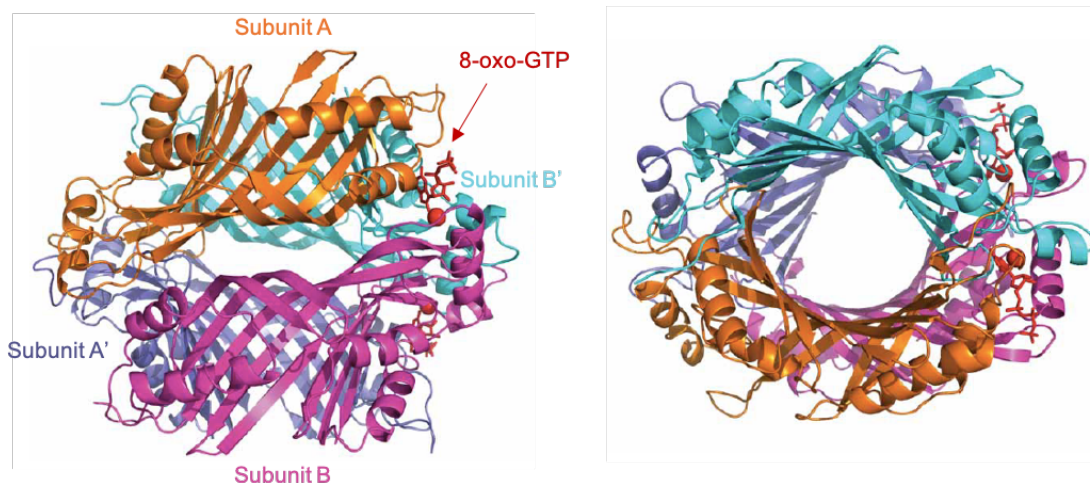


Figure 5-1: Crystal structure of *Ng*GYCH-IB in complex with 8-oxo-GTP.

Side (left) and top (right) views of the homotetramer enzyme with subunits (A, B, A' and B') shown in different colors. Bound Zn^{2+} ions are shown as red spheres and bound 8-oxo-GTP molecules in two of the four active sites of the homotetramer are shown in red stick representation.

In the 8-oxo-GTP bound structure of 2.8 Å resolution (PDB ID: 5K95), the O8 atom of 8-oxo-GTP completes a distorted tetrahedral geometry at the Zn^{2+} ion. The purine N7, which is protonated in 8-oxo-GTP, donates a hydrogen bond to the side chain of the invariant residue Glu243 (Figure 5-2). It was proposed that the latter interaction has a role for Glu243 in the protonation of N7 of the GTP substrate, an event necessary for the first hydration step of the GCYH-I reaction. The ribosyl 2'-OH group coordinates by a hydrogen bond (H-bond) to the carboxylate of Glu201 from subunit B'. The ribosyl C2' atom is within contact distance from the S_γ atom of the conserved and nitrosylated residue Cys149 (Figure 5-2). The S-nitrosothiol adopts a staggered anti-conformation with a $\text{C}\beta\text{--S}_\gamma\text{--N--O}$ dihedral angle of 103° . This conformation suggests a stable reduced thionitroxide radical form in which a hydrogen is bonded to the nitrogen atom ($\text{C}\beta\text{--S}_\gamma\text{--NH--O}\cdot$) (121). S-nitrosylation of Cys149 is also seen in the unoccupied active sites and with a similar staggered conformation. In the TRIS molecule bound structure of 1.9 Å resolution (PDB ID: 5K9G), TRIS molecule is seen coordinating the Zn^{2+} ion where, the Zn^{2+} ion forms a trigonal bipyramidal co-ordination shell with its five ligands: Cys147, Glu201 from subunit B', and the amino group of bound TRIS in the equatorial plane; and the O3 hydroxyl group of TRIS and the His159 as axial ligands (Figure 5-2). The S-nitrosyl group of Cys149 in this structure, is in a syn-conformation and exhibits a planar geometry with a small $\text{C}\beta\text{--S}_\gamma\text{--N--O}$ dihedral angle of $\sim 24^\circ$, close to the geometry of small-molecule S-nitrosothiols (122–127), indicating that it is the oxidized form $\text{C}\beta\text{--S}_\gamma\text{--N=O}$ (121).

When the two crystal structures of *Ng*GCYH-IB bounds with 8-oxo-GTP and TRIS molecule were superimposed several important observations were made. The bound TRIS molecule was found to be reminiscent of a tetrahedral transition-state intermediate proposed to occur in the GCYH-IA-catalyzed reaction prior to breakage of the C8-N7 bond and release of formate from GTP (Figure 5-2) (61). In this proposed intermediate, C8 is the tetrahedral center that is imitated by the tetrahedral carbon atom of TRIS. The N atom of TRIS occupies the same site as the O8 atom of 8-oxo-GTP, thus mimicking the state of the metal-coordinated hydroxyl ion after it performs the first nucleophilic attack on the C8 atom of the substrate. The C2 carbon of TRIS mimics the state of the second water molecule after it performs the second nucleophilic attack on C8. Notably, the O2 atom of TRIS makes a hydrogen bond with the conserved side chain His246 (Figure 5-2), and the position of O2 may represent the position of the second nucleophilic water molecule just before its attack on C8 and formation of the tetrahedral center. Furthermore, the metal-coordinating O3 atom of TRIS occupies an analogous position to the 2'-OH of 8-oxo-GTP and, thus, may represent the position of the 2'-OH of the substrate during a late step in the reaction after opening of the ribose ring. Finally, a 6 Å-wide water-filled cavity located behind the purine-binding pocket is seen in both structures and may serve as the putative binding site for the formate molecule released in the early steps of the GCYH reaction (Figure 5-2).

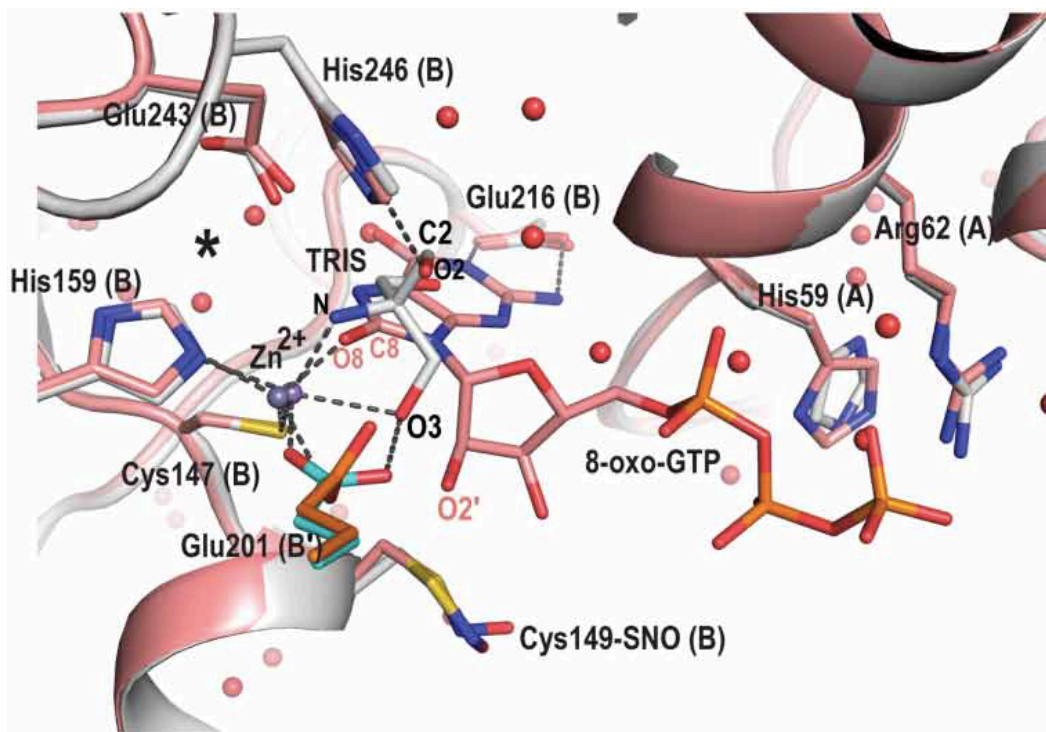


Figure 5-2: Active-site region of the superposition of the 8-oxo-GTP-bound structure (pink, PDB ID 5K95) and the TRIS-bound structure (gray, PDB ID 5K9G). Zn^{2+} is shown as a purple sphere, and water molecules as red spheres. Side chains interacting with 8-oxo-GTP are labeled and the subunits from which they originate are indicated in parentheses. TRIS and 8-oxo-GTP atoms discussed in the text are labeled in black and pink, respectively. The water-filled cavity serving as the putative formate binding site is marked with an asterisk.

Comparison of GCYH-IA and GCYH-IB binding pockets

A comparison of the crystal structures of *Ng*GCYH-IB and *Tt*GCYH-IA (both bound with 8-oxo-GTP) has identified three predominant regions of difference that could be exploited to improve inhibitor selectivity (Figures 5-3B). The largest difference is in the region named Pocket 1 (size $\sim 40 \text{ \AA}^3$), a site that is occupied by two water molecules when 8-oxo-GTP is bound to GCYH-IB (62). This pocket is expected to be the easiest to address synthetically, because it projects directly outward from O6 of the 8-oxoguanine nucleobase. Pocket 2 (size $\sim 100 \text{ \AA}^3$) is predominantly above the plane of the nucleobase (as oriented in Figure 5-3B). Pocket 3 is the opening of the active site and accommodates the ribose and phosphate groups. The glycosidic bond angle is anti when 8-oxo-GTP is bound to GCYH-IA and syn in GCYH-IB, resulting in a substantially different conformation of the inhibitor. Based on these crystallographically observed active-site differences, it was proposed that a new class of antifolate antibiotics can be developed by modifying the structure of 8-oxo-GTP so as to enhance potency against bacterial GCYH-IB and ablate binding to human (*Hs*) GCYH-IA, which exhibits 45% overall sequence identity to *Tt*GCYH-IA and identical active site residues and 3D structure (r.m.s.d. $0.86 \text{ \AA}$ over 817 C α atoms, Figure 5-3A).

George N. Samaan of Byron Purse Lab, Prof. Byron Purse and Prof. David A. Hecht designed a small set of guanosine analogue inhibitors (G1-3, Figure 5-4) with increasingly large substituents oriented towards the larger active site pockets 1 and 2 of GCYH-IB (Figure 5-4).

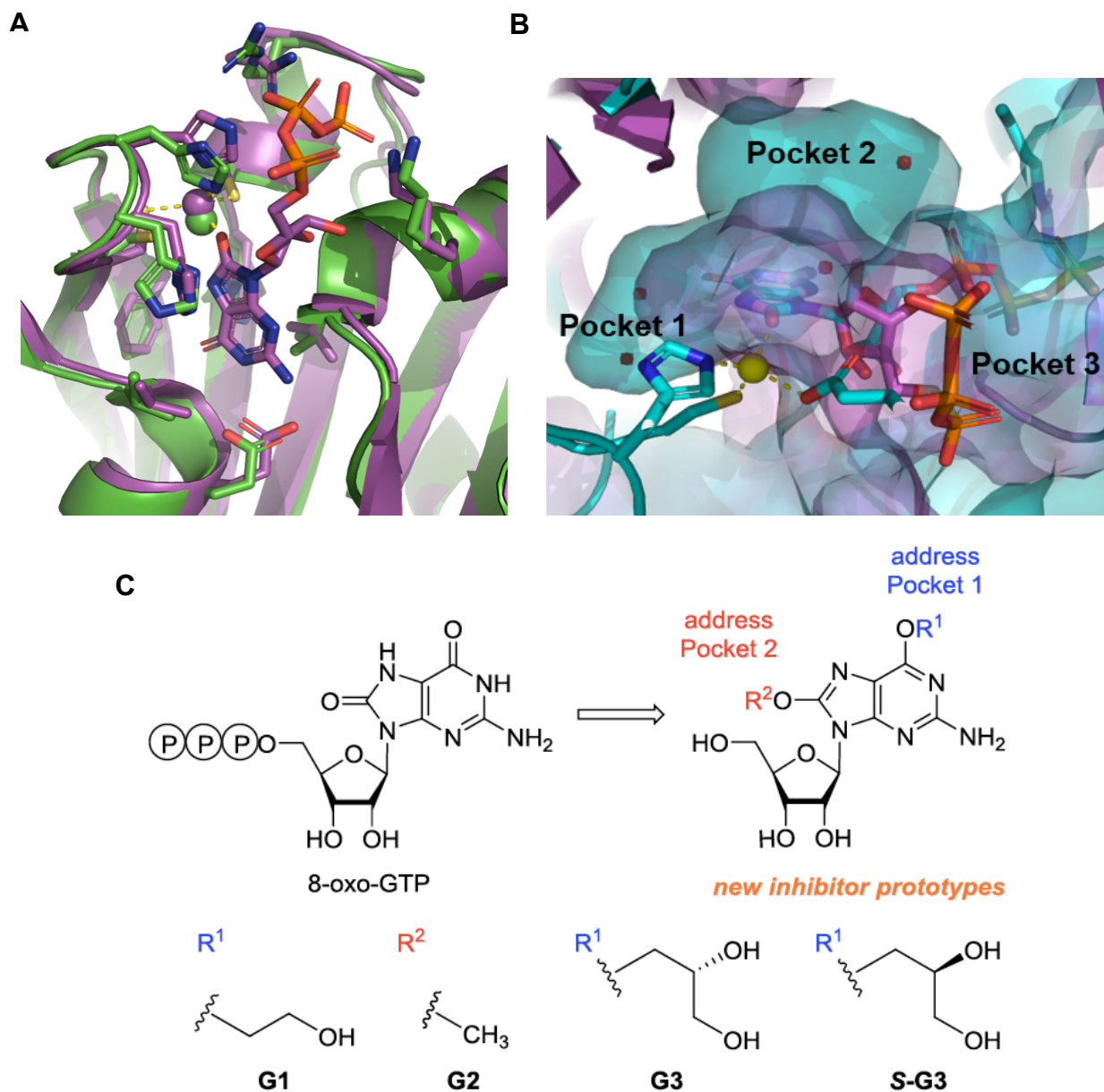


Figure 5-3: Basis for design of human GCYH-IA selective inhibitors.

A: Superposition of the crystal structures of *Tt*GCYH-IA in complex with 8-oxo-GTP (magenta, PDB ID 1WUQ) and *Hs*GCYH-IA (green, PDB ID 1FB1) in the active site region showing identical active site residues. Zn^{2+} ions are shown as spheres, active-site residues and the 8-oxo-GTP ligand bound to *Tt*GCYH-IA are shown in stick model. B: Superposition of *Ng*GCYH-IB (cyan, PDB ID 5K95) and *Tt*GCYH-IA (magenta, PDB ID 1WUQ) in the active site region showing bound 8-oxo-GTP and Zn^{2+} . Surface representations of the superposed active site cavities highlight the additional space available in Pockets 1 and 2 of GCYH-IB. The metal ion and water molecules are shown as yellow and red spheres, respectively. C: Prototype inhibitor designs to probe the filling of Pockets 1 and 2 of *Ng*GCYH-IB. For G1, G3, S-G3 $\text{R}^2 = \text{H}$. For G2 $\text{R}^1 = \text{H}$

In this chapter, I biochemically validated the presence of a S-nitrosothiol group on the conserved catalytic residue Cys149 of *Ng*GCYH-IB crystal structures using site-directed mutagenesis and biochemical methods. I then used the information revealed by the two *Ng*GCYH-IB crystal structures, site-directed mutagenesis, enzyme activity assays and the proposed reaction mechanism *Tt*GYCH-IA (61) to propose a reaction mechanism for *Ng*GCYH-IB. I also purified human GCYH-IA and assayed four inhibitors designed by Prof. Byron Purse and Prof. David Hecht labs for selective inhibition against human GYCH-IA over *Ng*GYCH-IB.

5.2 Materials and Methods

Overexpression and purification of wild-type and mutant *NgGYCH-IB* protein

Wild-type *NgGYCH-IB* was overexpressed as an N-terminally His₆-tagged protein in BL21(DE3) cells (New England Biolabs, Inc.) grown in Luria–Bertani medium with vigorous aeration as described previously (57) and purified by Ni-NTA chromatography. The His₆-tag was removed by cleavage with factor-Xa (New England BioLabs, product number P8010L) according to the manufacturer's protocol, followed by capture of the affinity tag on Ni-NTA resin. The His₆ tag-free protein was subjected to a final purification step by gel filtration chromatography using an Enrich SEC 650 column (Bio-Rad, Hercules, CA) pre-equilibrated with TRIS-free buffer containing 100 mM HEPES [N-(2-hydroxyethyl)piperazine-N0-(2-ethanesulfonic acid); pH 7.4], 100 mM KCl, and 1 mM DL-dithiothreitol (DTT). Protein purity was confirmed to be greater than 95% by SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

Preparation of delta 42 *HsGYCH-IA* protein

The human *GCYH-IA* gene lacking the N-terminal 42 amino acids (*HsGCYH-IA*) and cloned in pET28(+) expression vector was purchased from GENEWIZ (La Jolla, California, USA). The enzyme was overexpressed in *E. coli* C41(DE3) cells (New England Biolabs, Inc.). Culture was grown in Luria-Bertani medium containing 50 µg/mL Kanamycin at 37 °C with vigorous aeration for 3 hours. Expression was induced at A₆₀₀ = 0.6 by addition of IPTG to a final concentration of 1 mM and continued shaking for 3 hours at 37 °C. Cells were harvested by centrifugation at 8000 g for 15 minutes at 4 °C, and the resulting cell pellet was suspended and lysed in 50 mL of lysis buffer (50 mM Tris pH 8.0, 50 mM KCl, 1 mM β-mercaptoethanol (BME), 0.2 units of DNase I, one tablet of protease

inhibitor cocktail (Roche)) using a microfluidizer. The cell lysates were centrifuged at 14,000 g for 30 min at 4 °C, and filtered using a 0.22 µm filter before loading onto a 15 mL Ni-NTA column pre-equilibrated with Buffer A containing 100 mM Tris (pH 7.5), 300 mM KCl, 5% glycerol, 1% Triton, 2 mM BME and 1 mM phenylmethylsulfonyl fluoride (PMSF). The column was washed with 50 mL Buffer A, followed by 50 mL Buffer B (100 mM Tris pH 7.5, 300 mM KCl, 5% glycerol, 1% Triton, 2 mM BME, 1 mM PMSF and 20 mM imidazole) and 50 mL Buffer C (same composition as Buffer B excluding the PMSF). The protein was eluted from the Ni-NTA resin with Buffer C supplemented with 250 mM imidazole. Elution fractions were pooled and subjected to size-exclusion chromatography using a SEC650 column (Bio-Rad) pre-equilibrated with buffer containing 150 mM HEPES pH 7.5, 150 mM KCl and 1 mM DTT. Protein purity was verified by SDS-PAGE to be > 95%.

Enzyme activity assays

Enzyme activity was measured using either the standard fluorescence-based GTP cyclohydrolase assay or the UV–Vis absorption assay, as described previously (62).

Wild type and mutant NgGCYH-IB: The fluorescence assay was performed in 100 mM HEPES (pH 8.0), 100 mM KCl, 0.5 mM MnCl₂, 2 mM MgCl₂, 1 mM DTT and 0.5–5 µM of NgGCYH-IB in a total volume of 100 µl. Reactions were initiated by the addition of GTP (final concentration 100 µM) and incubated at 37°C for 50 - 60 min. Reactions were terminated by the addition of 12 µl of a 1% I₂, 2% KI solution in 1.0 M HCl to oxidize the H₂NTP to neopterin. Following a 15-min incubation at room temperature in the dark, excess iodine was quenched with the addition of 6 µl of 2% ascorbic acid and the samples were analyzed by fluorescence spectroscopy using a FluoroMax-3 instrument (Horiba

Jobin Yvon Scientific) at $\lambda_{\text{Ex}} = 365 \text{ nm}$. Emission spectra were recorded between 400 and 500 nm, and the neopterin peak at 446 nm was monitored. Neopterin was quantified using a linear calibration curve of the fluorescence signal from a pure neopterin standard.

For testing structure-based designed inhibitors on NgGCYH-IB and HsGYCH-IA:

This assay was modified to a semi high-throughput scale using 96-well plates and a plate reader. In the fluorescence-based assay, reactions were prepared the same way as described above but 2 μM enzyme and 0.5 mM MnCl_2 (for reactions with *NgGCYH-IB*) and 0.5 mM ZnCl_2 (for reactions with *HsGCYH-IA*), and either 10 μM GTP (for reactions with *NgGCYH-IB*) or 800 μM GTP (for reactions with *HsGCYH-IA*). The reactions were terminated after 10 minutes and treated with I_2 , KI and ascorbic acid as as described above. The samples were then analyzed by fluorescence spectroscopy using a Tecan infinite M200 spectrofluorimeter and the neopterin emission peak at 446 nm was monitored, with an excitation wavelength of 365 nm. In the UV-Vis absorption assay, reactions were carried out in a final volume of 70 μL containing the same reaction components and conditions as mentioned for the semi high-throughput fluorescence assay. The absorbance was measured at a wavelength of 330 nm with the same Tecan instrument set in absorbance mode.

Delta 42 HsGYCH-IA: The fluorescence assay was carried out in 100 mM HEPES (pH 7.5), 100 mM KCl, 2 mM MgCl_2 , 1 mM DTT, 0.5 mM ZnCl_2 , 2 μM enzyme, and variable concentrations of GTP (0-2000 μM) in a total volume of 100 μL . The reactions were incubated for 60 mins at 37°C before termination by the addition of 12 μL of 1% I_2 , 2% KI solution in 1.0 M HCl for 15 mins at room temperature followed by the addition of 6 μL of 2% ascorbic acid. The samples were analyzed using a Tecan infinite M200 instrument to read the emission spectra between 300 to 400 nm with an excitation wavelength of 365

nm. K_M measurement was calculated by fitting the data (average of triplicate measurements) to the following equation using GraphPad Prism version 8.1.1 software (La Jolla California, USA): $Y = V_{max} * X / (K_M + X)$.

Enzyme inhibition

Inhibition analysis was conducted by assessing enzyme activity in the presence of increasing concentrations of inhibitor compound (0-1000 μ M for G1 and S-G3, 0-1600 μ M for G2, and 0-800 μ M for G3) and pre-incubation for 30 minutes before addition of GTP to start the reactions. The reactions were carried out in the same conditions as described above, using 10 μ M GTP for *Ng*GCYH-IB reactions (K_M value previously determined to be $9.15 \pm 0.67 \mu$ M(62)), and 800 μ M GTP for *Hs*GCYH-IA reactions (K_M determined in this study to be $867 \pm 57 \mu$ M). For reactions in the presence of G1, G2, and S-G3 compounds, the absorbance assay was used to measure product formation. For reactions in the presence of the G3 compound, the fluorescence assay was used. The data were plotted as % activity (relative to the reaction in the absence of inhibitor) versus inhibitor concentration. IC_{50} measurements were calculated by fitting the data (average of triplicate measurements) to the following equation using GraphPad Prism version 8.1.1 software (La Jolla California, USA): $Y = 100 / (1 + (IC_{50} / X))$. Enzyme inhibition was done together with Ayesha Haque of Prof. Manal Swairjo Lab.

***In vitro* denitrosylation of GCYH-IB**

To prepare denitrosylated GCYH-IB, a sample containing 600 mg of freshly purified, active enzyme was made at a concentration of 28 μ M in buffer containing 100 mM HEPES (pH 8.0), 100 mM KCl, and 0.5 mM $MnCl_2$. Glutathione (GSH) was freshly dissolved in water and added to the protein sample to a final concentration of 280 μ M and

was incubated for 30 min in the dark. Excess GSH was removed in two buffer-exchange steps by loading onto Zeba Spin Desalting Columns [molecular mass cut-off (MWCO) 7000 Da, ThermoFisher Scientific, Waltham, MA] pre-equilibrated in buffer containing 100 mM HEPES (pH 7.4) and 100 mM KCl and by centrifuging at 1000 g for 2 min. The resulting GSH-treated protein was immediately assayed for activity using the fluorescence assay described above, and for its state of nitrosylation using the iodoTMT-switch assay (vide infra).

iodoTMT-switch assay

This assay was carried out using the Pierce™ S-nitrosylation Western Blot Kit (ThermoFisher Scientific, Waltham, MA) following a modified version of the manufacturer's protocol. Briefly, a 100 µl sample containing 1 mg/ml protein in HENS buffer (100 mM HEPES, 1 mM EDTA, 0.1 mM neocuproine, 1% (w/v) SDS, pH 7.4) was prepared, and 5 µl of a 100 mM solution of the irreversible blocking agent N-ethylmaleimide (NEM, prepared in ultrapure water) was added to block the protein-free thiols. After vortexing vigorously and incubating for 30 min at 50°C, excess NEM was removed in two buffer-exchange steps using Zeba Spin Desalting Columns (MWCO 7000 Da). The protein was then precipitated by adding six volumes of cold acetone (pre-equilibrated at -20°C) and incubating for 1 h at -20°C. The precipitated protein was pelleted by centrifuging at 10 000 g for 10 min, and the pellet was resuspended in 100 µl of HENS buffer and divided into two 50-µl batches. To one batch, 4 µl of 1.0 M sodium ascorbate (prepared in ultrapure water) and 1 µl of 20 mM iodoTMT6-126 labeling reagent (iodoTMT) were added to reduce and label the nitrosylated thiols, respectively. (The final protein, ascorbate, and iodoTMT concentrations were 30 µM, 75 mM, and 0.4 mM,

respectively.) For a negative control, the second batch was treated similarly except that the ascorbate was replaced with ultrapure water. Following incubation for 2 h at room temperature, excess labeling reagent and ascorbate were removed in two buffer-exchange steps using Zeba Spin Desalting Columns. The proteins were then precipitated by adding six volumes of cold acetone and incubating overnight at -20°C , followed by centrifugation at 10 000 g for 10 min. The precipitated proteins were each resuspended in 50 μl of HENS buffer and analyzed by western blotting. All steps were performed in the dark and the protein concentration after each precipitation/resuspension step was determined using a Bradford assay, absorbance at 280 nm, and bovine serum albumin (BSA)-calibrated SDS-PAGE on an Any kD Mini-Protean TGX gel (Bio-Rad, Hercules, CA).

Western blot analysis of iodoTMT-labeled proteins

Equal amounts of labeled proteins were loaded on a denaturing Mini-Protean TGX precast gel (Bio-Rad, Hercules, CA) and electrophoresed without any reducing agents for 15 min at 300 V. The proteins were then transferred to a PVDF membrane (0.45 mm, ThermoFisher Scientific) by electroblotting for 48 min at 60 V in a wet transfer apparatus (Bio-Rad, Hercules, CA). The membrane was blocked by incubating for 5 h in 5 ml of TRIS-buffered saline and Tween (TBST buffer: 20 mM TRIS, 150 mM NaCl, and 0.1% Tween 20, pH 7.5) containing 2% BSA and immunolabeled by incubation overnight in TBST solution containing mouse anti-TMT monoclonal antibody (IgG2b, ThermoFisher Scientific, product number 90075) at a dilution of 1:500. Following five washes in TBST, the membrane was probed with goat anti-mouse IgG-horseradish peroxidase conjugate antibody (ThermoFisher Scientific, product number Ab97040) at 1:10 000 dilution for 1 h.

Subsequently, the membrane was washed five times with TBST and developed with the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher Scientific). All steps were carried out at room temperature. To verify equal loading of proteins, a second, identical SDS–PAGE was performed in parallel on the same samples and that gel was stained with Coomassie to visualize total protein.

Oxidation of GCYH-IB by hydrogen peroxide

A 20- μ l sample of each of the freshly purified GCYH-IB (verified to be S-nitrosylated by the iodoTMT assay as described above) and the GSH-treated GCYH-IB was prepared in assay buffer [100 mM HEPES (pH 8.0), 100 mM KCl, 0.5 mM MnCl_2 , and 1 mM MgCl_2] at a final protein concentration of 25 μ M. Hydrogen peroxide (H_2O_2) was added to a final concentration of 0.25 mM, and the reactions were incubated in the dark for 5, 10, 15, 30, and 60 s before the addition of catalase (from bovine liver, Sigma–Aldrich) to a final concentration of 600 U/ml to quench excess H_2O_2 . The samples were immediately assayed for H_2NTP production using the fluorescence assay.

5.3 Results

S-nitrosylation of GCYH-IB is site-specific and preserves enzyme activity

The structural observation of S-nitrosylation of a catalytic active-site cysteine residue prompted the investigation of this post-translational modification biochemically. First, the presence of the SNO modification was confirmed in a freshly purified enzyme in solution using the iodoTMT-switch assay and before investigating the effect of the SNO on enzyme activity. In the iodoTMT-switch assay, the free thiols in the protein are first blocked using the irreversible blocking agent NEM. The S-nitrosylated cysteines are then reduced with ascorbate, and the newly formed free thiols are labeled with iodoTMT. The labeled thiols are then detected and quantified by western blot analysis using an anti-TMT antibody. To elucidate the effect of S-nitrosylation of Cys149 on enzyme activity, freshly purified wild-type enzyme was denitrosylated by treating it with reduced GSH (a widely used method for de-nitrosylation of proteins (128)) followed by removal of excess GSH, before assaying the resulting de-nitrosylated form for H₂NTP production using the fluorescence assay. To eliminate the effect of possible glutathione chelation of the catalytic metal, the GSH-treated enzyme was incubated in assay buffer containing 0.5 mM metal before adding a substrate. While the GSH-treated enzyme was fully denitrosylated, as confirmed by the iodoTMT-switch assay (Figure 5-4), its activity was found to be comparable with that of the untreated enzyme, with only 20% reduction (Figure 5-4), indicating that Cys149 supports catalysis in both the nitrosylated and reduced states.

In addition to Cys149, NgGCYH-IB contains two other cysteine residues in each subunit: the metal co-ordinating Cys147 in the active site and Cys190 located in a partially

solvated hydrophobic pocket 12 Å away from the active site. In the crystal structures, only Cys149 is seen S-nitrosylated. To confirm this observation, the nitrosylation state of the Cys149Ala mutant was assessed using the iodoTMT-switch assay. The results show that while the fully active, wild-type protein in solution, and as purified from *E. coli*, is S-nitrosylated, the Cys149Ala mutant protein purified under the same conditions lacks any S-nitrosylated thiols (Figure 5-4). These results demonstrate that the observed S-nitrosylation of GCYH-IB occurs post-translationally during heterologous expression in *E. coli* and that it is specific to Cys149. Although the physiological relevance of the observed S-nitrosylation of GCYH-IB in a heterologous system is unclear, the specificity of the nitrosylation to a conserved and essential active-site cysteine and the direct interaction of the thionitroxide group with the substrate analog seen in the crystal structure suggest that it may participate directly in catalysis.

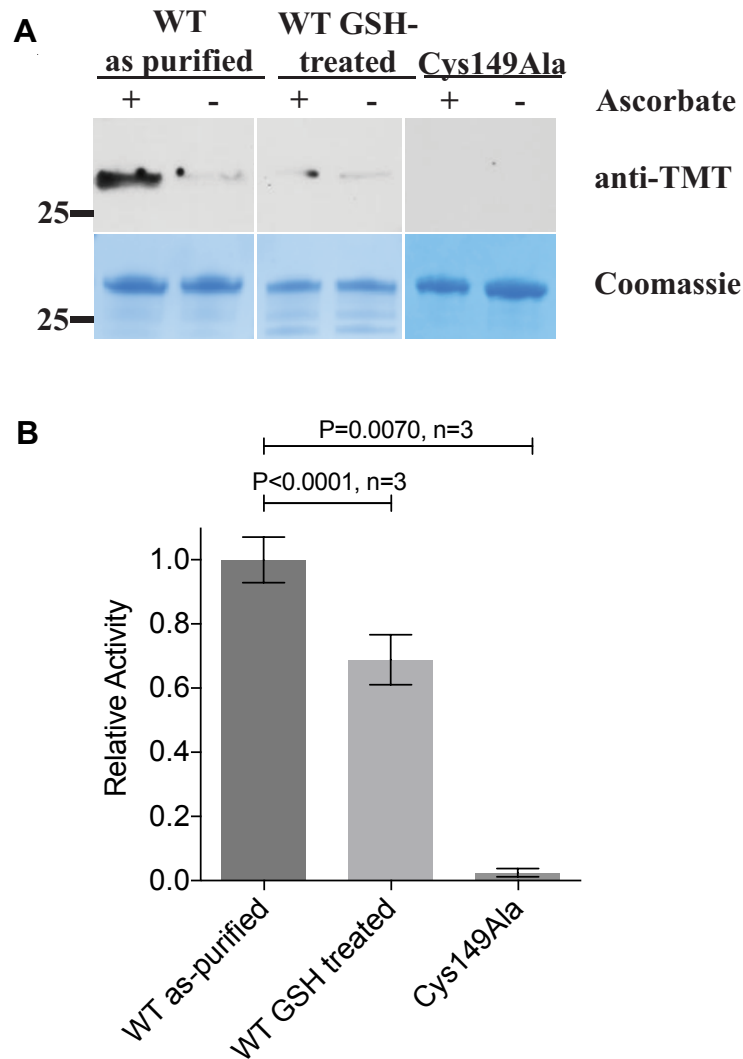


Figure 5-4: S-nitrosylation of NgGCYH-IB.

A: IodoTMT-switch assay of freshly purified wild-type GCYH-IB of the enzyme after treatment with 0.5 mM GSH and of the Cys149Ala mutant. Top, Western blot of TMT-labeled proteins visualized using an anti-TMT antibody. Bottom, Coomassie Brilliant Blue staining of the same gel showing equal loading in all lanes. B: Relative H₂NTP producing activities of the enzyme samples in A as measured using the fluorescence assay. The error bars indicate the standard error of triplicate measurements.

S-nitrosylation of GCYH-IB does not provide protection against oxidation

Since the nitrosylated cysteine is a catalytic residue, the possibility of the SNO modification serving a protective role against oxidative inactivation of the enzyme was interrogated. In this experiment, the activities of the freshly purified (S-nitrosylated) GCYH-IB and the GSH-treated (de-nitrosylated) enzyme were monitored following incubation with 0.5 mM hydrogen peroxide for 0-60s. The activities of both the nitrosylated and denitrosylated enzyme dropped by a similar extent exposure time to H₂O₂ (Figure 5-5), indicating no observable protection by the SNO against oxidative inactivation.

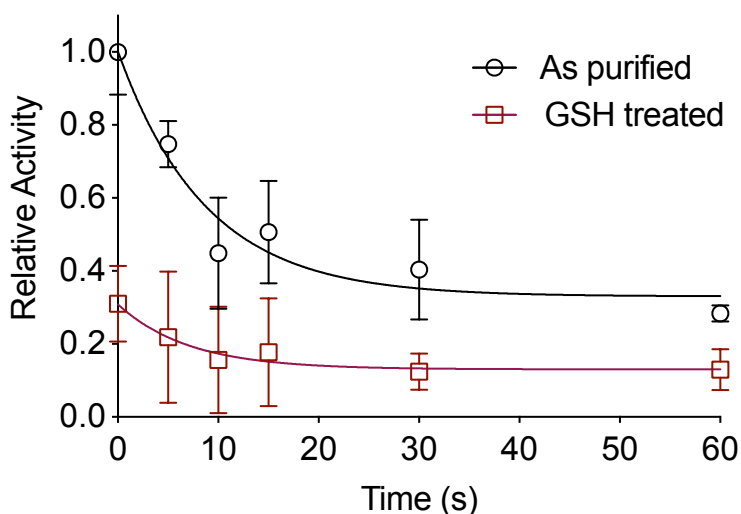


Figure 5-5: Effect of hydrogen peroxide on nitrosylated and denitrosylated *NgGCYH-IB*.

As purified or nitrosylated *NgGCYH-IB* and GSH-treated or denitrosylated *NgGYCH-IB* was assayed for activity in the presence of H₂O₂ over time. Each trace is an average of three independent acquisitions with background subtraction.

Role of Cys149, Glu152, Glu243, and His246 in catalysis

Of the active-site residues that interact with 8-oxo-GTP and TRIS in the structures, three residues make contacts that suggest a direct role in catalysis. These residues are Glu243 and Cys149, which interact with the guanine imidazole ring and the ribosyl moiety of 8-oxo-GTP, respectively, and His246, which co-ordinates the O2 atom of the transition-state mimic TRIS. Additionally, Glu152 is positioned where it might serve as an acid/base residue to modulate the protonation state of Cys149. Glu243 and His246 are conserved residues in all GCYH-IB sequences, whereas Cys149 is only conserved in bacterial GCYH-IB sequences and Glu152 is present in only a subset of bacterial sequences. To investigate the role of these residues in catalysis, we prepared site-directed mutants of Cys149, Glu152, Glu243, and His246, and measured the abilities of the mutant proteins to catalyze formation of the reaction product H₂NTP using a fluorescence assay as described in the methods section. All mutants were overexpressed in *E. coli* and freshly purified under the same conditions used to purify the wild-type enzyme. The structural integrity of the mutant proteins was checked by size-exclusion chromatography and their elution profiles were identical with that of the wild-type enzyme, showing a single peak corresponding to an intact homotetramer with an apparent molecular mass of 135 000 Da (Figure 5-6B). With the exception of the Glu152 mutant, which exhibited ~20% of the wild-type activity, all of the alanine mutants exhibited impaired ability to carry out full catalytic turnover to produce H₂NTP (Table 5-1 and Figure 5-7).

Notably, previous studies with the type IA enzyme have demonstrated the ability to uncouple the early steps in the reaction from the late steps by independently measuring formate and H₂NTP formation, and showed that some mutants compromised in H₂NTP

formation are capable of efficiently catalyzing the early steps in the reaction and release formate along with the intermediate 2,5-diamino-(6 β)-ribosylamino-4(3H)-pyrimidinone 50-triphosphate (DRPT)(119). Indeed, when assayed in collaborator Prof. Dirk Iwata-Reuyl's lab for production of [^{14}C]-formate, only the Glu152Ala and Glu243Ala mutants showed a similarly impaired ability in the production of formate, while the His246Ala mutant exhibited an almost 2X higher rate of formate production than H₂NTP, and the Cys149Ala mutant, for which we were unable to detect H₂NTP production, exhibited a robust rate of formate production roughly 10% that of the wild-type enzyme. Consistent with this pattern, Cys149Ser mutant was inactive for H₂NTP formation and exhibited formate production activity almost 20% that of wild type (62).

The observed catalytic activities of the Glu243 and Cys149 mutants suggest roles in the early and late stages of the chemical mechanism, respectively, conclusions consistent with the crystallographic information from the GCYH-IB structures containing 8-oxo-GTP and TRIS. In contrast, mutation of His246, which interacts with the O2 atom of TRIS in the TRIS-bound structure, an interaction suggestive of the transition state leading to the formate release (*vide supra*) in an early step in the reaction, results in enhanced production of formate relative to H₂NTP.

Table 5-1: Relative catalytic activities (%) of GCYH-IB mutants

Protein	Fluorescence H ₂ NTP assay
Wild-type	100
Cys149Ala	0
Cys149Ser	0
Glu152Ala	21*
Glu243Ala	0
His246Ala	0

* This measurement was conducted by Prof. Iwata-Reuyl Lab using a continuous UV-Vis assay as described in (37).

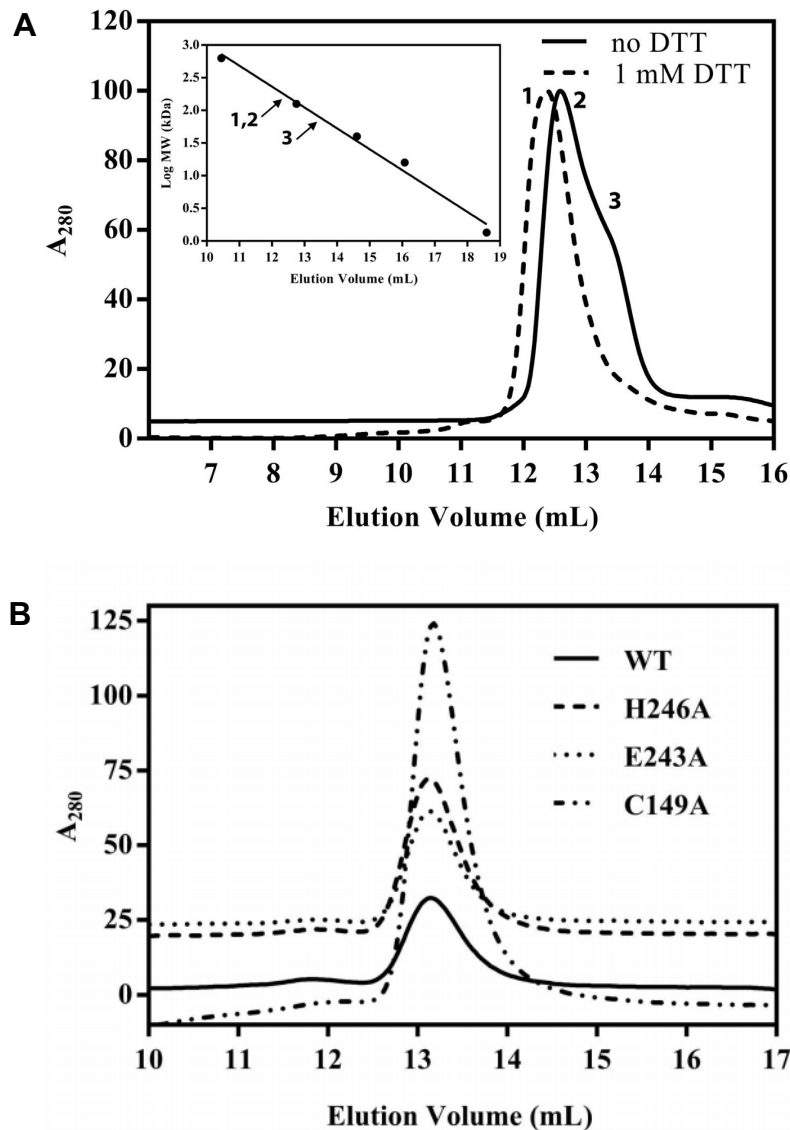


Figure 5-6: Effect of DTT on *NgGCYH-IB* structural integrity and sample SEC of *NgGCYH-IB* mutants.

A: SEC analysis in the presence and absence of DTT. The inset shows the molecular weight standard graph used to resolve the peaks. In the presence of DTT, a homogenous tetramer elutes with an apparent of 135kDa (peak 1 and peak2). In the absence of DTT, the enzyme elutes as a mixture with a lower apparent molecular weight species of 70kDa, indicating a destabilizing tetramer.

B: SEC profiles of a few *NgGCYH-IB* mutants in comparison to WT demonstrating the integrity of the biological homotetramer. The single peak corresponds to an apparent molecular weight of 135kDa.

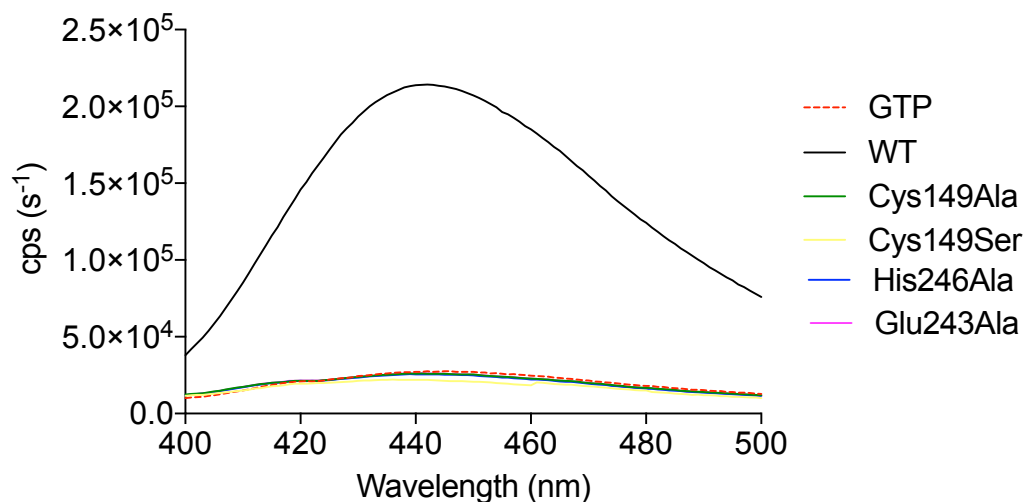


Figure 5-7: Sample activity data for WT and mutant *NgGCYH-IB*.

Fluorescence H_2NTP activity assay. Each trace is an average of three independent acquisitions with background subtraction.

Inhibition of GYCH-IB

We measured the half-maximal inhibitory concentrations (IC_{50}) of inhibitors G1–3 *in vitro* against heterologously expressed *NgGCYH-IB* and a *HsGCYH-IA* construct lacking the 42 N-terminal amino acids and previously reported to possess robust activity and improved solubility (59). Enzyme activity was measured using the absorbance-based assay quantifying product H_2NTP formation by its absorption at 330 nm; and the fluorescence-based assay that relies on post-reaction oxidation of the enzymatic product H_2NTP to the fluorescent neopterin and monitoring the neopterin emission peak at 446 nm with excitation at 365 nm (62). First, steady state kinetic analysis of *HsGCYH-IA* was performed using the fluorescence assay with GTP as substrate, and the analysis yielded a K_M of $867 \pm 57 \mu M$ (Figure 5-9). Inhibition assays were then conducted by pre-incubating

each enzyme with increasing concentrations of inhibitor compound (0–1600 μ M) for 30 min, and measuring enzyme activity using the absorbance assay (for G1, G2 and S-G3) or the fluorescence assay (for G3), and a substrate concentration set at or near the enzyme's K_M for GTP: 867 μ M for *HsGCYH-IA*, and 9 μ M for *NgGCYH-IB* (62) (Figure 5-8). All enzymatic assays were performed in triplicate.

IC_{50} values obtained from the inhibition data show that G1 has modest selectivity for *HsGCYH-IA*, G2 is not selective, and both G3 compounds are modestly selective for *NgGCYH-IB*, with G3 showing the greater selectivity (Table 5-2). G3 is three-fold more potent against *NgGCYH-IB*, a 31-fold reversal of selectivity as compared with our starting point, 8-oxo-GTP. Previous work shows that 8-oxo-GTP is 28-fold more potent against *TtGCYH-IA*, which harbors a nearly identical active site to *HsGCYH-IA* in sequence and 3D structure (61, 62). While these IC_{50} measurements are indicative of only modest potency, the 31-fold reversal of selectivity in favor of the bacterial enzyme is significant and supports our structure-guided hypothesis on inhibitor optimization. We note also that G1–3 lack the phosphate groups of 8-oxo-GTP, which engage in ion pairing with arginine and lysine residues of the enzyme, leading to the phosphorylated inhibitor's sub- μ M affinity (61).

Table 5-2: Measured half maximal inhibitory concentrations (IC₅₀) (μM) of designed inhibitors against HsGCYH-IA and NgGCYH-IB.

Values are presented with respect to their standard errors.

Inhibitor	<i>Hs</i> GYCH-IA	<i>Ng</i> GYCH-IB
G1	221 ± 10	413 ± 6
G2	514 ± 32	372 ± 25
S-G3	372 ± 14	164 ± 11
G3	409 ± 21	134 ± 11

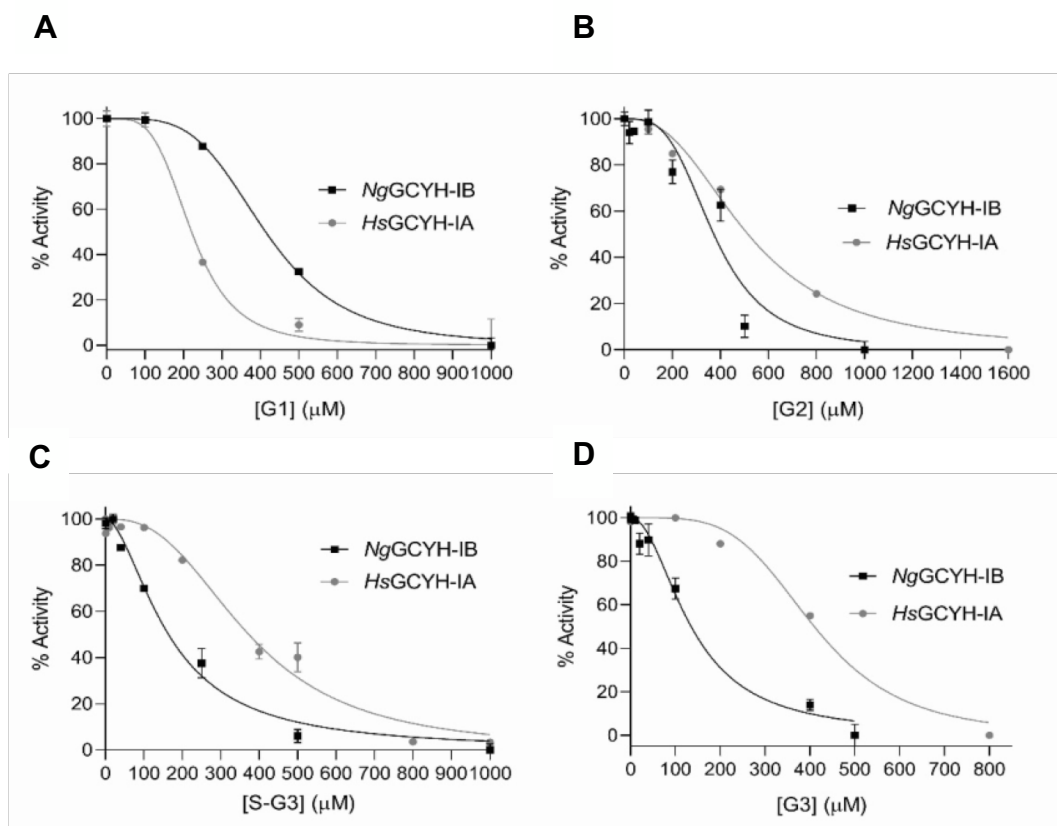


Figure 5-8: Relative activities of Human GCYH-IA and *NgGCYH-IB* in the presence of several inhibitors

Relative activity of *HsGCYH-IA* (grey circles) and *NgGCYH-IB* (black squares) in the presence of increasing concentrations of inhibitor compounds G1 (A), G2 (B), S-G3 (C) and G3 (D), measured using the absorbance assay (A-C) and the fluorescence assay (D), and used to obtain biochemical IC₅₀ values listed in Table 5-2. Data represent the average of triplicate measurements and error bars represent the standard deviation in the data.

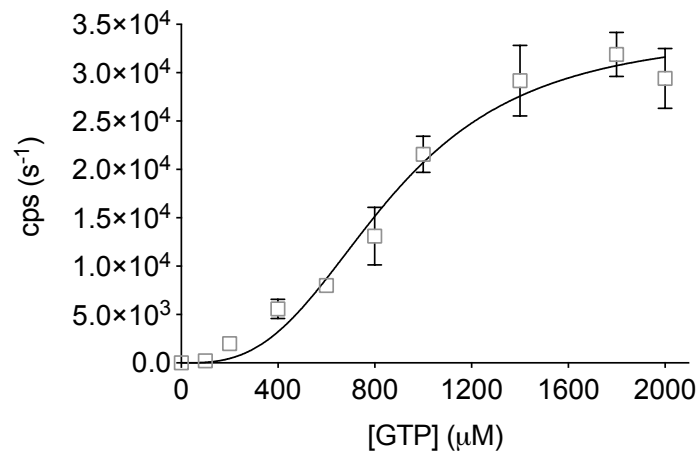


Figure 5-9: Steady state kinetics of human GCYH-IA

Activity of *HsGCYH-IA* with increasing concentrations of GTP measured using the fluorescence assay and used to obtain K_M of $867 \pm 57 \mu\text{M}$. Data represent the average of triplicate measurements and error bars represent the standard deviation in the data.

5.4 Discussion

S-nitrosylation of GCYH-IB

S-nitrosylation is a nitric oxide (NO)-mediated post-translational modification that regulates protein function (129–132). Structural and biochemical data show that GCYH-IB is S-nitrosylated at an essential, active-site catalytic cysteine and that the nitrosylated enzyme is active, if not more active than the de-nitrosylated form. Although many enzymes are known to be reversibly inactivated by S-nitrosylation of an active-site cysteine (e.g. the human mitochondrial caspases (133), protein tyrosine phosphatases (134), mammalian methionine adenosyltransferases (135), and dimethylarginine dimethylaminohydrolases (136)), to our knowledge, there are no reported examples of enzymes in which S-nitrosylation of a catalytic cysteine residue maintains (or enhances) activity. S-nitrosylation of active-site cysteines has also been shown to protect some enzymes from oxidative inactivation (e.g. protein tyrosine phosphatase IB (137) and galectin-2 (138)); however, such protection of S-nitrosylated *Ng*GCYH-IB from H₂O₂ mediated oxidative inactivation was not detected. This could be due to oxidation of one or both of the other two cysteine thiols in the protein, especially the metal ligand Cys147, thus compromising the structural integrity of the enzyme tetramer. Consistent with this interpretation, purification of the enzyme in the absence of DTT results in a destabilized tetramer as detected by gel filtration chromatography (Figure 5-6A). Additionally, GCYH-IB is transcriptionally up-regulated in *Ng* when the organism is subjected to oxidative stress by exposure to peroxide (139). The precise source of the NO that post-translationally modifies GCYH-IB during heterologous expression in *E. coli* remains unclear. S-nitrosylation in *E. coli* has been shown to be a prominent feature of anaerobic

respiration on nitrate where the nitrate reductase NarG acts as the major source of S-nitrosylating activity (132).

However, in this study the *E. coli* cultures were not provided with exogenous nitrate, and the cultures were grown aerobically. In general, biological S-nitrosylation of proteins occurs through 1) an autocatalytic mechanism in which the protein hydrophobic core accumulates NO and accelerates its oxidation to the more reactive N_2O_3 causing S-nitrosylation of a nearby cysteine thiol within the protein interior (140) or 2) oxidation of NO to the nitrosonium cation (NO^+) by reaction with a transition metal or transfer from another S-nitrosylated protein or small S-nitroso compound (e.g. S-nitrosogluthathione) to a solvent-exposed thiol (transnitrosylation) (141). The nitrosylated cysteine in GCYH-IB resides in a short helix on the surface of the protein, completely exposed to solvent, consistent with transnitrosylation, not autocatalysis.

Proposed catalytic mechanism of GCYH-IB

A chemical mechanism has been proposed for the GCYH-IA enzyme based on crystallography and a variety of biochemical studies, including mutagenesis, NMR analysis, and transient-state kinetics (58, 59, 61, 66, 118, 119, 142, 143). In the early steps in the mechanism, the reaction is initiated with reversible hydrolysis of GTP at C8 via a nucleophilic attack of a zinc-activated water molecule and cleavage of the C8–N9 bond to form an N-formyl intermediate, which subsequently undergoes a second hydrolysis reaction at C8 resulting in the cleavage of the C8–N7 bond and elimination of C8 as formate. In the second half of the reaction, the ribosyl moiety undergoes ring opening and an Amadori rearrangement followed by cyclization and loss of the 2'-oxygen as H_2O to give H_2NTP .

Our biochemical and structural data are consistent with GCYH-IB facilitating the same chemical steps as GCYH-IA. 8-Oxo-GTP, 7-deaza-GTP, and 20-deoxy-GTP do not act as substrates for GCYH-IB, consistent with the proposed GCYH-IA chemical mechanism in which oxidation of C8 to the carbonyl state and protonation of N7 (i.e. 8-oxo-GTP), although formally analogous to the N-formyl intermediate (III, Figure 5-10A), precludes the second nucleophilic attack of water due to the reduced electrophilicity of C8 as a consequence of electron delocalization in the 8-oxo-guanine ring. Likewise, replacing N7 with a carbon (i.e. 7-deaza-GTP) prevents the initial hydrolysis step due to reduced electrophilicity of C8. Finally, the absence of the 2'-OH (i.e. 2'-deoxy-GTP) prevents the Amadori rearrangement necessary for the formation of the pterin ring system (VIII to X, Figure 5-10B).

However, differences in the active-site architecture and the different modes of recognition of the ribosyl moiety by the two enzymes suggest that they utilize different catalytic strategies. Our proposal of the GCYH-IB-catalyzed mechanism is shown in Figure 5-10. In this proposal, a water molecule coordinated to the metal is first deprotonated by Glu243 to produce a metal-coordinated hydroxyl ion (I, Figure 5-10A) that carries out a nucleophilic attack at C8, accompanied by protonation of N7 by Glu243 (II, Figure 5-10A). In contrast, in GCYH-IA, protonation of N7 is carried out by a water molecule (61) coordinated to a His residue. Decomposition of the tetrahedral intermediate and breakage of the C8–N9 bond (II, Figure 5-10A) are mediated by a bound water molecule (W2 in 8-oxo-GTP-bound structure, Figure 5-2A), yielding the intermediate 2-amino-5-formylamino-(6 β)-ribosylamino-4(3H)-pyrimidinone 5'-triphosphate (III, Figure 5-10A). This intermediate has been experimentally verified in the reaction catalyzed by

GCYH-IA (118, 119) and is mimicked by 8-oxo-GTP except for the out-of-plane geometry of the CHO group relative to the base.

The water molecule (W2), H-bonded to His246, is deprotonated by Glu243 to yield a second nucleophilic hydroxyl ion that attacks the out-of-plane electrophilic carbonyl C8 to produce a second tetrahedral intermediate (IV, Figure 5-10A). This intermediate is mimicked by TRIS in the TRIS-bound crystal structure of GCYH-IB (Figure 5-2B). Collapse of this intermediate coupled with protonation of N7 by Glu243 and cleavage of the N7–C8 bond produces formic acid and the intermediate DRPT (V, Figure 5-10A) (144)].

Ring opening of the ribose moiety is facilitated by protonation of the ring oxygen by a water molecule coordinated to His246 coupled with Schiff base formation at C1'–N9 (VI, Figure 5-10B). Ring opening facilitates a conformational rearrangement that allows the 2'-hydroxyl group to displace formate from the metal, a process facilitated by Glu201, which is seen H-bonded to the 20-hydroxyl in the 8-oxo-GTP-bound structure (Figure 5-2A). The fate of the formate is unclear; the presence of a water-filled cavity behind 8-oxo-GTP (Figure 5-3) suggests that formate may be trapped there until the end of the catalytic cycle. Deprotonation of C2' by Cys149 (VII, Figure 5-10B) yields the enol-enamine and the protonated Cys149 thiol (VIII, Figure 5-10B). The proximity of the carboxyl group of Glu152 suggested that this step might be facilitated by proton transfer from Cys149 to Glu152, but the mutagenesis data, in which activity for the Glu152Ala mutant was decreased by only ~75% in both assays, does not support such a role in catalysis. Glu201 then deprotonates the 2'-OH with protonation at C1' by Cys149 (VIII, Figure 5-10B) to give a C2' keto group (IX, Figure 5-10B). Subsequent nucleophilic attack by N7 on the

electrophilic keto carbonyl at C2' is facilitated by deprotonation at N7 by Glu243 and protonation of the carbonyl by Glu201, giving the bicyclic pterin ring system (X, Figure 5-10B). Dehydration coupled with imine formation then occurs with protonation of the OH group by Glu243, forming dihydroneopterin triphosphate (H₂NTP).

The proposed mechanism is consistent with all of the mutagenesis and crystallographic data. Specifically, mutation of Glu243 essentially abolishes both the formate-releasing activity and complete turnover to H₂NTP, as is expected for a residue involved in the early steps of the reaction. Replacement of Cys149 with either Ala or Ser removes a base necessary for initiation of the Amadori rearrangement, thus precluding the formation of the pterin ring. However, mutation does not prevent the hydrolytic steps in the reaction as these occur prior to the direct participation of Cys149. Nevertheless, there is a decrease in the rate of hydrolytic activity (Table 5-1) for the two mutants, indicating that Cys149 does play some role in this process, potentially in substrate binding, as suggested by the 8-oxo-GTP-bound structure. The role of His246 is more complex; whereas the structural data suggest a role in the second hydrolytic step (*vide supra*), the mutagenesis data instead imply a more direct role in the late steps of the reaction and only a minor role in the steps leading to formate release. Thus, in our mechanistic proposal, His246 is involved in H-bonding in the early steps of the reaction but does not participate directly in any chemical steps (Figure 5-10A). His246 does participate in protonation of the ribose ring via an intervening water molecule in the second half of the reaction (Figure 5-10B).

Notably, the proposal is also consistent with the observation that Cys149 can be nitrosylated (Figure 5-11). S-nitroso groups on cysteine side chains can exist in several oxidation and protonation states, and computational quantum mechanical studies have

shown that the dihedral angle and the S–N bond length of the SNO moiety are functions of its chemical state (121). Accordingly, the staggered conformers seen in the 8-oxo-GTP occupied sites and in the empty active sites (dihedral angle $\sim 103^\circ$ and $\sim 122^\circ$, respectively) are consistent with the radical thionitroxide form C–S–NH–O $\cdot$, while the almost planar CSNO conformer (dihedral angle $\sim 24^\circ$) seen in the TRIS-occupied active site represents the oxidized C–S–N=O form. The latter species, constituting a minority of states, may be an artifact of oxidation by O $_2$ or X-ray exposure during data acquisition. Although detailed acid/base properties for these species have not been reported, they are observed to be involved in H-bonding interactions (Figure 5-2A) and presumed to readily undergo protonation/deprotonation reactions. It is not clear which atom of C–S–NH–O $\cdot$ serves as the site for deprotonating C2', but we propose the oxygen atom (Figure 5-11) since it has been explicitly highlighted as a putative basic site (145).

Since its discovery, GCYH-I has come to exemplify the chemical complexity possible for enzymatic catalysis, and it continues to astonish us some five decades later. Remarkably, this complex reaction is now known to be catalyzed by two enzymes that, despite their common origin, share only two amino acids in common and bind GTP in markedly distinct ways. As a further surprise, an essential catalytic cysteine in a GCYH-IB family member undergoes nitrosylation to form a thionitroxide species, a post-translational modification heretofore associated only with metabolic regulation, here utilized directly in catalysis. While the formation of this modification of GCYH-IB in *N. gonorrhoeae* awaits confirmation, the observations that the enzyme undergoes site specific nitrosylation in *E. coli*, and that nitrosylation enhances catalytic activity, offer an

exciting new insight into the chemistry of GCYH-I and potentially new roles for nitrosylation in biology.

Selective inhibition of *Ng*GYCH-IB

Two of the analogues, G3 and S-G3, inverted the selectivity of the parent inhibitor 8-oxo-GTP by up to 31-fold in the case of G3, now providing three-fold greater potency against *Ng*GCYH-IB as compared with the human enzyme. While these new inhibitors are not potent enough to be drug leads, their structure-guided design and the observation of the predicted change in selectivity to target the bacterial enzyme supports the premise that GCYH-IB can be exploited as a target for a new class of antibiotics.

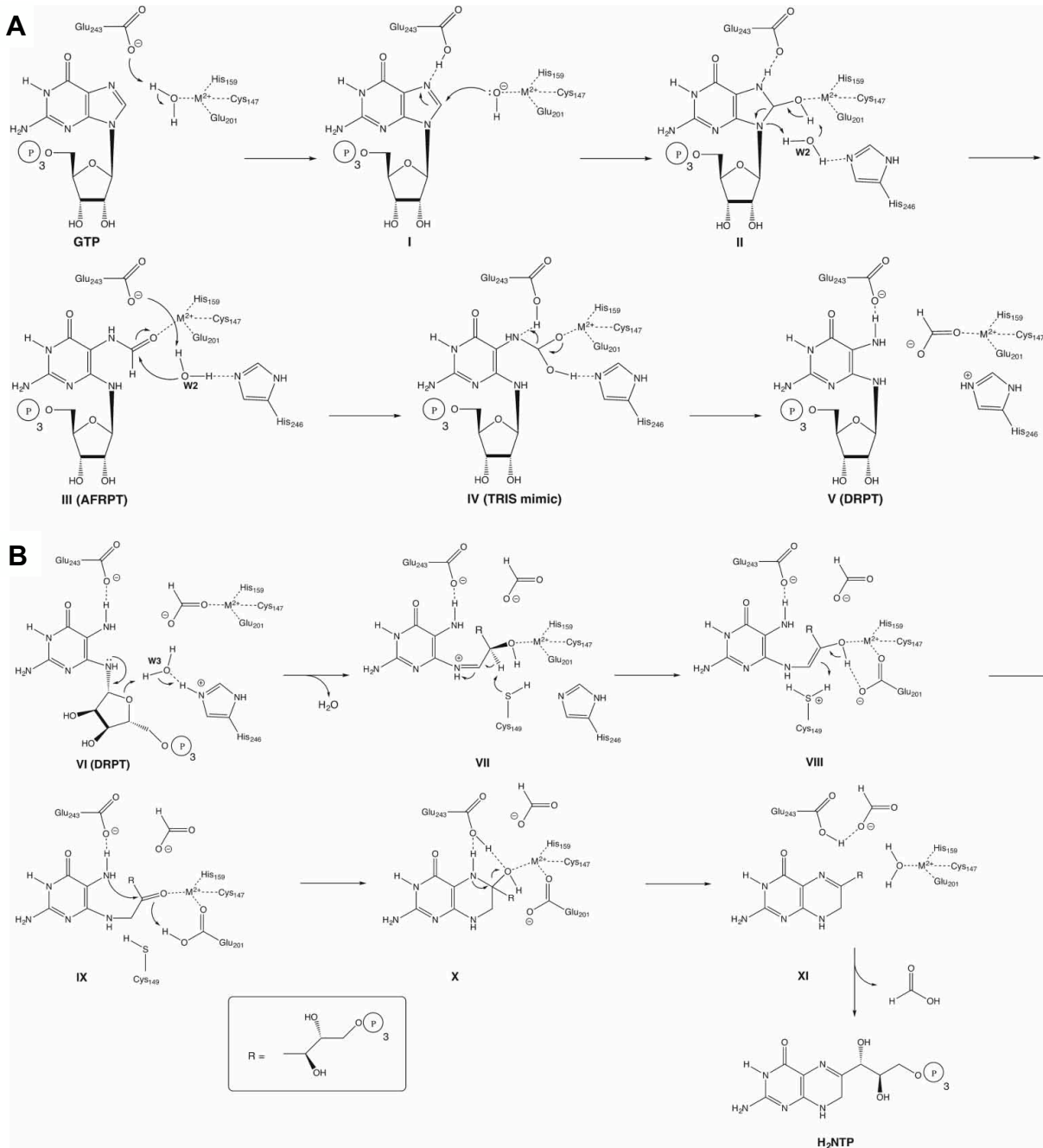


Figure 5-10: Proposed mechanism for the GCYH-IB-catalyzed reaction.

(A) The early hydrolytic steps in the reaction leading to the formation of formic acid and the intermediate DRPT. (B) The later steps in the reaction involving the conversion of DRPT to H₂NTP.

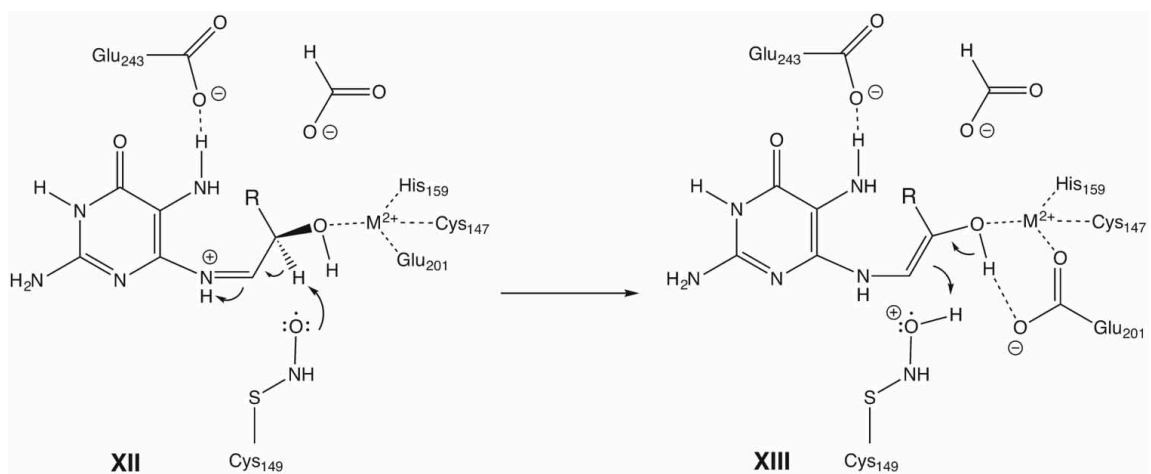


Figure 5-11: Proposed mechanism for the participation of the thionitroxide of Cys149 in catalysis.

5.5 Future Directions

The next phase of this study would be to synthesize compounds that utilize Pocket 1 (similar to G3) and Pocket 3 (using phosphate surrogates) in the design. Utilization of Pocket 2 has not been very successful according to the current study and it presents a challenge from a synthesis point of view as the pocket is located above the plane of the nucleobase. Compounds synthesized to address Pocket 1 and Pocket 3 would be tested *in vitro* against *Ng*GYCH-IB and human GYCH-IA to calculate IC₅₀ value, as done in this study. Compounds that show promising selectivity to *Ng*GYCH-IB over human GYCH-IA and low IC₅₀ values, can be used for minimal inhibitory concentration (MIC) studies. MIC studies would entail the use of liquid *Ng* bacterial cultures (146) in a high throughput 96-well microplate manner, where a serial dilution of a test compound will be applied to *Ng* cultures containing the same amount of bacterial cells. MIC can be determined by assessing the turbidity of the cultures after treatment with the compound. Compounds that perform well in MIC studies can be subjected to a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay that can measure cytotoxicity of the compounds in a human cell line such as HeLa cells. The MTT assay works by relying on metabolically active cells that contain NADPH dependent oxidoreductase enzymes to reduce the yellow colored MTT to purple colored formazan, thus the purple color reflects the number of viable cells under the conditions tested (147). In both MIC and MTT assay-based cytotoxicity studies, Ceftriaxone the antibiotic that is currently in use for treatment of Gonorrhea, can be used as a control (<https://www.cdc.gov/std/gonorrhea/treatment.htm>).

5.6 Acknowledgment

Chapter 5, in part, is a reprint of the material as it appears in Paranagama, N., Bonnett, S. A., Alvarez, J., Luthra, A., Stec, B., Gustafson, A., Iwata-Reuyl, D., & Swairjo, M. A. (2017). Mechanism and catalytic strategy of the prokaryotic-specific GTP cyclohydrolase-IB. *The Biochemical journal*, 474(6), 1017–1039 and Samaan, G. N., Paranagama, N., Haque, A., Hecht, D. A., Swairjo, M. A., & Purse, B. W. (2020). Structure-based design of guanosine analogue inhibitors targeting GTP cyclohydrolase IB towards a new class of antibiotics. *Bioorganic & medicinal chemistry letters*, 30(2), 126818. The dissertation author was the first and second author of these two papers respectively.

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