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Elucidating the role of Cosmc in the regulation of T-synthase biosynthesis

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An abstract of
a dissertation submitted to the Faculty of the Graduate School of Emory University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
Graduate Division of Biological and Biomedical Science
Biochemistry, Cell and Developmental Biology.

2011

Abstract

Elucidating the Role of Cosmc in the Regulation of T-synthase Biosynthesis
By Rajindra Prasad Aryal

Cosmc (Core 1 β3-galactosyltransferase Specific Molecular Chaperone) plays an important role in the regulation of O-glycan biosynthesis for animal cell glycoproteins. T-synthase (Core 1 β3-galactosyltransferase) is the key Golgi residing enzyme that initiates the formation of extended O-glycans by catalyzing the addition of galactose to the Tn antigen (GalNAcα1-Ser/Thr) to form the core 1 O-glycans (Galβ1-3GalNAcα1-Ser/Thr). The core 1 O-glycan is the precursor structure for many extended O-glycans. Lack of Cosmc function *in vivo* results in an inactive oligomeric complex of T-synthase that is eventually degraded, leading to loss of T-synthase activity and expression of aberrant O-glycans. Although Cosmc is required for the formation of catalytically active T-synthase *in vivo,* its mechanism is largely unknown. We developed an *in vitro* functional assay and showed that Cosmc specifically promotes the activity of partially denatured T-synthase independently of other factors, and that Cosmc does not bind native T-synthase. Furthermore, we generated active Cosmc conjugated beads, and demonstrated that Cosmc directly interacts with only non-native T-synthase to form a relatively stable noncovalent tight complex. Unexpectedly, the T-synthase within the complex is reactivated. ATP did not dissociate this complex suggesting that it does not regulate the Cosmc chaperone cycle. Furthermore, Cosmc interaction and release of reactivated T-synthase is not regulated by redox, calcium and pH. The primary factor that shifted the equilibrium between Cosmc and reactivated T-synthase was excess denatured T-synthase itself. These studies, along with others, suggest that newly synthesized T-synthase interacts with Cosmc in the ER to promote its folding in that compartment. This process leads to the formation of a transient complex between Cosmc and a catalytically active T-synthase. The T-synthase within Cosmc complex is released in the presence of more newly synthesized T-synthase, which interacts with Cosmc for another round of refolding. These findings have significantly contributed in our understanding of the molecular mechanism of Cosmc function, which may in turn lead to further understanding of protein O-glycan biosynthesis and several different diseases, such as Tn syndrome, IgA nephropathy, Henoch-Schönlein purpura, and malignant transformation.

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Acknowledgements

I would like to recognize my advisor, Dr. Richard D. Cummings, for his support and guidance during my graduate education. Under his guidance I have not only learned how to think about science, but have also grown my passion for it. I have developed a set of skills that allow me to ask important biological questions and answer them with appropriate controls and critically interpret them. From the very beginning of my graduate studies, Dr. Cummings largely allowed me to find my own way in science, which made me an independent and a very confident scientist. I am thankful to Dr. Cummings for his support of my personal and professional development as well as his interest and concern for the well being of my family and me.

I am grateful to the members of my thesis Committee: Dr. Keith Wilkinson, Dr. Xiaodong Cheng, Dr. Ichiro Matsumura, and Dr. Haian Fu for their continued support, suggestions, and constructive criticism that helped immensely, not only to shape my research project in the right direction, but also to transform myself as a scientist.

I am indebted to the members of the Cummings lab for all the support that they provided to me. Specially, I would like to thank Dr. Tongzhong Ju for his continued support and suggestions for better shaping the project. I learned from him how to work effectively at the bench. Dr. Ju was always there to help me and inspire me. I want to express my gratitude especially to Sandy Cummings for her great support both for personal and professional development as well as making Atlanta home for us. Also, I would like to thank Dr. Eric Ortlund and his lab for their assistance with the size exclusion chromatography study.

I have been lucky enough to have the support and inspiration from many individuals and organizations at every level of my education and they should be mentioned here as well. Banpala School, Rastrya Secondary School, and Pioneers' English Boarding school Waling Syangja, are the schools I attended in Nepal. I would like to thank those teachers who have instilled in my mind the value of education and its power to change society in many ways. Furthermore, I am indebted to Tribhuvan University (TU), Nepal, for providing me with six years of course work that were instrumental in building my strong academic foundation.

I would like to recognize my family, especially my parents and grandmother for sacrificing so much for me, as well as for their persistent faith in my abilities, which are really important for this accomplishment. I would like to thank my wife Krishna Aryal for her relentless support and encouragement to accomplish this goal. And just one and a half years ago we were blessed to welcome son Suraj Aryal in our family. He has already inspired me to work harder and do my best with an aim to contribute to human health.

This work is dedicated to my grandmother, Naina Devi, and my parents, Lok Nath and Tika Devi Aryal.

Table of Contents

List of Abbreviations

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
β4-GalT	β1-4-galactosyltransferase
Cosmc	Core 1 β3-GalT specific molecular chaperone
ER	Endoplasmic reticulum
Gal-3	galectin-3
GalNAc	N-acetylgalactosamine
Gal	Galactose
GlcNAc	N-acetylglucosamine
GnHCl	Guanidinium hydrochloride
HPC	Human protein C
UDP	Uridine diphosphate.
Bip	Binding protein
NMR	Nuclear magnetic resonance
HSP	Heat shock protein
HSP	Henoch Schönlein Purpura
AFM	Atomic force microscopy
RAP	Receptor associate protein
IgAN	IgA Nephropathy

LRP	Lipoprotein receptor related protein
VNTR	Variable number of tandem repeats
TACA	Tumor-associated carbohydrate antigens
MALDI-TOF	Matrix-assisted laser-desorption/ionization – time-of-flight mass spectrometry
PDI	Protein disulfide isomerase
NEF	Nucleotide exchange factor
NEM	N-ethylmaleimide
TM	Transmembrane domain
GT	Glycosyltransferase
TPR	Tetratricopeptide repeat
UPR	Unfolded protein response
ERAD	Endoplasmic reticulum–associated protein degradation

List of Figures

List of Tables

CHAPTER 1: Introduction

1.1 Protein folding

The primary structure of a protein contains all of the information needed to specify its unique secondary and tertiary three-dimensional functional structure (1). Under appropriate conditions, small proteins can spontaneously fold to their native structures (1). How conformationally diverse linear polypeptides efficiently and rapidly attain their diverse native functional structures is an important biological question (2,3). Leventhal suggested that the protein folding process is not a random search but rather a unique well-defined folding pathway leading to the most theromodynamically stable native state (4). After more than half a century of studies, the general features of protein folding pathways still have not been worked out, but several models have been proposed to explain protein folding on a biological time scale. In contrast to the idea of a unique folding pathway, the funnel landscape model (5,6) and the similar zipping and assembly model both hypothesize the existence of many parallel microscopic routes (7-9). In these models, protein folding initiates with the independent assembly of partially folded structural subunits at different sites of a polypeptide, followed by successive convergence along increasingly restricted pathways to reach the native state (7-9). In the diffusion-collision model, the formation of microdomains occurs in the first step within a polypeptide sequence, which is then followed by diffusion and collision of these microdomains in search of a stable native structure (10). In the hierarchy condensation model, neighboring structural intermediates form that assemble into successively larger structural units leading ultimately to the native structure (11). It is notable that problems

in protein folding pathways can result in aggregation of a misfolded protein, as observed in a wide range of debilitating diseases, including Alzheimer's and Parkinson's (12).

The dominant forces that drive protein folding are mostly hydrophobic interaction and conformational entropy (13). Hydrophobic interaction is the burial of nonpolar amino acids of a polypeptide away from water in a nonpolar core (14,15), and as a result entropy of water increases. Upon initiation of refolding of a polypeptide chain in an aqueous environment, the folding is initiated by the formation of a large number of secondary structures in a dead-time ~ few microseconds (16) as a result of hydrophobic collapse (13). The corresponding structure that exist as a result of hydrophobic collapse is also referred to as molten globule (16,17). These correct secondary structural elements may grow into the native structure by a process which incorporates some of the characteristics of the above-mentioned models (9).

1.2 *In vitro* verses *in vivo* protein folding

Proteins fold to their unique functional three-dimensional structures both *in vivo* and *in vitro* despite the significant differences in the folding environments. Nuclear magnetic resonance (NMR) studies demonstrate that native structures of medium-size proteins both *in vitro* and *in vivo* are very similar (18,19). Both *in vivo* and *in vitro* protein folding has been assumed to follow fundamentally similar pathway (20). It is assumed, therefore, that protein folding inside a cell and in a test tube occurs via a similar pathway. However, non-native conformations containing secondary structures with an unstable hydrophobic core or exposed hydrophobic side chains can accumulate in the protein-folding pathway and tend to aggregate *in vivo* (21,22). *In vitro* folding

experiments are carried out in a dilute solution in a lower temperature aimed to reduce aggregation and utilize complete protein sequences to study protein folding. However proteins in cells fold in the very crowded and concentrated cellular environment of proteins and other macromolecules (200-400mg/ml), including chaperones, cofactors, ribosomes, proteasomal degradation machinery and posttranslational processes such as glycosylation (19). Newly synthesized polypeptides are presented vectorially from ribosomes during synthesis and their nascent chains need to compete with other potentially interacting molecules within the concentrated cellular environment. Furthermore, incomplete polypeptides generated during the course of translation have potentially high risk of aggregation, since incomplete polypeptides cannot fold into stable native structures (23). Fluorescent techniques developed to study protein folding and aggregation in cells in real time indicate that the highly crowded environment of the cell greatly enhances the aggregation of partially folded proteins (24). This tendency to aggregate is a general characteristics of all proteins, so cells must have evolved some mechanisms to overcome this problem (25).

1.3 Assisted protein folding

While protein folding *in vitro* is dependent on its primary structure (1), it has become increasingly clear that within a cellular environment a vast majority of the proteins fold into three-dimensional functional structures with the assistance of molecular chaperones (20).

1.3.1 Molecular chaperone

The term “molecular chaperone” was first used by Laskey in 1978 to describe the function of nucleoplasmin to promote chromatin assembly (26). From a broader perspective Pelham used the term to describe molecules that bind and stabilize exposed hydrophobic sequences of unfolded proteins and prevent them from inappropriate interactions and aggregation (27). Later, the work of Ellis in 1988 further extended the use of the terminology to describe the roles of chaperones in correct posttranslational assembly of oligomeric structures (28). A molecular chaperone is now defined as any protein that selectively interacts with non-native proteins and promote their folding to their native functional structures, but are not associated with the final functional structure (23). Chaperones do not contain steric information for protein folding, but they facilitate folding by inhibiting unproductive pathways to irreversible aggregation (29). Chaperones are involved in many cellular functions, like refolding of stress induced denatured proteins, folding of newly synthesized protein, intracellular protein transport, oligomeric assembly and assisting proteolysis (23). Based on their ability to bind and promote protein folding of non-native proteins, chaperones are classified as either general or specific. This classification is briefly discussed here.

1.3.1.1 General Chaperones

During stress or in the course of translation, unfolded proteins need to be protected against aggregation by general chaperones through direct transient interaction with exposed hydrophobic sequences. Small heat shock proteins (sHsp), Hsp60, Hsp70,

Hsp90 are the predominant classes of general chaperone involved in protein-folding pathway (30).

Hsp70 Chaperone

The Hsp70 family is present in most organisms and contains a large number of chaperones that function to minimize misfolding of newly synthesized proteins (30). Hsp70 chaperones contain an N-terminal ATP binding domain and C-terminal substrate binding domain, both important for protein folding (30). The substrate-binding domain transiently associates with short stretches of hydrophobic amino acids that, in the substrate's native structure, are buried inside the core of the protein or in a subunit interface (31-33).

The chaperone cycle of Hsp70 is extensively regulated by many factors, including ATP and cochaperones like Hsp40 (30). Hsp40 binds to polypeptides and the Hsp40-polypeptide complex associates with Hsp70, delivering the bound polypeptide to the substrate binding domain of Hsp70 (34). Furthermore, Hsp40 stimulates the ATPase cycle of Hsp70 which leads to the formation of high affinity bound Hsp70-ADP substrate complex. Following ATP hydrolysis, nucleotide exchange factors, such as Bag, associate with Hsp70 to mediate exchange of ADP for ATP (35). Bound ATP induces a conformational change in the substrate binding domain of Hsp70 that reduces its affinity for the substrate, leading to the release of the substrate from high affinity bound form of Hsp70-ADP (36). The chaperone cycle is completed when Hsp70-ATP complex binds to another non-native protein, which is then followed by ATP hydrolysis that leads to the formation of high affinity bound form of Hsp70-ADP substrate complex.

Hsp90

The Hsp90 family of chaperones is one of the most conserved families of chaperones (30). Members of the Hsp90 family are known to assist folding protein kinases, stereoid hormone receptors and cytoskeleton proteins (30). In the cell, Hsp90 functions downstream of the Hsp70 chaperone system (37). Although the activities of Hsp90 and Hsp70 are similar, the former works by associating with many cochaperones (38) and chaperones, as well as other factors, many of which contain an alpha helical tetracopeptide repeat (TPR) domain (39). Some TPR domains make connections between Hsp90 and Hsp70, promoting the substrate transfer to Hsp90 (39). The Hsp90 chaperone cycle is ATP-driven and several cofactors regulate the cycle (40). ATP hydrolysis is important for substrate release from Hsp90 and this process is assisted by cochaperone p23 (41).

Small heat shock proteins (sHsp)

Another important family of chaperone involved in protein folding is small heat shock proteins (sHsp), which range in size from 12- to 43-kDa, are ubiquitous, and contain a unique conserved C-terminal alpha crystalline domain (42). Under stress, small heat shock proteins form stable complexes with denatured proteins preventing their aggregation in an ATP independent manner (43,44). Small heat shock proteins are functional oligomers with conserved structural organization containing dimeric alpha crystalline domain as a basic subunit (45,46). Mass spectrometry study showed that with the increase in temperature oligomeric sHsp dissociates to dimeric subunits, which then bind to unfolded proteins causing a shift in the equilibrium of sHsp to higher hetero

molecular structures of sHsp: substrate complexes (47). Under favorable conditions sHsp deliver their unfolded proteins to other chaperone networks, such as the Hsp70 chaperones system, for subsequent refolding (48).

Chaperonins

Chaperonins are large double-ringed multisubunit complexes of ~800kDa in the cytoplasm (23). The best studied chaperonin is GroEL-GroES, a bacterial homolog of Hsp60 chaperone (49). The GroEL consist of an apical domain containing exposed hydrophobic sequences, an equitorial ATPase domain and hinge region(30). GroES, a cochaperonin of GroEL, is a hepatameric ring (50), which covers the ends of GroEL cavity (49). The apical domain binds to a substrate with its exposed hydrophobic amino acids residues (49). The asymmetric nature of binding of ATP and GroES to GroEL triggers changes in the conformation of the apical domain which leads to major changes in the hydrophobic lining of the wall to hydrophilic as well as a substantial increase of the size of the cavity (51). Additionally the binding of ATP to GroEL causes the bound substrate to be released to the interior of the cavity for productive folding guided by hydrophilic environment of the cavity which is known to stabilize native state of a protein (52). ATP induced conformational changes facilitate dissociation of GroES from GroEL which leads to release of the folded protein (30).

1.3.1.2 Specific molecular chaperones

For some proteins to fold into their unique three dimensional functional structure, a highly specific molecular chaperone is required (53-55). Why there is a need for

specific molecular chaperones in addition to the large number of general chaperones is not well understood. However, it has been suggested that specific molecular chaperone must have co-evolved with their substrate to correct a problem of the particular protein in its folding pathway to reach to its native structure (55). Such chaperones represent another level of quality control mechanism (53). Some of the well-studied specific molecular chaperones are briefly described here.

Hsp47 is a collagen specific molecular chaperone that transiently interacts with newly synthesized procollagen molecules and facilitates folding, assembly and transport of the triple helical collagen in the ER (56) in an ATP independent manner (57). Hsp47 preferentially binds to (Pro-Arg-Gly)n-repeats of triple helical structure and prevent it from aggregation (58,59). The bound substrate is released from Hsp47 in the lower pH of the Golgi apparatus (57).

The correct folding of low-density lipoprotein receptor related protein (LRP) is facilitated by an ER localized Receptor associated protein (RAP), which is a specific chaperone for LRP(60,61). RAP binds tightly to LRP family members, preventing unproductive aggregation of LRP and the binding of LRP to other ligands (62). The RAP chaperone cycle is regulated by pH: it associates with LRP in the ER and releases the LRP in the acidic environment of the Golgi apparatus(61,63).

There are many protein specific chaperones and some of them, which are important for protein folding and assembly of macromolecular structures, are described in Table I.

Table I

Chaperone	Client	Function
LolA	*E. coli* lipoproteins	Sorts membrane localization of lipoproteins (64)
AHSP(α-hemoglobin stabilizing protein)	α-hemoglobin (α-Hb)	Promotes folding of α-Hb and facilitate binding to β-Hb to form αβ-Hb (65)
Tubilin cofactors	α and β tubulins	Facilitate the formation of α/β tubulin heterodimer assembly (66)
CALR3	ADAM3	Promote ADAM3 maturation (67)
Shr3p	Amino acid permeases(AAPs)	Stabilize and promote folding of AAPs and acts as a packaging chaperone CopII vesicles (68,69)
NinaA	Drosophila Rhodopsin Rh1	Forms a stable complex with Rh1 and possibly escort its substrate through the secretary pathway (70)
Invariant chain	MHC class II	Promote correct assembly of α and β chains of MHC class II complex and prevent premature ligand binding (71)
Tapasin	MHC class I	Assembles peptide and MHC class I molecule in the ER (72)
MTP (microsomal triglyceride transfer	Apolipoprotein B (ApoB)	Lipoprotein assembly in the ER (73)

protein)		
Calmegin	α and β fertilin	Facilitate hetero-dimerization of α and β fertilin (74)
TorD	TorA	Required for the expression of TorA (75)
CCS-1 (Copper chaperone for Superoxide dismutase)	SOD-1(Superoxide dismutase I)	Delivers copper to copper/zinc superoxide dismutase (SOD1) (76)
MESD(Mesodermal development protein)	LRP5 and LRP6	Promote folding and localization of LRP5 and LRP6 by reducing receptor aggregation (77)

Table 1.1 Substrate specific molecular chaperones

Cosmc, a chaperone of our interest and the focus of this dissertation, is also a specific molecular chaperone for T-synthase (78).

1.4 A brief summary of information known about T-synthase and its specific chaperone Cosmc prior to this dissertation

T-synthase (Core 1 β3-galactosyltransferase)

T-synthase is a glycosyltransferase that specifically transfers galactose from UDP-Gal onto the Tn structure (GalNAcα1-Ser/Thr) to form the core 1 O-glycan, also known as the T-antigen (Galβ1-3GalNAcα1-Ser/Thr) (79). The *T-synthase* gene is on chromosome 7 (7p14-p13) and encodes a predicted protein of 42kDa (80,81). The T-synthase enzyme purified from rat liver microsomes exists as a disulfide bonded homodimer of 84/86kDa as well as monomer of 42/43kDa and both forms are active (80), although the isolated monomeric form might associate non-covalently to form active dimers. The T-synthase is a unique enzyme and shares minimal homology with the conserved motif of other β3-galactosyltransferases. Remarkably, the mammalian T-synthase is not glycosylated on Asn residues (N-glycans), whereas most other glycosyltransferases contain one or more N-glycosylation sites (-Asn-X-Ser/Thr-) and are glycosylated (82). The T-synthase enzyme is developmentally important because T-synthase knockout in mouse is embryonic lethal (83). The human lymphoblastoid T-cell line Jurkat and human colon cancer cell line LSC contain transcripts for T-synthase, but do not contain functional enzyme, suggesting their lack of T-synthase activity is post transcriptionally regulated (78,84). As described below, this post-transcriptional

regulation was found to occur through the action of the specific molecular chaperone Cosmc that was lacking in those cells due to genetic mutation.

Cosmc (Core 1 β3-galactosyltransferase Specific Molecular Chaperone)

The *Cosmc* gene is X-linked (Xq24) and contains a single exon; its cDNA predicts a protein containing 318aa (78). It contains a single N-glycosylation sites (-Asn-X-Ser/Thr-) at Asn 313 in the extreme C-terminus, but that site is not efficiently used. Expression of active T-synthase in the T-cell line Jurkat and LSC cells requires co-expression with wild-type Cosmc to overcome the mutant Cosmc (78,84). Similarly, insect cells such as Hi-5 and Sf-9, which lack a Cosmc homolog, require coexpression of wild-type Cosmc for the expression of functional vertebrate T-synthase enzymes (78). Importantly, during the initial purification of the T-synthase from rat liver microsome, a small amount of a novel protein, subsequently identified as Cosmc, was found by proteomic analyses to be copurifying during early steps with the T-synthase (80), although it was not present in the final purified enzyme. Similarly, pull down experiments using epitope tagged versions of both proteins in Hi-5 insect cells and human 293T cells showed that a small amount of T-synthase coprecipitates with Cosmc (78). The coexpression of recombinant Cosmc with T-synthase reduces the levels of inactive higher oligomeric species of T-synthase in Sf-9 insect cells and LSC cells; in the absence of Cosmc, T-synthase is mostly a covalent disulfide-bonded, catalytically inactive, oligomer (84). Tn syndrome patients have somatic mutations in *Cosmc* in hemapotoietic stem cells and lack a functioning T-synthase in some blood cell populations (85). Unlike wild type Cosmc, the mutant Cosmc from Tn-syndrome patients

could not restore T-synthase activity when coexpressed with T-synthase in insect cells (85). Additionally, human Cosmc localizes in the ER whereas functionally active dimeric form of T-synthase localizes in the Gogi apparatus (84).These lines of evidence led to the hypothesis that Cosmc is a specific molecular chaperone for T-synthase (78).

1.5 Protein folding in the ER

Cosmc is an ER localized molecular chaperone, so an understanding of the ER environment and its role in assisting global protein maturation is important. Newly synthesized proteins traveling through secretory pathway first emerge in the ER, where they fold correctly before moving further in the pathway. The ER is a highly oxidizing environment, containing high levels of calcium and N-glycosylating enzymes as well as chaperones and other machinery important for protein folding and export (86). There are two major chaperone systems that have been studied for their role in protein folding in the ER. The first group includes BiP, which is an ER Hsp70 homolog, and Grp94, which is an ER Hsp90 homolog. These chaperones have been shown to interact with non-native proteins and facilitate their folding (87). The mechanism of action of the BiP and Grp94 homologs, Hsp70 and Hsp90, respectively, are discussed in the general chaperones section of this work. The second well-studied group of chaperones, including calnexin and calreticulin, are lectin-based chaperones that bind to N-glycan structures on nascent glycoproteins (87). Each of these chaperones independently recognize both monoglucosylated N-glycans and non-native proteins, which they retain in the ER for subsequent rounds of refolding (87). Exit from the ER occurs when the folded protein is free of monoglucosylated structures, a state controlled by α-glucosidases and UDP-

glucose glycoprotein glucosyl transferase (UGGT) (88). Within the secretory pathway, O-glycosylation through the addition of GalNAc residues to Ser/Thr residues occurs post-translationally in the Golgi apparatus, while N-glycosylation of newly synthesized proteins occurs co-translationally in the ER. Co-translationally translocated newly synthesized proteins are subjected to encounter with variety of chaperones, including BiP, Grp94, protein disulfide isomerase (PDI), peptidyl prolyl isomerase (PPI) (89) as well as calnexin and calreticulin (87).

The PDI family of chaperone facilitates isomerization and correct disulfide bond formation, which are important both for protein folding and for preventing aggregation of nonative proteins (90). The family is characterized by a thioredoxin domain containing a CXXC catalytic site (91) that cycles between oxidized and reduced state to form correct disulfide bond within the substrate (90). Another important family of proteins that defines the rate of protein folding is PPI (92). Its members bind non-native proteins and catalyze cis-trans isomerization of prolyl amide bonds, which an important rate determining step of the protein folding process (93,94).

1.5.1 Unfolded Protein Response (UPR)

The accumulation of unfolded proteins in the ER is detrimental to cells, so eukaryotic cells have evolved pathways to cope with them (95). ER protein homeostasis is maintained by activating intracellular signal transduction pathways, leading to an increase in the protein folding capacity and a decrease in the load of protein folding in the ER (96). Together, this response is called the unfolded protein response (UPR) (96). In mammalian cells, the UPR induces upregulation of many chaperones and components of

ER associated degradation machinery together with attenuation of protein synthesis (97). The ER resident chaperone BiP senses the folding capacity of the ER and transduces the signals accordingly (95). Unfolded proteins are retro-translocated to the cytoplasm by the endoplasmic reticulum–associated protein degradation (ERAD) pathway; once in the cytoplasm, they can be ubiquitinated and degraded by proteasomal machinery (98).

1.5.2 Protein glycosylation

Glycoproteins are found in the secretary pathway and the plasma membrane, as well as in the cytosol. Protein glycoslyation is a complex co- or posttranslational modification and over one half of a cell's total proteins are glycosylated (99). In humans the most common types of protein glycosylation are N-linked and O-linked, which occurs mostly in the ER and Golgi, the so-called secretory pathway (100). N-linked glycosylation is a common form of protein glycosylation in the secretory pathway and the process is initiated by the co-translational addition of preassembled tetradecasaccharaide $Glc_3Man_9GlcNAc_2$ from the dolichol pyrophosphate oligosaccharide donor ($Glc_3Man_9GlcNAc_2$-P-P-Dol) to any nascent chain containing the -Asn-Xaa-Ser/Thr- sequon in the ER (101). Following the joint action of α-glucosidase I, α-glucosidase II, calnexin, calreticulin and α-mannosidases, fully folded glycoproteins containing a $Man_8GlcNAc_2$ structure leave the ER and enter the Golgi apparatus for additional modifications and trimming by a myriad of glycosyltransferases and glycosidases (102,103). By contrast, the biosynthesis of O-glycans is initiated post-translationally either in the Golgi compartment or in the late ER (104,105). The O-glycosylation process is initiated by a covalent modification of a serine or threonine residue of a protein by the

addition of a sugar molecule, which is then followed by sequential actions of several other highly regulated down stream glycosyltransferases to build complex O-glycans (106). Protein glycosylation is important for many important cellular tasks including protein conformation and stability, protein localization, cell-cell interaction or cell-matrix interaction, cell signaling, the immune response and development (107,108).

1.5.3 Glycosyltransferase

Glycosyltransferases catalyze the transfer of an activated sugar from a sugar donor onto specific acceptor molecule, resulting in the formation of a glycosidic bond (109). Glycosyltransferases comprise a highly divergent group of enzymes (110), which are ubiquitously expressed in all cell types and are most prevalent in the ER and Golgi apparatus. Whereas some glycosyltransferases have evolved from a common ancestral gene by gene duplication and exon shuffling followed by divergence (111), there is very little similarity among most glycosyltransferases, suggesting that many glycosyltransferases arose independently (112). Therefore, sequence analysis cannot predict whether a protein is a glycosyltransferase. However, among some known glycosyltransferase families structural analysis reveals conserved three-dimensional architectures, clssified as GT-A, GT-B and GT-C folds (113). GT-A folds consist primarily of an $\alpha/\beta/\alpha$ sandwich with some sort of similarity to the Rossmann fold, contain an DXD motif, and require a divalent cation for activity (113). GT-B folds consist of an active site formed by two distinct Rossmann fold-like domains connected by a linker, and require metal ions but do not contain a DXD motif (113). Based on the

crystal structure of a sialyltransferase, the GT-C fold family has been described as displaying a slightly different α/β/α sandwich and lacking a DXD motif (109).

The Golgi-localized glycosyltransferases are type II transmembrane proteins that possess catalytically active lumenal domains, short stem regions, hydrophobic transmembrane domains and short cytoplasmic domains (114). However, these characteristics are not shared by the plasma membrane-, cytoplasmic-, or nuclear-localized glycosyltransferases (114). The general process by which glycosyltransferase are localized to the Golgi is not fully understood. Two models have been proposed: the bilayer thickness model and the oligomerization/kin recognition model (115). In the bilayer thickness model, the length of the transmembrane domain (TM) of a protein and the thickness of different regions of lipid bilayer within the secretory pathway define the localization of the protein, anywhere from the Golgi apparatus to the plasma membrane (116,117). In the oligomerization/kin recognition model, glycosyltransferases of specific pathways oligomerize to form homo- or hetero-oligomeric complexes within the Golgi cisternae and are retained together in the Golgi apparatus (112,118). Oligomerization might be promoted by several factors, including the environment of the Golgi apparatus, concentration of glycosyltransferases, and interactions of cytoplasmic domains, lumenal domains, or transmembrane and stem regions (115). Hetero-oligomerization would be ideal for retaining glycosyltransferases in the Golgi apparatus due to the low abundance of individual glycosyltransferases, and the possible requirement of even distribution of these enzyme within the Golgi cisternae (118). Furthermore, it is reported that several glycoslytransferases can homodimerize or homooligomerize, which might be important for retention of these enzymes in the Golgi apparatus (115). Additionally, it has been

proposed that the interaction of cytoplasmic domain of the enzyme with underlying matrix in the cytoplasm may help the enzyme to retain in the Golgi (119).

1.6 Mucin type O-glycosylation

O-glycosylation is among the most common posttranslational modifications on many secreted or membrane bound proteins and this process is initiated by the addition of a N-acetylgalactosamine (GalNAc) to serine and threonine residue (GalNAcα1-Ser/Thr – termed the Tn antigen). by a family of polypeptide N-acetylgalactosaminyltransferases (ppGalNAcTs) (120,121). This GalNAc residue is then normally modified by several other downstream glycosyltransferases to produce a tremendous variety of glycan structures (122). One of the common modifications is the addition of galactose (Gal) to GalNAcα1-Ser/Thr to generate the core 1 disaccharide Galβ1-3GalNAcα1-Ser/Thr (called the T antigen) (80). The T antigen can be further modified by the subsequent addition of many other carbohydrate units, including N-acetylglucosamine (GlcNAc) and sialic acid (Sia) (121,123).

1.6.1 Mucin structure

A common characteristic of mucins is the presence of variable number of tandem repeats (VNTR), that are O-glycosylated, and the repeats are rich in serine, threoine and proline and is highly variable between species (124-126). However, many glycoproteins lacking such VNTRs can also be O-glycosylated on select serine and/or threonine residues. The O-glycans are attached to the polypeptide by O-glycosidic bonds to the hydroxyl group of serine or threonine resembling a bottlebrush configuration (**Figure**

1.1). VNTRs provide a high degree of multivalency for sugar attachment and thereby introduce a great degree of variability between mucin molecules (126). Secreted mucin monomers can form extremely large oligomers of several million Daltons via intermolecular disulphide bonds between their cysteine-rich domains (127). Membrane-bound mucin and secreted mucin both contain large numbers of O-glycans and both forms may be up to ~80% carbohydrate by weight, comprised primarily GalNAc, GlcNAc, galactose, fucose and sialic acid and small amount of mannose and sulfate (128). Structural analysis of mucins using atomic force microscopy (AFM) (129), nuclear magnetic resonance (NMR) (130), light scattering and circular dichroism studies (131) have shown that clustering of oligosaccharides results in extended protein structure up to hundreds of nanometers in length (132).

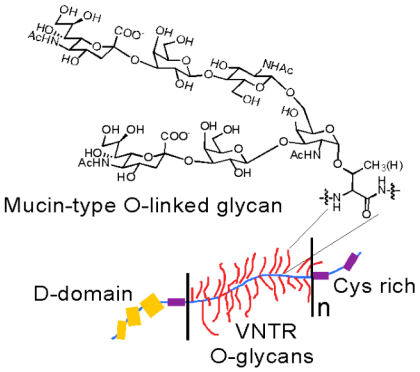

Figure 1.1. Schematic representation of mucin type O-linked glycosylation. Simplified secreted mucin structures. Highly O-glycosylated variable number of tandem repeat (VNTR) region, cysteine rich region and D domains are shown. The mucin structure is adapted and redrawn from Varki *et al.,* 2009. Esseential of glycobiology. Sugar structures are adapted and redrawn from Hang HC *et al.*, 2005. The chemistry and biology of mucin type O-linked glycosylation.

1.6.2 Biosynthesis of Mucin-type O-glycosylation

The Tn antigen is the common precursor to all mucin-type O-glycans (133). It has been hypothesized that ppGalNAcTs may bind a secondary structural motif rather than a primary amino acid sequence of the target polypeptide (123). Following its formation, the Tn antigen may be further modified by any of the several downstream Golgi-resident glycosyltransferases to form core1, core 2 and core 3 (**Figure 1.2**) (79). Sialyl-Tn is synthesized by a CMP-Neu5Ac:GalNAc α2,6-sialyltransferase (ST6GAlNAc-I), which transfers a sialic acid residue in α2,6-linkage to Tn antigen (79). Though the Tn structure is made by many ppGalNAcTs, the next step in mucin biosynthesis is catalyzed by a single enzyme: core 1 β1-3 galactosyltransferase or T-synthase. The T-synthase is a type II transmembrane golgi resident protein that catalyzes the formation of the core 1 O-glycan Galβ1-3GalNAcα-1-Ser/Thr (T antigen) (79). This disaccharide T-antigen is the precursor to many kinds of other complex O-glycan structures that are important in many biological processes, including animal development, lymphocyte homing (134) and leukocyte homing (135).

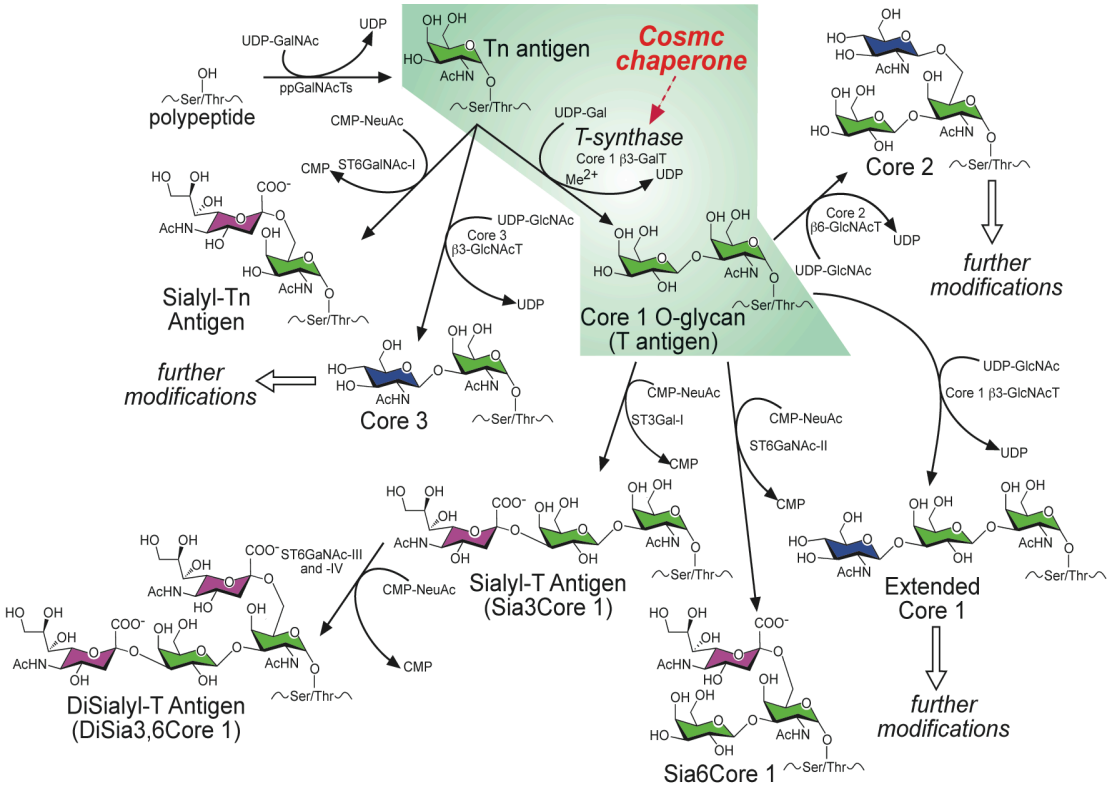

Figure. 1.2. Proposed role of T-synthase and its chaperone Cosmc in the biosynthesis of O-Glycans. The Tn antigen, the product of many ppGalNaCT2, is a precursor for the T-synthase, which modifies the Tn antigen to make the common core 1 O-glycan (T antigen). T-antigen is further modified by many glcosyltransferases to generate diversity of O-glycans. But for the synthesis of functional T-synthase Cosmc is essential and in the absence of Cosmc results in the expression of Tn and Sialyl-Tn antigen. Adapted and redrawn from Ju *et al.*, 2011. The Tn antigen-Structural Simplicity and biological complexity.

1.7 Significance of Cosmc in relation to human diseases

In the absence of functional Cosmc, inactive T-synthase accumulates in the ER, and is then retrotranslocated back to the cytosol and degraded by proteosomal machinery (84). Absence of functional T-synthase, which can be due to defective T-synthase, or a somatic mutation of chaperone Cosmc, results in the expression of Tn and Sialyl-Tn antigen (Neu5Acα2-6GalNAcα1-Ser/Thr) (78,136). Expression of aberrant mucin-type O-glycans such as Tn antigen or Sialyl-Tn antigen has been correlated with a number of diseases which are briefly described here.

1.7.1 Tn syndrome (Mixed-field Polyaggultinability)

Tn syndrome is a rare autoimmune disease characterized by the expression of abnormal glycans Tn- and Sialyl-Tn antigens in a subpopulation of some blood cells of all lineages, (79,120). Clinically, patients with Tn syndrome can develop leukopenia, thrombocytopenia, and a chronic hemolytic anemia (120). Tn syndrome may be caused by the lack of T-synthase activity resulting in elevation of the Tn antigen (81,83). Tn syndrome is also associated with an acquired somatic mutation in *Cosmc* (85).

1.7.2 Cancer

Expression of Tn and Sialyl-Tn structures is uncommon in normal mucin and mostly found in those mucins which are derived from tumors (79). Tn and Sialyl-Tn antigen are two of the most common tumor associated carbohydrate antigens (TACA), being expressed by more than 80% of the human carcinoma and present on secreted and surface glycoprotein and mucins (136,137). Tn and Sialyl-Tn are markers for poor

prognosis in cancer (137,138). Expression of the Tn antigen correlates with metastatic potential and poor prognosis in cervical (138), lung adenocarcinoma (139), breast and gastric and colorectal carcinomas (140-142). Whether Tn and Sialyl-Tn antigen promote cancer or are a consequence of it is still largely unknown. Understanding how these tumor-associated antigens Tn and silayl Tn arise might elucidate mechanisms of cancer metastasis and aid in the design of drugs for cancer therapy. Tn and Sialyl-Tn antigens arise from somatic mutation of *Cosmc,* which provides the first evidence to pinpoint genetic mutation in the glycosylation pathway resulting in expression of the Tn and Sialyl-Tn antigens (136,143).

1.7.3 IgA nephropathy and Henoch Schönlein Purpura

Both IgA nephropathy (IgAN) and Henoch Schönlein Purpura (HSP) are autoimmune diseases. IgA nephropathy (IgAN) is the most common type of glomerulonephritis worldwide (144). The pathologic hallmark of the disease is caused by the predominant deposition of IgA1- containing immune complexes in the glomerular mesangium (145), leading to renal failure. HSP is the most common type of vasculitis, and males are more often affected than females (146). The pathology of the diseases is a consequence of widespread vasculitis due to IgA1 deposition in the vessel walls and the renal mesangium (147). Both IgA nephropathy and Henoch Schönlein Purpura are known to exhibit increased expression of altered O-glycans (Tn and Sialyl-Tn) in the hinge region of IgA1 (148,149). These diseases correlate significantly with defects in the mucin-type O-glycan biosynthetic pathway, since IgA1 is a unique class of human antibody that carries such O-glycan modifications. One of the causes proposed for altered

O-glycosylation in these disorders is the loss of activity of the enzyme T-synthase (79). It is not clear at this point whether Cosmc is involved in these diseases, but several studies suggest that the existence of low level of transcript both for Cosmc and T-synthase in the B cells obtained from an IgA nepropathy patient (79).

1.8 Focus of this dissertation

As discussed in the introduction, mucin type-O-glycosylation is a common type of protein O-glycosylation and O-glycans are important in a wide variety of cellular processes. T-synthase is an important branchpoint enzyme in the pathway that catalyses the addition of galactose to Tn antigen to synthesize T antigen. The T antigen is the precursor disaccharide structural unit in the biosynthetic pathway of most mucin-type O-glycans. In the absence of T-synthase, T-antigen cannot be synthesized and the whole pathway is disrupted. Previous studies implicated Cosmc as an ER localized specific molecular chaperone required for the folding and maturation of T-synthase. The active dimeric form of T-synthase leaves the ER and localizes in the Golgi apparatus, while in the absence of functional Cosmc, either by mutation or altered expression, inactive T-synthase aggregates in an oligomeric complex, leading to the expression of abnormal truncated O-glycans Tn and Sialyl-Tn antigens. However, the molecular mechanism by which Cosmc assists the folding of T-synthase is largely unknown. The goal of this thesis is to understand the role of Cosmc in the folding and maturation of T-synthase. Here I provide evidence that Cosmc is a genuine chaperone for T-synthase and demostrate that Cosmc, independently of other factors, can restore the activity of denatured T-synthase. Furthermore, I explored the potential chaperone cycle of Cosmc. Results presented in this

dissertation are important for future studies to define the molecular mechanism of Cosmc function *in vivo*. These results support the hypothesis that Cosmc is central to the regulation of mucin-type O-glycan biosynthetic pathways by regulating the biosynthesis of active T-synthase enzyme.

1.9 References

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Chapter 2: The Endoplasmic Reticulum Chaperone Cosmc directly promotes *in vitro* folding of T-synthase

This research was originally published in The Journal of Biological Chemistry.

Authors: Rajindra P. Aryal, Tongzhong Ju, and Richard D. Cummings.
The Endoplasmic Reticulum Chaperone Cosmc directly promotes *in vitro* folding of T-synthase. *The Journal of Biological Chemistry.* 2010*;* 285: 2456-2462.

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2.1 Abstract

The T-synthase is the key β3-galactosyltransferase essential for biosynthesis of core 1 O-glycans (Galβ1-3GalNAcα1-Ser/Thr) in animal cell glycoproteins. Here we describe the novel ability of an endoplasmic reticulum-localized molecular chaperone termed Cosmc to specifically interact with partly denatured T-synthase *in vitro* to cause partial restoration of activity. By contrast, a mutated form of Cosmc observed in patients with Tn syndrome has reduced chaperone function. The chaperone activity of Cosmc is specific, does not require ATP *in vitro*, and is effective toward T-synthase but not another β-galactosyltransferase. Cosmc represents the first ER chaperone identified to be required for folding of a glycosyltransferase.

2.2 Introduction

Proteins correctly fold into unique functional three-dimensional structures within very crowded intracellular environments, including the cytoplasm and the oxidizing environment of the endoplasmic reticulum (ER) (1-3). While *in vitro* studies have demonstrated that proteins can fold independently under physiological conditions based on the primary amino acid sequence of the polypeptide (4), protein folding *in vivo* typically involves assistance of other proteins termed molecular chaperones, which recognize and selectively bind non-native structures and prevent aggregation (5-10). Chaperones are typically either general or specific in their client recognition. For example, relatively general and less-specific chaperones in client recognition include BiP/GRP78 and GRP94 along with co-chaperones such as Hsp40, and lectin chaperones such as calnexin/calreticulin and ERp75 (8,11-13). Client-specific chaperones include

Hsp47, which assists in collagen assembly (14) and Shr3p, an ER chaperone in yeast required for assembly and correct tertiary structures of amino acid permeases (15).

Previous studies indicate that a unique and specific molecular chaperone in the ER is Cosmc, which is required for the formation of active core 1 β3-galactosyltransferase (core 1 β3GalT, T-synthase) (16,17), an essential enzyme required for core 1 O-glycan (Galβ1-3GalNAcα1-Ser/Thr) biosynthesis on animal glycoproteins (18,19). Acquired mutations in *Cosmc*, which is encoded by the X-chromosome gene (Xq24) in humans, leads to loss of T-synthase activity and expression of the abnormal Tn (GalNAcα1-Ser/Thr) and Sialyl-Tn (NeuAcα2-3GalNAcα1-Ser/Thr) antigens (17,20,21), that are also known as tumor-associated carbohydrate antigens or TACAs (22-24). Disruption of the *T-synthase* in mice results in embryonic lethality primarily due to defective angiogenesis and lymphangiogenesis (18,19). In cultured cells lacking Cosmc, inactive T-synthase forms oligomeric aggregates in the ER and is eventually degraded by ubiquitin-dependent pathways in the cytosolic proteasome (16,17). Cosmc appears to bind directly to T-synthase and to ATP (16), but the mechanism by which Cosmc participates in correct folding of the T-synthase, and the possibility that Cosmc is specific for folding of the T-synthase, have not been explored.

To aid in understanding this interesting chaperone system, here we describe an *in vitro* reconstitution approach to explore Cosmc interactions with the unfolded T-synthase. Understanding the molecular mechanisms of the regulation of T-synthase should lead to a greater appreciation of human diseases and disorders involving altered expression and activity of the T-synthase and potential new therapeutic strategies for Tn antigen-related diseases.

2.3 Experimental Procedure

Materials. GalNAc-α-phenyl, UDP-Gal, GlcNAc-β-S-pNp, β4-GalT from bovine milk, and firefly luciferase were obtained from Sigma-Aldrich. GRP78 (BiP) protein (active) was purchased from Abcam. Luciferase assay substrate and the luciferase assay buffer were purchased from Promega. UDP-6-[^{3}H]Gal (40–60 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. Insect cells (Hi-5 and Sf-9) were obtained from American Type Culture Collection. Sep-Pak C18 Cartridges were obtained from Waters Corporation. Restriction enzymes were obtained from New England Biolabs, Inc. pVL 1393 vector and transfection kit were obtained from BD Biosciences. Ni-NTA Superflow beads were obtained from Qiagen. SDS-PAGE gels were obtained from Invitrogen. Centricon 10kDa molecular weight cut-off membrane was obtained from Millipore.

Preparation of Expression Construct.

Soluble N-terminal 6xHis-tagged soluble Cosmc (6xHis-sCosmc) was prepared as described (16). For constructing plasmids for encoding E152K mutated form of Cosmc, a fragment from the digestion of the plasmid encoding the soluble form of the 6xHis-tagged Cosmc, as described (16), was replaced by the fragment obtained by the digestion using *SacI/NheI* for full length E152K mCosmc as described (25). The construct was confirmed by sequencing and termed 6xHis-msCosmc.

Expression and purification of 6xHis-sCosmc, 6xHis-smCosmc, and soluble N-terminus HPC4-tagged Core 1 β3-Gal-T (T-synthase).

Soluble T-synthase was made by co-expressing N-terminal HPC4 epitope-tagged soluble T-synthase (HPC4-sT-syn) as described (26) with wild-type full length Cosmc as described (25) in Hi-5 cells using the Baculovirus system. After 96 h post-infection in Hi-5 cells, the media was collected and the epitope-tagged protein was absorbed on HPC4 antibody conjugated Ultralink resin, washed, and eluted with elution buffer containing 10mM EDTA. Using Centricon 10kDa cut-off membranes, protein was concentrated in 5mM Tris-HCl buffer, 30mM NaCl, pH 7.8. Both 6xHis-sCosmc and 6xHis-msCosmc were made as described (16). Reducing SDS-PAGE was carried out followed by Coomassie staining to determine purity (27). Gels were imaged on FluorChem Camera (Alpha Innotech) and density of the bands was quantified using software FluorChem^{TM} V.5.0.2.4 (Alpha Innotech).

***In vitro* reconstitution of heat-denatured T-synthase and heat-denatured β4-GalT.**

Recombinant HPC4-sT-syn and β4-GalT (~0.25μg in 32μl) were denatured by heating over time at approximately 54°C or 62°C respectively in 10mM HEPES buffer pH 7.8 containing 12mM $MgCl_2$ then cooled to room temperature. Renaturation was initiated by the addition of 6xHis-sCosmc and ATP to a final concentration of approximately 2.27μM and 5mM, respectively, in the reconstitution buffer except in **Figure 2.1E, F**, where the amount of 6xHis-sCosmc and BiP were both at a final concentration of 1.8μM. After preincubation at room temperature for 45 min, T-synthase activity was measured using the methods used previously except varying the concentration of UDP-Gal to 0.2mM final concentration and without Triton-X100 (26). In **Figure 2.1E, F** preincubation was carried out at room temperature for 75 min and T-

synthase activity was measured. β4-GalT activity was determined using pNP-β-S-GlcNAc as the acceptor as described (28). Relative T-synthase or β4-GalT activities were calculated using 100% as the activity of the untreated enzymes.

Chemical denaturation and renaturation of T-synthase

HPC4-sT-syn (35μg) was denatured in 1ml of 6M guanidinium hydrochloride (GnHCl), pH 7.2 for 90 min at room temperature. The sample was concentrated to ~70μl using Centricon 10,000 cut off membranes. The sample was diluted 100 times in reconstitution buffer (10mM HEPES, 150mM NaCl, 12mM $MgCl_2$ pH 7.8) then 34μl aliquots of the diluted sample were used for reconstitution reactions. Reconstitution was initiated by the addition of 6xHis-sCosmc and ATP where the final concentration was 2.27μM and 5mM, respectively. Parallel control reconstitution experiments were initiated either by the addition of BSA or galectin-3 where the final concentration was 2.27μM and 5mM ATP, respectively. The reaction was incubated for 45 min at room temperature followed by assay for T-synthase activity.

Luciferase renaturation assay

Approximately 10nM commercial luciferase (firefly) in the reconstitution buffer (10mM HEPES buffer containing 12mM $MgCl_2$ at pH 7.8) was denatured at 43°C for 7 min and cooled to room temperature. Both ATP and BiP were added to a final concentration of 5mM and 1.8 μM, respectively. Renaturation was carried out for approximately 75 min, and the luciferase activity was immediately measured by the addition of 100μl of assay reagents. The light produced was measured by a Top Count

NXT Microplate Scintillation and Luminescence Counter. Luciferase activities were calculated using 100% as the activity of the untreated enzymes.

Time dependency for restoration of HpC4-sT-syn.

Reconstitution of heat denatured HPC4-sT-syn was carried out at 37°C. For each reaction 0.25μg in 32μl recombinant HPC4-sT-syn was denatured by heat at approximately 54°C in 10mM HEPES buffer containing 12mM $MgCl_2$ at pH 7.8 for 2 min and cooled to 37°C. For reconstitution at 37°C, 6xHis-sCosmc, ATP, and other T-synthase reagents preincubated at 37°C were added. Reconstitution of HPC4-sT-syn was carried out simultaneously for different time points, and the renaturation stopped at different times by diluting the reaction approximately 15 times with cold water, following by assays for T-synthase activity.

2.4 Results

2.4.1 Cosmc promotes renaturation of denatured T-synthase *in vitro*

To investigate whether the chaperone function of Cosmc can be measured *in vitro*, we expressed recombinant soluble 6xHis-tagged Cosmc (6xHis-sCosmc) and HPC4-tagged soluble T-synthase (HPC4-sT-syn), and purified each to apparent homogeneity (**Figure 2.1A**), of apparent M_r of 35 and 39kDa, respectively. Note that to generate the enzymatically active HPC4-sT-syn in Hi-5 insect cells, we co-expressed the HPC4-sT-syn with a construct encoding full-length, membrane-bound Cosmc, which insects lack. Since the full-length Cosmc is not secreted it does not contaminate preparations of the co-expressed HPC4-sT-syn. To generate the 6xHis-sCosmc, we

expressed a construct encoding that protein alone in Hi-5 cells. For these *in vitro* renaturation studies, we denatured HPC4-sT-syn either thermally or chemically. The denaturation status of HPC4-sT-syn was monitored by assaying T-synthase activity after being heated over time, or being treated with 6M guanidinium hydrochloride (GnHCl) for 90 min, as shown in **Figure 2.1B** and **C**, respectively. Treatments with either heat or GnHCl caused significant loss of enzyme activity. In exploring the ability of Cosmc to restore activity to denatured HPC4-sT-syn, the renaturation assay was initiated by the addition of purified 6xHis-sCosmc into the denatured enzyme, which was followed by assaying enzyme activity. This involved measuring product formation with the donor UDP-[^{3}H]Gal toward the acceptor GalNAcα1-O-phenyl (29). Incubation with 6xHis-sCosmc caused significant restoration of activity of denatured HPC4-sT-syn either by heat treatment or GnHCl (**Figure 2.1D** and **G**, respectively). While addition of 6xHis-sCosmc induced significant restoration of heat-denatured HPC4-sT-syn, addition of a control protein, recombinant galectin-3, at equal amounts to 6xHis-sCosmc had no effect on restoring the activity (**Figure 2.1D**). Human galectin-3 was chosen as one of the controls, since like Cosmc it is also a non-glycosylated recombinant protein, and has a similar size (~30 kDa) to Cosmc (30,31). Importantly, addition of recombinant human BiP, a general ER chaperone, did not promote the renaturation of heat denatured HPC4-sT-syn (**Figure 2.1E**). Addition of BiP did not inhibit the reconstitution of heat denatured HPC4-sT-syn by 6xHis-sCosmc (**Figure 2.1E**), indicating that BiP-containing buffer lacks inhibitory activity in the renaturation process. By contrast, addition of BiP to heat denatured luciferase (**Figure 2.1F**) caused partial restoration of luciferase activity, and BiP alone lacks contaminating luciferase activity (**Figure 2.1F**). For HPC4-sT-syn

denatured by GnHCl treatment, incubation with 6xHis-sCosmc also caused restoration of activity, whereas addition of control proteins, bovine serum albumin (BSA) or galectin-3, had little effect (**Figure 2.1G**).

We examined Cosmc-dependent renaturation of heat-denatured HPC4-sT-syn over time of heating. The results showed that the longer HPC4-sT-syn was heated, there was a reduction of the amount of restoration of enzyme activity by the same amount of 6xHis-sCosmc (**Figure 2.1H**). This reduction is likely caused by precipitation of denatured T-synthase with prolonged heating. 6xHis-sCosmc alone had no contaminating T-synthase enzyme activity (**Figure 2.1D, E,** and **G**), but we observed that at time zero before heating, the addition of 6xHis-sCosmc to the recombinant HPC4-sT-syn led to elevated enzyme activity. This is likely to be due to the presence of some partly-denatured HPC4-sT-syn in the starting preparation purified from insect cells. These results strongly support the conclusion that Cosmc functions as a chaperone for the denatured T-synthase and it can act independently of other co-chaperones in this *in vitro* assay.

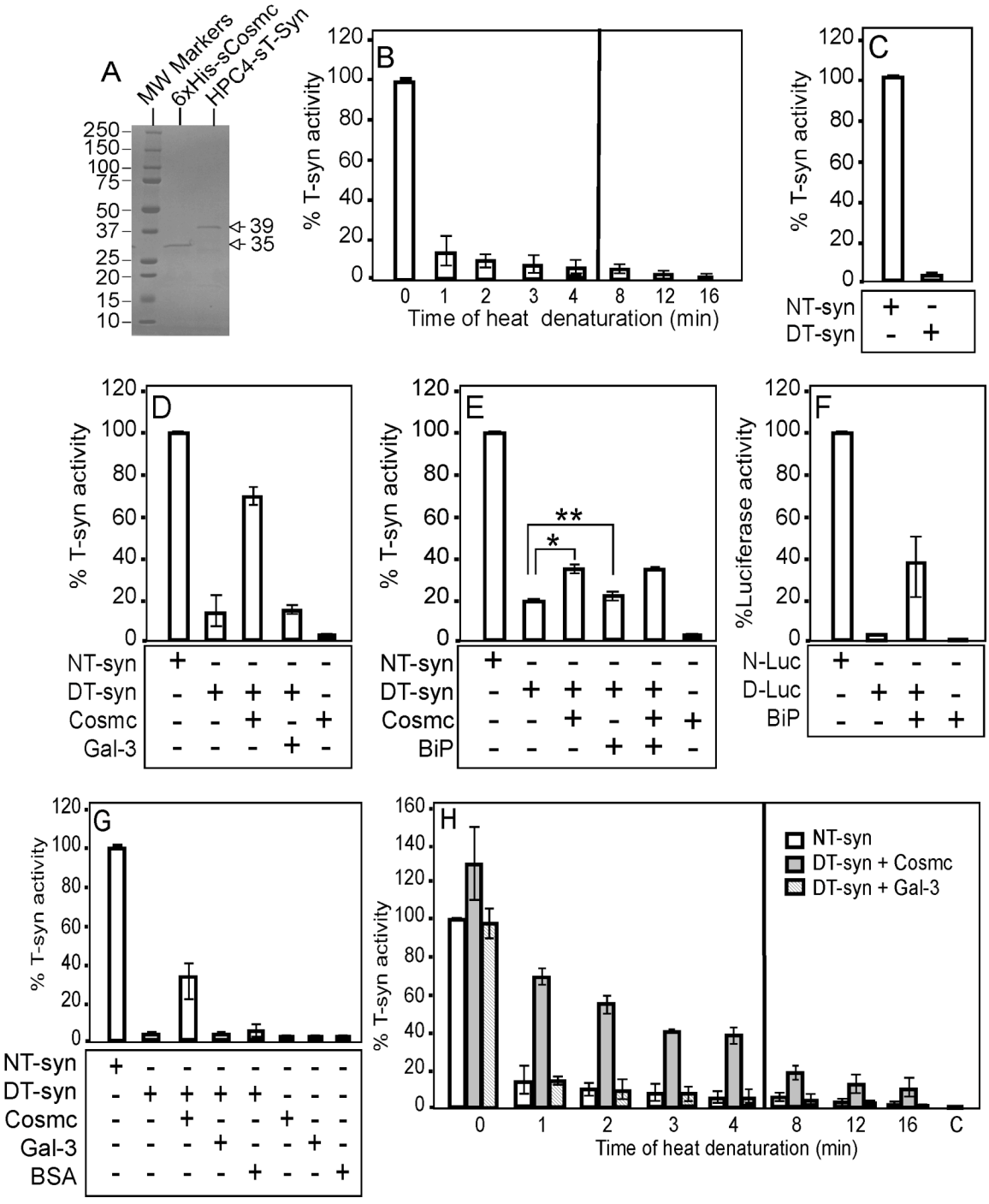

A
MW Markers
6xHis-sCosmc
HPC4-sT-Syn
250
150
100
75
50
37
25
20
15
10
39
35
B
% T-syn activity
Time of heat denaturation (min)
C
NT-syn
DT-syn
D
Cosmc
Gal-3
E
BiP
**
*
F
%Luciferase activity
N-Luc
D-Luc
G
BSA
H
NT-syn
DT-syn + Cosmc
DT-syn + Gal-3
C

Figure 2.1. Cosmc dependent-reconstitution of active T-synthase from denatured T-synthase. (A) SDS-PAGE analysis of purified recombinant human 6xHis-sCosmc and HPC4-sT-syn. The human N-terminal 6xHis-tagged soluble Cosmc (6xHis-sCosmc) and the N-terminal HPC4 epitope-tagged soluble T-synthase (HPC4-sT-syn) were expressed in Hi-5 cells and purified directly from the media. Protein (2 μg each) was subjected to polyacrylamide gel electrophoresis and stained by Coomassie blue, showing one major band in each lane (arrows). The lanes represent protein standards (Lane 1), 6xHis-sCosmc (Lane 2), and HPC4-sT-syn (Lane 3). (B) HPC4-sT-syn was heat-denatured in reconstitution buffer over time and percent specific activities of native, and each preparation of denatured, HPC4-sT-syn were determined. The vertical line separates experiments performed at two different times. (C) Purified soluble human HPC4-sT-syn was treated with guanidinium hydrochloride (GnHCl) and the percent specific activities of both treated (DT-syn) and untreated (NT-syn) T-synthase were determined. (D) Reconstitution of the heat-denatured HPC4-sT-syn, which was heated for 1min at 54°C, was initiated by the addition of 6xHis-sCosmc and the percent of restored T-synthase activity was determined, while galectin-3 (Gal-3), a control protein for specificity, did not support reconstitution. (E) Reconstitution of the heat denatured HPC4-sT-syn was initiated by the addition of ER general chaperone BiP, which did not support the reconstitution of heat denatured HPC4-sT-syn while (F) Reconstitution of BiP appears to restore the activity of heat denatured luciferase. N-Luc = native luciferase, D-Luc = denatured luciferase. (G) Reconstitution of GnHCl-denatured HPC4-sT-syn was initiated by the addition of recombinant 6xHis-sCosmc, Gal-3, or BSA, as indicated, and percent T-synthase activity was determined. (H) Restoration of activity of denatured HPC4-sT-

syn heated over time by addition of 6xHis-sCosmc was measured. 6xHis-sCosmc was added to denatured HPC4-sT-syn at each time point. A parallel reconstitution experiment of denatured HPC4-sT-syn was initiated by the addition of Gal-3 and percent T-synthase activity was determined. C = 6xHis-sCosmc alone. Vertical line separates experiments performed at two different times. In B, D and H, each assay was performed in duplicate and three replicate experiments were performed, and data represents the average of all experiments. In C and H, each assay was performed in duplicate and four replicate experiments were performed. In E and F at least three replicate experiments were performed, and data represents the average of all experiments. Error bars = +/- 1 SD from the average. * and ** represent P values $P<0.01$ and $P=0.62$, respectively.

2.4.2 Cosmc promotes renaturation of heat-denatured T-synthase in a dose-dependent manner

To further characterize the role of 6xHis-sCosmc in the reconstitution of heat-denatured HPC4-sT-syn, we performed *in vitro* reconstitution experiments of heat-denatured HPC4-sT-syn (0.18μM) with varying concentrations of 6xHis-sCosmc. The ability of 6xHis-sCosmc to aid in reconstitution of heat-denatured HPC4-sT-syn was concentration-dependent (**Figure 2.2A**). In this *in vitro* assay, significant restoration of HPC4-sT-syn activity occurred when the molar ratio of 6xHis-sCosmc and HPC4-sT-syn was ~1:1 (~0.14μM 6xHis-sCosmc), as shown in **Figure 2.2A**. We also measured the time-dependency of restoration of activity of heat-denatured HPC4-sT-syn by 6xHis-sCosmc. In this experiment, HPC4-sT-syn was denatured by treatment at ~54°C for 2 minutes, and then incubated at 37°C with 6xHis-sCosmc in the presence of all components of the HPC4-sT-syn activity assay for the indicated times. Therefore, the renaturation and measurement of HPC4-sT-syn activity was carried out simultaneously. Within the first five minutes, which was the minimal feasible time for conducting this type of experiment, we observed that maximum HPC4-sT-syn activity was regained (**Figure 2.2B).** The results show that inactive HPC4-sT-syn regained activity within 5 minutes after the addition of 6xHis-sCosmc.

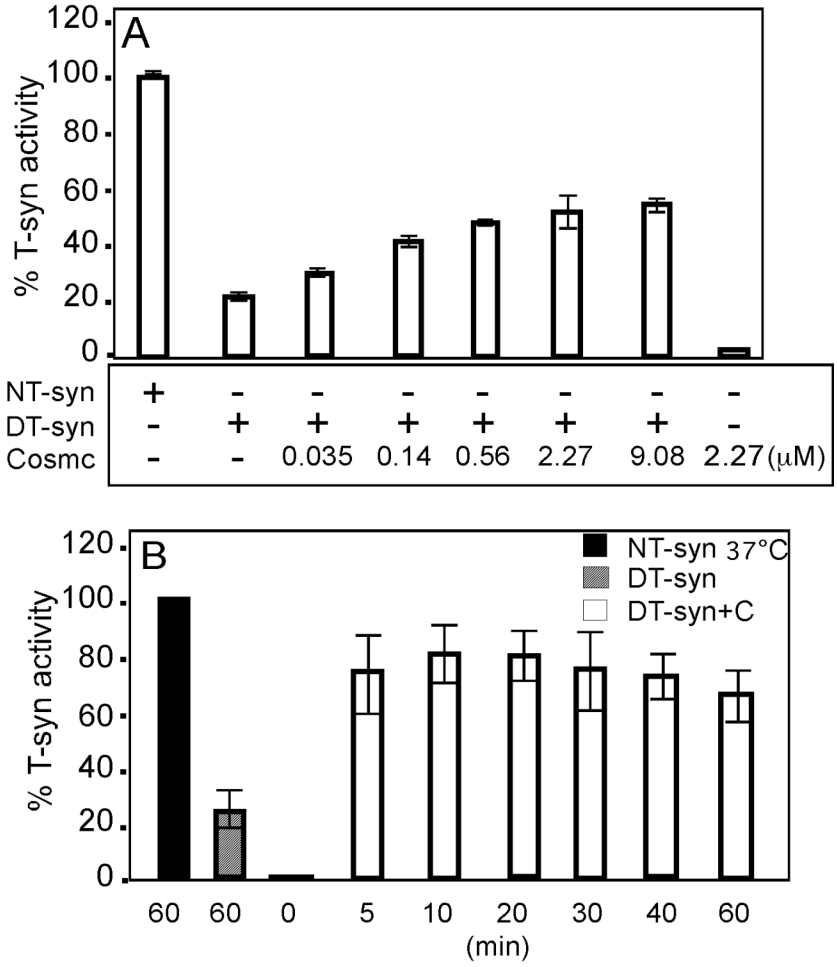

A
% T-syn activity
120
100
80
60
40
20
0
NT-syn + - - - - - - -
DT-syn - + + + + + + -
Cosmc - - 0.035 0.14 0.56 2.27 9.08 2.27 (μM)
B
% T-syn activity
NT-syn 37°C
DT-syn
DT-syn+C
60 60 0 5 10 20 30 40 60
(min)

Figure 2.2. Cosmc restoration of activity of heat-denatured T-synthase is concentration dependent. (A) Purified HPC4-sT-syn (NT-syn) was heat-denatured in reconstitution buffer. Renaturation of the activity of the heat-denatured HPC4-sT-syn (DT-syn) was initiated by the addition of increasing concentrations of 6xHis-sCosmc as indicated to DT-syn preparations and percent T-synthase activity of each reaction was determined. (B) Time-dependency of restoration of T-synthase activity from DT-syn by 6xHis-sCosmc, in which reconstitution and assay of T-synthase was conducted at 37°C and the percent restored activity was determined at different time points as indicated. In A, each assay was performed in duplicate and two replicate experiments were performed, and data represents the average of all experiments. In B, each assay was performed in duplicate and three replicate experiments were performed, and data represents the average of all experiments. Error bars = +/- 1 SD from the average.

2.4.3 Mutated form of Cosmc does not restore the activity of heat-denatured T-synthase as efficiently as soluble Cosmc

Cosmc with Glu (E) to Lys (K) mutation at position 152 (E152K) was originally identified in a patient with Tn syndrome (25) (**Figure 2.3A**). Co-expression of this mutated Cosmc with human HPC4-sT-syn in insect cells indicated that 6xHis-msCosmc (E152K) has little ability to function as a chaperone in a cell-expression system for forming active T-synthase (25). To investigate the characteristics of mutated Cosmc *in vitro,* we expressed and purified the tagged, soluble form of mutated Cosmc (6xHis-msCosmc) in Hi-5 cells (**Figure 2.3B**). 6xHis-msCosmc (~35kDa) is not as stable as wild type 6xHis-sCosmc and appears to be partly degraded (~50%) to a smaller form (~27kDa). Therefore, the SDS-PAGE and Coomassie-stained gel shown in Figure 2.3B utilized twice as much 6xHis-msCosmc (8 μg) compared to 6xHis-sCosmc (4 μg). A scan of the gel shows that the amount of material at ~35kDa in size for both 6xHis-msCosmc (Lane 2) and 6xHis-sCosmc (Lane 3) are similar. Using this preparation of 6xHis-msCosmc, we compared its ability to 6xHis-sCosmc to assist renaturation of heat-denatured HPC4-sT-syn, and tested the effect over time. To compensate for the partial degradation observed for 6xHis-msCosmc, we used twice the concentration of 6xHis-msCosmc as compared to 6xHis-sCosmc. While 6xHis-sCosmc was effective in assisting renaturation of heat-denatured HPC4-sT-syn, 6xHis-msCosmc was weakly active and had no significant activity in restoring HPC4-sT-syn activity heated for 3 minutes or more (**Figure 2.3C**). These results show that mutated Cosmc is not as effective as soluble wild-type Cosmc in this *in vitro* assay in assisting refolding activity of T-synthase.

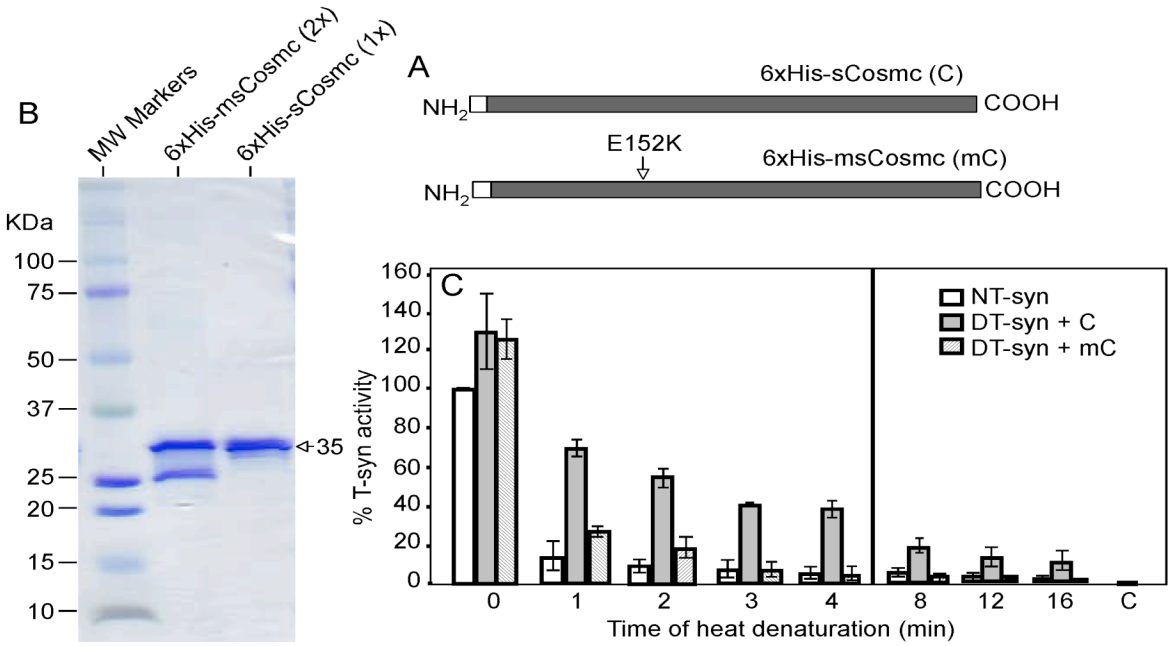
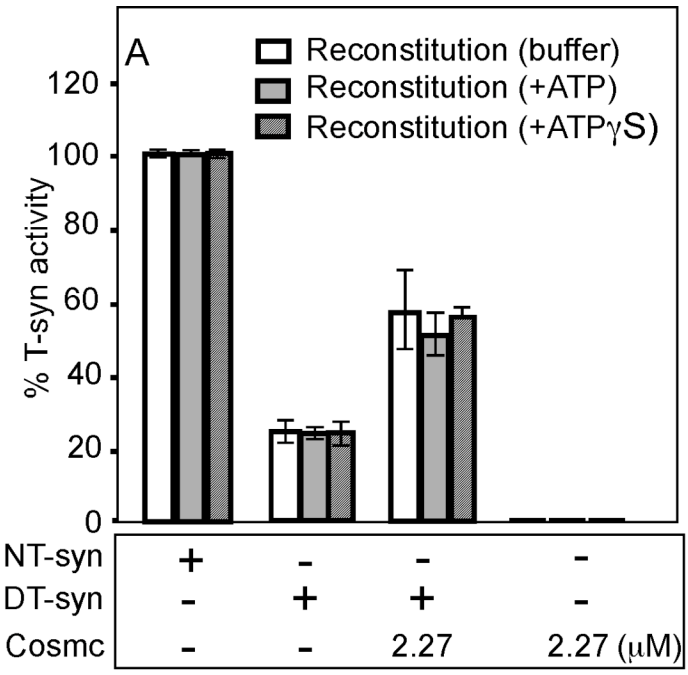

B
MW Markers
6xHis-msCosmc (2x)
6xHis-sCosmc (1x)
KDa
100
75
50
37
25
20
15
10
35
A
6xHis-sCosmc (C)
NH2
COOH
E152K
6xHis-msCosmc (mC)
NH2
COOH
C
NT-syn
DT-syn + C
DT-syn + mC
% T-syn activity
160
140
120
100
80
60
40
20
0
0
1
2
3
4
8
12
16
C
Time of heat denaturation (min)

Figure 2.3. Mutated Cosmc has little effect on restoration of denatured T-synthase activity. (A) Depiction of the 6xHis-sCosmc and 6xHis-msCosmc constructs, where the latter has a point mutation E152K. (B) SDS-PAGE analysis of purified recombinant soluble human 6xHis-sCosmc and 6xHis-msCosmc, as indicated. Lane 1 = molecular weight standards, Lane 2 = 6xHis-msCosmc (~8 μg), Lane 3 = Human N-terminal 6xHis-sCosmc (~4 μg). A densitometry scan of the protein bands in Lanes 2 and 3 showed that the amount of protein corresponding to the apparent M_r of ~35kDa for both 6xHis-smCosmc and 6xHis-sCosmc were similar. (C) Renaturation of heat-denatured HPC4-sT-syn (DT-syn) was initiated by addition of 6xHis-msCosmc and with recombinant 6xHis-sCosmc, and percent T-synthase activity was determined. In C, each assay was performed in duplicate and three replicate experiments were performed, and data represents the average of all experiments. Error bars = +/- 1 SD from the average. Vertical line separates experiments done at two different time points.

2.4.4 Cosmc does not require ATP for its in vitro renaturation of heat-denatured T-synthase

Our previous study showed that Cosmc, but not the T-synthase, has ATP-binding activity (16), which is consistent with a possible ATP-dependent chaperone function for the human Cosmc. It is interesting that in prior studies, the mouse homolog of Cosmc (NP_067525), which was studied before the function of Cosmc was identified, was also reported to have ATP binding activity (32). To explore whether ATP has any effect in the renaturation of heat-denatured T-synthase by Cosmc, we studied the effects of 6xHis-sCosmc *in vitro* in the presence of ATP, the non-hydrolyzable analog ATPγS, and buffer without nucleotide. The 5mM final concentration of nucleotide were chosen in this *in vitro* experiment because that amount was used in reconstitution of denatured luciferase by Hsp70 chaperone system (33). We first investigated the role of ATP where the 6xHis-sCosmc concentration was approximately 12-times higher than the concentration of HPC4-sT-syn. There was not a significant difference between the reconstitution of HPC4-sT-syn in the three different conditions (**Figure 2.4A**). To further explore the potential effect of ATP, we varied the concentrations of 6xHis-sCosmc in the presence of ATP and ATPγS as indicated. The data showed that neither ATP nor ATPγS had a significant difference in the restoration of activity of denatured HPC4-sT-syn (**Figure 2.4B**). These results indicate that Cosmc does not require ATP for the folding of T-synthase in this *in vitro* assay.


A
Reconstitution (buffer)
Reconstitution (+ATP)
Reconstitution (+ATPγS)
% T-syn activity
120
100
80
60
40
20
0
NT-syn + - - -
DT-syn - + + -
Cosmc - - 2.27 2.27 (μM)

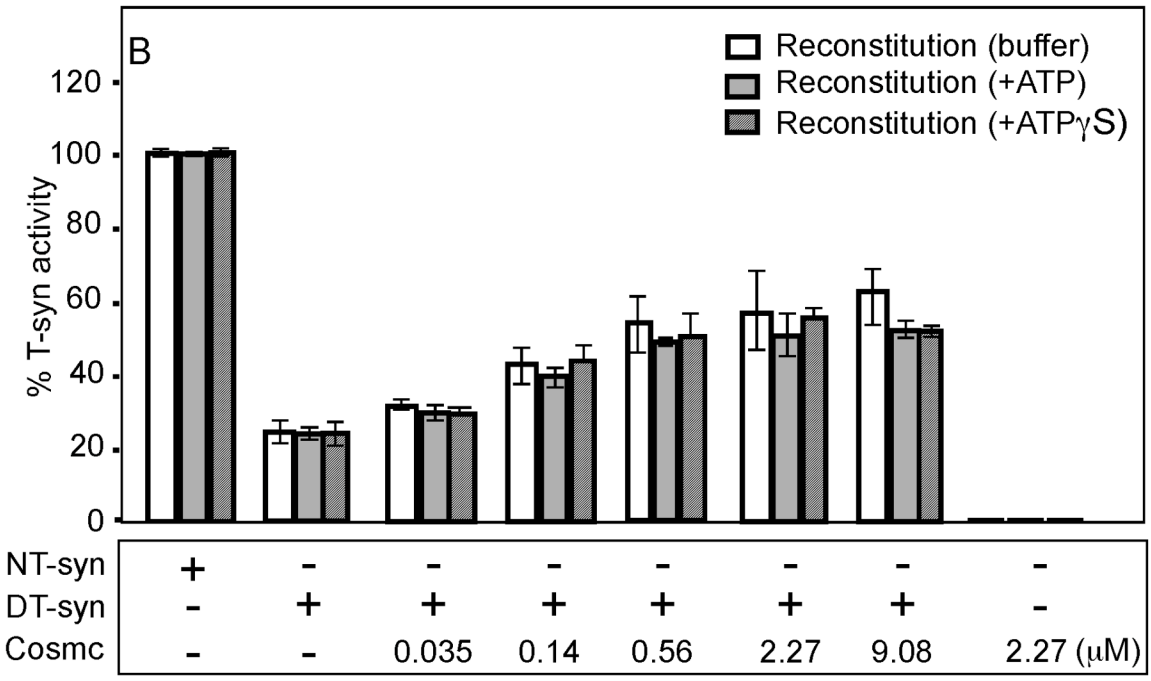

B
Reconstitution (buffer)
Reconstitution (+ATP)
Reconstitution (+ATPγS)
% T-syn activity
120
100
80
60
40
20
0
NT-syn + - - - - - - -
DT-syn - + + + + + + -
Cosmc - - 0.035 0.14 0.56 2.27 9.08 2.27 (μM)

Figure 2.4. Cosmc can restore the activity of heat-denatured T-synthase independently of ATP *in vitro*. (A) Purified HPC4-sT-syn (NT-syn) was heat-denatured in reconstitution buffer. Reconstitution of denatured HPC4-sT-syn (DT-syn) activity was initiated by the addition of 6xHis-sCosmc in reconstitution buffer with ATP, non-hydrolyzable ATP (ATPγS), or without nucleotide, where final concentration of nucleotides is 5mM and percent T-synthase activity was determined. (B) Reconstitution of heat-denatured HPC4-sT-syn by the addition of increasing concentration (μM) of 6xHis-sCosmc in reconstitution buffer with ATP, ATPγS, or without nucleotide, where final concentration of nucleotides is 5mM and percent T-synthase activity was determined. Each assay was performed in duplicate and two replicate experiments were performed, and the data represents the average of all experiments. Error bars = +/- 1 SD from the average.

2.4.5 Cosmc cannot restore the activity of denatured β1-4-Galactosyltransferase (β4-GalT)

To further examine whether Cosmc is specific to the T-synthase, we investigated the nature of its specificity *in vitro* by conducting reconstitution experiments for another partially denatured enzyme in parallel to the experiments conducted with denatured HPC4-sT-syn. To this end, we selected the well-studied enzyme UDPGal:GlcNAc β1-4-galactosyltransferase (β4-GalT) (34-37). Like T-synthase, β4-GalT is also a Golgi-localized β-glycosyltransferase, uses the same donor substrate UDP-Gal, and is also an inverting β-galactosyltransferase. The i*n vitro* reconstitution reaction was initiated by addition of either 6xHis-sCosmc or galectin-3 to heat-denatured β4-GalT and the activity of this enzyme was assayed. No reconstitution of denatured β4-GalT by either 6xHis-sCosmc or galectin-3 was observed, whereas in parallel control experiments, 6xHis-sCosmc was effective in restoring partial activity of heat-denatured HPC4-sT-syn (**Figure 2.5A**). We further investigated the ability of Cosmc to function as a potential chaperone for β4-GalT, which was heated over time in the expectation that a diversity of intermediate folded forms might be present ranging from slightly unfolded to fully unfolded forms. The β4-GalT was heated over time at 62°C, which resulted in time-dependent loss of its activity (**Figure 2.5B**). However, addition of 6xHis-sCosmc or galectin-3 had no significant effect on restoring activity of the β4-GalT. Taken together, these results show that Cosmc is specific for the T-synthase and is not able to restore activity of a related Golgi β-galactosyltransferase.

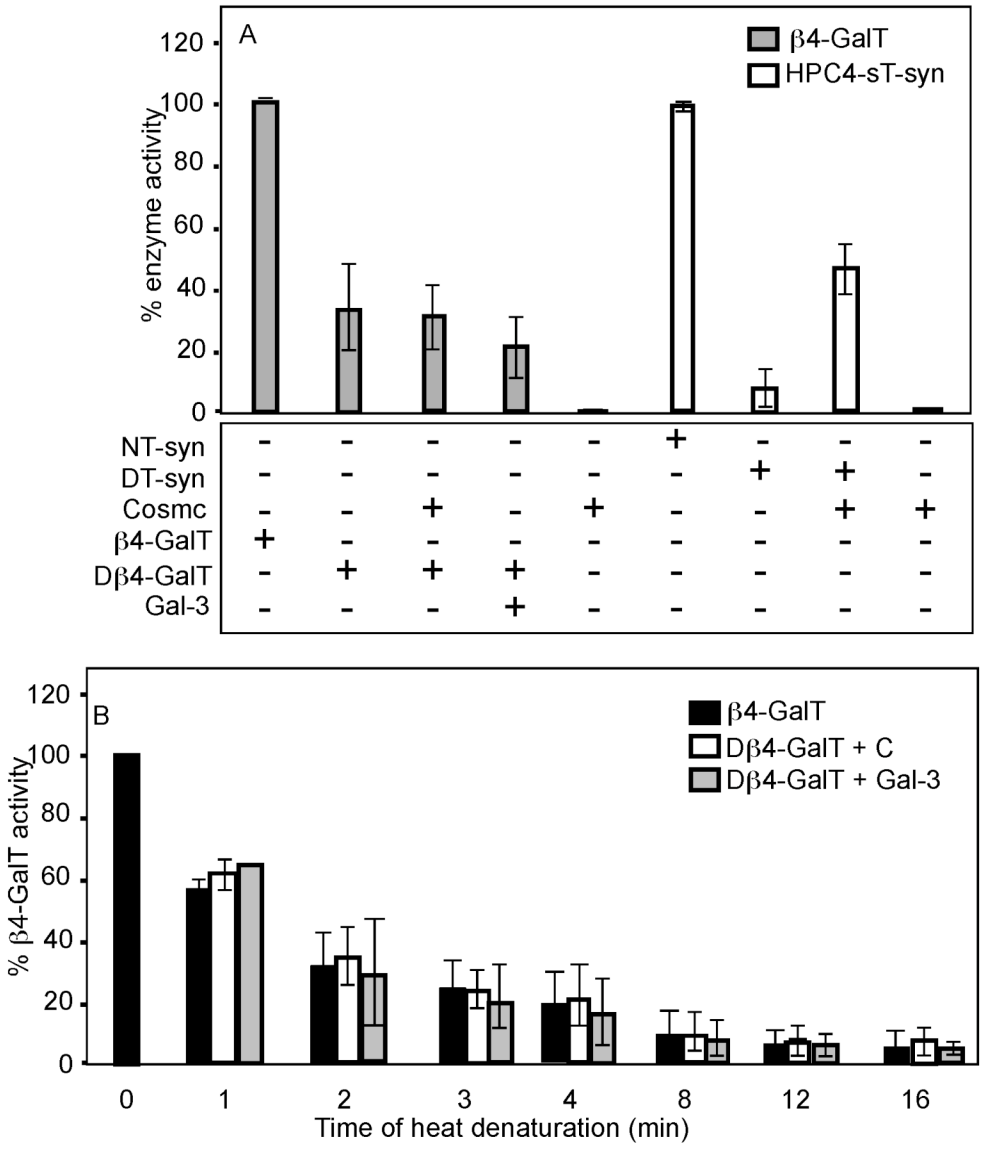

A
β4-GalT
HPC4-sT-syn
% enzyme activity
120
100
80
60
40
20
0
NT-syn - - - - - + - - -
DT-syn - - - - - - + + -
Cosmc - - + - + - - + +
β4-GalT + - - - - - - - -
Dβ4-GalT - + + + - - - - -
Gal-3 - - - + - - - - -
B
β4-GalT
Dβ4-GalT + C
Dβ4-GalT + Gal-3
% β4-GalT activity
120
100
80
60
40
20
0
0 1 2 3 4 8 12 16
Time of heat denaturation (min)

Figure 2.5. Cosmc is unable to restore activity to denatured β4-GalT. (A) β4-GalT was heat-denatured and reconstitution of denatured β4-GalT (Dβ4-GalT) was initiated by the addition of recombinant 6xHis-sCosmc or Gal-3 and percent β4-GalT activity was determined. In parallel, reconstitution of the heat-denatured HPC4-sT-syn (DT-syn) was initiated by the addition of recombinant 6xHis-sCosmc and percent T-synthase activity was determined. (B) β4-GalT was heat-denatured over time and reconstitution of denatured β4-GalT was initiated by the addition of recombinant 6xHis-sCosmc (C) or Gal-3 and percent β4-GalT activity was determined. Each assay was performed in duplicate and two replicate experiments were performed, and data represents the average of all experiments. Error bars = +/- 1 SD from the average.

2.5 Discussion

Cosmc is the first known ER molecular chaperone required for expression of a glycosyltransferase that functions in the Golgi apparatus, but the mechanism of its action has not been clear. Recently, we reported that ER-localized Cosmc can be co-immunoprecipitated with T-synthase (16), and we hypothesized that Cosmc might play a direct role in the biosynthesis of the active form of T-synthase by interacting with newly synthesized non-native T-synthase. The studies described here provide novel information as to how this ER chaperone functions and show that Cosmc directly interacts with partly unfolded and inactive T-synthase to partially restore enzyme activity. We showed previously that expression of active T-synthase in cells requires ER-localized Cosmc (16,17), and the lack of Cosmc function results in inactive T-synthase aggregates (16).

The biochemical studies and the *in vitro* functional assays using purified 6xHis-sCosmc show that Cosmc can restore activity from inactive forms of T-synthase following a brief period (<5 min) of incubation, that the addition of ATP had no effect in this assay format, and that restoration *in vitro* occurs independently of other chaperones/co-chaperones. Importantly, addition of the general ER chaperone BiP did not restore the activity of heat denatured T-synthase, whereas BiP does support renaturation of luciferase. It has been shown previously that recombinant Hsc70 can support *in vitro* refolding of heat-denatured luciferase (38). Furthermore, 6xHis-msCosmc E152K cannot restore the denatured HPC4-sT-syn *in vitro* as efficiently as 6xHis-sCosmc and 6xHis-sCosmc does not effect restoration of activity for another well-characterized β-galactosyltransferase (β4-GalT). Overall, our results provide experimental evidence *in vitro* that Cosmc is a specific chaperone for folding and maturation of the T-synthase.

Moreover, these studies have a broad impact in understanding the expression of Tn and Sialyl-Tn (22-24) which is associated with mutations in *Cosmc* and consequent deficiency of active T-synthase in human diseases, such as Tn syndrome (25) and human tumors (20,21).

The approach taken here is modeled after studies on other chaperones showing their ability to restore the activity of thermally or chemically denatured substrate. Typically, the ability of a chaperone to restore activity to denatured substrates can result in restoration of from 1-2% to nearly 80% of initial activity. For example, the small heat shock protein α/β crystallin causes ~14% restoration of a client protein citrate synthase and addition of ATP enhances that to 25% (39). Treatment of the Rubisco enzyme with 6M GnHCl results in considerable loss of secondary structure, and addition of chaperone Cpn60 (GroEL) with its co-chaperone Cpn10 (GroES) can rescue its enzymatic activity up to ~80% of initial (40). Additionally, chaperones such as Hsp70 or its prokaryotic homolog DnaK/DnaJ and GroEL-GroES, can reactivate both thermally and chemically denatured substrates (40-42). We observed that 6xHis-sCosmc can cause up to ~75% restoration of heat-denatured HPC4-sT-syn activity depending on how long the protein has been heat-treated (**Figure 2.1D and H**), and that it could restore ~30% of activity to HPC4-sT-syn denatured by GnHCl treatment (**Figure 2.1G**). Thus, Cosmc restoration of activity to denatured T-synthase is relatively similar to that seen for other chaperones with other clients *in vitro*, and probably reflects the heterogeneity of mis-folded forms of the enzyme generated by these denaturing conditions.

While Cosmc can restore the activity of denatured T-synthase activity independently of other co-chaperones *in vitro,* it is possible that co-chaperones play an

important role *in vivo* within the crowded environment of the ER regulating the kinetics of T-synthase refolding *in vivo*. We previously reported that inactive oligomeric T-synthase accumulating in cells in the absence of Cosmc is associated with GRP78 (16). Thus, while other chaperones may associate with T-synthase, their interactions appear to be non-productive in helping to form active enzyme, but may be important in ER stress responses or eliminating/removing the inactive and oligomeric T-synthase from the ER lumen. Future studies are required to examine the chaperone folding complex and assess whether co-chaperones may function with Cosmc. Some known chaperones require co-chaperones and others do not. For example, chaperones like Hsp60, Hsp70, and Hsp90 require co-chaperones for their functional cycle (5), while small heat shock proteins Hsp18.1, Hsp17.7, Hsp27, and Hsp25 do not require co-chaperones and can function independently as molecular chaperones (43,44).

The E152K mutated form of *Cosmc* found in Tn syndrome patients appears to lack any significant activity *in vivo*, since there is no detectable activity of T-synthase in the patient (25,45). However, the mutated Cosmc is expressed. Therefore, we tested whether this mutated Cosmc can restore the activity of denatured T-synthase using our *in vitro* reconstitution experiment. Clearly, while the 6xHis-msCosmc could not restore the activity of heat-denatured HPC4-sT-syn as efficiently as 6xHis-msCosmc (**Figure 2.3C**), there was a small but detectable level of restored activity. It is possible, but unlikely, that 6xHis-msCosmc (E152K) generated recombinantly in insect cells might differ structurally or in other respects from the protein expressed *in vivo*, or that the 6xHis tag might contribute in some indirect way to this partial functionality of the mutated Cosmc. The mutated protein expressed *in vivo* might not be folded correctly, localize differently,

interact with different interacting partner/s, or the steady state level of the protein could be lower, resulting in its no detectable function *in vivo*.

We previously reported that Cosmc binds to ATP-Sepharose, and can be crosslinked to α- ^{32}P-azido-ATP (16). The ability of Cosmc to bind ATP is consistent with prior reports showing that the mouse homolog of Cosmc also appears to have ATP binding activity (32). The functional cycles of many chaperones for assisting protein folding are enhanced by their interaction with nucleotides such as ATP/ADP (5,46). Our results indicate that ATP is not required *in vitro* for the renaturation of heat-denatured HPC4-sT-syn (**Figure 2.4A and B**). Consistent with our data, several other ATP-independent chaperones have been identified, including Hsp47 (47), small Hsp (48), calnexin (49), SecB (50) and α-crystallin (51). Thus, the role, if any, of ATP in Cosmc function remains to be determined. It is possible that ATP binding by Cosmc may be important in a cycle of binding and release of T-synthase *in vivo*, through interactions with other proteins or perhaps co-chaperones.

The studies described here show that inactive and partly mis-folded T-synthase can be partly restored to active form by incubation with 6xHis-sCosmc and suggest that Cosmc activity is specific for T-synthase and not active toward another inverting β-galactosyltransferase. In other studies, we have generated mice deficient for *Cosmc* and found that embryonic cells from such null animals completely lack T-synthase activity but have apparently other normal protein glycosylation except for loss of core 1 O-glycans (Yingchun Wang, Tongzhong Ju, and Richard D. Cummings, unpublished observations). The experimental results here are consistent with such observations and imply the apparently specific requirement of Cosmc for forming active T-synthase.

Experiments are underway using mutants of Cosmc and T-synthase to define the molecular sites responsible for Cosmc/T-synthase inter-molecular interactions.

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Acknowledgements

We thank Dr. Sean Stowell for providing recombinant Galectin-3, Anthony Luyai for suggestions and critique of this manuscript, and Dr. Jamie Heimburg-Molinaro for help in preparing and editing the manuscript. This work was supported by NIH RO1 grant (RO1 GM068559) to R.D.C.

CHAPTER 3: Cosmc directly facilitates folding of T-synthase to promote correct protein O-glycosylation

This chapter has been submitted for publication.

Authors: Rajindra P Aryal, Tongzhong Ju, Richard D. Cummings. Role of Cosmc in Promoting Folding and Activity of T-synthase.

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3.1 Abstract

T-synthase is the key enzyme for biosynthesis of core 1 O-glycans in all animal cell glycoproteins. Cosmc is an ER resident chaperone that assists the correct folding of T-synthase but its mechanism of action is poorly understood. Here we demonstrate that Cosmc directly interacts with non-native, but not native, T-synthase to form a relatively stable complex. We found that Cosmc does not require ATP for its functional cycle *in vitro* indicating that Cosmc is an ATP-independent chaperone. Cosmc function is not regulated by redox, calcium, or pH 6.3-7.4. More importantly, reactivated T-synthase dissociates from the Cosmc complex in the presence of an excess of non-native T-synthase. These data suggest that during the biosynthesis of T-synthase, Cosmc directly interacts and promotes the folding of newly synthesized non-native T-synthase within the crowded environment of the ER and forms a transient catalytically active complex. Cosmc releases the catalytically active T-synthase when the newly synthesized non-native T-synthase enters the ER and associates with Cosmc for another round of refolding. These findings not only aid in understanding the molecular mechanism of the Cosmc chaperone function, but also shed light on the molecular basis of acquired human diseases associated with altered O-glycan expression involving inactive Cosmc/T-synthase, such as Tn syndrome, IgA nephropathy, Henoch-Schönlein purpura, and malignant transformation.

3.2 Introduction

Mucin-type O-glycosylation is one of the most common posttranslational modifications to animal glycoproteins and is important in many biological processes (1). The first step in this pathway is catalyzed by a family of polypeptide-N-acetylgalactosaminyltransferases (ppGalNAcTs) that covalently modify Ser/Thr by the addition of N-acetylgalactosamine (GalNAc) (2,3). The resulting structure is known as the Tn antigen (GalNAcα1-Ser/Thr) and is the common precursor structure to all mucin-type O-glycans (3,4). Although 24 different ppGalNAc transferases are involved in the first step, the next step can only be catalyzed by a single enzyme called T-synthase, or core 1 β1-3 galactosyltransferase, that adds galactose onto Tn antigen to form the core 1 disaccharide O-glycan Galβ1-3GalNAcα1-Ser/Thr (Thomsen-Friedenrich antigen or T antigen) (4,5). The T antigen can be further modified by several downstream glycosyltransferases in the Golgi compartment to form complex O-linked glycans that are critically important in many cellular processes such as animal development, lymphocyte homing (6), and leukocyte homing (7,8).

The generation of functional T-synthase requires a specific molecular chaperone termed Cosmc, which promotes the activity of T-synthase both *in vivo* and *in vitro* (9,10). In the absence of functional Cosmc, enzymatically inactive T-synthase aggregates in the ER and retrotranslocates back to the cytosol where it is ubiquitinated and degraded by proteasomal machinery (11). Cells lacking Cosmc or T-synthase express the Tn and Sialyl-Tn antigens, which are known as tumor-associated carbohydrate antigens (TACA) predominantly found in many carcinomas (9,12,13). Acquired somatic mutations in *Cosmc* give rise to Tn Syndrome and human cancers (12,14,15). Importantly, T-synthase

activity is present in most cell types and T-synthase is coordinately expressed with Cosmc (11). T-synthase is developmentally important and the deletion of this enzyme in mice is embryonic lethal (16). Similar to the T-synthase, Cosmc knockout mice die at embryonic days10.5 to 12.5 (17). Therefore, a clear picture of how Cosmc works to make functional T-synthase and how this interaction is regulated is crucial for our understanding of several human diseases.

In principle, chaperones interact specifically with unfolded but not with folded protein structures, facilitating the formation of native structures (18) and do not form part of the three dimensional structure of functional protein (19). Several families of chaperones are responsible for protein folding in the ER; those most studied are BiP (Grp78), protein disulfide isomerase (PDI), and calnexin and calreticulin families and these chaperones have different types of chaperone cycles for substrate binding and release. For example, BiP belongs to the family of Hsp70 chaperones that bind and release substrates through the allosteric movement of both a conserved ATPase N-terminal domain and a peptide binding C-terminal domain (20,21). ATP is critical for the binding and release of non-native proteins by Hsp70 and this process is assisted by co-chaperone Hsp40 and nucleotide exchange factors (NEF) (21). Hsp40 regulates the ATPase activity of Hsp70 (22). In the ATP bound state, Hsp70 has low affinity for the substrate and when ATP is hydrolyzed to ADP, the resulting ADP bound state of Hsp70 has higher affinity for the substrate (19). PDI chaperones function by a different mechanism. PDI helps to form disulfide bonds, which are required for the folding of newly synthesized proteins that travel through the secretory pathway. PDI has two thioredoxin like motifs (CXXC) and is a redox-regulated chaperone (23). Based on the

redox state of the environment, the CXXC motif of PDI can make, shuffle, or reduce the disulfide bonds of newly synthesized proteins (24). Interestingly, the lectin-based chaperone system, calnexin and calreticulin, assist the folding of only glycoproteins and depend mostly on the monoglucosylated core glycans (25). Hsp47 is critical for collagen biosynthesis and interacts transiently with procollagen in an ATP-independent manner, and the release of collagen seems to be regulated by changes in pH, especially the intraorganelle pH (26-29). Similarly, RAP binds and promotes the folding of the LDL receptor at neutral pH and then dissociates from the substrate at the lower pH of the Golgi (30,31).

Previous studies have shown that Cosmc is an ER localized specific molecular chaperone for T-synthase (11,32). However, Cosmc chaperone function in the ER is not well understood. In the present study, we examined how Cosmc acts to promote formation of functional T-synthase. Our results show that Cosmc acts as a specific molecular chaperone and it binds non-native T-synthase and promotes it acquisition of activity in a chaperone cycle involving binding and release of native/non-native T-synthase.

3.3 Materials and Methods

Preparation of different versions of Cosmc and T-synthase

Soluble N-terminal 6×His-tagged Cosmc (His-sCosmc) and soluble N-terminal HPC4-tagged T-synthase (HPC4-sT-syn) were prepared as described (10). N-terminal 6×His-tagged soluble Cosmc (His-sCosmc) and N-terminal HPC4-tagged soluble T-synthase (HPC4-sT-syn) were produced in pVL1393 vector as described (11). To obtain

active T-synthase preparations, Asialo-BSM beads were prepared as described (5) and 500 ml of media containing secreted HPC4-sT-syn was produced as described (10) and mixed with 500 µl of Asialo-BSM conjugated beads in the presence of 20 mM $MnCl_2$ and incubated for 3-4 hours. Beads were washed 3 times with wash buffer containing 20 mM $MnCl_2$ and protein was eluted by 1 M NaCl in buffer lacking Mn^{2+} and concentrated using Centricon 10kDa cutoff. HPC4-sCosmc preparation was prepared as described (10,11). Soluble N-terminal 6×His-tagged T-synthase (His-sT-syn) was prepared in pVL1393 using the similar strategy for making His-sCosmc as described (10).

Preparation of anti-HPC4 and Cosmc conjugated beads

Anti-HPC4 and His-sCosmc conjugated UltraLink Biosupport beads were prepared following the manufacturer's protocol (Pierce) using ~1.5 µg/µl of the beads unless otherwise stated. In parallel, control beads without protein were prepared.

Characterization of recombinant HPC4-sT-synthase using Asialo-BSM beads

HPC4-sT-syn (1.2 µg) was prepared in 300 µl of buffer (50 mM Tris-HCL, 20mM $MnCl_2$, 150 mM NaCl, 0.1% Triton X-100, pH 7.0). Two thirds of the preparation was mixed with 10 µl Asialo-BSM beads, equilibrated in the buffer, and incubated for 20 min on ice. Beads were pelleted by centrifugation at 200xg for 1 min and supernatant was mixed with 10 µl fresh Asialo-BSM beads and the process was repeated four times. Supernatant (~200 µl) was collected. All beads were pooled and washed five times with 1 ml of washing buffer. All washes were pooled and concentrated to 100 µl using Centricon 10kDa cutoff. In parallel, the same experiment was conducted for beads alone.

One tenth of the preparation was assayed for T-synthase activity, which was carried out for 30 min, and one fifth of the total reaction was assayed to determine the activity. One tenth of the sample was directly boiled and used for SDS-PAGE and western blotting.

***In vitro* reconstitution experiments**

Recombinant HPC4-sT-syn (0.25 μg) was heat denatured at ~55°C for 2 minutes in reconstitution buffer (10 mM HEPES, 12 mM $MgCl_2$ at pH 7.8) and cooled to RT followed by addition of 5 mM ATP final concentration. Renaturation was initiated by the addition of His-sCosmc to a final concentration of 2.27 μM unless otherwise stated and incubated for 45 min. T-synthase activity was measured as described (5,33). Specific T-synthase activity of native T-synthase was considered 100% activity, and all activity experiments were calculated accordingly.

***In vitro* reconstitution experiments with Cosmc beads and pull down experiments**

Recombinant HPC4-sT-syn (0.45 μg) was heated at ~55°C in reconstitution buffer and cooled to RT. Cosmc beads (20 μl of 50% slurry) were diluted in 100 μl buffer containing 0.1% Triton X-100 (pH 7.8) with 5 mM ATP or ATPγS final concentration. In this preparation, renaturation was initiated by the addition of denatured T-synthase and incubated for 45 min at RT. Beads were pelleted by centrifugation (200xg) for 1 minute and washed 5 times with 400 μl of wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, pH 7.8). Supernatant was collected and all washes were pooled and concentrated to 100 μl. Input, bound, and unbound materials were analyzed by western blotting against HPC4. Bound and unbound (supernatant and wash) were assayed for

activity. Total activity of native T-synthase was considered 100% activity (Counts/h) and the activity of all experiments was defined accordingly.

N-Ethylmaleimide (NEM) reaction

Recombinant His-sCosmc (28 μM) and NEM (24 mM) final concentration were prepared in reaction buffer (100 mM phosphate, 150 mM NaCl, pH 7.2). After incubating overnight at 4°C, the reaction was passed through a PD10 column and equilibrated with 5 mM Tris-HCl containing 30 mM NaCl, pH 7.8. The protein was concentrated and the molecular mass of both NEM modified and unmodified Cosmc were determined using mass spectrometric analysis (MALDI-TOF).

Sodium Arsenite reaction

Recombinant His-sCosmc (15.5 μM) was incubated with DTT (290 μM) for 1 hour on ice. The reaction mixture was passed through a PD10 column equilibrated with 5 mM Tris-HCl, 30 mM NaCl, pH 7.8. This preparation of recombinant His-sCosmc (2.4 μg) was used to refold heat-denatured HPC4-sT-syn (0.25 μg) in the presence of 13 μM Sodium Arsenite final concentration.

Release experiments

HPC4-sT-syn (2 μg) was heat denatured at ~55°C for 2 minutes and then cooled to RT. Renaturation was initiated by the addition of 40 μl His-sCosmc beads (50% slurry, 0.5 μg/μl of the beads) and prepared reconstituted active HPC4-sT-syn associated with 6×His-sCosmc beads as described above. In this preparation, 25 μl containing 1.5 μg of

heat denatured His-sT-syn (10 mM HEPES, 150 mM NaCl, 12 mM $MgCl_2$, pH 7.8), which was heated at 60°C for approximately 2 minutes, was added. As a control, approximately 3 μg of BSA, which was heat denatured at ~65°C for 15 minutes was used. The reaction mixture was incubated at RT for 30 minutes, mixing every 4-5 minutes. Beads were centrifuged for 1 minute at 1500xg and allowed to settle on ice for 5 min. Four fifths of the supernatant was allocated for SDS-PAGE and western blotting for HPC4. Gels were silver stained to determine the amount of protein used for the elution. Beads were boiled and one fifth of the mixture was used for SDS-PAGE and western blot analysis. Dose studies were conducted using 0.2, 0.5, and 2.0 μg of heat denatured His-sT-syn.

Quantification of Western blot data

Western blot films were scanned using a Cannon scanner and bands were quantified using an Alphatech system.

Plasmid Construction

Cysteine (C) to Serine (S) mutant version of HPC4-sT-syn (HPC4-msT-syn) was made using site directed mutagenesis (Quickchange, Stratagene) using the soluble version of HPC4-sT-syn as the template(34). HPC4-msT-syn has the point mutation C233S. The following primers were used:

5’GATGCATTTAAAACAGACAAGTCTACACATAGTTCCTCCATTGAAGAC3’

5’GTCTTCAATGGAGGAACTATGTGTAGACTTGTCTGTTTTAAATGCATC3’

Mutation was further confirmed by sequencing.

Recombinant Protein Expression and Purification

HPC4-msT-syn was expressed and purified as described (10). The protein was expressed and purified as described (10).

In-solution refolding followed by pull down experiments

His-sT-syn (0.45 μg) was heat denatured at 60°C in reconstitution buffer (10 mM HEPES buffer containing 12 mM $MgCl_2$ at pH 7.8) for 2 minutes and cooled to room temperature. Reconstitution was initiated by the addition of HPC4-sCosmc (~2 μg) and ATP (5 mM final concentration). The reaction mixture was incubated for 45 minutes followed by addition of wash buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM $CaCl_2$, 0.1% Triton X-100, pH 7.8). In this preparation, 20 μl of anti-HPC4 beads were added and incubated for 1 hour at 4°C. Beads were pelleted, washed with 400 μl wash buffer containing 1 mM $CaCl_2$ for 5 times. All beads, $1/3^{rd}$ of supernatant and $1/7^{th}$ of the first wash were assayed for activity, and total activity of bound, supernatant, and wash was calculated accordingly. In this case total activity (Bound, Supernatant, and Wash) of native T-synthase was considered 100% and the activity in other experiments was calculated accordingly.

Treatment of Cosmc by 6 M GnHCl

His-sCosmc (152 μg) was treated with 1 ml 6M GnHCl final concentration and incubated overnight. The preparation was diluted to 4 ml in buffer (5 mM Tri-HCl, 30 mM NaCl, pH 7.8). The preparation was concentrated and washed 5 times with buffer

and further incubated for 1 hour at 4°C and the preparation was used for refolding experiments.

Crosslinking

Proteins were prepared in 10mM HEPES, 150mM NaCl, 12mM $MgCl_2$ pH 7.8 and crosslinking was carried out using bis(sulfosuccinimidyl)suberate (BS3) (Thermoscientific) at 1mM final concentration. Reaction was incubated for 30 minutes at RT and quenched by using 1M Tris-HCl for 15 minutes, which was followed by SDS PAGE analysis, and WB.

Chromatography

Sup610/300 column chromatography was used in all experiments. Column was equilibrated with equilibrium buffer (10mM HEPES, 150mM NaCl, 12mM $MgCl_2$ pH 7.8) and calibrated by using Biorad standards, BSA, and a recombinant form of human galectin-3. Proteins were prepared in the equilibrium buffer and incubated at least two hours before loading into the column. 400 microliter per fraction was collected for further analsysis.

3.4 Results

3.4.1 Cosmc does not promote the activity of native T-synthase but promotes the refolding of denatured T-synthase

To investigate the refolding of denatured soluble N-terminal HPC4-tagged T-synthase (HPC4-sT-syn) by soluble N-terminal 6×His-tagged Cosmc (His-sCosmc) *in*

vitro, we first characterized its activity with asialo-bovine submaxillary mucin (Asialo-BSM). It has been shown that active T-synthase binds to Asialo-BSM in the presence of Mn^{2+} (5). We reasoned that if our preparation contained active HPC4-sT-syn, we would recover it in the Asialo-BSM bound fraction. The majority of the protein remained in the bound fraction with Asialo-BSM (**Figure 3.1A and 1C**) while the HPC4-sT-syn preparation did not bind to beads alone (**Figure 3.1B and 1D**). These results demonstrate that the majority of the protein is active. These data support previous results of His-sCosmc restores the activity of heat-denatured HPC4-sT-syn, and indicate that substantial restoration of activity in the heat-denatured material in a Cosmc-dependent fashion is not due to cofirmational change of a population of misfolded T-synthase in the initial preparation.

To further explore this possibility and the general role of Cosmc in the biosynthesis of active T-synthase, we developed a functional assay to investigate the chaperone function of Cosmc *in vitro* and showed that His-sCosmc directly promotes the activity of both chemically or heat denatured HPC4-sT-syn independently of other factors (10). The addition of His-sCosmc to the purified preparation of HPC4-sT-syn slightly increases the activity of T-synthase (10). Alexander *et al* similarly reported that the addition of recombinant purified soluble FLAG-tagged Cosmc slightly enhances the activity of purified FLAG-tagged T-synthase (35). Here we asked whether Cosmc promotes the activity of a purified enzymatically active T-synthase preparation. We used Asialo-BSM beads to purify only enzymatically active T-synthase. We successfully purified the HPC4-sT-syn secreted into the media to apparent homogeneity (**Figure 3.1E).** Interestingly, His-sCosmc does not further enhance the activity of Asialo-Bsm –

purified active HPC4-sT-syn (NT-syn) (**Figure 3.1F, Lanes 1 and 2**). However, His-sCosmc could significantly restore the activity of heat-denatured HPC4-sT-syn (DT-syn) (**Figure 3.1F, Lanes 3 and 4**). These results suggest that Cosmc specifically facilitates folding of denatured T-synthase independent of other factors but does not enhance the activity of native T-synthase.

Furthermore, we asked whether the soluble form of T-synthase exist as a dimer or monomer by crosslinking experiments. Interestingly, we found that in reducing SDS-PAGE the T-synthase behaved as a monomeric species of ~38kDa, but in the presence of the crosslinker BS3, the T-synthase was extensively cross-linked to a dimeric species of ~75kDa (**Figure 3.1G).** Control studies were done with BSA, which showed that its mobility was shifted to slower migrating species upon modification with BS3, but no apparent dimeric species were found. These results suggest that the recombinant soluble form of T-synthase is a non-covalent dimer in solution. Whether Cosmc assist the assembly of the dimeric form of T-synthase or T-synthase self dimerizes is not yet known.

In order to determine whether His-sCosmc is oligomeric in solution, chemical crosslinking and gel filtration experiments were carried out. Chemical crosslinking experiments shows that the soluble form of His-sCosmc in reducing SDS-PAGE migrated as a ~35kDa species, but could be crosslinked with BS3 to generate apparent crosslinked dimers of ~70kDa, along with some higher molecular cross-linked species **(Figure 3.1H)**. Again, control studies with BSA showed no evidence for efficient cross-linking to dimeric species. Interestingly, in gel filtration experiments, Cosmc behaved as two major species, one corresponding to a monomer and the other to a hexamer **(Figure**

3.1I). These results suggest that dimeric form of His-sCosmc may exist in an equilibrium of monomers and hexamers, with the possibility that unstable dimeric species might be intermediates in this possible equilibrium, but further studies are required to resolve the issue. In addition, the relevance of different forms of His-sCosmc in terms of T-synthase folding is not known yet.

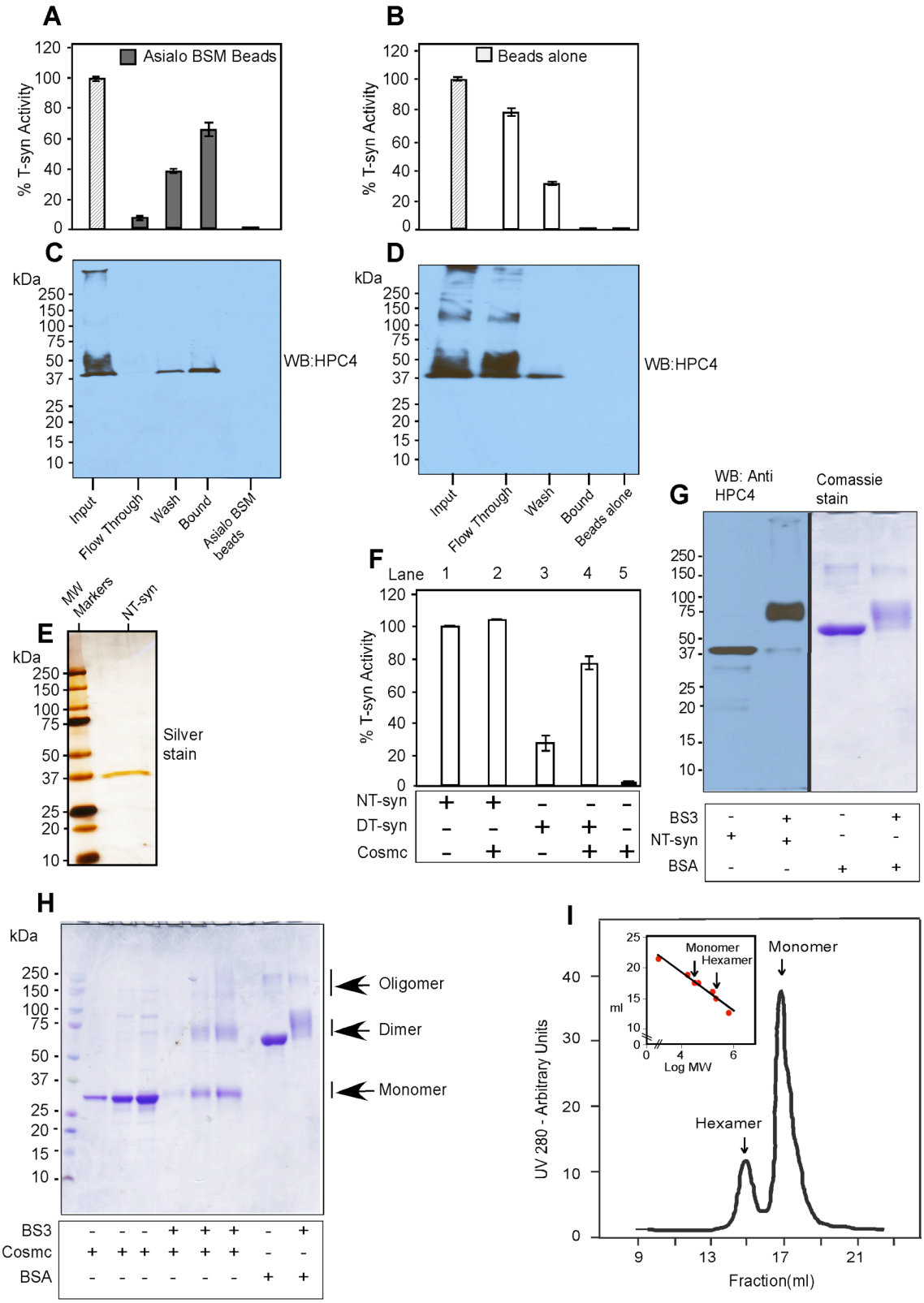

A
Asialo BSM Beads
% T-syn Activity
B
Beads alone
% T-syn Activity
C
kDa
WB:HPC4
Input
Flow Through
Wash
Bound
Asialo BSM beads
D
kDa
WB:HPC4
Input
Flow Through
Wash
Bound
Beads alone
E
MW Markers
NT-syn
kDa
Silver stain
F
Lane 1 2 3 4 5
% T-syn Activity
NT-syn
DT-syn
Cosmc
G
WB: Anti HPC4
Comassie stain
BS3
NT-syn
BSA
H
kDa
Oligomer
Dimer
Monomer
BS3
Cosmc
BSA
I
Monomer
Hexamer
ml
Log MW
Monomer
Hexamer
UV 280 - Arbitrary Units
Fraction(ml)

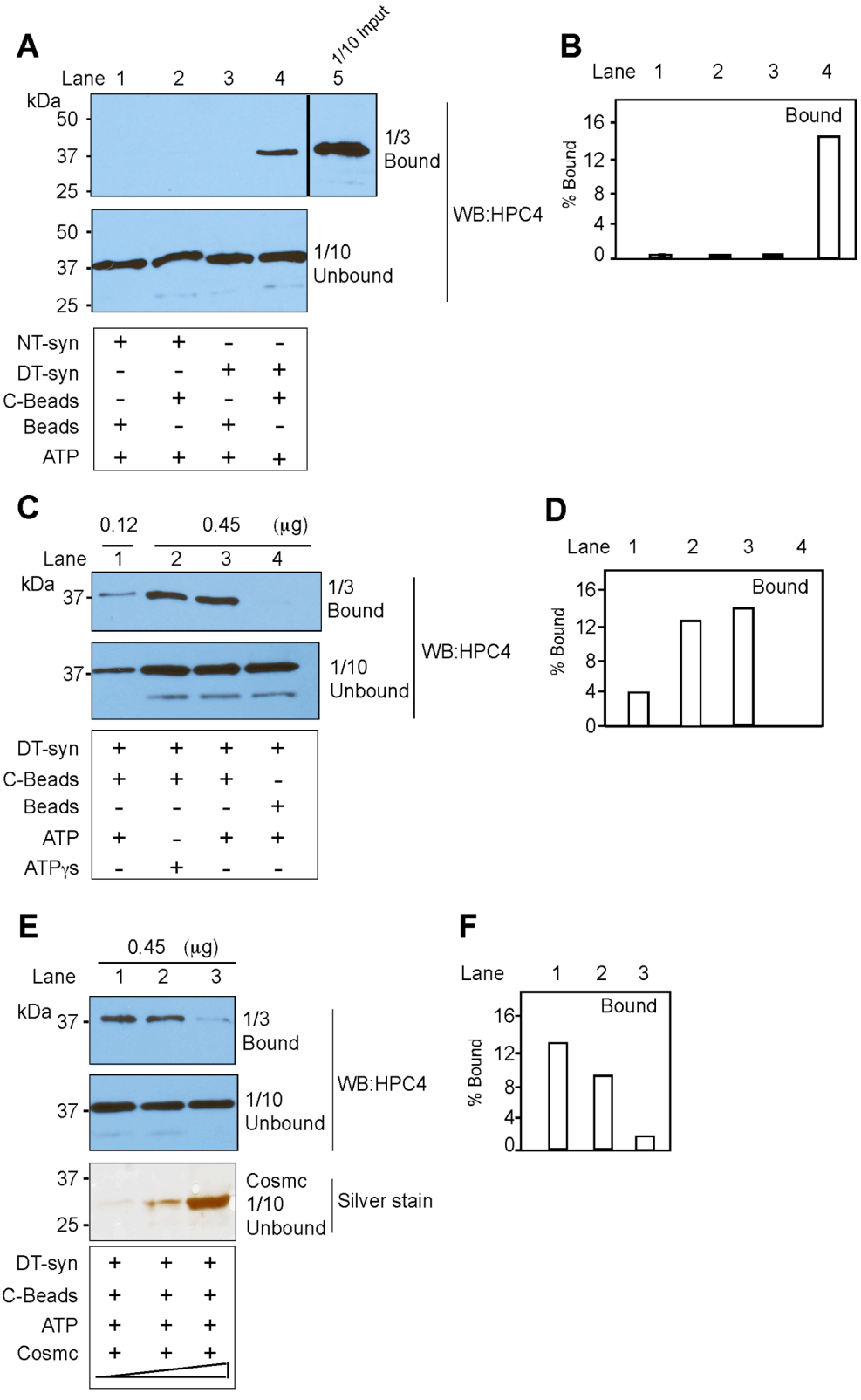
Figure 3.1. Characterization of recombinant HPC4-T-synthase and His-sCosmc. Human recombinant N-terminal HPC4 epitope-tagged soluble T-synthase (HPC4-sT-syn) was co-expressed with wild type membrane-bound Cosmc in Hi-5 insect cells. Also human N-teminal His6 epitope-tagged soluble Cosmc (His-sCosmc) was expressed in Hi-5 insect cells. Both tagged proteins were purified directly from the media. (**A-D**) HPC4-sT-syn preparation was characterized in terms of its activity by incubating with Asialo-BSM beads or beads alone. Pulls down experiments were carried out and HPC4-sT-syn bound to Asialo-BSM beads was determined either by measuring the activity of T-synthase (**A** and **B**) or Western blotting against HPC4 (**C** and **D**). In **A** and **B**, two replicate experiments were performed, and the data represents the average of the two independent experiments. Error bars, ±1 S.D. from the average. **C** and **D** show a representative example of two independent experiments. (**E**) Recombinant HPC4-sT-syn (NT-syn) purified with Asialo BSM beads was resolved by SDS-PAGE and visualized by silver stain. (**F**) *In vitro* refolding shows that Cosmc restores the activity of Asialo BSM purified heat denatured HPC4-sT-syn (DT-syn) but not the activity of the native active HPC4-sT-syn (NT-syn). Each experiment was performed in duplicate, two replicate experiments were performed, and the data represents the average of all experiments. Error bars, ±1 S.D. from the average. (**G**) HPC4-T-synthase (0.25µg) and BSA (6µg) were crosslinked and analyzed by SDS-PAGE and WB. Anti HPC4 antibody was used to probe HPC4-T-synthase. Control BSA was crosslinked parallelly and analyzed by SDS-PAGE followed by Comassie stain. (**H**) Different concentrations of soluble His-sCosmc (2, 4 and 6µg) and BSA (6µg) were crosslinked and analyzed by sing SDS-PAGE followed by comassie statin. (**I**) Size exclusion chromatography (SEC) was performed,

using 300µg of the Cosmc in 100µl of the total volume. Eluted protein was detected by using in line detection absorbance at 280nM (dashed line). Elution profile of the molecular weight standard and Cosmc (Monomer (M) and Hexamer (O)) are shown in the inset.

3.4.2 Cosmc forms a stable complex with non-native but not with native T-synthase

Molecular chaperones bind to non-native proteins, but not to native proteins, to form a stable complex leading to productive folding (36,37). To investigate the nature of Cosmc binding to T-synthase, we designed an assay that may mimic the postulated folding of T-synthase by ER-localized Cosmc. We predicted that membrane-bound Cosmc directly interacts with a newly synthesized unfolded T-synthase forming a stable complex to promote its folding, independently of other chaperones. In our assay, we replaced both membrane-bound Cosmc and newly synthesized T-synthase with covalently coupled His-sCosmc Ultralink beads and heat-denatured HPC4-sT-syn, respectively, and performed a direct interaction study. We performed pull down experiments by incubating heat-denatured or native HPC4-sT-syn with His-sCosmc conjugated beads to determine whether HPC4-sT-syn is associated with His-sCosmc conjugated beads by Western blotting with anti-HPC4 antibody. Interestingly, some portion of denatured HPC4-sT-syn (DT-syn) was directly associated with His-sCosmc conjugated beads (**Figure 3.2A, Lane 4;** quantification in **Figure 3.2B, Lane 4**) but we did not detect any association with native HPC4-sT-syn (NT-syn) (**Figure 3.2A, Lane 2**). As controls, denatured (**Figure 3.2A, Lane 3**) and native (**Figure 3.2A, Lane 1**) HPC4-sT-syn does not nonspecifically bind to the beads. To further characterize the binding of DT-syn with His-sCosmc conjugated beads, we performed the binding experiment with varying concentrations of DT-syn with a constant amount of His-sCosmc conjugated beads. We found that the binding of DT-syn with His-sCosmc conjugated beads was concentration dependent (**Figure 3.2C, Lanes 1 and 3;** quantification in **Figure 3.2D, Lanes 1 and 3**). As a control, DT-syn does not bind nonspecifically to beads (**Figure**

3.2C, Lane 4). Furthermore, we asked whether His-sCosmc could compete with His-sCosmc conjugated beads in terms of the binding of DT-syn. For these experiments, we incubated varying concentrations of His-sCosmc conjugated beads with a constant amount of DT-syn. We observed that the binding of DT-syn with His-sCosmc conjugated beads was competitively inhibited with His-sCosmc in a dose dependent manner (**Figure 3.2E, Lanes 1, 2, and 3;** quantification in **Figure 3.2F, Lanes 1, 2 and 3**). This finding suggests the existence of a relatively stable complex between Cosmc and non-native T-synthase but not with native T-synthase, which is consistent with our hypothesis that Cosmc functions as a chaperone for T-synthase.


A
1/10 Input
Lane 1 2 3 4 5
kDa
50
37
25
1/3 Bound
1/10 Unbound
WB:HPC4
NT-syn + + - -
DT-syn - - + +
C-Beads - + - +
Beads + - + -
ATP + + + +
B
Lane 1 2 3 4
Bound
% Bound
16
12
8
4
0
C
0.12 0.45 (µg)
Lane 1 2 3 4
kDa
37
1/3 Bound
1/10 Unbound
WB:HPC4
DT-syn + + + +
C-Beads + + + -
Beads - - - +
ATP + - + +
ATPγs - + - -
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Cosmc 1/10 Unbound
Silver stain
DT-syn + + +
C-Beads + + +
ATP + + +
Cosmc + + +
F
Lane 1 2 3
Bound
% Bound
16
12
8
4
0

Figure 3.2. Cosmc directly interacts with denatured T-synthase but not with Native T-synthase. Reconstitution was initiated by the addition of His-sCosmc conjugated beads to the preparation of heat denatured HPC4-sT-syn and incubated for 45 mintues. After incubation, beads were pelleted, washed, and analyzed by western blotting for HPC4. A parallel experiment was conducted with native HPC4-sT-syn. (**A**) Denatured HPC4-sT-syn (DT-syn) directly interacts with His-sCosmc but not with native HPC4-sT-syn (NT-syn). Bound and unbound materials were analyzed to determine the amount of HPC4-sT-syn bound to His-sCosmc beads. A vertical line separates the data from different parts of the same gel and of the same experiment. (**B**) Quantification of bound material from A. (**C**) His-sCosmc directly interacts with denatured HPC4-sT-syn in a concentration dependent manner. Two different concentrations of DT-syn were incubated with a constant amount of His-sCosmc conjugated beads. After pull down followed by washing, bound and unbound materials were analyzed by western blotting for HPC4. (**D**) Quantification of bound material from **C**. (**E**) Soluble His-sCosmc can compete with His-sCosmc conjugated beads for binding to DT-syn. Reconstitution of heat denatured HPC4-sT-syn was initiated by the addition of His-sCosmc beads in the presence of different concentration of soluble His-sCosmc (4 μg, 10 μg, and 30 μg). Data shown are a representative example of two independent experiments. (**F**) Quantification of bound material from **E**.

3.4.3 Cosmc associates with reconstituted active T-synthase but not with native T-synthase

We recently reported that Cosmc promotes the refolding of heat- or chemically-denatured T-synthase independently of other factors (10). However, whether reconstituted active T-synthase is released from Cosmc has not been determined. Now, we have successfully prepared stable active immobilized Cosmc beads to investigate, using *in vitro* reconstitution, the chaperone function of Cosmc. This procedure allows us to follow the steps involved in restoration of the activity of T-synthase. When Cosmc refolds heat- or chemically-denatured T-synthase, we envision two possibilities. First, Cosmc facilitates the refolding of denatured T-synthase and the refolded active T-synthase is released from Cosmc. Second, the refolded active T-synthase remains associated with Cosmc. To distinguish between these two possibilities, we performed *in vitro* reconstitution of heat-denatured HPC4-sT-syn with His-sCosmc conjugated beads and analyzed both the bound and unbound fractions by assaying the T-synthase activity as outlined (**Figure 3.3A**). Strikingly, the majority of the reconstituted active HPC4-sT-syn, but not native HPC4-sT-syn (NT-syn), was associated with His-sCosmc beads (C-beads) (**Figure 3.3B, Lanes 4 and 2**). As controls, NT-syn and denatured HPC4-sT-syn (DT-syn) do not nonspecifically bind to the beads as measured by T-synthase activity (**Figure 3.3B, Lanes 1 and 3**). Next, we conducted a dose response analysis by incubating varying concentrations of DT-syn with a constant amount of C-beads and found increased activity associated with C-beads with an increased amount of DT-syn (**Figure 3.3C, Lanes 3 and 4**). Furthermore, addition of increasing concentrations of His-sCosmc during the course of the reconstitution reaction decreases the activity of T-

synthase associated with C-beads (**Figure 3.3D, Lanes 2, 3 and 4**). These data indicate that Cosmc forms a complex with catalytically active reconstituted or reactivated T-synthase.

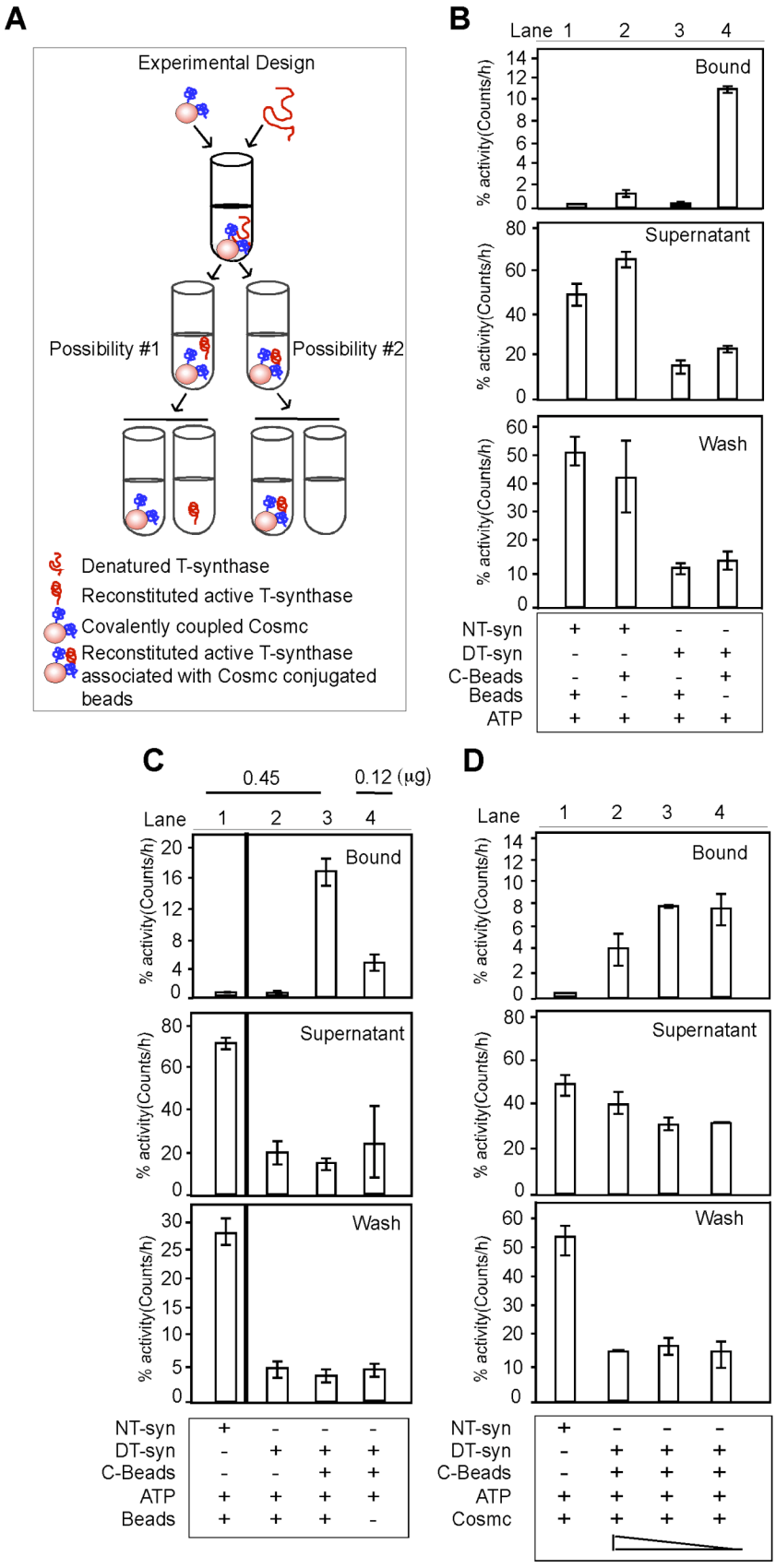

A
Experimental Design
Possibility #1
Possibility #2
Denatured T-synthase
Reconstituted active T-synthase
Covalently coupled Cosmc
Reconstituted active T-synthase associated with Cosmc conjugated beads
B
Lane 1 2 3 4
Bound
Supernatant
Wash
% activity(Counts/h)
NT-syn + + - -
DT-syn - - + +
C-Beads - + - +
Beads + - + -
ATP + + + +
C
0.45
0.12 (µg)
Lane 1 2 3 4
Bound
Supernatant
Wash
% activity(Counts/h)
NT-syn + - - -
DT-syn - + + +
C-Beads - - + +
ATP + + + +
Beads + + + -
D
Lane 1 2 3 4
Bound
Supernatant
Wash
% activity(Counts/h)
NT-syn + - - -
DT-syn - + + +
C-Beads - + + +
ATP + + + +
Cosmc + + + +

Figure 3.3. Cosmc is associated with catalytically active T-synthase. (**A**) Experimental design depicting *in vitro* reconstitution followed by pull down experiments. HPC4-sT-syn was heat denatured (DT-syn) in reconstitution buffer for 2 min at 55°C. After cooling to room temperature, refolding was initiated by the addition of His-sCosmc conjugated beads (C-beads) and allowed to refold for 45 min. Beads were pelleted and washed. Beads, supernatant, and wash were analyzed for T-synthase activity. A parallel experiment was carried out with native HPC4-sT-syn (NT-syn). (**B**) His-sCosmc directly interacts with reconstituted catalytically active HPC4-sT-syn but not with native HPC4-sT-syn. (**C**) His-sCosmc reconstitutes heat denatured HPC4-sT-syn (DT-syn) in a dose dependent manner. (**D**) Soluble His-sCosmc competes with His-sCosmc beads for reconstitution of DT-syn. Reconstitution of DT-syn was initiated by adding a constant amount of His-sCosmc beads containing different concentrations of His-sCosmc as described in **Figure 3.2E**. Representative example of two independent experiments done in duplicate is shown. Error bars, ±1 S.D. from the average of duplicate experiments.

To further confirm that reactivated T-synthase associates with Cosmc, we investigated the nature of the interaction by conducting solution based refolding experiments. This approach aims to address the issues of both conjugation of Cosmc to beads and tags, which might have adverse effects on the release function. For this purpose, we switched the tags and made soluble N-terminal 6×His-tagged T-synthase (His-sT-syn) and the soluble N-terminal HPC4-tagged Cosmc (HPC4-sCosmc) that were expressed and purified to apparent homogeneity as shown by Coomassie stained gels (**Figure 3.4B and C**). Taking advantage of these two epitope-tagged versions of the proteins, HPC4-sCosmc and His-sT-syn, we performed solution-based refolding experiments as outlined (**Figure 3.4A**). We first incubated HPC4-sCosmc with denatured His-sT-syn and allowed it to refold for 45 minutes, followed by pull-down experiments using anti-HPC4 beads to ask whether any active HPC4-sT-syn is still associated with His-sCosmc. Consistent with our earlier findings, reconstituted active His-sT-syn remained associated with HPC4-sCosmc (**Figure 3.4D, Lane 3**). These data clearly demonstrate that our *in vitro* reconstitution experiments using His-sCosmc conjugated beads serve as an excellent tool to explore the interaction between Cosmc and T-synthase. Taken together, these results show that reactivated, but not native T-synthase, remains associated with Cosmc.

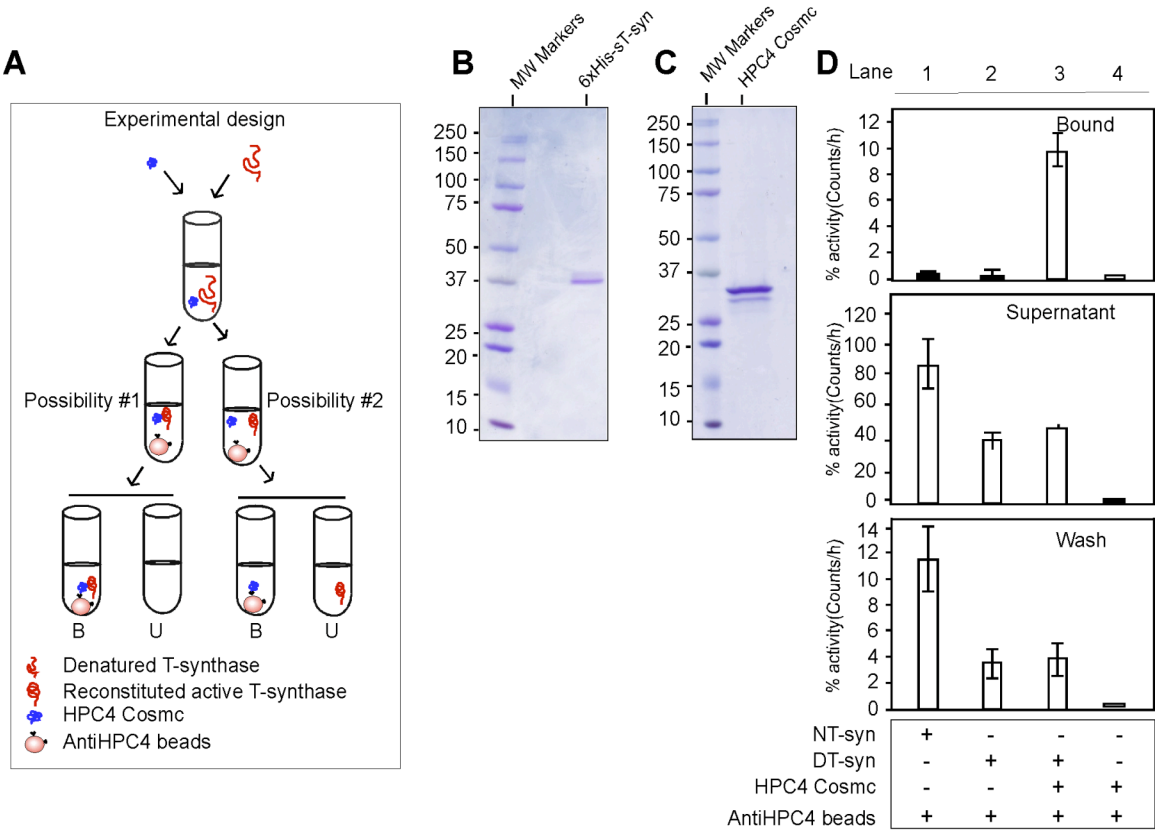

A
Experimental design
Possibility #1
Possibility #2
B
U
B
U
Denatured T-synthase
Reconstituted active T-synthase
HPC4 Cosmc
AntiHPC4 beads
B
MW Markers
6xHis-sT-syn
250
150
100
75
50
37
25
20
15
10
C
MW Markers
HPC4 Cosmc
250
150
100
75
50
37
25
20
15
10
D
Lane
1
2
3
4
Bound
% activity(Counts/h)
Supernatant
% activity(Counts/h)
Wash
% activity(Counts/h)
NT-syn
DT-syn
HPC4 Cosmc
AntiHPC4 beads

Figure 3.4. Reconstituted active T-synthase remains associated with Cosmc in solution. (**A**) Schematic diagram of *in vitro* reconstitution experiment followed by pull down experiment using HPC4-sCosmc and His-sTsyn. His-sTsyn was heat denatured for 2 min at 60°C in reconstitution buffer and allowed to cool to RT. Refolding was initiated by the addition of HPC4-sCosmc to the preparation of denatured T-synthase and allowed to refold for 45 minutes. HPC4-sCosmc was pelleted using anti-HPC4 Beads. Beads, supernatant, and wash were analyzed for T-synthase activity. (**B and C**) Reducing SDS-PAGE analysis of purified human recombinant 6×His-sT-syn and HPC4-sCosmc, respectively. (**D**) *In vitro* reconstitution experiment carried out using HPC4-sCosmc and DT-syn followed by pull down experiment. T-synthase activity was assayed for both bound and unbound fractions. Graph is a representative example of two independent experiments done in duplicate. Error bars, ±1 S.D. from the average of duplicate experiments.

3.4.4 ATP does not cause release of reconstituted active T-synthase associated with Cosmc

For most chaperones, ATP binding triggers a change in conformation of the chaperone leading to release of the substrate (18,36). Our previous study showed that Cosmc binds to ATP, consistent with the study of Inoue *et al* suggesting that ATP might be important in the process of substrate binding and release (38). Although we reported that neither ATP nor ATPγS had a significant difference in the refolding of heat denatured HPC4-sT-syn by His-sCosmc (10), we did not determine if refolded T-synthase is released with ATP or ATPγS. To investigate this issue, we took advantage of the functional Cosmc conjugated beads for *in vitro* refolding experiments with ATP or ATPγS. Using this approach we showed that there is no significant difference in the activity of both bound and unbound material (**Figure 3.5A, Lanes 3 and 4)**. In a parallel experiment, we analyzed the amount of protein in the bound and unbound fractions by western blotting for HPC4-sT-syn and saw no significant differences (**Figure 3.5B, Lanes 2 and 3;** quantification in **Figure 3.5C, Lanes 2 and 3**). These data suggest that Cosmc remains associated with reconstituted active T-synthase with or without ATP at least *in vitro*.

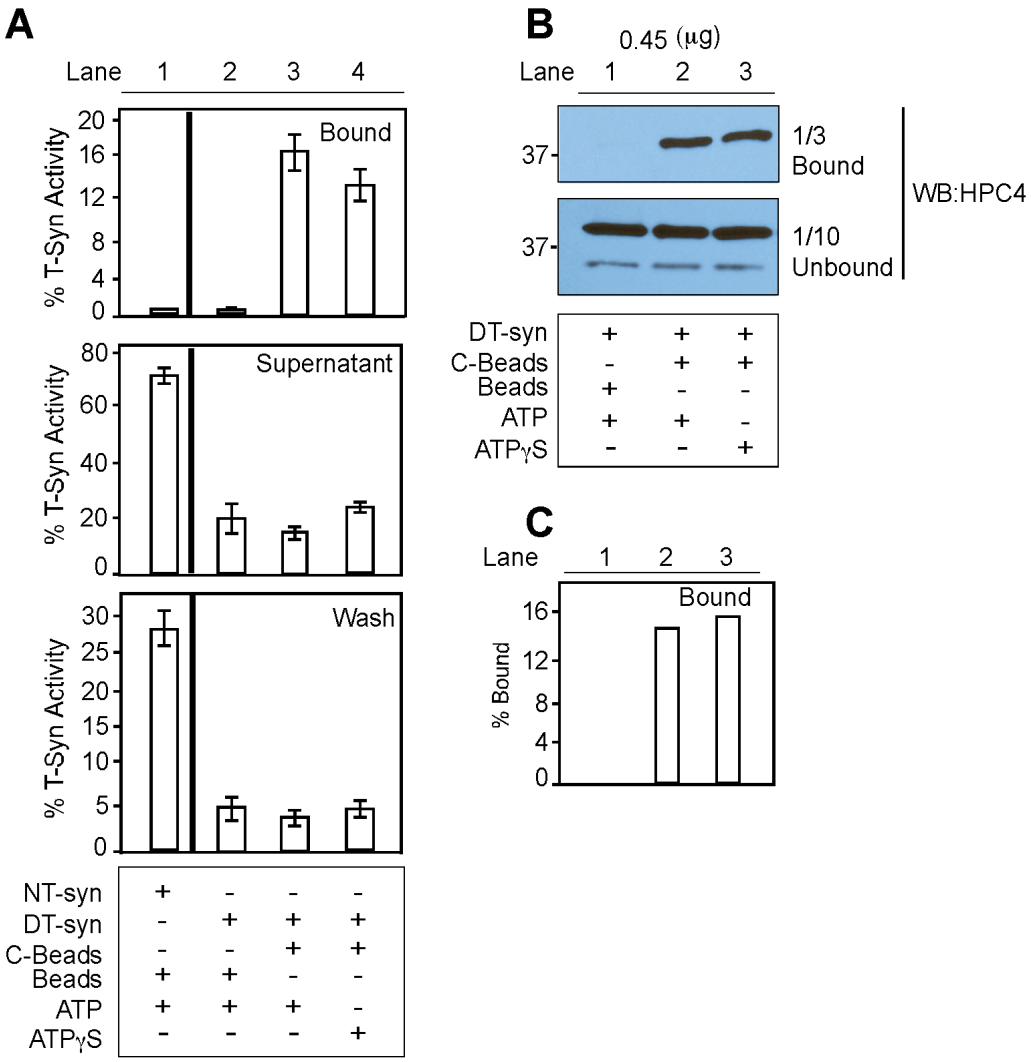

A
Lane 1 2 3 4
% T-Syn Activity
Bound
Supernatant
Wash
NT-syn
DT-syn
C-Beads
Beads
ATP
ATPγS
B
0.45 (μg)
Lane 1 2 3
37
1/3 Bound
1/10 Unbound
WB:HPC4
DT-syn
C-Beads
Beads
ATP
ATPγS
C
Lane 1 2 3
% Bound
Bound

Figure 3.5. ATP is not important in the functional cycle of Cosmc. Reconstitution of heat denatured HPC4-sT-syn was carried out in the presence of ATP or ATPγS to investigate whether ATP regulates substrate binding and releasing. As described in **Figure 3.3A**, *in vitro* reconstitution followed by pull down experiments were carried out and analyzed by western blot and assaying the activity. (**A**) ATP does not cause the release of reconstituted active HPC4-sT-syn from His-sCosmc conjugated beads. The graph shows representative example of two independent experiments done in duplicate. Error bars, ±1 S.D. from the average of duplicate experiments. (**B**) ATP does not affect the binding of DT-syn to His-sCosmc, representative example of two independent experiments. (**C**) Quantification of Western blot data in **B**.

Furthermore, we considered the possibility that His-sCosmc preparation might have residual contaminating ATP from the cells used for Cosmc expression since Cosmc binds to ATP (11). We treated the purified Cosmc with 6 M guanidinium hydrochloride (GnHCl) to cause unfolding, which should result in ATP dissociation, if there were any present. We then allowed His-sCosmc to refold and performed *in vitro* refolding experiments. We found that this reactivated Cosmc was capable of promoting the folding of heat-denatured HPC4-sT-syn (DT-syn) (**Figure 3.6**). Taken together these results suggest that Cosmc is an ATP-independent chaperone *in vitro.*

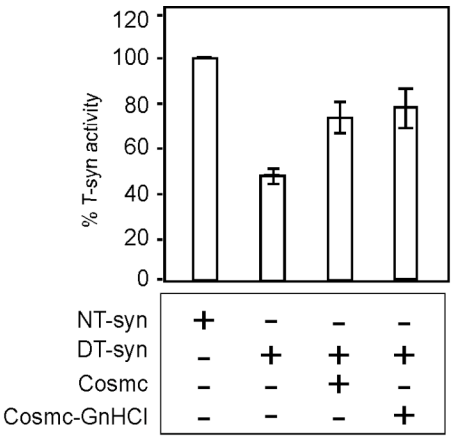


Figure 3.6. 6M GnHCl treated Cosmc can refold heat denatured T-synthase in the absence of ATP. Reconstitution of DT-syn was carried out either by Guanidinium hydrochloride (GnHCl) treated or untreated His-sCosmc. HPC4 sT-syn was heat denatured and after cooling, reconstitution was initiated by the addition of Cosmc followed by measuring T-synthase activity. Graph is a representative example of four replicate experiments. Error bars, ±1 S.D. from the average.

3.4.5 Cosmc folds T-synthase independently of intermolecular disulfide bond formation

We next asked how Cosmc remains associated with catalytically reactivated T-synthase after its folding *in vitro* when native T-synthase will not bind. First we considered the possibility that Cosmc functions as a catalyst for intermolecular disulfide bond formation with T-synthase. To answer this question, we first tested whether His-sCosmc can function in the millimolar range of reduced (GSH) and oxidized (GSSG) glutathione with a GSH:GSSG ratio ranging from reducing 4:1 to oxidizing 1:4 environment. Interestingly, both a reducing and oxidizing environments can support the refolding of denatured T-synthase (DT-syn) by Cosmc (**Figure 3.7A**). Furthermore, if we increase the reducing environment by four fold (GSH:GSSG 16:1), we still saw a significant amount of restoration of DT-syn (**Figure 3.7B**). Additionally, we modified all accessible cysteines of His-sCosmc by N-Ethylmaleimide (NEM), to prevent the potential reformation of disulfide bridges between Cosmc and T-synthase. Analysis by MALDI-TOF (matrix-assisted laser-desorption/ionization – time-of-flight mass) spectrometry showed that Cosmc has two free cysteines (**Figure 3.7C),** and this NEM-derivatized His-sCosmc (NEM-Cosmc) can restore the activity of DT-syn (**Figure 3.7D**). Next we tested the possible role of vicinal thiols in the refolding of denatured T-synthase by Cosmc, since Cosmc appears to have two free cysteines. Our *in vitro* studies showed that the presence of arsenite, which reacts with dithiols and forms a relatively stable cyclic compound blocking the availability of the thiols, did not inhibit the refolding of DT-syn (**Figure 3.7E**). These results suggest that the refolding of heat-denatured T-

synthase by His-sCosmc is not redox regulated and suggest that Cosmc refolds denatured T-synthase without the formation of intermolecular disulfide bonds.

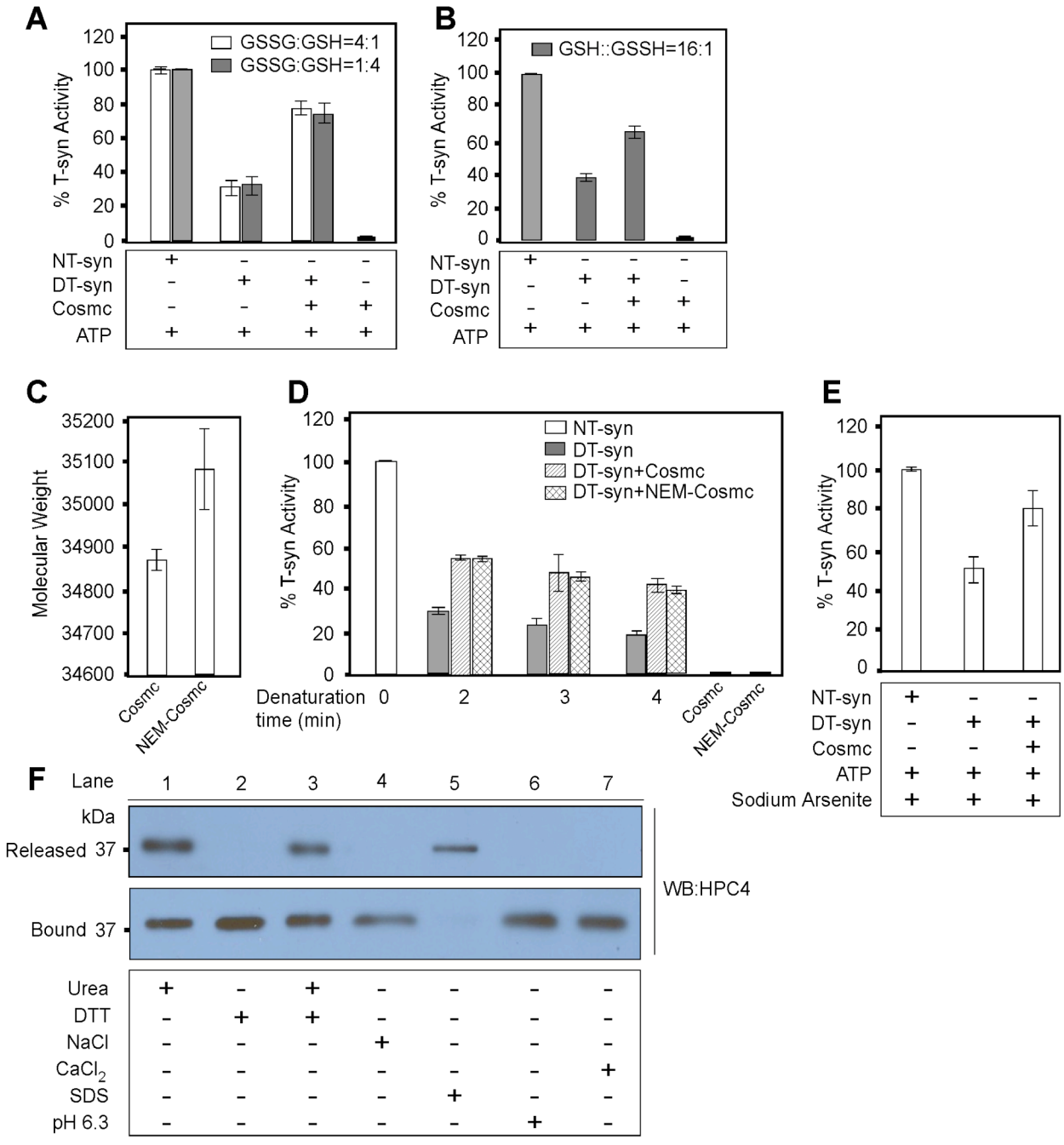

A
% T-syn Activity
GSSG:GSH=4:1
GSSG:GSH=1:4
NT-syn
DT-syn
Cosmc
ATP
B
% T-syn Activity
GSH::GSSH=16:1
C
Molecular Weight
Cosmc
NEM-Cosmc
D
% T-syn Activity
NT-syn
DT-syn
DT-syn+Cosmc
DT-syn+NEM-Cosmc
Denaturation time (min)
E
% T-syn Activity
Sodium Arsenite
F
Lane
kDa
Released 37
Bound 37
WB:HPC4
Urea
DTT
NaCl
CaCl2
SDS
pH 6.3

Figure 3.7. Cosmc folds T-synthase independently of intermolecular disulfide bond formation. (**A-C**) Reconstitution of heat denatured HPC4-sT-syn (DT-syn) was carried out with purified His-sCosmc in either an oxidizing environment or reducing environment for 45 min and directly assayed for T-synthase activity. (**A**) Reconstitution of DT-syn in reducing environment GSH: GSSG (4:1) or oxidizing environment GSSG: GSH (4:1). Data shown are the average of three different experiments each performed in duplicate. (**B**) Reconstitution was carried out in the reducing environment containing GSH:GSSG (16:1). Data shown are the average of duplicate experiments. (**C**) Mass spectrometry analysis of His-sCosmc with or without NEM modification. Data shown are the average of four independent mass spectrometry measurements. (**D**) Purified His-sCosmc or NEM modified His-sCosmc were incubated with DT-syn heated for different time points at 55°C and allowed to refold, and measured T-synthase activity. Data shown are the average of two independent experiments performed in duplicate. (**E**) Refolding experiment carried out as in **B**, in the presence of 13 mM Sodium Arsenite. Data shown are the average of duplicate experiments. (**F**) Reconstituted active HPC4-sT-syn associated with His-sCosmc beads was prepared as described in **Figure 3.3A** except that reconstitution was carried out with 40 µl of His-sCosmc beads (50 % slurry, ~0.5 µg/µl of the beads). The bound materials was treated separately with 8 M Urea, 10 mM DTT, 2 mM $CaCl_2$, 1 M NaCl, 0.1% SDS and pH 6.3 containing reconstitution buffer for 45 minutes at RT. Unbound and Bound fractions were analyzed by western blotting for HPC4. The data shown are the representative example of two independent experiments. Error bars, ±1 S.D. from the average.

Interestingly, T-synthase but not Cosmc contains a thioredoxin (CXXS) like motif and this motif is reported to have thiol/disulfide oxidoreductase activity (39). We asked whether this motif plays a role in this refolding process. For this purpose, we expressed and purified a mutant version of T-synthase (HPC4-mT-syn) (CXXS to SXXS) (**Figure 3.8A**). Our *in vitro* refolding studies show that His-sCosmc can restore the activity of the mutant version of heat denatured T-synthase (DT-syn), suggesting that the CXXS motif of T-synthase might not be involved in the refolding process (**Figure 3.8B**). These data indicate that disulfide bonds do not form between Cosmc and T-synthase during the refolding process *in vitro*.

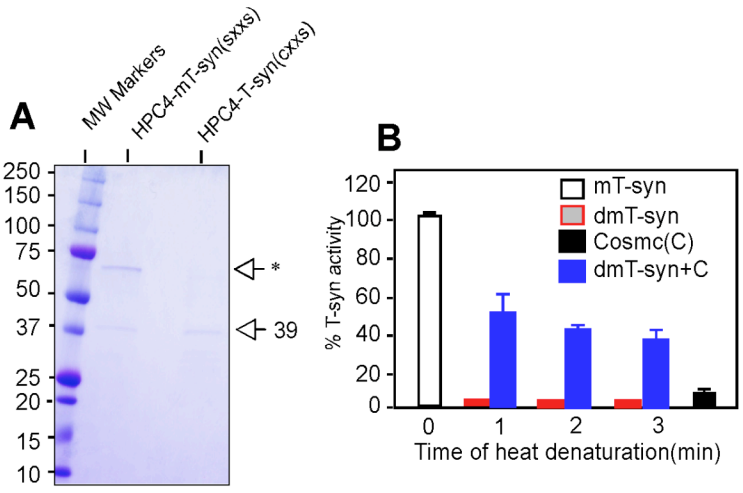


Figure 3.8. CXXS motif of T-synthase is not necessary for its *in vitro* refolding by Cosmc. (**A**) Reducing SDS-PAGE analysis of purified human recombinant HPC4-sT-syn (CXXS) and HPC4-msT-syn (SXXS). (**B**) *In vitro* reconstitution experiment of heat denatured HPC4-msT-syn, which was heated overtime at 55°C. Reconstitution was initiated by the addition of His-sCosmc and incubated for 45 minutes followed by assaying the T-synthase activity. Graph is a representative example of two experiments done in duplicate. Error bars, ±1 S.D. from the average.

Next we asked what causes the release of the T-synthase from His-sCosmc conjugated beads. For this purpose, we treated the complex (reconstituted catalytically active T-synthase bound on the immobilized Cosmc beads) with 8 M urea, 10 mM DTT, 1 M NaCl, or 0.1% SDS. We found that treatment by 8 M urea or 0.1% SDS caused the release of reconstituted active T-synthase from His-sCosmc-conjugated beads (**Figure 3.7F, Lanes 1 and 5**), but 10 mM DTT or 1 M NaCl did not (**Figure 3.7F, Lanes 2 and 4**), which provides further evidence that the interaction between Cosmc and T-synthase is tight but noncovalent. Furthermore, some ER chaperones such as Hsp47 and RAP are regulated by pH (26,31) and other chaperones such as Hsp90, PDI, ERp72, Calreticulin, and p50 are regulated by calcium (40). Therefore we tested the potential role of physiological pH 6.3 and calcium to release catalytically active T-synthase from His-sCosmc. We found that neither pH 6.3 nor calcium caused release of T-synthase from His-sCosmc **(Figure 3.7F, Lanes 6 and 7)**, suggesting that this interaction is not regulated by pH or calcium.

3.4.6 Denatured T-synthase is sufficient for the release of reconstituted active T-synthase bound to Cosmc

The formation of Golgi localized active T-synthase requires ER localized Cosmc (11). Therefore, we asked what factors might cause the release of reconstituted active T-synthase from Cosmc *in vitro*. Since we showed that Cosmc directly interacts with denatured T-synthase but not with native T-synthase, we explored whether denautured T-synthase itself could release the refolded T-synthase from Cosmc. Here, we designed an experiment to explore mechanistically the postulated release of reconstituted active T-

synthase from His-sCosmc beads *in vitro* as outlined (**Figure 3.9A**). First, we asked whether the addition of denatured T-synthase to His-sCosmc-beads bound with renatured T-synthase can alter the equilibrium between His-sCosmc and catalytically active T-synthase to promote dissociation of bound T-synthase. To test this we used two different versions of T-synthase: HPC4-sTsyn and His-sT-syn. First, we prepared reconstituted active HPC4-sT-syn associated with His-sCosmc-beads in *in vitro* reconstitution experiments. We added denatured His-sT-syn and determined whether any HPC4-sT-syn was released. Interestingly, the denatured His-sT-syn promoted the release of HPC4-sT-syn from His-sCosmc-beads as determined by western blot against HPC4 (**Figure 3.9B, Lane 1 released;** quantification in **Figure 3.9C, Lane 1**). As controls, denatured BSA and buffer alone did not cause a significant amount of release (**Figure 3.9B, Lanes 2 and 3 released;** quantification in **Figure 3.9C, Lanes 2 and 3**). Next, we tested whether the release of the reconstituted active T-synthase is concentration dependent in the presence of varying amounts of denatured T-synthase. We demonstrated that the release was dose dependent (**Figure 3.9D, Lanes 2, 3 and 4;** quantification in **Figure 3.9E, Lanes 2, 3 and 4**), suggesting that denatured T-synthase specifically releases the reconstituted active T-synthase and provides evidence for the dynamic and reversible nature of the interaction between Cosmc and T-synthase during the chaperone cycle.

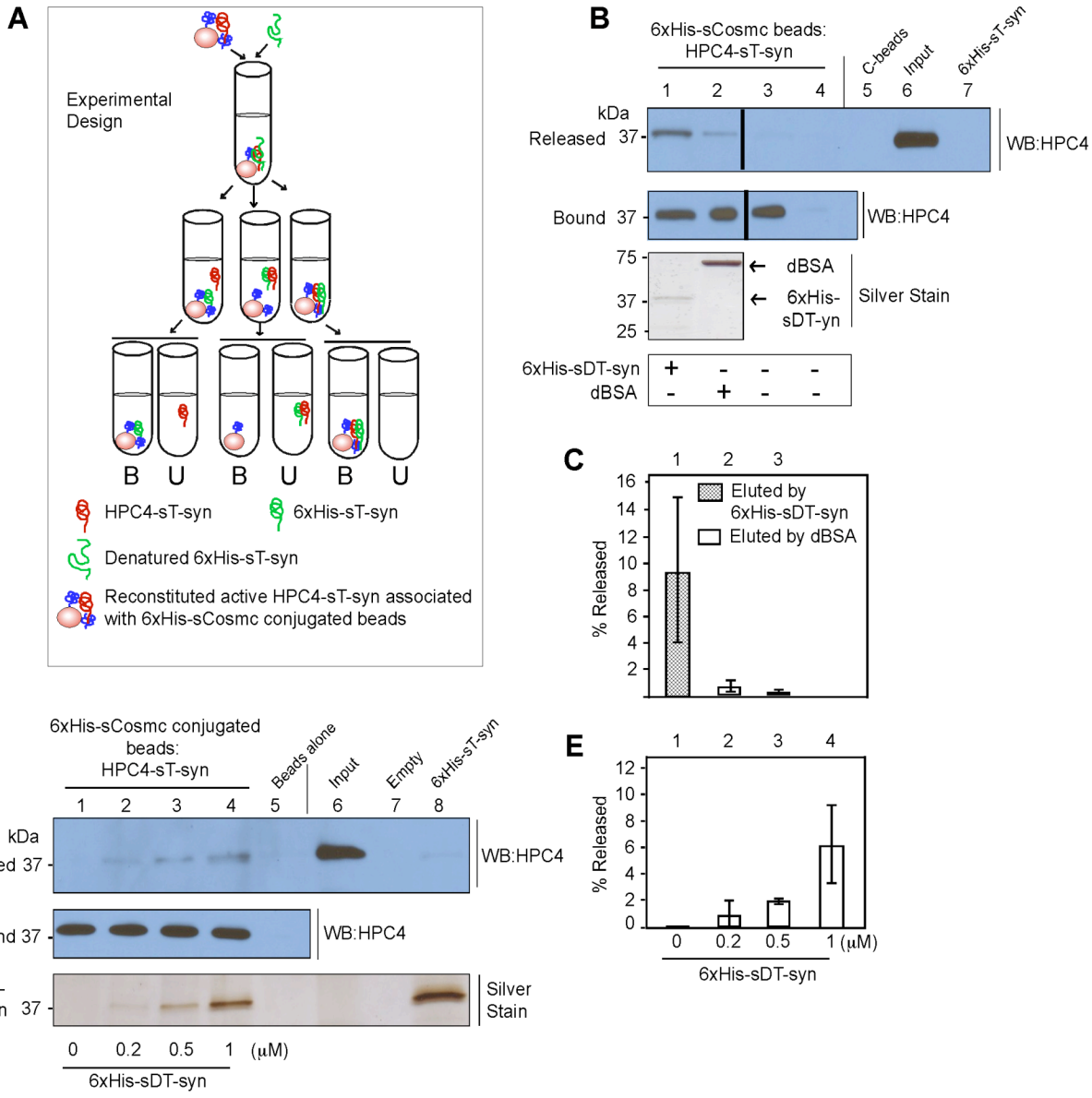
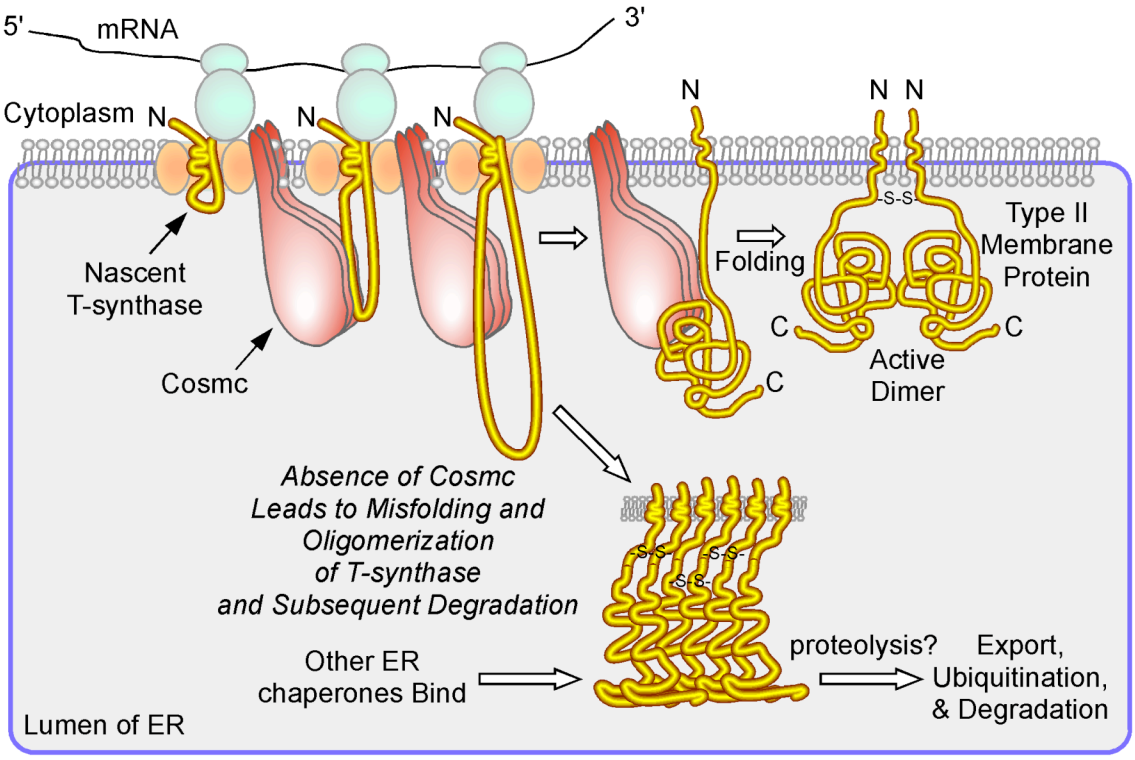

A
Experimental Design
B U B U B U
HPC4-sT-syn
6xHis-sT-syn
Denatured 6xHis-sT-syn
Reconstituted active HPC4-sT-syn associated with 6xHis-sCosmc conjugated beads
B
6xHis-sCosmc beads: HPC4-sT-syn
C-beads
Input
6xHis-sT-syn
1 2 3 4 5 6 7
kDa
Released 37
WB:HPC4
Bound 37
WB:HPC4
75
37
25
dBSA
6xHis-sDT-syn
Silver Stain
6xHis-sDT-syn + - - -
dBSA - + - -
C
1 2 3
% Released
Eluted by 6xHis-sDT-syn
Eluted by dBSA
D
6xHis-sCosmc conjugated beads: HPC4-sT-syn
Beads alone
Input
Empty
6xHis-sT-syn
1 2 3 4 5 6 7 8
kDa
Released 37
WB:HPC4
Bound 37
WB:HPC4
6xHis-sT-syn 37
Silver Stain
0 0.2 0.5 1 (μM)
6xHis-sDT-syn
E
1 2 3 4
% Released
0 0.2 0.5 1 (μM)
6xHis-sDT-syn

Figure 3.9. Denatured T-synthase can cause release of the reconstituted active T-synthase from Cosmc. (**A**) Schematic diagram of *in vitro* release experiment. (**B**) Denatured T-synthase can alter the equilibrium between Cosmc and reconstituted active T-synthase but not of denatured BSA or buffer alone. Reconstituted active HPC4-sT-syn associated with Cosmc beads were incubated with heat denatured His-sT-syn, heat denatured BSA, or buffer alone. Supernatant and bound fractions were analyzed by western blotting against HPC4. Silver stain gel shows the amount of T-synthase and BSA used in the experiment. A vertical line separates the data taken from the different part of the same gel and of the same experiment. (**C**) Quantification of eluted fraction (U) of western blot data as compared to Input from B. Data shown is an average of three independent experiments. Error bars, ±1 S.D. from the average of triplicate experiments. (**D**) Denatured His-sT-syn releases active HPC4-sT-syn from Cosmc in a concentration dependent manner. Reconstituted active HPC4-sT-syn associated with Cosmc beads were incubated with different concentrations of DT-syn. Eluted fractions (Released) and the amount of protein remained in the beads (Bound) were analyzed by WB for HPC4. Silver stain gel shows the amount of heat denatured His-sT-syn used. (**E**) Quantification of eluted fraction (Released) as compared to Input. Data shown is an average of two independent experiments. Error bars, ±1 S.D. from the average of two independent experiments.

3.5 Discussion

Previously we reported that Cosmc is an ER localized specific molecular chaperone that is required for the expression of functional T-synthase, which resides in the Golgi apparatus (11,32). However, we do not know, how Cosmc works to produce functional T-synthase. The present study provides a mechanistic explanation of Cosmc functions as a chaperone for T-synthase folding. In this study, we demonstrated that soluble Cosmc directly interacts with denatured, but not native soluble T-synthase. The interaction between soluble Cosmc and soluble denatured T-synthase results in the formation of stable complexes *in vitro* through noncovalent interactions. Furthermore, the reconstituted T-synthase in the Cosmc complex is catalytically active. Interestingly, the addition of denatured T-synthase itself can cause the partial release of the T-synthase from Cosmc, but ATP, calcium, and pH, which are known regulators of many ER chaperones, do not cause the release of T-synthase. Taken together, the current *in vitro* data are consistent with the model that Cosmc alone is sufficient to bind to and facilitate folding of denatured T-synthase *in vitro,* and denatured T-synthase independently of other factors can cause the dissociation of T-synthase, suggesting a potential model for Cosmc function in the biosynthesis of T-synthase (**Figure 3.10**).



Simplified fate folding kinetics of T-synthase in the presence and absence of Cosmc

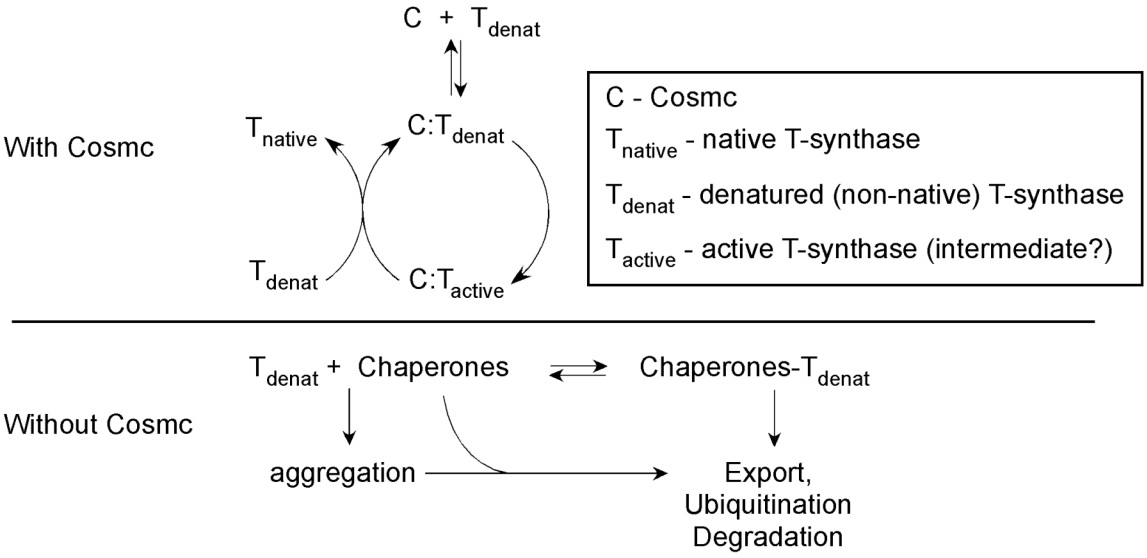


Figure 3.10. A model of Cosmc function in the biosynthesis of T-synthase.

ER-localized Cosmc directly interacts with cotranslationally translocated non-native T-synthase and promotes its refolding independently of other factors. Cosmc forms a transient complex with active T-synthase and active T-synthase is released from Cosmc complex when the Cosmc interacts with other cotranslationally translocated non-native T-synthase for another cycle of folding. Active dimeric T-synthase exits to the Golgi apparatus. In the absence of functional Cosmc, T-synthase aggregates and is degraded by proteasomal machinery.

In our earlier publications we demonstrated that Cosmc promotes the activity of T-synthase *in vivo* (9,41). *In vitro* reconstitution studies suggest that Cosmc, independently of ATP and other factors, can facilitate folding of heat- or chemically-denatured T-synthase into a catalytically active form (10). We also reported that in the absence of functional Cosmc, inactive T-synthase aggregates into oligomeric complexes, which are ubiquitinated and degraded by proteasomal machinery (11). Now, we have demonstrated that Cosmc preferentially binds to non-native, but not native T-synthase (**Figure 3.2A, Lanes 4 and 2; Figure 3.3B, Lanes 4 and 2)**. Also we showed that Cosmc does not promote the activity of native T-synthase (**Figure 3.1F, Lanes 1 and 2)** suggesting that there may be a unique non-native structural element(s) of the non-native T-synthase that interacts with Cosmc. Native T-synthase shows no detectable binding with Cosmc suggesting that these structural element(s) might be inaccessible in the native T-synthase. The structural features of heat denatured T-synthase that are recognized by Cosmc and stoichiometry of the reactions are not clear at this point. These results further support our conclusion that Cosmc functions as a chaperone for T-synthase, and indicates that ER localized Cosmc could preferentially bind to newly synthesized non-native T-synthase to promote its folding *in vivo*. The ability of Cosmc to associate with non-native T-synthase appears to be similar to that of well-characterized molecular chaperones including Hsp70 (42), Cpn60 (GroEL) (43), DnaK (44), and Hsp18.1 (45) with their substrates. Alexender et al (2006) reported that the addition of recombinant purified soluble FLAG-tagged Cosmc slightly enhances the activity of purified FLAG-tagged T-synthase (35) and concluded that Cosmc and T-synthase form a heterodimer or heterooligomer for maximal activity. Alternatively, this result could be due to the

presence of a fraction of partly denatured inactive T-synthase in the purified preparation of flag-tagged T-synthase. The addition of Cosmc to that preparation helped to restore the activity of the denatured protein.

Importantly, we have demonstrated the physical existence of a relatively stable complex between Cosmc and heat denatured T-synthase (**Figure 3.2A, Lane 4** and **Figure 3.7F)** suggesting that Cosmc prevents irreversible aggregation of denatured T-synthase by direct interaction. Similar stable complexes between chaperones and their substrates have been reported, such as alpha-crystalline and carbonic anhydrase (46), and GroEL and Rubisco I (43). We also showed that Cosmc associates with the reconstituted catalytically active form of T-synthase (**Figure 3.3B, Lane 4** and **Figure 3.4D, Lane 3**), which appears to be inconsistent with one of the characteristics of molecular chaperones that they do not form part of the final functional structure (47). However, it is possible that the complex that we observed might be a transitional stage in the folding process comprising of Cosmc and catalytically active folding intermediates of T-synthase.

We demonstrated that Cosmc refolds denatured T-synthase even in reducing environments (**Figure 3.7A and B**), suggesting that the two proteins within the complex do not have an intermolecular disulfide bond(s). Furthermore, treatment of the complex with 8 M urea or 0.1% SDS dissociate the complex (**Figure 3.7F, Lanes 1 and 5**), but 10 mM DTT or 1 M NaCl (**Figure 3.7F, Lane 2 and 4**) do not, suggesting that the interaction is mostly hydrophobic. This evidence indicates that Cosmc is not a redox-regulated chaperone. *In vivo* studies have shown that Cosmc resides in the ER and T-synthase localizes in the Golgi apparatus with other glycosyltransferases (11,32), which clearly indicates that Cosmc releases T-synthase after its refolding. This idea prompted us

to explore other potential factors that might regulate the chaperone cycle of Cosmc. Since ATP regulates many chaperone cycles and Cosmc binds to ATP (11,38), we tested the possibility that ATP might be important in this process, but we saw no effect of ATP or ATPγS in our *in vitro* experiments (**Figure 3.5A, Lanes 3 and 4; Figure 3.5B Lanes 2 and 3**). Furthermore, Cosmc denatured by 6M GnHCl and refolded could fold heat denatured T-synthase (**Figure 3.6**) suggesting that the Cosmc preparation does not contain contaminating ATP. These results suggest that Cosmc is an ATP independent chaperone, at least for the *in vitro* assay we have developed. Also, some ER chaperones such as Hps47 are regulated by pH where the chaperone binds and releases the substrate by exploiting the change in pH from the ER to the Golgi environment (26). Additionally, chaperones such as Hsp90, PDI, ERp72, Calreticulin, and p50 are regulated by calcium (40). Our *in vitro* data show that within the physiological range of pH and calcium, the complex of Cosmc and reconstituted T-synthase do not dissociate (**Figure 3.7, Lanes 6 and 7**) which suggest that Cosmc is not a pH or calcium regulated chaperone.

To better understand how catalytically active T-synthase associates with Cosmc, we postulate that when the newly synthesized T-synthase translocates into the ER, membrane bound Cosmc directly interacts and subsequently promotes the folding of T-synthase forming a transient complex and then catalytically active T-synthase is released from the complex in the presence of newly synthesized non-native T-synthase. Our data indicate that non-native T-synthase could shift the equilibrium between His-sCosmc and catalytically active T-synthase to promote dissociation of bound T-synthase (**Figure 3.9B, Lane 1; Figure 3.9D, Lanes 2, 3 and 4**). It is possible that the new form of reconstituted active T-synthase may have relatively low affinity for Cosmc as compared

to non-native T-synthase. This difference may drive the binding and releasing cycle of Cosmc for the folding of T-synthase. The molecular details of the interaction between Cosmc and denatured T-synthase are under further investigation.

Earlier we reported that T-synthase purified from rat liver microsomes is mostly dimeric (5) and the soluble form of active HPC4-T-synthase exists mostly as a dimer as detected by cross-linking experiments (**Figure 3.1G**). As we showed in our earlier publication, in the absence of functional Cosmc, T-synthase aggregates in an inactive oligomeric complex (11). These data indicate that Cosmc might be important for assembly of the dimeric form of T-synthase suggesting that Cosmc is also an assembly chaperone. It is possible that Cosmc helps to facilitate both folding and homodimerization of newly synthesized T-synthase and that active dimeric forms of T-synthase may be released from Cosmc, These ideas will be investigated in the future.

Acknowledgements

We thank Dr. Jamie Heimburg-Molinaro for manuscript editing. We also thank Sandy Cummings for technical support. This work was supported by NIH RO1 grant (RO1 GM068559) to R.D.C.

3.6 References

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CHAPTER 4: Discussion and Future directions

4.1 Cosmc refolds T-synthase from unfolded state *in vitro*

Prior *in vivo* work in the Cummings lab has demonstrated that Cosmc is a specific chaperone for T-synthase, localized in the ER (1,2). In the presence of functional Cosmc, an active dimeric form of T-synthase exits to the Golgi and localizes in the Golgi apparatus (2). Dysfunctional Cosmc results in the expression of inactive, oligomeric T-synthase aggregates which are subsequently polyubiquitinated and degraded by proteasomal machinery (2). In this thesis, we aimed to recapitulate the chaperone function of Cosmc *in vitro* to better understand the mechanism of chaperone functions. To this end, we developed a functional *in vitro* assay to investigate the chaperone function of Cosmc. Using this approach, we showed that Cosmc directly promotes refolding of both thermally and chemically denatured T-synthase. This characteristic of Cosmc function is consistent with many established chaperones.

We showed in this thesis work that Cosmc refolds denatured T-synthase independent of other co-chaperones *in vitro* (3). Many known general chaperones, including Hsp70, Hsp90, Hsp60, etc. are regulated by co-chaperones (4). Functionally, co-chaperones play important roles including providing specificity, presenting clients to chaperones, nucleotide exchange and hydrolysis, substrate binding and releasing as well as physically linking two chaperone networks, for example Hsp70 and Hsp90 (5). It is not known whether Cosmc requires such regulatory factors *in vivo*. Previous work in the Cummings lab has shown that active T-synthase associates with Hsp40 and misfolded T-synthase associates with grp78 suggesting that Cosmc might be working with the Hsp70

system network (2). However, the direct physiological relevance of Hsp70 and Hsp40 in terms of T-synthase folding is unclear. It is also possible that Hsp40 might be a co-chaperone for Cosmc or Cosmc itself could even be a co-chaperone for other chaperone systems. It is notable that all chaperones do not need co-chaperones for their chaperone functions. Therefore, the role of Cosmc to assist T-synthase folding without the assistance of a co-chaperone is not unique.

ATP regulates general chaperones, such as Hsp70, Hsp90, and Hsp60 (4). Cosmc binds to ATP (2). Therefore we questioned the role of ATP in this *in vitro* reconstitution experiment. We showed that Cosmc refolds T-synthase independently of ATP and non-hydrolyzable ATPγS suggesting that ATP does not regulate Cosmc function *in vitro* (3). Furthermore, we showed in this thesis work that ATP is not important for the chaperone cycle of Cosmc. However, it is possible that ATP might be important *in vivo* in many ways such as regulation of Cosmc oligomerization.

4.2 Cosmc is a *bona fide* chaperone for T-synthase

A molecular chaperone is defined as any protein that selectively binds non-native but not native protein to promote folding to its native structure and does not become part of it (4,6,7). The experiments reported in this dissertation provide further experimental evidences in support of chaperone function of Cosmc. We showed that Cosmc directly facilitates folding of the non-native T-synthase to a catalytically active form but Cosmc does not promote the activity of native T-synthase. Also we demonstrated that Cosmc selectively binds to cryptic structural units exposed upon T-synthase denaturation and does not bind native T-synthase suggesting that cryptic structural units are buried or not

available in the fully folded active T-synthase. Interestingly, we were able to detect a relatively stable noncovalent complex between Cosmc and T-synthase. These features of Cosmc are in accordance with the current molecular chaperone concept (4,7).

Furthermore, prior *in vivo* studies from the Cummings lab have shown that ER localized Cosmc is required for the formation of active T-synthase (2). For example, cell lines lacking functional Cosmc, including human colorectal carcinoma LSC, melanoma LOX cells, and Jurkat cells all lack T-synthase activity (2). However, this activity can be restored by expressing wild type Cosmc (1,2). Furthermore, expression of human recombinant T-synthase in insect cells, which lack orthologs of Cosmc, does not produce active T-synthase unless co-expressed with Cosmc (1). Importantly, Cosmc knockout mice also completely lack T-synthase activity and die in early embryonic days (8). Studies done in LSC cells using recombinant forms of both proteins demonstrate that in the absence of Cosmc, T-synthase appears as higher molecular weight oligomeric species but the expression of Cosmc results in the expression of the monomeric form of T-synthase in reducing gels (2). *In vitro* translational studies also suggest that Cosmc is required during the translation of T-synthase (9). This evidence further supports that Cosmc is a chaperone for T-synthase.

In this dissertation work, we showed that the general ER resident chaperone BiP could not refold heat denatured T-synthase *in vitro* (3) which is consistent with *in vivo* studies expressing T-synthase in the Cosmc-deficient background (1,2). For example, Cosmc knockout mice presumably retain normal chaperones but still cannot make functional T-synthase suggesting that Cosmc is essential for T-synthase biogenesis (8). However, while Cosmc is required for T-synthase, Cosmc could not restore other

glycosyltransferases *in vitro* (3). Similarly, Cosmc deficient cell lines or Cosmc knockout embryos do not appear to have any problem within the enzyme working for the glycosylation pathways and are found to be defective only in mucin type O-glycan biosynthetic pathways, but not other glycan structures (8,10), suggesting that Cosmc is a specific chaperone for T-synthase. However, Cosmc might have other clients outside the glycosylation pathway but we do not have any evidence at this point.

Importantly, invertebrate T-synthase contains multiple N-glycans whereas mammalian T-synthase does not have any N-glycosylation sites (11). Active invertebrate T-synthase can be generated without Cosmc but mammalian T-synthase requires Cosmc (11). This implies that the calnexin/calreticulin chaperone system can only work for invertebrate T-synthase containing N-glycans. Therefore it is possible that Cosmc compensates for the role of N-glycans and promotes T-synthase folding.

4.3 Chaperone cycle of Cosmc

Several *in vivo* studies suggest that Cosmc localizes in the ER and T-synthase localizes in the Golgi apparatus (2,12) which indicates that Cosmc folds T-synthase and releases it. Cosmc does not contain any canonical ER localization signal, but recently Cummings lab has demonstrated the importance of the cysteine residue within the transmembrane domain (TMD) of Cosmc for ER localization (13). T-synthase localizes in the Golgi apparatus possibly through the signal as defined by the thickness of the TMD (14). Additionally, purified active T-synthase from rat liver microsomes is devoid of Cosmc (15) suggesting that Cosmc does not form part of active T-synthase. Furthermore, coexpression of recombinant soluble T-synthase containing a signal sequence with

membrane bound wild-type Cosmc in insect cells results in the secretion of active T-synthase in the media which can be further purified to apparent homogeneity (3). Additionally, recombinant Cosmc does not promote the activity of recombinant native T-synthase. Taken together, these data suggest that Cosmc folds and then releases T-synthase, which is consistent with other documented molecular chaperones and their client interactions. In this dissertation work, we aimed to study the mechanism of this chaperone client interaction. For this purpose, we prepared active Cosmc conjugated beads for use in *in vitro* refolding experiments. We demonstrated that Cosmc selectively interacts with non-native T-synthase but not with native T-synthase to promote refolding independently of other factors. Unexpectedly, reconstituted active T-synthase found in the bound fractions and not in the released fractions. We demonstrated in this work that Cosmc and T-synthase interact directly and the nature of this interaction is noncovalent which is consistent with many known chaperone-client complexes.

Furthermore, the Cosmc chaperone cycle is not regulated by ATP, redox, pH, or calcium. However, the only factor that we found to release the reconstituted active T-synthase bound to Cosmc was denatured T-synthase itself. It is possible that reconstituted active T-synthase within the Cosmc complex could be a folding intermediate of T-synthase where the catalytic part has already folded correctly, but not all of the parts of T-synthase. Therefore, the unfolded region(s) of T-synthase may be able to maintain interaction with Cosmc. The question of how reconstituted active T-synthase is released from Cosmc in the presence of denatured T-synthase is still not understood. There are several possibilities: I) Cosmc might have a higher affinity for denatured T-synthase compared to reconstituted active T-synthase. This difference might help to compete off

the active T-synthase from Cosmc. The released T-synthase then further folds to reach its native state. II) Cosmc might be working as both a folding and assembly chaperone. It is important to note that both the soluble and membrane bound forms of T-synthase exist as a dimer. In this case we can hypothesize that Cosmc folds nascent T-synthase and then helps to assemble the dimeric form of T-synthase, which is then released from Cosmc. However, it is also possible that dimerization is a downstream event that occurs after the release of T-synthase. Based on this work and several other studies, a working model for Cosmc functions have been proposed (**Figure 4.1**).

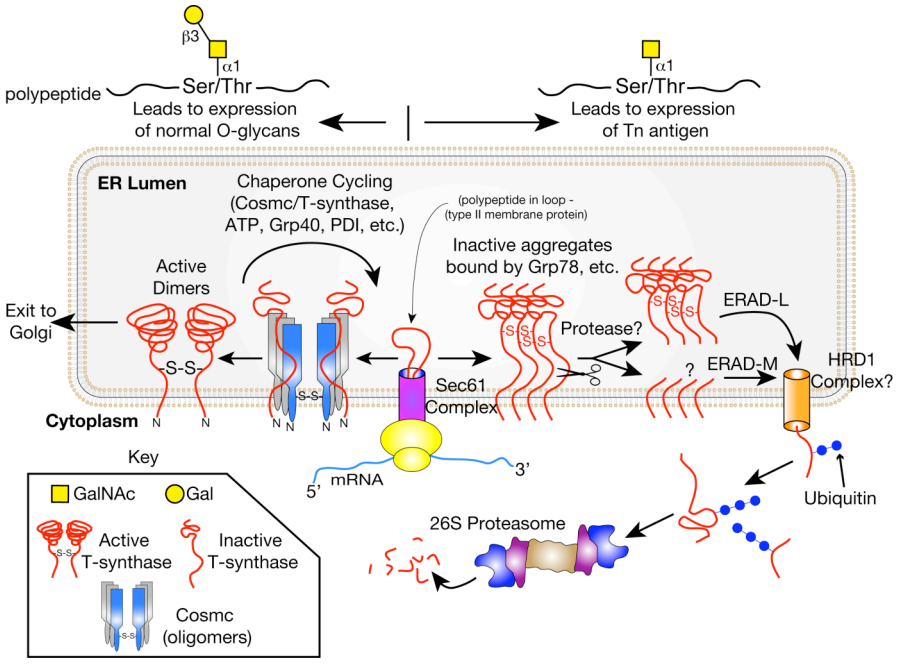

Fig. 4.1. Working model of the mechanism of Cosmc function in the regulation of the T-synthase in the ER. Cosmc is an ER localized chaperone, which may exist as an oligomeric complex. Cosmc directly interacts with newly synthesized non-native T-synthase, facilitating folding of the T-synthase. In the absence of functional Cosmc, T-synthase aggregates into non-productive aggregates and associates with other chaperones, e.g. Grp78, and is subsequently ubiquitinylated and degraded in the 26S proteasome system. The active dimeric form of T-synthase exits to the Golgi. (Adapted and redrawn from Ju *et al*, 2008, Regulation of protein O-glycosylation by the endoplasmic reticulum–localized molecular chaperone Cosmc.)

4.4 Future studies

We demonstrated that Cosmc directly interacts with non-native T-synthase to form a relatively stable complex *in vitro*. Further studies are warranted in order to better understand the molecular nature of the interaction between Cosmc and T-synthase. A primary future goal will be to identify the elements or the region within T-synthase that directly interacts with Cosmc. For this we will take a multifaceted approach. One approach is to synthesize a series of overlapping peptides of ~30 amino acids within the lumenal domain of human T-synthase. The peptides can be used for many different experimental purposes. Some of the experiments to identify the potential peptide(s) that interacts with Cosmc are as follows:

Binding experiment in microarray format

The peptides will be immobilized onto NHS-activated glass slides and the array probed with biotinylated recombinant soluble Cosmc. Bound material will be identified using Alexa488-labeled streptavidin.

Pull down experiment

A mixture of the peptides will be used in pull-down experiments using Cosmc conjugated beads. The bound fraction will be eluted and mass spectrometry analysis will be performed to identify eluted peptide(s).

Elution of bound T-synthase from Cosmc beads

In this thesis work we have shown that denatured T-synthase can cause the elution of renatured T-synthase associated with Cosmc beads in our *in vitro* assay approach in chapter 3. We will use this approach, and use T-synthase peptides to elute bound fractions. We will analyze the bound fraction by western blot.

If we identify the peptide region of T-synthase that interacts with Cosmc, we will test the ability of the peptide to interact with Cosmc *in vivo* using 293T cells which contain high endogenous T-synthase activity. Plasmid coding for the interacting peptide with signal sequence will be made and 293T cells will be transfected. As a control, peptides where the sequence is scrambled will be used in the experiment. Additionally, it is possible that expressing peptide alone may not result in stable peptide so we will design construct coding peptide with another membrane bound ER resident protein. We predict that the peptide will compete with endogenous T-synthase for binding with Cosmc, which might result in a recovery of less active T-synthase. We will detect T-synthase activity levels by our fluorescent T-synthase assay. We will also analyze changes in glycan structure by using anti-Tn antibody and where we expect that peptide will result in more Tn and Sialyl-Tn antigen in the infected cells. This approach is important to not only confirm the interaction, but also will be a great tool to "knockdown" T-synthase with an aim to study the biological functions of O-glycans. Additionally, this peptide will be used for co-crystallization with Cosmc, which will be really crucial to understand the molecular nature of the interaction.

We anticipate identifying the peptide domain of T-synthase recognized by Cosmc since Cosmc binds to denatured T-synthase and forms a relatively stable complex.

However, it is also possible that Cosmc may recognize some non-native structural elements originated from different parts of the T-synthase. In this case we will create several constructs, which are 50 amino acids shorter in increments from both the N- and C-terminus of the lumenal domain. Both *in vivo* and *in vitro* pull down experiments will be performed. The possible outcomes of this experiment could be that only the full-length protein binds and none of the mutant forms bind; that would imply secondary structure or whole domain which is recognized by Cosmc is on the last 50 amino acids. We will use this approach to identify the domain of T-synthase that interacts with Cosmc. If the domain that has the information is not within 50 amino acids, then we will use a synthetic approach to make several overlapping peptides within the region and use the binding experiment as described above.

Alternatively, we will do pull down experiments using our Cosmc conjugated beads with T-synthase partially digested by trypsin or chymotrypsin and Mass spectrometry analysis will be performed to identify the fragment of T-synthase that is responsible for the interaction.

Characterization of the released T-synthase

Since both wild type and mutant T-synthase lacking the TMD exist as dimeric species, other future experiments may address whether the T-synthase is an active monomeric or dimeric species following its restoration in activity and release from Cosmc. Cross-linking experiments as well as gel filtration experiments will be used to define its existence as a dimeric or monomeric species.

Characterization of Oligomeric Cosmc

In this thesis work we demonstrated that soluble Cosmc forms oligomeric species using three independent approaches– cross-linking, native gel, and gel filtration experiments. Cross-linking experiments showed that Cosmc behaves as a monomeric/ dimeric and oligomeric species. In gel filtration we found that the elution volume of Cosmc corresponding to hexameric and monomeric species. This is interesting, since other molecular chaperones, including Hsp90 and Hsp104 and others occur as dimeric species that assemble into functional hexamers (16,17). In this regard, future studies may want to determine whether the monomer/dimer or oligomer is functional and whether binding to denatured T-synthase changes the nature of oligomerization as well as the ability to restore functional enzyme activity. We will use gel filtration of Cosmc mixed with either denatured or native T-synthase and analyze whether binding alters oligomerization. Similarly, if we find the peptide region as we describe above that interacts with Cosmc, we will use the peptide in this gel filtration experiment and analyze the elution profile of Cosmc as described above. We will also study the nature of monomers/hexamers after isolation. Isolated monomers or hexamers will be further incubated for different periods of time (h) and will be crosslinked and analyzed through the gel filtration column. We anticipate that soluble Cosmc is in equilibrium between hexameric/ dimeric/monomeric forms and we expect to find reconstituted active T-synthase forming a relatively stable noncovalent complex with Cosmc. It is possible that in the presence of non-native T-synthase, hexameric Cosmc may dissociate into dimers and/or monomers and the monomeric form of Cosmc may stabilize non-native T-synthase and promote its folding and assembly. During the process Cosmc itself might

form dimers, bringing reconstituted T-synthase together for the dimerization and so formed stable dimers might be released from Cosmc.

In conclusion, our study explored the molecular mechanism of the chaperone function of Cosmc for T-synthase and we demonstrated that Cosmc is central to the regulation of mucin-type O-glycosylation by regulating T-synthase. It is obvious that we have not uncovered the complete picture of the mechanism of Cosmc function for T-synthase in the ER. As we continue to explore the molecular mechanism of Cosmc function for T-synthase, we hope to use these findings to develop novel specific and efficient therapies for several types of cancers and Tn syndrome and possibly IgA nephropathy.

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