IDENTIFYING TRIGGER FACTOR FUNCTIONS AND SUBSTRATES

by

HONGLEI WANG

(Under the direction of Marly K. Eidsness and Robert A. Scott)

ABSTRACT

In all kingdoms of life, proteins synthesis is carried out by the ribosome. Nascent chains leave the ribosome through a narrow tunnel in the large subunit, 50S. In bacteria, the first protein to interact with newly synthesized polypeptides is a ribosome-associated chaperone, trigger factor. The function of trigger factor on the ribosome has been well studied, but little is know the role of unbound trigger factor. Research in this thesis focuses on two proteins, trigger factor and tryptophanase, and their relationship. Tryptophanase was identified as a specific substrate of trigger factor using the two dimensional electrophoresis. Part of tryptophanase was found missing in *tig*-deletion *E. coli* strain. *In vitro* refolding experiment indicated that the recovered activity of tryptophanase can be improved 3 times with trigger factor. This indicates the potential function of trigger factor to facilitate the assembly of tryptophanase tetramer.

INDEX WORDS: Two Dimensional Electrophoresis, Ribosome, Protein Synthesis, Molecular Chaperone, Protein Refolding

IDENTIFYING TRIGGER FACTOR FUNCTIONS AND SUBSTRATES

by

HONGLEI WANG

B.S., NanKai University, China, 2001

Ph. D, The University of Georgia, 2007

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

Athens, Georgia, 2007

IDENTIFYING TRIGGER FACTOR FUNCTIONS AND SUBSTRATES

by

HONGLEI WANG

Major Professor:	Robert Scott
Committee:	I_Jonathan Amster Jeffrey Urbauer

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
August 2007

DEDICATION

To my parents.

ACKNOWLEDGEMENTS

I would express my deepest gratitude to my major Professors, Dr. Marly Eidsness and Dr. Scott for their encouragement, support and guidance during my six years of study at The University of Georgia. Without their extensive knowledge, experience and creative ideas, I would not have been able to finish this project.

I would also express my sincere gratitude to my committee members, Professor Jonathan Amster and Professor Jeffrey Urbauer , for their precious inspiration.

I would also thank Professor Robert Phillips for giving advice and providing materials for tryptophanase assay.

I would thank Professor James Prestegard for serving in my previous committee and for introducing a very important technique in protein science, NMR, to me.

I would also thank Professor John Stickney for his support in the past year.

I would thank my co-workers, members of the Eidsness group: Jihye Shim, Jing Xu, Caroline Watson and YiXuan Zhu for providing a comfortable working environment.

I would also thank members of the Scott group: Darin M. Cowart, Sungguan Hong, Xiayu Huang, Gina P. Lipscomb, Mike (Chenxi) Li, Xin Li, Minnie Murugesan and Guanqun Yuan for providing a lot of help to me.

I thank my parents in China for supporting me all these years.

TABLE OF CONTENTS

Page

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

2DE	Two Dimensional Electrophoresis
BSA	Bovine Serum Albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CTD	C-terminal domain of trigger factor
DTT	Dithiothreitol
EDTA	Ethylenediamine Tetraacetic Acid
FKBP	FK506 binding protein
FPLC	Fast Protein Liquid Chromatography
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GdnHCl	Guanidine Hydrochloride
GFP	Green Fluorescent Protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSQC	Heteronuclear Single Quantum Correlation
IEF	Isoelectric Focusing
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Kan	Kanamycin
KJE	DnaK/DnaJ/GrpE
LB	Luria Bertani
MALDI	Matrix-Assisted Laser Desorption/Ionization
MS	Mass Spectrometry
MW	Molecular Weight

NMR	Nuclear magnetic resonance
NP40	Nonidet P40
NTD	N-terminal domain of trigger factor
pI	Isoelectric Point
PLP	Pyridoxal-5-phosphate
PPIase	Peptidyl-prolyl cis/trans isomerase
SDS	Sodium Dodecyl Sulfate
SEC	Size Exclusion Chromatography
SOPC	S-o-nitrophenyl-L-cysteine
SRP	Signal Recognition Particle
TEMED	N,N,N',N' - tetramethylethylene diamine
TF	Trigger Factor

Chapter I:

Introduction & Literature Review

SUMMARY

Trigger factor was first discovered during a study of the secretion machinery of *E. coli.* Trigger factor was found to stabilize the loose conformation of the precursor form of outer membrane protein A (OmpA) and trigger its membrane translocation.[1-3] Later, peptidyl prolyl isomerase (PPIase) activity was identified as an important *in vitro* function of trigger factor.[4] *In vivo*, trigger factor was found to cooperate with other chaperones, such as GroEL, DnaK and signal recognition particle (SRP).[5-7] The location of trigger factor on the ribosome and its chaperone properties makes trigger factor the first chaperone to interact with nascent polypeptides.[8] Trigger factor is a unique chaperone in bacteria because of its combined functions of PPiase and chaperone activities.

STRUCTURE OF TRIGGER FACTOR

The structure of trigger factor is modular;[9] limited proteolysis results in three individual domains: the N-terminal domain (residues 1-145, NTD), the FKBP-like middle domain (residues 146-251, FKBP) and the C-terminal domain (residues 252-432, CTD). (Fig.1.1.A) CD spectra showed that all three domains contain ordered secondary structure. Both NTD and CTD were composed mainly of α-helical structure. β-sheet structure was identified to dominate in the FKBP domain, which is consistent with its similarity to the structure of FKBP12 with five antiparallel β-sheet and one short α-helix.[10, 11] FKBP domain is the most stable domain in the full-length trigger factor,

and CTD is the least stable domain. The combination of NTD and FKBP+CTD domain CD spectra coincides with the spectrum of full length trigger factor. Connecting NTD did not enhance the stability of the FKBP domain. CTD and FKBP domains slightly stabilized each other in the constructed FKBP+CTD domain. The stability of CTD was shown to be improved by interaction with NTD in the full length trigger factor. The FKBP domain harbours all PPIase activity of trigger factor (K_{cat}/K_m (TF) = 1.01 $uM^{-1}s^{-1}$ and K_{cat}/K_m (TF-FKBP) = 0.62 $uM^{-1}s^{-1}$). But PPIase activity is not required for the chaperone activity of TF.[12]

The NMR structure of the TF-FKBP domain from *Mycoplasma genitalium* was published in 2002.[13] 101 amino acids (151-251 of the intact trigger factor sequence) were assigned. The structure of the TF-FKBP domain is similar to human FKBP12 except that the first strand of β-sheet in FKBP12 is missing or becomes loose in TF-FKBP. The aromatic active site is conserved, and FKBP12 and TF-FKBP employ similar internal motions. In contrast, the substrate binding site of TF-FKBP is narrower than it is in FKBP12, which could cause strict stereoselectivity. The major difference between the two proteins is located at the flap region and the 40S bulge, which may be relevant for substrate binding and specificity. (Fig.1.1.B) Trigger factor doesn't bind FK506, the substrate of FKBP12, due to steric hindrance around the binding site. Later X-ray structures of intact trigger factor confirmed the NMR structure.[14, 15] In the X-ray structure of full length trigger factor, the FKBP domain is outside the substrate binding cradle formed by the NTD and CTD, suggesting that the FKBP domain may be the last part of TF when it is on the ribosome to interact with nascent chains coming from

the channel exit.[15] In the structure of trigger factor from *Vibrio cholerae,* a loop from CTD was found to be plugged into the FKBP domain. This binding was thought to control the access of substrate to the active site of the FKBP domain. On the other hand, since the role of the FKBP domain in TF is still mysterious, the interaction between the FKBP domain and CTD could be evidence of the possible function of the FKBP domain to regulate the binding or release of the substrate in trigger factor.

CTD is the least stable domain of trigger factor in isolation. The X-ray crystal structure showed that the linker (residues 111-133) connecting NTD and the FKBP domain stabilize the CTD. Without this linker, CTD from some organisms could not be expressed separately.[15, 16] So this linker is sometimes considered as part of CTD.[14] The most recent report shows the CTD of trigger factor from *Thermotoga maritim* exists as a homo-dimer in solution.[17] The sequence identity of CTD from *Thermotoga maritim* and *E. coli* is only 12%, which could explain the different stabilities of CTD in isolation. At first the structure of CTD was described as part of the back and two arms of a crouching dragon (full length trigger factor).[15] (Fig.1.1.C) The arms are composed of six α-helices. From another perspective, this arm and back structure could also be illustrated as a trivet structure with a large cavity in the center.[18] (Fig.1.1.D) This cavity together with NTD forms a cradle hanging over the channel exit of the ribosome. This cradle encloses 3500Å^2 of hydrophobic surface presumably to protect newly synthesized polypeptides from aggregation or misfolding caused by the crowded cytosolic environment in the cell. A 14-kDa protein could be fitted in this space.[15]

A most important function of trigger factor is its chaperone activity for nascent

polypeptides when TF associates with the ribosome. The structure of the NTD was well studied because trigger factor is bound to the ribosome through the NTD. NTD consists of a four-stranded antiparallel β-sheet and two long α-helices. The β-sheet is located at one side of the molecule, and two α-helices are at the other side. (Fig.1.1.E) Two (sometimes three) α-helices surround the signature loop.[15, 17, 19, 20] Unbound NTD was found as homo-multimers in solution, and the crystal structure of isolated NTD was resolved mainly from the dimer fraction.[17] The study of co-crystallized TF with ribosome showed that trigger factor monomer binds to ribosome via a loop in NTD. This loop (**GFRXGXXP**) is highly conserved across organisms, and is flexible. The loop region contacts rRNA and ribosomal proteins L23 and L29 on the ribosome via hydrogen bonds and hydrophobic interactions. The most important contacts were formed by the interaction of the NH groups from the loop towards the phosphate oxygen from rRNA and carboxylate oxygens of Glu from L23, then the hydrogen bond between neighboring groups from rRNA and L23 closed the circular hydrogen bond, which is significant for trigger factor binding to ribosome. The importance of circular hydrogen bond is consistent with the fact that both Arg of TF and Glu of L23 residues are conserved universally and the mutation of Glu on L23 reduces the affinity of TF for the ribosome.[8] The glycine residues are probably responsible for flexibility of the loop. The proline group interacts with a Tyr residue of L23, which contributes to the TF binding. The conserved Phe residue is solvent-exposed in the structure of unbound TF, but flips into loop centered to stabilize its conformation in the structure of ribosome-bound TF.[19] Ribosome-bound TF-NTD displays a rigid confirmation compared to NTD structure in

unbound TF.[14, 15, 21] The long α-helix was broken into two shorter helices, which were drawn 6Å apart. The resulting α-helix on top was shifted by 40 degree away from the β-sheet. The β-sheet is not packed with the broken α-helix any more, which exposes its hydrophobic surface to the channel exit on the ribosome. In some species, such as *D. radiodurans*, TF interacts with the backbone of L29 and pulls the α-helix away. But this interaction is not important for *E. coli* trigger factor.[8] Other than L29, in some species, L24 also interacts with TF through hydrogen bonds to orient TF on the ribosome. These interactions also cause a conformational change of the binding loop. The internal space of the loop becomes larger, and the loop folds back to produce a hook conformation, which may be a common conformation of the ribosome-bound loop. The hook confirmation is stabilized by the flipped Phe residue. The conformational rearrangement of TF upon ribosome binding pulls two major α-helices farther away and exposes the hydrophobic cradle for the incoming newly synthesized polypeptide from the ribosome channel. (Fig.1.1.F)

The shape of full length trigger factor resembles a “crouching dragon”. The “tail” (NTD) and the “arms” (CTD) together form the binding cradle for nascent polypeptides. A long linker connecting NTD and FKBP domains becomes the “back” of the dragon to support the binding pocket. CTD stays between the NTD and FKBP domain. The FKBP domain is the “head” floating above the cradle. No crystal structure of full length trigger factor bound to ribosome is available because full length trigger factor interferes with 50S packing.[15] Most crystal structures of TF-NTD bound to the ribosome indicated that TF binds to the ribosome as a monomer.[14, 15, 19, 20] Neutron scattering experiments

showed TF could bind to ribosomes as a dimer.[22] Whether TF monomer or dimer binds to ribosomes remains a mystery. An X-ray crystal structure of ribosome-bound TF-NTD enabled modeling of full length TF monomer onto ribosome from *D. radiodurans*.[15, 19] The binding cradle composed of NTD and CTD hangs above the ribosome. The hydrophobic β-sheet was positioned right over the tunnel exit.[20] The whole TF molecule is drawn towards L24 by the interaction between residues of NTD and L24. This shift opens up a channel with hydrophobic residues lining the inside surface.[19] (Fig.1.1.G) Nascent polypeptides may go through this conduit from the ribosome into the binding space between the CTD arms. The crevice may expose the newly synthesized polypeptide to another ribosome binding chaperone, signal recognition particle (SRP). The recognition domain of SRP may monitor nascent polypeptides as they pass through the hydrophobic channel and look for the signal sequence. Without the signal sequence, nascent peptides would go through the channel into the CTD, and eventually towards the FKBP-like head region. Folding of nascent peptide may change the conformation of TF and make it dissociate from the ribosome.[15] After dissociation, TF may or may not continue to bind polypeptides. It was shown that TF could bind polypeptides for a relatively long time after dissociation from ribosome until the nascent protein folds into a "safe" conformation.[23]

Since the size of trigger factor is too large for NMR spectrometry, its solution structure is not available. There exists a monomer/dimer equilibrium for TF in solution.[24] TF dimer may form by interaction of NTD and CTD.[14, 17, 21, 24] The X-ray structure of truncated TF from *Vibrio cholerae* indicated that the shape of the dimer may resemble right-handed double strands of DNA.[14] There are two grooves on each side of molecule.

The major groove is surrounded by TF-FKBP and NTD and floored by CTD from both monomers. There are holes between interfaces to expose the center of this model. This architecture of TF dimerization is based on truncated trigger factor, so the model may not reflect the true structure of the TF dimer, but it is the only available dimer model based on real X-ray data. And this model may be important to understand the chaperone activity of ribosome free trigger factor *in vivo* and *in vitro*.

PPIASE AND CHAPERONE ACTIVITY OF TRIGGER FACTOR

For most peptide bonds, the *trans*- conformation of the peptide bond is much more stable than the *cis*- conformation. Although the *trans*- peptidyl-prolyl bond is the stable conformer, the free enthalpy difference between *cis* and *trans* conformation is only 2.0 kJ mol^{-1} for prolyl bonds compared to 10 kJ mol^{-1} for nonproline bonds.[25] About 30% of peptidyl prolyl bonds in proteins of known structure are in the *cis*- conformation, which makes prolyl isomerization possible. Because of its partial double bond character, peptide bonds are planar. The *cis*- and *trans*- conformations are separated by a high activation energy barrier of 75-100 kJ/mol. Thus, prolyl isomerization is a rate-limiting process in protein folding.[26] Peptidyl prolyl *cis/trans*-isomerases (PPIases) accelerate the *cis/trans* isomerization by binding to the transition state to decrease the energy barrier.[27, 28] (Fig. 1.2)

PPIases includes three sub-families: cyclophilins, FKBPs and parvulins. Their amino acid sequences are unrelated, even at active sites.[28-30] Cyclophilins are highly conserved during evolution.[31] Cyclophilins are the target of the immunosuppressant drug, cyclosporin A.[32] Trigger factor is a PPIase of the FKBP family.[9] Except for trigger

factor, members of FKBP family are inhibited by immunosuppressant drugs, FK506 and rapamycin.[33]

PPIases are well conserved from bacteria to animals. PPIases are involved in important process, such as signal transduction, immunosuppression, cell cycle regulation and protein folding.[34-38] They play important roles in protein folding and assembly, but in many cases, deletion of PPIases does not affect cell growth.[12, 39-41] Deletion of all immunophilins except parvulin in *Saccharomyces cerevisiae* did not affect the yeast growth under normal and stressed conditions.[42] It was concluded that PPIases may be important for protein folding of specific substrates. Protein folding in the periplasm is different from folding in cytosol. The only case with periplasmic PPIases playing essential roles in general protein folding is in *E. coli*. Double deletion of PPiD and SurA is lethal for cell growth.[38] There are ten PPIases in *E. coli*, five in the cytosol and five in the periplasm. (Table I)

Trigger factor is a special PPIase. The NMR structure of TF-FKBP from *Mycoplasma genitalium* shows that it adopts a similar fold with human FKBP and other member of the FK506 binding protein family.[13] But trigger factor could not be inhibited by either FK506 or cyclosporine A.[4] The unique feature of trigger factor is that it is the only ribosome-bound PPIase.[4] Trigger factor may represent a sub-class of the FKBP family. Its PPI activity is lower than that of other PPIases.[4] Isolated TF-FKBP and full length trigger factor show similar PPIase activities toward a tetrapeptide substrate.[9, 43, 44] It seems that NTD and CTD do not improve TF's PPIase activity towards short peptide substrate. In contrast, in the refolding experiment of S-carboxymethylated

S54G/P55N-variant of RNase T1 (RCM-T1), intact trigger factor is a much more efficient PPIase (20-100 folds) to protein substrate compared to other PPIases or isolated TF-FKBP.[9] The high catalytic activity is due to the tight binding of trigger factor to substrates. NTD and CTD of trigger factor contribute to the chaperone activity because TF-FKBP alone does not show the extreme folding activity. Trigger factor prefers hydrophobic residues, like other FKBPs.[4] Peptide scanning shows that TF-FKBP domain or any construct including this part has affinity for peptides on cellulose membrane. However, this specificity was found to be independent of proline residues.[45] The importance of PPIase activity for trigger factor was studied by site-directed mutagenesis within the FKBP domain.[12] Two variants, TF-F198A and TF-F233Y were inactive with peptide substrates. TF-F233Y can still assist RCM-T1 refolding with lower efficiency, but TF-F198A exhibits no catalytic activity to protein substrate. Deficiency of PPIase activity did not affect chaperone activity of trigger factor to protein substrate (GAPDH), suggesting that the chaperone activity is independent of prolyl isomerization. The PPIase activity of trigger factor may be important only for specific proteins. Mutation of F198 and F233 did not change the binding specificity of trigger factor for polypeptides. Both wild type and TF variants recognize the same peptides. PPIase activity is not essential for trigger factor's *in vivo* function. Both wild type TF and TF-F198A suppress protein aggregation in *ΔtigΔdnaK* cells at 30℃.[12] However, TF-F233Y was found less effective at keeping cells alive at 6℃ than wild type trigger factor.[46] Prolyl isomerization may become too slow to sustain life in a cold environment. So Trigger factor could be the catalyst to accelerate praline isomerization-limited steps for certain proteins at low temperature.

Compared to its PPIase activity, trigger factor chaperone activity has been well studied. As a ribosome-bound protein, trigger factor is probably the first chaperone nascent peptides meet, but trigger factor is not essential for cell growth, since deletion of *tig* gene is not lethal.[6] 26-39% of nascent polypeptides interact with DnaK in *Δtig* cells compared to 9-18% in wild type. DnaK would compensate the absence of TF by binding to more newly synthesized polypeptides. Normally, DnaK prefers substrates of 30-75kDa.[47] Upon deletion of trigger factor, DnaK interacts with more small peptides. In contrast, trigger factor and DnaK may cooperate in folding of mutidomain proteins. Cell free experiments indicated that in the absence of TF, the DnaK/DnaJ/GrpE (KJE) system could not improve the specific activity or delay the folding rate of large proteins, such as firefly luciferase and β-galactosidase.[48] The chaperone activity of KJE towards multidomain proteins may be modulated by trigger factor. Thus, the function of trigger factor on ribosomes may include binding to small proteins folding fast and mediating large polypeptide flux through the KJE system. Double deletion of trigger factor and DnaK causes serious aggregation of cytosolic proteins. The lethality of double deletion of DnaK and TF genes depends on growth temperature.[49] *ΔtigΔdnaK* cells are viable at low temperature.

Overproduction of GroEL/GroES would compensate the loss of DnaK and TF.[49, 50] Aggregated proteins from *ΔtigΔdnaK* cells were analyzed by 2D-electrophoresis. 40% of aggregated proteins were large proteins of ≥ 60KDa.[51] 29 identified proteins were found to physically interact with DnaK. There were only two proteins shown to interact with trigger factor by cross-linking experiments. Since it is hard to identify substrates of TF due to its low affinity for substrates (a KD value of 120 uM has been reported), there are

probably more polypeptides directly interacting with trigger factor.[45] Peptide library scanning indicated that 77% of TF-binding peptides also bind to DnaK,[51] so the trigger factor and DnaK may have similar binding specificities to polypeptides. So they could backup each other's absence *in vivo*. Binding study showed that trigger factor prefers aromatic residues (Phe, Tyr, Trp and His) and positively charged residues (Arg and Lys), which is comparable to DnaK binding motif.[45] Its binding to peptides does not depend on presence of proline residues. TF has no preference for α-helices or β-sheets. All three domains of TF were shown to interact with nascent chains by photo-crosslinking experiments.[18] The interaction between the FKBP domain and substrate may depend on specific binding site on the amino acid sequence of substrate.

The binding affinity of TF for peptides normally is low, but ribosome binding may improve the substrate affinity of TF. The chaperone activity of TF on the translating ribosome was monitored by fluorescence spectroscopy.[23] Results from this study implicated a mechanism in which: 1) the dimer/monomer equilibrium of trigger factor in solution provides TF monomer for ribosome; 2) upon binding, TF adjusts its conformation to extend the binding pocket for incoming nascent polypeptides; 3) without ongoing translation, TF is binding to and released from ribosome with a time interval of about ten seconds; 4) during translation, TF associates with nascent peptides for various times (up to 30s); 5) multiple TF monomers could bind to longer substrates simultaneously; 6) TF dimerizes and gets ready for another cycle after dissociating from folded polypeptides.[23] Other chaperones may be needed to assist further folding of multidomain proteins.

Mutation of the ribosome binding motif does not significantly change the *in vivo*

function of trigger factor.[50] This result suggested additional function of trigger factor other than its chaperone activity on ribosome. *In vitro* refolding experiments showed that trigger factor improves the activity recovery of denatured proteins, such as green fluorescence protein (GFP), D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and lysozyme.[52-54] Trigger factor was found to suppress aggregation during refolding of denatured proteins. But at high concentrations, trigger factor has trouble to release the substrate. Adding DnaK/DnaJ/GrpE or GroEL/GroES with ATP can help substrates dissociate from trigger factor.[52, 55] These results may represent folding arrest activity of trigger factor via non-specific hydrophobic interactions.[52] Trigger factor does not bind nucleotide, so how TF regulates its chaperone activity is not clear. A real-time binding study cast light on a possible regulation mechanism for TF *in vitro* chaperone activity.[23] Ribosome binding opens up the hydrophobic binding site on TF and activates its chaperone activity. Nascent chain binding can also stabilize the "open" conformation of TF, and its folding may trigger its release from TF. Dimerization of free TFs would bury and protect the inner hydrophobic pocket from aqueous solution. So non-specific substrates, such as GFP or GAPDH, may not be allowed to fold when they bind to TF or their folding may not be able to trigger the conformation change of TF for releasing. As discussed above, the deletion of TF is not lethal for cell growth. So it is likely that TF is important for certain protein folding or assembly. Identifying specific substrate of TF is neccessary for studying the function of free TF in solution.

STRUCTURE AND FUNCTION OF TRYPTOPHANASE

Tryptophanase can catalyze α,β-elimination, converting of tryptophan to indole, pyruvate and ammonia.[57] This activity of tryptophanase requires a co-factor, pyridoxal-5'-phosphate (PLP). Tryptophanase binds PLP in a ratio of 1:1.[58] Apo-tryptophanase is easily purified and crystallized.[59] Holo-enzyme can be produced by dialysis of apo-tryptophanase against buffer with PLP. Extensive dilution causes dissociation of PLP from protein, which suggests that the conformation of holo-tryptophanase may not be stable. K^+ or NH_4^+ is required for tryptophanase activity.[60] In the presence of K^+, there exists a pH dependent equilibrium for holo-tryptophanase between protonated, enzymatically inactive form and deprotonated, active form. Na^+ or imidazole inhibits tryptophanase activity by converting tryptophanase to its inactive conformation.[61] The active form includes a Schiff base without the hydrogen bond formed between the phenolic hydrogen and the nitrogen of lysine. (Fig. 1.3.A) The general mechanism for tryptophanase activity is: 1) substrate interacts with PLP to form a new bond to replace the azomethine linkage between PLP and apo-enzyme; 2) PLP-substrate is deprotonated and forms a quinonoid intermediate; 3) β substituent is eliminated from intermediate; 4) pyruvate and ammonia are formed.[61-67] (Fig. 1.3.A) The structure of tryptophanase was studied long time ago.[58, 68-70] Tryptophanase has a tetrameric structure. There exists dimer/tetramer equilibrium for apo-tryptophanase in solution with K^+ or Na^+ at low temperatures (<10℃). Tetramer of apo-protein is not stable and dissociates to dimers at high pH. Association of dimers is through hydrophobic interaction. Dissociation of dimer to monomer induced by sodium dodecyl sulfate or high pH is not

reversible.[58, 71] Unstable monomers form large aggregate.[69] PLP can stabilize tryptophanase at low temperature by converting apo-tryptophanase to holo-tryptophanase. This binding requires essential cation, K^+ or NH_4^+. It was shown that association of dimers and binding of PLP occur simoutaneously.[58] But tetramerization is the rate limiting step in activity recovery of holo-tryptophanase.[70] Fixation of four PLPs on holo-tryptophanase via reduction of Schiff base by sodium borohydride removed its sensitivity to cold temperature.[72] The same method was applied to make different tryptophanase hybrids, H_3A_1 and H_2A_2 (H represents protomer and A represents apo-protomer).[73] Hybrids with one or two apo-protomers are stable as tetramer at 4℃. With these hybrids, study on binding of PLP to apo-tryptophanase was performed. Binding of the last PLP was shown to be 35 times slower than binding of the first PLP. So binding of one PLP may induce conformational change of neighbour apo-promoter and make it more difficult to bind the next PLP. Structural study with a sensitive cysteine as a probe for conformational change confirmed the difference in conformations of apo- and holo-tryptophanase.[71, 73, 74] The transfer between conformations may be regulated by binding potassium ion.[74] This conformational change may help PLP to reorient its pyridine ring for maximizing orbital overlap in the transition state.[75] The X-ray crystal structures indicated more details around PLP binding site and active site of tryptophanase. The first X-ray crystal structure is for holo-tryptophanase from *proteus vulgaris*.[76] (Fig. 1.3.B) The tryptophanase monomer is composed of an N-terminal arm, a small domain and a large domain. The small domain has a four-stranded antiparallel β-sheet. The internal side of β-sheet faces the large domain. Four α-helices located on the other side are exposed to

solvent. In the center of the large domain, there is a seven-stranded β-sheet surrounded by three α-helices on the side of interdomain interface and six α-helices on the outer side. PLP binding site is in the interdomain cleft. The active site is formed by "catalytic dimer". This catalytic dimer is stabilized by binding PLP and monovalent cation. The double bond between lysine and PLP is not on the same plane with PLP pyridine ring. It is tilted by about 30º. This conformation is stabilized by salt bridge and hydrogen bonds formed between PLP and residues on the large domain. The monovalent cation locates between two subunits of the catalytic dimer. K^+ is coordinated with Gly53 O and Asn271 O from one subunit, Glu70 OE1 and Glu70 O from the other subunit of catalytic dimer and two water molecules. These potassium ions were referred to as "activating ion" because they are important to activate tryptophanase.[77] Since K^+ is far away from PLP, K+ could affect PLP binding and tryptophanase activity via inducing conformational change of whole tryptophanase.[58] Cys298 in *E. coli* tryptophanase was thought to be close to active site and important for PLP binding.[78, 79] Cys292 of *P.vulgaris* tryptophanase, equivalent to Cys298 in *E. coli* trytpophanase, was found about 7Å from the phosphate group of PLP. PLP binding may change the hydrophobic environment around Cys292 and lower the solvent accessibility for Cys292 side-chain.[80]

The overall structure of apo-tryptophanase from *E. coli* is similar with holo-tryptophanase from *Proteus vulgaris*.[80] In the X-ray crystal structure of tryptophanase from *E. coli*, two additional potassium binding sites were found in the interface of two "catalytic dimer"s.[80] (Fig. 1.3.C,D) These potassium ions between catalytic dimers may contribute to stabilization of the tryptophanase tetramer. No crystal

structure of tryptophanase with substrate is available yet. But two sulfate molecules were found at the active site of *E. coli* tryptophanase structure. One occupies the position of PLP, and the other one interacts with Arg419, which could bind the α-carboxyl of tryptophanase substrate.[81] Thus, PLP and tryptophan (TRP) were modeled into *E. coli* tryptophanase structure based on the positions of the two sulfates.[80] (Fig. 1.3.E) An enzymatic mechanism was proposed based on this model. According to this mechanism, the basic cavity of tryptophanase will deprotonate the α-amino group of substrate to make a good nucleophile when the substrate enters the active site. Substrate binding holds two domains of tryptophanase in a "closed" conformation. This conformation in turn pushes the α-amino group on the substrate closer to the lysyl-PLP bond to form an external imine. The nucleophilic attack of lysyl-PLP produces a PLP-TRP external aldimine intermediate. Then Tyr74 donates a proton to PLP-TRP to make the indole a better leaving group.[63] Following β elimination, Lys270 attacks the PLP-α-aminoacrylate intermediate to bind PLP again and release the unstable α-aminoacrylate. Pyruvate and ammonia are the final products from dissociation of α-aminoacrylate.

REFERENCES

1. Crooke, E., et al., *ProOmpA is stabilized for membrane translocation by either purified E. coli trigger factor or canine signal recognition particle.* Cell, 1988. **54**(7): p. 1003-11.
2. Crooke, E., et al., *ProOmpA spontaneously folds in a membrane assembly competent state which trigger factor stabilizes.* Embo J, 1988. **7**(6): p. 1831-5.
3. Crooke, E. and W. Wickner, *Trigger factor: a soluble protein that folds pro-OmpA into a membrane-assembly-competent form.* Proc Natl Acad Sci U S A, 1987. **84**(15): p. 5216-20.
4. Stoller, G., et al., *A ribosome-associated peptidyl-prolyl cis/trans isomerase identified as the trigger factor.* Embo J, 1995. **14**(20): p. 4939-48.
5. Kandror, O., et al., *Trigger factor is involved in GroEL-dependent protein degradation in Escherichia coli and promotes binding of GroEL to unfolded proteins.* Embo J, 1995. **14**(23): p. 6021-7.
6. Deuerling, E., et al., *Trigger factor and DnaK cooperate in folding of newly synthesized proteins.* Nature, 1999. **400**(6745): p. 693-6.
7. Valent, Q.A., et al., *Early events in preprotein recognition in E. coli: interaction of SRP and trigger factor with nascent polypeptides.* Embo J, 1995. **14**(22): p. 5494-505.
8. Kramer, G., et al., *L23 protein functions as a chaperone docking site on the ribosome.* Nature, 2002. **419**(6903): p. 171-4.
9. Zarnt, T., et al., *Modular structure of the trigger factor required for high activity in*

protein folding. J Mol Biol, 1997. **271**(5): p. 827-37.

10. Van Duyne, G.D., et al., *Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex.* Science, 1991. **252**(5007): p. 839-42.
11. Michnick, S.W., et al., *Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin.* Science, 1991. **252**(5007): p. 836-9.
12. Kramer, G., et al., *Trigger factor peptidyl-prolyl cis/trans isomerase activity is not essential for the folding of cytosolic proteins in Escherichia coli.* J Biol Chem, 2004. **279**(14): p. 14165-70.
13. Vogtherr, M., et al., *NMR solution structure and dynamics of the peptidyl-prolyl cis-trans isomerase domain of the trigger factor from Mycoplasma genitalium compared to FK506-binding protein.* J Mol Biol, 2002. **318**(4): p. 1097-115.
14. Ludlam, A.V., B.A. Moore, and Z. Xu, *The crystal structure of ribosomal chaperone trigger factor from Vibrio cholerae.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13436-41.
15. Ferbitz, L., et al., *Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins.* Nature, 2004. **431**(7008): p. 590-6.
16. Merz, F., et al., *The C-terminal domain of Escherichia coli trigger factor represents the central module of its chaperone activity.* J Biol Chem, 2006. **281**(42): p. 31963-71.
17. Martinez-Hackert, E. and W.A. Hendrickson, *Structures of and interactions between domains of trigger factor from Thermotoga maritima.* Acta Crystallogr D Biol Crystallogr, 2007. **63**(Pt 4): p. 536-47.

18. Lakshmipathy, S.K., et al., *Identification of nascent chain interaction sites on trigger factor.* J Biol Chem, 2007.

19. Schlunzen, F., et al., *The binding mode of the trigger factor on the ribosome: implications for protein folding and SRP interaction.* Structure, 2005. **13**(11): p. 1685-94.

20. Baram, D., et al., *Structure of trigger factor binding domain in biologically homologous complex with eubacterial ribosome reveals its chaperone action.* Proc Natl Acad Sci U S A, 2005. **102**(34): p. 12017-22.

21. Kristensen, O. and M. Gajhede, *Chaperone binding at the ribosomal exit tunnel.* Structure, 2003. **11**(12): p. 1547-56.

22. Blaha, G., et al., *Localization of the trigger factor binding site on the ribosomal 50S subunit.* J Mol Biol, 2003. **326**(3): p. 887-97.

23. Kaiser, C.M., et al., *Real-time observation of trigger factor function on translating ribosomes.* Nature, 2006. **444**(7118): p. 455-60.

24. Patzelt, H., et al., *Three-state equilibrium of Escherichia coli trigger factor.* Biol Chem, 2002. **383**(10): p. 1611-9.

25. Stewart, D.E., A. Sarkar, and J.E. Wampler, *Occurrence and role of cis peptide bonds in protein structures.* J Mol Biol, 1990. **214**(1): p. 253-60.

26. Kiefhaber, T., et al., *Folding of ribonuclease T1. 2. Kinetic models for the folding and unfolding reactions.* Biochemistry, 1990. **29**(12): p. 3061-70.

27. Kofron, J.L., et al., *Determination of kinetic constants for peptidyl prolyl cis-trans isomerases by an improved spectrophotometric assay.* Biochemistry, 1991. **30**(25):

p. 6127-34.

28. Zhao, Y. and H. Ke, *Crystal structure implies that cyclophilin predominantly catalyzes the trans to cis isomerization.* Biochemistry, 1996. **35**(23): p. 7356-61.

29. Harding, M.W., et al., *A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase.* Nature, 1989. **341**(6244): p. 758-60.

30. Ranganathan, R., et al., *Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent.* Cell, 1997. **89**(6): p. 875-86.

31. Pahl, A., K. Brune, and H. Bang, *Fit for life? Evolution of chaperones and folding catalysts parallels the development of complex organisms.* Cell Stress Chaperones, 1997. **2**(2): p. 78-86.

32. Liu, J., C.M. Chen, and C.T. Walsh, *Human and Escherichia coli cyclophilins: sensitivity to inhibition by the immunosuppressant cyclosporin A correlates with a specific tryptophan residue.* Biochemistry, 1991. **30**(9): p. 2306-10.

33. Bierer, B.E., et al., *Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin.* Proc Natl Acad Sci U S A, 1990. **87**(23): p. 9231-5.

34. Hennig, L., et al., *Selective inactivation of parvulin-like peptidyl-prolyl cis/trans isomerases by juglone.* Biochemistry, 1998. **37**(17): p. 5953-60.

35. Clardy, J., *The chemistry of signal transduction.* Proc Natl Acad Sci U S A, 1995. **92**(1): p. 56-61.

36. Schreiber, S.L. and G.R. Crabtree, *The mechanism of action of cyclosporin A and*

FK506. Immunol Today, 1992. **13**(4): p. 136-42.

37. Lu, K.P., S.D. Hanes, and T. Hunter, *A human peptidyl-prolyl isomerase essential for regulation of mitosis.* Nature, 1996. **380**(6574): p. 544-7.

38. Lazar, S.W. and R. Kolter, *SurA assists the folding of Escherichia coli outer membrane proteins.* J Bacteriol, 1996. **178**(6): p. 1770-3.

39. Dartigalongue, C. and S. Raina, *A new heat-shock gene, ppiD, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in Escherichia coli.* Embo J, 1998. **17**(14): p. 3968-80.

40. Kleerebezem, M., M. Heutink, and J. Tommassen, *Characterization of an Escherichia coli rotA mutant, affected in periplasmic peptidyl-prolyl cis/trans isomerase.* Mol Microbiol, 1995. **18**(2): p. 313-20.

41. Kok, R.G., et al., *A gene of Acinetobacter calcoaceticus BD413 encodes a periplasmic peptidyl-prolyl cis-trans isomerase of the cyclophilin sub-class that is not essential for growth.* Biochim Biophys Acta, 1994. **1219**(3): p. 601-6.

42. Gothel, S.F., et al., *Cyclophilin and trigger factor from Bacillus subtilis catalyze in vitro protein folding and are necessary for viability under starvation conditions.* Biochemistry, 1998. **37**(38): p. 13392-9.

43. Dolinski, K., et al., *All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 13093-8.

44. Stoller, G., et al., *An 11.8 kDa proteolytic fragment of the E. coli trigger factor represents the domain carrying the peptidyl-prolyl cis/trans isomerase activity.*

FEBS Lett, 1996. **384**(2): p. 117-22.

45. Hesterkamp, T. and B. Bukau, *Identification of the prolyl isomerase domain of Escherichia coli trigger factor.* FEBS Lett, 1996. **385**(1-2): p. 67-71.

46. Patzelt, H., et al., *Binding specificity of Escherichia coli trigger factor.* Proc Natl Acad Sci U S A, 2001. **98**(25): p. 14244-9.

47. Schiene-Fischer, C., et al., *Evaluation of similarities in the cis/trans isomerase function of trigger factor and DnaK.* Biol Chem, 2002. **383**(12): p. 1865-73.

48. Teter, S.A., et al., *Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains.* Cell, 1999. **97**(6): p. 755-65.

49. Agashe, V.R., et al., *Function of trigger factor and DnaK in multidomain protein folding: increase in yield at the expense of folding speed.* Cell, 2004. **117**(2): p. 199-209.

50. Vorderwulbecke, S., et al., *Low temperature or GroEL/ES overproduction permits growth of Escherichia coli cells lacking trigger factor and DnaK.* FEBS Lett, 2004. **559**(1-3): p. 181-7.

51. Genevaux, P., et al., *In vivo analysis of the overlapping functions of DnaK and trigger factor.* EMBO Rep, 2004. **5**(2): p. 195-200.

52. Deuerling, E., et al., *Trigger Factor and DnaK possess overlapping substrate pools and binding specificities.* Mol Microbiol, 2003. **47**(5): p. 1317-28.

53. Suno, R., et al., *Trigger factor from Thermus thermophilus is a Zn2+-dependent chaperone.* J Biol Chem, 2004. **279**(8): p. 6380-4.

54. Huang, G.C., et al., *Chaperone and antichaperone activities of trigger factor.* Eur J

Biochem, 2002. **269**(18): p. 4516-23.

55. Huang, G.C., et al., *Assisted folding of D-glyceraldehyde-3-phosphate dehydrogenase by trigger factor.* Protein Sci, 2000. **9**(6): p. 1254-61.

56. Liu, C.P., S. Perrett, and J.M. Zhou, *Dimeric trigger factor stably binds folding-competent intermediates and cooperates with the DnaK-DnaJ-GrpE chaperone system to allow refolding.* J Biol Chem, 2005. **280**(14): p. 13315-20.

57. W. A. Wood, I.C.G., and W. W. Umbreit, *FUNCTION OF PYRIDOXAL PHOSPHATE: RESOLUTION AND PURIFICATION OF THE TRYPTOPHANASE ENZYME OF ESCHERICHIA COLI.* J. Biol. Chem., 1947. **170**(1): p. 313.

58. Morino, Y. and E.E. Snell, *The subunit structure of tryptophanase. I. The effect of pyridoxal phosphate on the subunit structure and physical properties of tryptophanase.* J Biol Chem, 1967. **242**(23): p. 5591-601.

59. Newton, W.A., Y. Morino, and E.E. Snell, *Properties of Crystalline Tryptophanase.* J Biol Chem, 1965. **240**: p. 1211-8.

60. Newton, W.A. and E.E. Snell, *Catalytic Properties of Tryptophanase, a Multifunctional Pyridoxal Phosphate Enzyme.* Proc Natl Acad Sci U S A, 1964. **51**: p. 382-9.

61. Morino, Y. and E.E. Snell, *The relation of spectral changes and tritium exchange reactions to the mechanism of tryptophanase-catalyzed reactions.* J Biol Chem, 1967. **242**(12): p. 2800-9.

62. Phillips, R.S., T.V. Demidkina, and N.G. Faleev, *Structure and mechanism of tryptophan indole-lyase and tyrosine phenol-lyase.* Biochim Biophys Acta, 2003.

1647(1-2): p. 167-72.

63. Phillips, R.S., N. Johnson, and A.V. Kamath, *Formation in vitro of hybrid dimers of H463F and Y74F mutant Escherichia coli tryptophan indole-lyase rescues activity with L-tryptophan.* Biochemistry, 2002. **41**(12): p. 4012-9.

64. Kiick, D.M. and R.S. Phillips, *Mechanistic deductions from multiple kinetic and solvent deuterium isotope effects and pH studies of pyridoxal phosphate dependent carbon-carbon lyases: Escherichia coli tryptophan indole-lyase.* Biochemistry, 1988. **27**(19): p. 7339-44.

65. Kiick, D.M. and R.S. Phillips, *Mechanistic deductions from kinetic isotope effects and pH studies of pyridoxal phosphate dependent carbon-carbon lyases: Erwinia herbicola and Citrobacter freundii tyrosine phenol-lyase.* Biochemistry, 1988. **27**(19): p. 7333-8.

66. Phillips, R.S., E.W. Miles, and L.A. Cohen, *Differential inhibition of tryptophan synthase and of tryptophanase by the two diastereoisomers of 2,3-dihydro-L-tryptophan. Implications for the stereochemistry of the reaction intermediates.* J Biol Chem, 1985. **260**(27): p. 14665-70.

67. Phillips, R.S., E.W. Miles, and L.A. Cohen, *Interactions of tryptophan synthase, tryptophanase, and pyridoxal phosphate with oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan: support for an indolenine intermediate in tryptophan metabolism.* Biochemistry, 1984. **23**(25): p. 6228-34.

68. Gopinathan, K.P. and R.D. DeMoss, *Reversible dissociation of tryptophanase into protein subunits.* Proc Natl Acad Sci U S A, 1966. **56**(4): p. 1223-5.

69. Cowell, J.L. and R.D. DeMoss, *Trytophanase from Aeromonas liquefaciens. Subunit structure and aggregation of the enzyme into enzymatically active polymeric species.* J Biol Chem, 1973. **248**(18): p. 6262-9.

70. Raibaud, O. and M.E. Goldberg, *The dissociated tryptophanase subunit is inactive.* J Biol Chem, 1976. **251**(9): p. 2820-4.

71. Morino, Y. and E.E. Snell, *The subunit structure of tryptophanase. II. A correlation of ultracentrifugal and chemical studies.* J Biol Chem, 1967. **242**(23): p. 5602-10.

72. Skrzynia, C., J. London, and M.E. Goldberg, *The tryptophanase from Escherichia coli K12. 3. Further characterization of hybrids between the apo- and holoenzyme.* J Biol Chem, 1974. **249**(7): p. 2325-6.

73. Raibaud, O. and M.E. Goldberg, *Structural and functional interdependence of the protomers of Escherichia coli K 12 tryptophanase during binding of pyridoxal 5'-phosphate.* J Biol Chem, 1976. **251**(9): p. 2814-9.

74. Raibaud, O. and M.E. Goldberg, *The reactivity of one essential cysteine as a conformational probe in Escherichia coli tryptophanase. Application to the study of the structural influence of subunit interactions.* Eur J Biochem, 1977. **73**(2): p. 591-9.

75. Dunathan, H.C., *Conformation and reaction specificity in pyridoxal phosphate enzymes.* Proc Natl Acad Sci U S A, 1966. **55**(4): p. 712-6.

76. Phillips, R.S. and P.D. Gollnick, *Evidence that cysteine 298 is in the active site of tryptophan indole-lyase.* J Biol Chem, 1989. **264**(18): p. 10627-32.

77. Honda, T., et al., *Characterization of the reactivity of sulphydryl groups in*

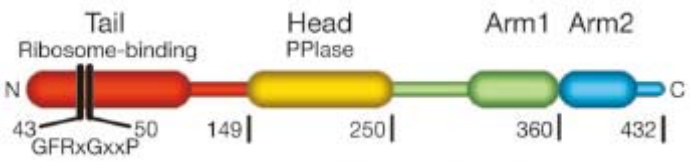
tryptophanase by a dual-monitoring high-performance liquid chromatographic system with a site-directed fluorescent reagent. J Chromatogr, 1986. **371**: p. 353-60.

78. Ku, S.Y., P. Yip, and P.L. Howell, *Structure of Escherichia coli tryptophanase.* Acta Crystallogr D Biol Crystallogr, 2006. **62**(Pt 7): p. 814-23.

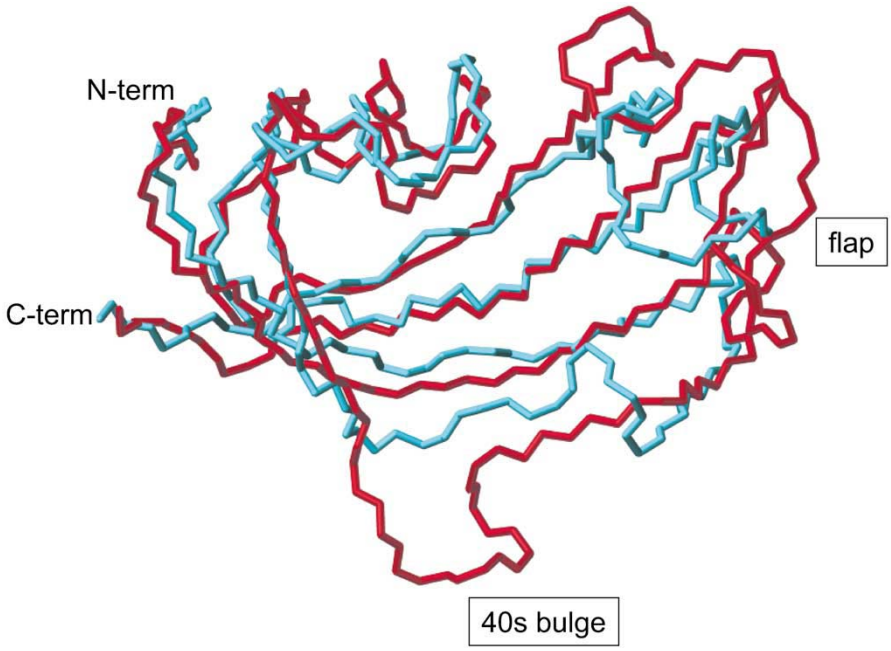
79. Hogberg-Raibaud, A., O. Raibaud, and M.E. Goldberg, *Kinetic and equilibrium studies on the activation of Escherichia coli K12 tryptophanase by pyridoxal 5'-phosphate and monovalent cations.* J Biol Chem, 1975. **250**(9): p. 3352-8.

80. Phillips, R.S. and K.J. Doshi, *Cleavage of Escherichia coli tryptophan indole-lyase by trypsin at Lys406 affects the transmission of conformational changes associated with monovalent cation activation.* Eur J Biochem, 1998. **255**(2): p. 508-15.

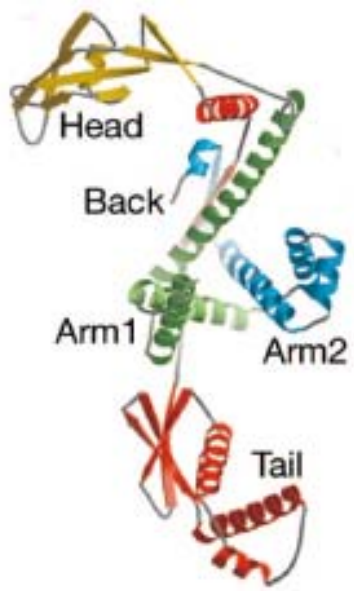
Figure 1.1



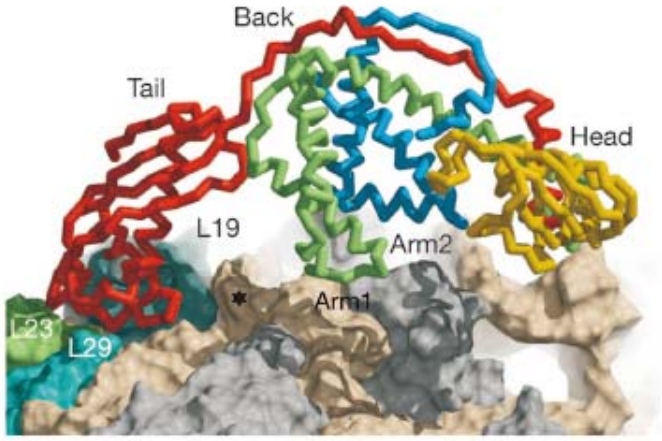
(A) Domain assignment in TF sequence. (Figure taken from [15])



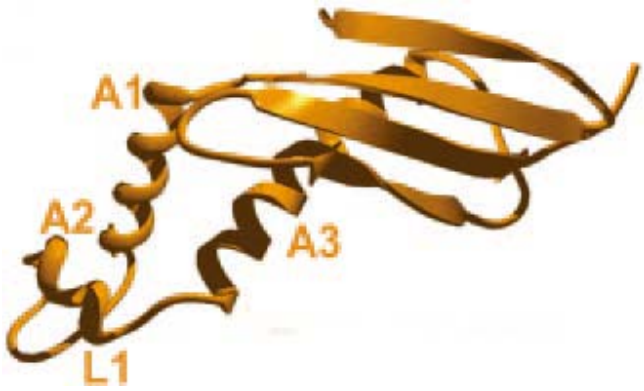
(B) Superposition of the lowest-energy solution structure of TF-FKBP (blue) and the X-ray structure of FKBP(red). (Figure taken from [13]



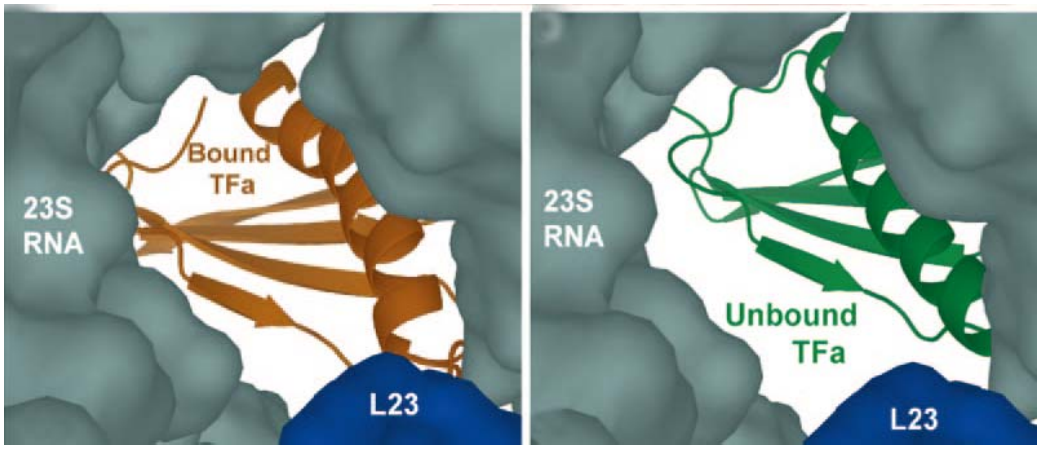
(C) Structure of full-length trigger factor. (Figure taken from [15])



(D) Close-up side view of trigger factor on the ribosome. (Figure taken from [15])

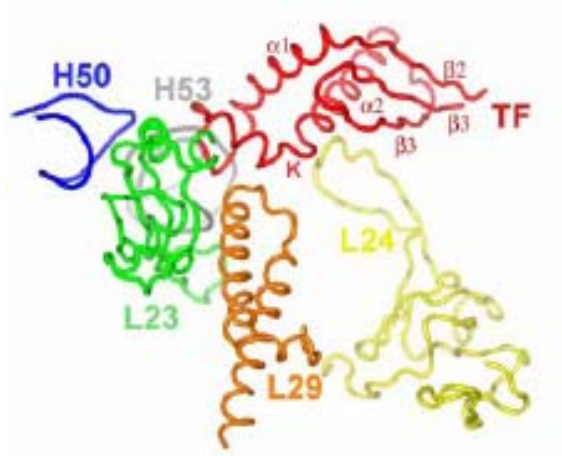


(E) Structure of ribosome-bound TF-NTD. (Figure taken from [20])



(F) Conformational rearrangements in TF-NTD upon its association with the ribosome.

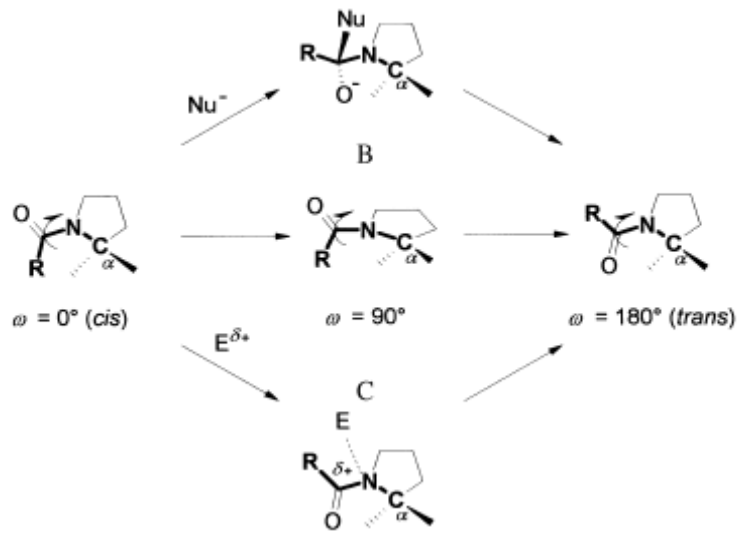
(Figure taken from [20])



(G) TF interaction with the ribosomal proteins L23 (green), L24 (yellow), and L29 (orange).

(Figure taken from [19])

Figure 1.2



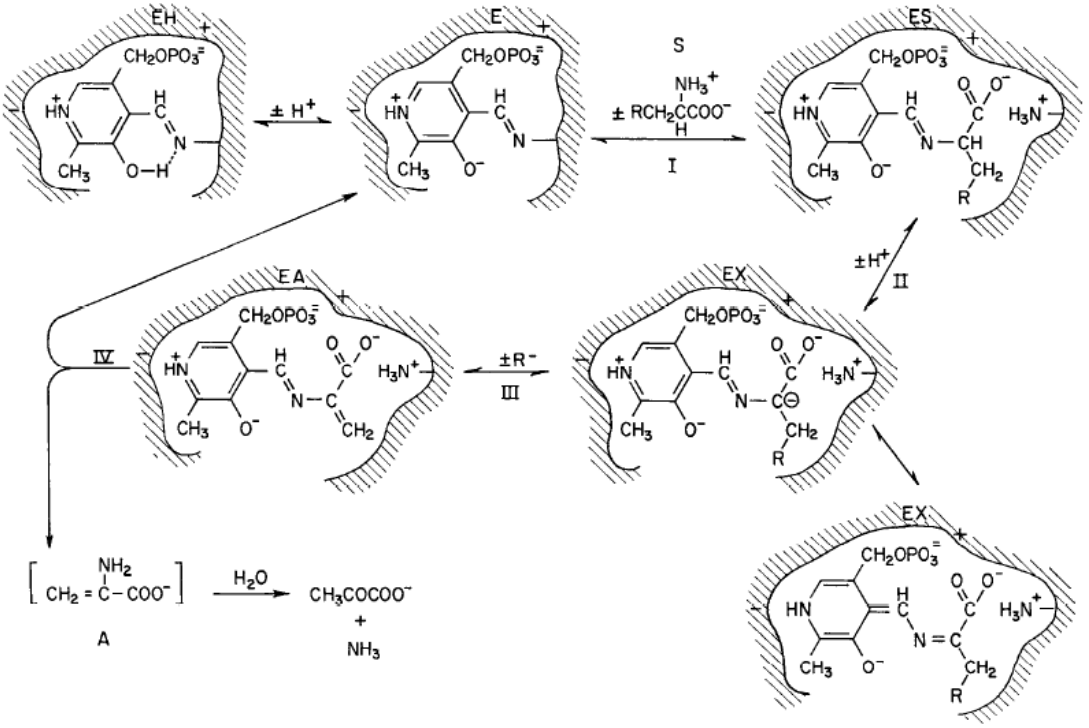
Three mechanisms of catalysis for PPIase-catalyzed peptidyl-prolyl *cis/trans* isomerization. A: Nucleophilic attack of the carbonyl carbon atom; B: formation of a transition state perpendicular to the plane of the proline ring; C: electrophilic attack of the imide nitrogen to weaken the partial double bound. (Scheme taken from [56])

Table 1

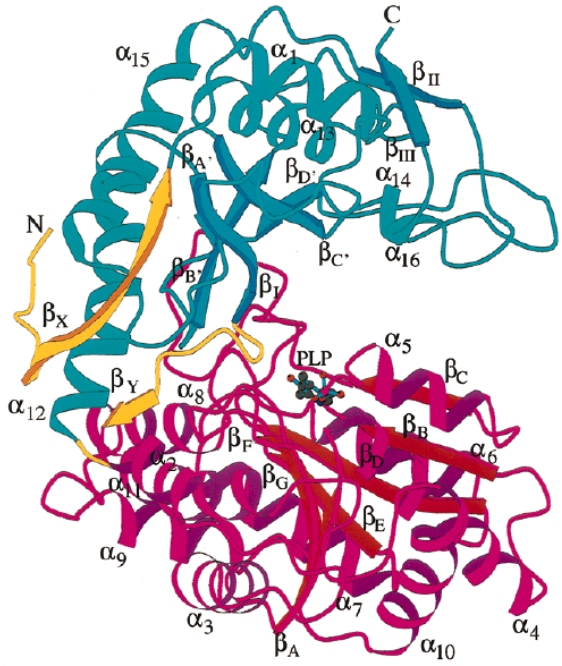
Ten PPIases found in *E. coli.* Five are in cytosol and five are in periplasm

PPIase Family	Gene Name	MW (KDa)	Location	PI	Access No.	Function, Characteristics
Parvulin	PPIC	10.1	cytosol	9.8	P39159	inhibited by juglone, smallest PPIase
Cyclophilin	PPIB	17.7	cytosol	5.5	P23869	inhibited by cylcosporine
FKBP	SlpA	16	cytosol	4.1	P22563	inhibited by FK506, histidine rich
FKBP	FKBP20	20.9	cytosol	4.7	P30856	inhibited by FK506 histidine rich;reversibly inhibited by Ni2+
FKBP	Trigger Factor	48.2	cytosol	4.8	P22257	binds ribosomes; not inhibited by FK506;cold stress induced
Cyclophilin	PPIA	17.5	periplasm	8.5	P20752	inhibited by cyclosporine
FKBP	FKBP22	22.1	periplasm	4.7	POA9L3	inhibited by FK506; cold stress induced
FKBP	FKBP29	28.9	periplasm	9	P45523	inhibited by FK506; dimer
Parvulin	SurA	47.3	periplasm	7	P21202	chaperone outer membrane porins
Parvulin	PPID	68.2	periplasm	4.8	P77241	largest PPI;chaperone outer membrane proteins

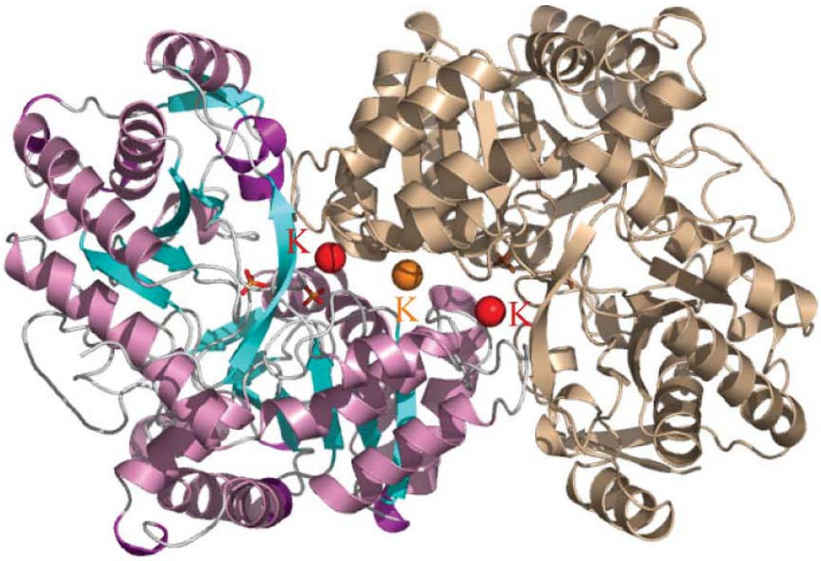
Figure 1.3



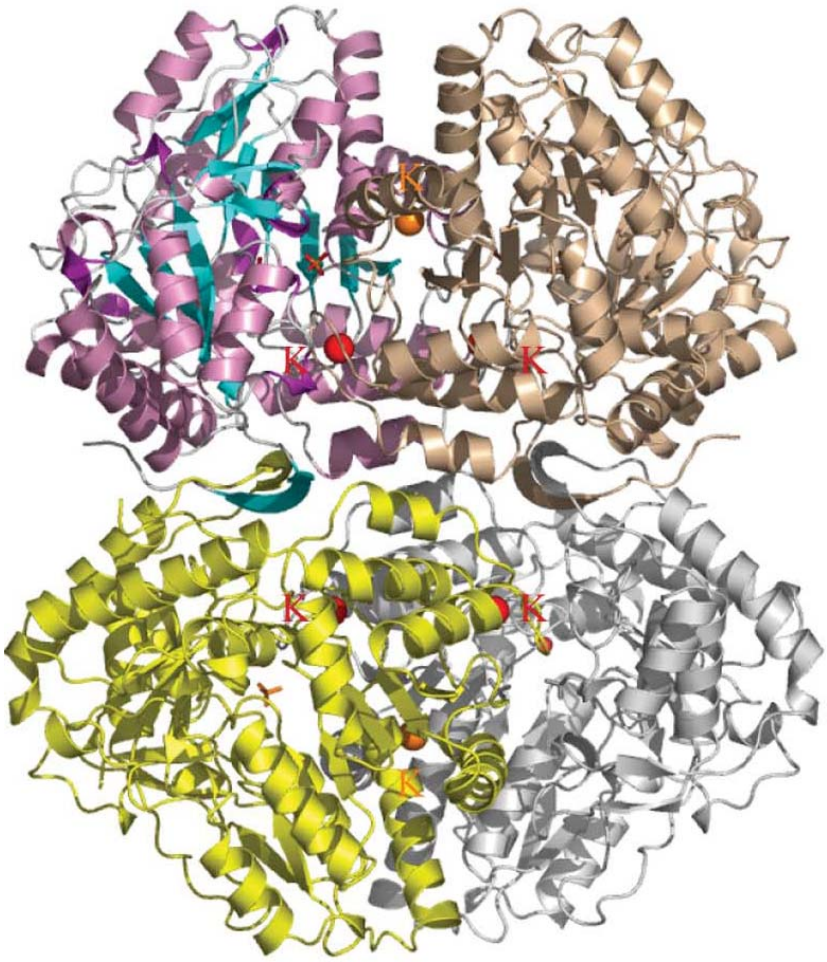
(A) Mechanism for the β-elimination reaction catalyzed by tryptophanase. (Scheme taken from [61])



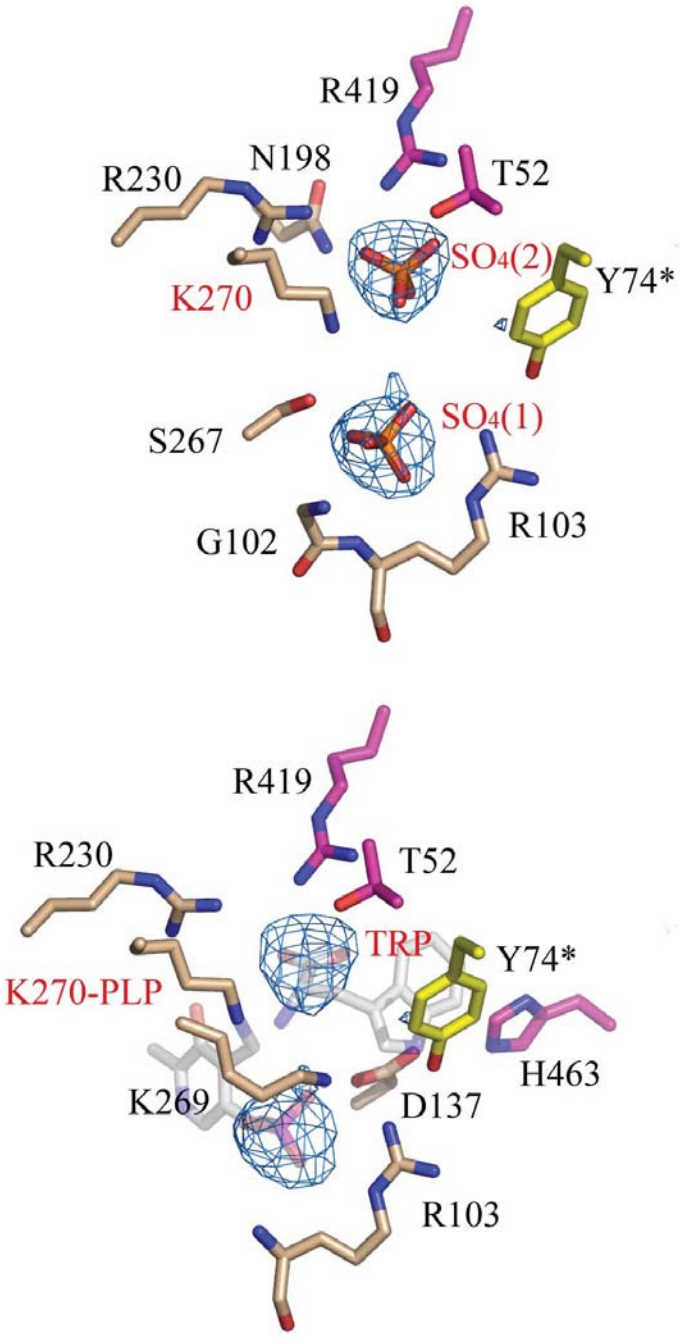
(B) Crystal structure of *Proteus vulgaris* tryptophanase. (Figure taken from [76])



(C) “Catalytic dimer” of *E. coli* tryptophanase. (Figure taken from [80])



(D) *E. coli* Tryptophanase tetramer. (Figure taken from [80])



(E) Model for the active site of *E. coli* tryptophanase. (Figure taken from [80])

Chapter II:

Identifying *Escherichia coli* PPIases and Potential Substrates of Trigger Factor Using Proteomics

INTRODUCTION

The minimal growth temperature for *E. coli* is assumed to be ≥ 7 ℃.[1] It is unclear what causes death of *E. coli* at low temperature. Denaturation and aggregation of proteins in a cold environment may be one reason.[2, 3] Low temperature may cause denaturation of cold-sensitive proteins. Hydrophobic interactions, which are important for stabilizing the tertiary structure of proteins, become weak during cold shock. On the other hand, the strong energetic interaction between water and buried polar residues in folding state would accelerate unfolding of proteins.[4, 5] *E. coli* adapts to low temperature by inducing expression of cold shock proteins to stabilize cold-sensitive proteins after a transient phase when most protein synthesis is inhibited.[6-8]

CspA is the major cold shock protein that is induced immediately and dramatically (13% of total protein synthesis) upon cold shock.[6, 7, 9] Overexpression of CspA promotes transcription of other cold shock proteins.[7] At low temperature, induction of cold shock proteins may assist protein synthesis and prevent aggregation using different enzymatic machinery than that used at normal temperature.

At low temperatures, proline isomerization in proteins is strongly decelerated. For example, a prolyl isomerization with a half time in the range of a few seconds at 37 °C would increase to several hundred seconds at 10 °C, too slow to sustain life. Therefore,

more PPIases may be needed to accelerate proline-limited folding steps at low temperatures. Many PPIases were found to be involved in cold adaptation in various organisms. The PPIase FKBP18 in *Thermococcus sp* SK-1 was shown to be induced when the growth temperature was shifted from the optimum, 75 ℃, to 60 or 65 ℃.[10] A PPIase with pI 4.8, in the psychrophilic archaeon, *Methanococcoides burtonii*, was found to be 3-fold enhanced at 4 ℃ compared to the growth at the optimum temperature of 23 ℃.[11] An 18-kDa FKBP-type PPIase in the hyperthermophilic methanoarchaeon *Methanococcus jannaschii* was found to be up-regulated in response to cold shock from its optimum growth temperature of 85 °C to 65 °C.[12] The cellular content of FKBP22 in *Shewanella sp.* SIB1 was found to be increased greatly upon cold shock.[13] Synthesis of PPiB in *Bacillus subtilis* was found to increase in response to cold stress.[14] Expression of cold-adapted chaperones Cpn60 and Cpn10 from the psychrophilic bacterium *Oleispira antarctica* was reported to increase the amount of PPiB 54 times in *E. coli* compared to in wild type cells.[3] This indicated that PPiB is a cold-sensitive protein and requires assistance from other chaperones for stabilization in a cold environment. On the other hand, since PPiB accounts for 80% of the PPIase activity in *E. coli*, up-regulation of PPiB after cold shock suggested the importance of PPIase activity to *E. coli* at low temperature.[3] Trigger factor is a cold shock chaperone in *E. coli*.[15] When *E. coli* is grown at 16 °C, the concentration of trigger factor in the cell is 2- to 3-fold higher than in cells grown at 37 °C. Trigger factor is induced two hours after cold shock from 37 °C to 10 °C. Deletion of trigger factor results in a decrease of viability of cells by about 5-fold at 4 °C. Overexpression of trigger factor enhances the viability of *E.*

coli at 4 °C.[15]

Although trigger factor is required to keep cells alive at temperatures below 8 °C, it is not essential for *E. coli* cell growth at any temperature above 8 °C.[16-18] No specific substrates for trigger factor have been reported. Trigger factor and DnaK were shown to share the same substrate pool.[17] Double deletion of *tig* and *dnak* genes resulted in aggregation of proteins.[17] 40% of these aggregated proteins are large proteins (≥60KDa). 72% of the total aggregated proteins are thermolabile proteins. The chaperone activity of trigger factor and DnaK may target large proteins with multi-domains which are vulnerable to misfolding or aggregation during thermostress.[17]

Our hypothesis is that survival of *E. coli* cells at low temperature requires PPIases to accelerate protein folding. Deletion of the most efficient PPIase, trigger factor, would induce overexpression of other PPIase(s) at low temperature. In our experiments, we identified three PPIases of *E. coli* on 2D gels. We studied the response of other cytosolic PPIases to deletion of the *tig* gene in *E. coli*. We also discovered a potential substrate of trigger factor.

EXPERIMENTAL PROCEDURE

Strains and culture conditions

Growth in rich media

Escherichia coli strains MC1061 and *Δtig::kan* MC1061 (constructed by Dr. Bijoy Mohanty in the laboratory of Professor Sidney Kushner, Department of Genetics, University of Georgia) were grown in Luria Bertani (LB) media, with kanamycin added

only for the mutant strain. Fresh LB plates were streaked with glycerol stocks of MC1061 and *Δtig::kan* MC1061 stored at -80 °C. Next, 3 mL of liquid LB media was inoculated with a single colony from the plate after overnight growth at 37 ℃ shaken at 250 rpm. 500 µL liquid culture was used to inoculate 100 mL cell cultures that were grown at 37 °C with shaking at 250 rpm until OD_{600nm} = 0.6. After growth for an extra hour, cells from 25-mL culture were collected by centrifugation at 6000 rpm in a Beckman GS-15R (Bioexpression and Fermentation Facility, The University of Georgia). Cell pellets were frozen at -80 °C until use.

Growth in minimal media

M9 medium was prepared by dissolving 6.0 g Na_2HPO_4, 3.0 g KH_2PO_4 and 0.5 g NaCl in one l L water. 1 mL of 1M $MgSO_4$, 100 µL of 1M $CaCl_2$, 20 mL of 5% (w/v) NH_4Cl and 10 mL of 40% (w/v) glucose were added as supplements to every 1 L of M9 prior to growth. ^{15}N-labeled NH_4Cl was used when growing *Δtig::kan* MC1061 cells. The procedure was similar to growth in rich media except that when OD_{600nm} = 0.6, *Δtig::kan* MC1061 cells were transferred to a shaker pre-cooled at 4 °C and continued to grow for another 5 h.

Sample preparation for soluble proteins

Cell pellets from 25-mL culture were washed with 1 mL PBS buffer (3.0 mM KCl, 1.5 mM KH_2PO_4, 68 mM NaCl and 9.0 mM NaH_2PO_4) twice. Cells were resuspended in sonication buffer (10 mM Trizma base, 5 mM $MgCl_2$, pH = 7.4) and disrupted by sonication with 4x5-s pulses of 30% power on Sonic Dismembrator Model 550 (Fisher Scientific). Solid urea was added to the sample to a final concentration of 1 mg/mL,

followed by equal volume of lysis buffer (9 M urea, 2.5 mM EDTA, 1% DTT, 4% CHAPS). Samples were incubated at room temperature for at least one hour. Then solid particles, lipid and aggregated proteins were removed to avoid blocking gel pores by centrifugation at 14,000 x g for 30 min on a Beckman Avanti J-25I centrifuge. Soluble proteins were quantified by the Bradford method.[58] 500 μg soluble proteins were loaded onto 24-cm 2DE gels.

Isolation of aggregated proteins

Aggregated proteins were isolated according to the protocol by Tomoyasu et al.[59] Pellets of aggregated proteins and membrane proteins from 25-mL cell culture were washed twice with 400 μL washing buffer (10 mM potassium phosphate, pH 6.5, 1 mM ETDA). Washed pellets were collected by centrifugation (15,000 x g 20 min, 4 °C, Beckman Avanti J-25I centrifuge). 80 μL of 10% (v/v) NP40 was added to remove the contaminating membrane proteins. NP40-insoluble fractions were collected by centrifugation (15,000 x g, 30 min, 4 °C) and washed with 400 μL of washing buffer. Pellets were resuspended in 200 μL washing buffer and analyzed by 2D electrophoresis.

Two-dimensional gel electrophoresis

First dimension

Rehydration solution (8 M urea, 2 % CHAPS, trace bromophenol blue) with IPG buffer (GE Healthcare, 5 μL/mL) and solid DTT (final concentration was 2.8 mg/mL) was added to protein samples. The total volume was 500 μL. 24-cm pH 4-7 immobiline strips (GE Healthcare) were rehydrated in rehydration solution with cell extract for 12 h. Isoelectric Focusing (IEF) was carried out on an IPGphor Isoelectric Focusing system

(GEHealthcare). Proteins were focused for 1 h at 500 V, 1 h at 1000 V and 12 h at 8000 V on the IPGphor. Following completion of IEF, strips were rehydrated for 15 min with 0.1 g DTT and 10 mL SDS equilibration buffer (50 mM Tris-Cl, pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, trace bromophenol blue), then rehydrated for 15 min with 0.25 g iodoacetimide and 10 mL of fresh SDS equilibration buffer. Then rehydrated strips were loaded onto home-made 12.5% acrylamide gels.

Second-dimension

The gel caster of Ettan DALT II system (GEHealthcare) was used to cast second dimension gels. Blank cassette inserts and separating sheets were loaded into the caster first, then a certain number of empty 1-mm gel cassettes with separating sheets were loaded. The gel caster was closed and tightened. Homogeneous gel solution was loaded into the caster through a plastic funnel and feeding tube. To make four 25-cm 12.5% gels, 400 mL of gel solution was made by mixing 125 mL AccuGel (40%, National Diagnostics), 100 mL of 4X resolving solution (1.5 M Tris Base, pH = 8.8), 4 mL 10% SDS and 169 mL of purified water (US Filter System). 2 mL of 10% ammonium persulfate and 132 μL of TEMED (National Diagnostics) were added to the gel solution immediately prior to use. About 100 mL of displacing solution (0.375 M Tris-HCl, pH 8.8, 50% glycerol and trace amount of bromophenol blue) were added to the balance chamber until the gel solution was about 1 cm or 2 cm below the final desired gel height. 1 mL of water-saturated butanol was pipetted onto each gel immediately. The gels were polymerized for at least one hour. After polymerization, the top surface of each gel was rinsed with distilled water to remove the butanol and unpolymerized acrylamide. The

gels were either used right away or stored in 4 °C refrigerator with gel storage solution (0.375 M Tris-HCl, pH 8.8, 0.1% SDS) on top to keep gels from drying out.

The rehyrated strips from first dimension were sealed onto the second-dimension gel with sealing solution (0.25 mM Tris, 192 mM glycine, 0.1% SDS, bromophenol blue, 0.5% agarose). The gels were carefully slid into the tank filled with 8-9 L of SDS electrophoresis buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Blank cassettes were inserted into unused slots. Gel running conditions were: 5 W per gel for the first 15 min, then 15 W per gel at 15 °C until the blue dye front reached the bottom of the gels. After the run was complete, gels were removed from the cassette and rinsed with purified water three times, then fixed in fixation buffer (10% acetic acid, 40% ethanol) overnight. The next day, the gels were stained with PIERCE Imperial Protein stain for about two hours, then destained for at least four hours.

Mass spectrometry

Spots of interest were cut out of gels and chopped into small pieces. Gel pieces were destained with ammonium bicarbonate in 50% acetonitrile for 10 min. This step was repeated four times to completely dehydrate gel sample. Re-swelling of gel particles was carried out in 50 μL of 10 mM DTT in 40 mM NH_4HCO_3 at 55 ℃ for one hour. After cooling to room temperature, samples were spun down and solvent was drawn out. 50 μL 0f 55 mM iodoacetamide in 40 mM NH_4HCO_3 was added to gel samples to block –SH groups and prevent disulfide bond formation. Samples were incubated at room temperature for 45 min. Then the gel pieces were washed with 40 mM NH_4HCO_3 and 100% CH_3CN twice. 10 ng/μL trypsin was added to rehydrated gel particles on ice for 45

min. After washing with 40 mM NH_4HCO_3 the digestion reaction was incubated at 37 ℃ overnight. Digested products were extracted with 5% formic acid / 50% CH_3CN. The resulting peptides were washed with Nutip (Glygen Corp.) and loaded onto a MALDI target. The target was submitted to the Chemical and Biological Sciences Mass Spectrometry Facility for analysis.

RESULTS AND DISCUSSION

Identifying and quantifying PPIases on 2D gels for wild type *E. coli* and tig deletion strain

We used two-dimensional electrophoresis (2DE) to separate *E. coli* cytosolic proteins. The proteins were separated first by isoelectric focusing with 13-cm or 24-cm immobiline strips with pH ranges of 3-10 or 4-7 (GE Healthcare). The second dimension separation was performed on 10 %, 12.5 %, or 14 % polyacrylamide SDS denaturing gels. At the beginning, the gels were mainly stained with the silver staining method. This method is most sensitive compared to normal Coomassie or Sypro Ruby dyes. Glutaraldehyde may modify lysines and interrupt trypsin digestion, interfering with mass spectrometric peptide identification. Glutaraldhyde may also cause cross-linking of lysines and reduce the efficiency of extracting peptides from gel matrixes.[32] So we used a glutaraldehyde-free sensitizer. There was no significant change of staining sensitivity with the glutaraldehyde-free protocol.

Since the typical product of trypsin digesion is 600-3000 Da, we only collected peaks within 600-6000 m/z range with mass spectrometry. Data were analyzed by

"peptide mass fingerprint" on www.matrixscience.com. Five PPIases in *E. coli* are cytoplasmic and five are periplasmic (Table I, Chapter 1). Only two cytosolic PPIases, PPiB and trigger factor, are reported to be identified on 2DE maps in Swissprot-2DPAGE database (http://ca.expasy.org/ch2d/). Four periplasmic PPIases are reported in this database. Only one, FKBP22, is in the pI range of 4-7. We identified three PPIases, PPiB, trigger factor, and FKBP22, on both pH 4-7 and 3-10 gels (Figure 2.1). We could not identify any PPIase with high pI. The score for trigger factor was always good. The significance level was usually less than 5%, which means there is a 5% probability that the hit occurred randomly. But we had trouble identifying small proteins (MW < 30 kDa). For example, the Mascot scores for FKBP22 and PPiB were always low and normally not the top ranked hits when we searched the database with raw data. To improve the search results, we had to compare the mass values from spots of small proteins to mass values of "blank" gel pieces, and remove the background peaks from trypsin autolysis and polyacrylamide. Setting a narrow search window, e.g. 0-30 kDa, improved scores of small proteins somewhat. We also used more samples for running 2DE gels. But loading 1 mg cell extracts (compared to 200 μg) did not improve the results for small proteins as much as for large proteins. When we changed the staining method from silver stain to GelCode Blue stain, which is more compatible with mass spectrometry, the scores for large protein, like trigger factor, were improved significantly, but the scores for small proteins improved only slightly. After applying all methods mentioned above, the scores for small proteins were not improved significantly.

We considered that there may be some limitation of the scoring algorithm causing

low scores for small proteins. Peptide mass fingerprint methods have difficulty identifying small proteins because of limited trypsin cleavage sites in short sequences. After trypsin digestion and extraction, less than 50% of peptides were predicted to be detected by mass spectrometry.[33] Our results (Table 2.1) are consistent with this prediction. About half of the theoretical peptides from trypsin digestion of FKBP22 were identified. And 40% of trypsin digestion products from PPiB were detected. Mascot uses the MOWSE scoring algorithm, which is dependent on protein sizes.[34] For large proteins (> 40 kDa), only 10% of theoretical peptides from trypsin digestion are required for identification, but about 30% of theoretical peptides for small proteins (< 30 kDa) are usually needed for identification. Usually, proteins can be identified by using five peptides in this scoring system. So if there are about 20 theoretical peptides from digestion of a large protein, missing one or two peptides (out of ten) during sample preparation would not affect the confidence of the identification. But for a small protein with ten theoretical peptides, missing one or two peptides (out of five) would lead to wrong or no hits. This may cause serious difficulty for identification of small proteins using the Mascot "peptide mass fingerprint" program. So this may be the reason why we had trouble identifying small PPIases, like PPiB and FKBP22. To improve identification of small proteins on 2D gels with the "peptide mass fingerprint" program, large amounts of samples and more careful handling would be necessary for good search result.

Although trigger factor is not essential for growth of *E. coli* cells, it is important for viability of *E. coli* at low temperature (< 15 ℃).[15] We hypothesized that other PPIases

may be up-regulated in *E. coli* cells upon deletion of the *tig* gene at low temperature. We applied quantitative proteomics to study other PPIases' response to deletion of trigger factor. PPiB is the only detectable cytoplasmic PPIase on 2D gels so far for *E. coli* proteomes, so it was chosen for comparison in this study. We used silver staining to visualize protein spots, because it was much more sensitive than Sypro Ruby or Coomassie in our hands. But the linearity of the relationship between protein level and silver staining intensity is poor.[35] Quantitative analysis of silver stained gels with software would not provide accurate results.

So we used an isotopic labeling method for comparing protein expression levels in different strains. Both wild type MC1061 and *Δtig:kan* MC1061 were grown in M9 minimal media with glucose as carbon source and ammonium chloride as nitrogen source. ^{15}N-labeled ammonium chloride was used in media to grow *Δtig:kan* MC1061. Cell cultures were grown at 37 ℃ until OD_{600nm} = 0.6 to ensure enough cells for detection on 2D gels. Then growth was either continued at 37 ℃ for another hour or the temperature was lowered to 4 ℃ and growth was continued for 5 h. The cold response of trigger factor is similar to that of most cold shock proteins.[6, 7] Induction of trigger factor occurs 2-3 h after cold shock, and the expression of trigger factor continues for another 2 h.[15] So 5 h of growth at low temperature should be enough for induction of trigger factor or other cold-responsive proteins. Prior to cell harvest, final optical density (OD_{600}) values were measured to quantify cells. Based on OD_{600} values, the amount of cells resulting from growth at 4 ℃ was much less than at 37 ℃.

Equal amounts of cells from each strain under the same growth condition were

mixed. Cell pellets were collected and processed using the protocol in the Experimental section. Proteins were separated on 24-cm 12.5% acrylamide gels with pH 4-7 immobiline strips. 1 mg of cell extracts was loaded each time. Each spot would include polypeptides from growth in media with natural abundance of nitrogen isotopes (99.6% ^{14}N) and ^{15}N-enriched media ($^{15}N > 96\%$). High levels of ^{15}N increase the masses of all peptides. Also the highest peak will not be the first peak in the isotopic distribution because of the change of ^{14}N:^{15}N ratio. Since the same processes were applied to proteins from both sources, the original ratios of proteins from two strains are maintained in peptide fragments analyzed by mass spectrometry. So the relative abundance of peptides from two sources can be measured by the ratio of intensities of two highest peaks in the two distributions. We identified the spot position of PPiB on similar gels before and there were few protein spots around PPiB. Although the quality of gels was worse than previous gels, it was not difficult to find the PPiB spot. As discussed above, there are only eleven theoretically detectable polypeptides from trypsin digestion of PPiB. If the digestion efficiency is 50%, only about five mass peaks could be collected in mass spectrometry. Six peaks of PPiB from growth at 37 ℃ and four peaks from growth at 4 ℃ were identified by mass spectrometry. There was some noise around 2299 m/z, which made it difficult to compare the intensities of the natural abundance and isotope-labeled peak at 2299. All other peaks provided useful quantitative information for peptides from the two sources. The sizes of the PPiB spot on both gels were similar, but the intensities of MS peaks for PPiB from the cold-shock proteome were much less than PPiB grown at normal condition (37 °C) (Figure 2.2). This is reasonable because wild type *E. coli* cells

dies at 4 °C and only 60% of overall proteins observed at 37 °C can be detected after 150 min of cold incubation at 4 °C.[8] The cold incubation at 4 °C in our experiments is 300 min. Thus, more cells are expected to die. The ratios of peak pairs indicated that there were about 50% less PPiB expressed in the *Δtig::kan* MC1061 strain than wild type strain under both conditions.

Cold shock did not induce the overexpression of PPiB in the *tig* deletion strain to compensate for the absence of trigger factor. This result contradicts previous observations in another organism. In *Bacillus subtilis,* trigger factor and PPiB were shown to complement each other functionally when cells were grown in minimal media.[36] On the other hand, trigger factor and PPiB are the only two cytosolic PPIases in *Bacillus subtilis* and trigger factor harbors most of the PPIase activity.[36] But in *E. coli*, there are five cytosolic PPIases and trigger factor only accounts for about 5% of the total PPIase activity, while PPiB harbors about 80% of the total PPIase activity.[37] Thus, even though PPIases can become essential for viability of cells under harsh (cold) environments, deletion of trigger factor may not significantly affect the total PPIase activity in *E. coli.* Up-regulation of other PPIases may not be necessary under this circumstance. Our result shows that the PPIase activity of trigger factor may not be important to cell viability at cold temperatures.

Tryptophanase is identified as a specific substrate of Trigger Factor

We applied traditional proteomics (2D electrophoresis, 2DE) to search for specific substrates of trigger factor and probe the possible function of trigger factor. 2DE is known to provide accurate pI and molecular weights of proteins. The choice of protein

dye is of critical importance in 2DE for accurate identification of proteins. Gels stained with GelCode Blue stain (Pierce) provided best results in these experiments. Detection of proteins by this method is nearly equivalent to the silver staining method. Silver staining is still the most sensitive staining technique, but many spots from silver-stained gels could not be identified by mass spectrometry due to interference of silver. We were able to identify more spots with GelCode blue stain. Unlike silver staining, the Coomassie dye in GelCode Blue does not interfere with mass spectrometric analysis, which was much improved. Overnight fixation with ethanol/acetic acid aqueous solution is necessary for this method to reach the optimum dying result. 2DE gels comparing proteomes from MC1061 and *Δtig::kan* MC1061 (*tig* deletion strain) are shown in Figure 2.3. Trigger factor is indicated on the gel image for MC1061, and this spot is missing on the 2D map for the *tig* deletion strain. Thus, the deletion of the *tig* gene was confirmed by 2DE analysis. We found that the absence of trigger factor did not change *E. coli* protein expression very much. We compared the gels using ImageMaster. Most spots were matched between the two gels. This is consistent with previous reports that trigger factor is not essential for *E. coli* cell growth at 37 ℃.[16, 18]

By careful investigation of the 2D maps, we discovered some spots were missing around the region of pI 5.5 and MW 50 kDa on the map for the *tig* deletion strain (Figure 2.4.A and B). Five gel spots on the same horizon were excised and identified by mass spectrometry peptide mass fingerprinting (after in-gel trypsin digestion). MS data were processed and searched against NCBI, MSDB, and SwissProt database with “Peptide Mass Fingerprint” of the Mascot search engine. The results from different databases were

all very similar. Two missing spots (T1, T2) from the map of *Δtig::kan* MC1061 were identified as tryptophanase. The Mascot search results with NCBI showed that the sequence coverage of tryptophanase for T1 and T2 was 34% and 48% respectively, which are pretty good. We are confident that these spots represent tryptophanase. Surprisingly, another three spots on the right (T3-T5 and T3'-T5') observed on both maps were also identified as tryptophanase. Quantitative analysis of gel spot intensities was performed on these spots using ImageMaster. In the gel displaying the proteome from wild type *E. coli*, T3-T5 represented 84.2% of the total amount of tryptophanase and T5 alone represented 43.2% based on gel intensity analysis（Figure 2.4.E). In the gel for the *tig* deletion strain, T5' represented 55.8% of total amount of tryptophanase. So the three spots observed on both gels represent most of tryptophanase in cell extracts. Spot T5 (or T5') on the far right is always most abundant. Its pI is about 6.0, which is close to the calculated pI based on the amino acid sequence of tryptophanase.

There were no apparent mass differences within these spots on 2D maps, so multiple spots are not the result of truncation of tryptophanase. Protein isoforms are characterized by a consecutive pI shift on the 2DE gel. Post-translational modification could cause multiple horizontally displaced spots for the same protein on 2D maps. The pI intervals between the three major spots are almost identical. The three-spot pattern for tryptophanase was observed on 2DE maps for proteomes of *E. coli* grown under various conditions.[38] So this appears to be a standard pattern for *E. coli* tryptophanase on 2DE gels.

Phosphorylation is known to cause a pI shift on 2DE maps.[39] Thirteen possible

phosphorylation sites on tryptophanase sequence were found in PROSITE database (http://www.expasy.org/prosite/).[40] Hypothetical phosphorylation on possible serine, threonine or tyrosine residues is predicted by ProMoST (proteomics.mcw.edu/promost).[41] The pattern of spots predicted on 2DE maps is very similar to what we observe (Figure 2.4.C). We compared our MS data with hypothetical peptide sequences with possible phosphorylation produced by PEPTIDEMAP (http://prowl.rockefeller.edu/prowl/peptidemap.html). Two of matched peptides were found including possible phosphorylation motif from PROSITE search result above. However, both peptides were found at their unphosphorylated m/z in all tryptophanase spots. Thus, even though phosphorylation could occur in tryptophanase, it is not the cause of the pI shift we observed.

On the other hand, PLP is a phosphate form of pyridoxal. Binding of PLP could also cause the pI shifts. Dissociation of PLP from the tryptophanase subunit upon unfolding is the predominant form, so T5 could be monomer without PLP. The dissociation of coenzyme is preferred upon disassembly of the tetrameric structure of tryptophanase, but the dissociation is very slow.[25] PLP has been shown to inhibit various enzymes by binding lysine at the active center through a Schiff base linkage.[45-47] Dissociation of the Schiff base is rapid at low pH, and the Schiff base is most stable at pH 8-9.[48] Sample preparation for 2D electrophoresis was with 8 M urea, which is a weak base and could stabilize the linkage between PLP and lysine. The reaction of PLP and lysine may involve an equilibrium between covalent and non-covalent forms.[49]

So we should consider that the multiple spots on 2DE may represent multiple

PLP-tryptophanase forms. The Schiff base linkage may be labile during trypsin digestion, which included 1 h reduction with DTT at 55 °C and overnight digestion at 37 °C. Following trypsin digestion, TFA was used to wash the peptide solution. As a result of hydrolysis in acidic solution, PLP would likely dissociate from tryptophanase peptides prior to mass spectrometric analysis. By comparing peptides detected by MS, we found a peptide as a result of digestion at Lys270 in T5 (or T5') that was missing in all other spots (Figure 2.4.D). Lys270 binds PLP in native holo-tryptophanase. This could mean that covalent or non-covalent bound PLP may block the access of trypsin to approach Lys270 or -269. Normally, PLP only binds to one or two lysine residues in a protein, although there are always more lysine residues available.[47] Amines with lower pK_a favor Schiff base formation, and unprotonated lysine is susceptible to attack by PLP.[50] The hydrophobic environment and nearby groups with positive charges are two possible reasons for the lower pK_a.[51] Lys269 is adjacent to Lys270, so they are in a similar protein environment. Perhaps these provide multiple sites for PLP binding. Interaction of Lys269 and PLP was found important for formation of quinonoid intermediate.[52] The crystal structure of *E. coli* tryptophanase showed that the active site is in a deep basic cavity. Both PLP and tryptophan could be modeled into this site in a closed conformation.[53]

But shouldn't all PLP be dissociated by treatment with urea? The exact mechanism of urea in protein unfolding remains unknown. But 8 M urea only yields partially unfolded protein.[54] Incubation for one hour at room temperature with 8 M urea is not enough to inhibit the activity of tryptophanase, suggesting that PLP is retained. PLP

could still be near the active site of tryptophanase in 8 M urea. So after unfolding, the closed conformation could loosen allowing the attachment of the second PLP to Lys269. The second PLP binding would shift the pI in the acidic direction. Different numbers of PLP bound to tryptophanase subunit could result in T3 and T4 spots with different pIs on 2DE maps, which is consistent with what we observed.

Sucrose density gradient centrifugation experiments revealed that two species of tryptophanase could be isolated form crude cell extracts. The smaller form, which could represent the dissociation product from trytpophanase tetramer, is the major form of tryptophanase in crude cell extracts. Since there exists an equilibrium between dimer and tetramer apotryptophanase and the holoenzyme is much more stable, the two species of tryptophanase in crude cell extracts represent holoenzyme and apoenzyme.[27, 55] The conformational difference between holoenzyme and apoenzyme could cause different post-translational modification.

For example, apotryptophanase contains 16 free –SH groups, but only 8 of them are active, and the reactivity of free –SH groups in holotryptophanase is much less than in apotryptophanase.[29] Cys298, which is essential for activity of trytophanase, is reactive in apotryptophanase, but masked in holotryptophanase.[44] Dissociation of the tetrametric structure of tryptophanse could expose more free -SH groups. Different oligomeric states and conformations of trytpophanase could expose different groups and cause different post-translational modification, and lead to the complex pattern of tryptophanase spots on the 2DE map. The incorrect interaction between tryptophanase monomers was shown to be slow, but irreversible, resulting in aggregation.[56] Some

loose confirmation or monomer of tryptophanase might only be saved from aggregation when there is sufficient trigger factor in the cells.

Aggregated proteins were analyzed by 2DE maps (Figure 2.5). The big spot (AG) on both gels was identified as tryptophanase. Aggregation started after about 30 min in 4 M guanidine hydrochloride (GdnHCl).[57] Chaperonin GroE could not suppress aggregation of tryptophanase.[30] It may be normal that some aggregation cannot be avoided. There were two spots around the big spots. One missing spot (M) on the gel for MC1061 (wt) was identified as a tryptophanase fragment. The peptide coverage is not high (24%), but most peptide fragments in this region were preserved except that several near the ends were missing. Apparently, after aggregation, the sequence integrity could not be maintained and only fragments of the whole sequence could be identified by mass spectrometry. Thus, some missing tryptophanase in the soluble *Δtig::kan* MC1061 sample could be due to aggregation of tryptophanase in the absence of trigger factor. In contrast to the dimer-tetramer equilibrium, the dissociation of dimer to monomer is irreversible.[1] This might be due to formation of large aggregates from monomers due to exposure of some active groups masked in the oligomer structure or incorrect interactions. In the presence of trigger factor, the tendency of tryptophanase monomer to aggregate or to proteolytically degrade might be ameliorated by unbound trigger factor in the cell cytosol. In this scenario, the other spots for tryptophanase would be missing on 2D maps for the *tig* deletion strain.

REFERENCES

1. Shaw, M.K., A.G. Marr, and J.L. Ingraham, *Determination of the minimal temperature for growth of Escherichia coli.* J Bacteriol, 1971. 105(2): p. 683-4.
2. Kandror, O., A. DeLeon, and A.L. Goldberg, *Trehalose synthesis is induced upon exposure of Escherichia coli to cold and is essential for viability at low temperatures.* Proc Natl Acad Sci U S A, 2002. 99(15): p. 9727-32.
3. Strocchi, M., et al., *Low temperature-induced systems failure in Escherichia coli: insights from rescue by cold-adapted chaperones.* Proteomics, 2006. 6(1): p. 193-206.
4. Privalov, P.L., et al., *Cold denaturation of myoglobin*. J Mol Biol, 1986. 190(3): p. 487-98.
5. Graziano, G., et al., *A reassessment of the molecular origin of cold denaturation*. J Biochem (Tokyo), 1997. 122(2): p. 395-401.
6. Jones, P.G., R.A. VanBogelen, and F.C. Neidhardt, *Induction of proteins in response to low temperature in Escherichia coli.* J Bacteriol, 1987. 169(5): p. 2092-5.
7. Jones, P.G., et al., *DNA gyrase, CS7.4, and the cold shock response in Escherichia coli.* J Bacteriol, 1992. 174(18): p. 5798-802.
8. Perrot, F., et al., *Protein synthesis in Escherichia coli at 4 degrees C.* Electrophoresis, 2000. 21(8): p. 1625-9.
9. Goldstein, J., N.S. Pollitt, and M. Inouye, *Major cold shock protein of Escherichia coli.* Proc Natl Acad Sci U S A, 1990. 87(1): p. 283-7.
10. Ideno, A., et al., *FK506-binding protein of the hyperthermophilic archaeum,*

Thermococcus sp. KS-1, a cold-shock-inducible peptidyl-prolyl cis-trans isomerase with activities to trap and refold denatured proteins. Biochem J, 2001. 357(Pt 2): p. 465-71.

11. Goodchild, A., et al., *A proteomic determination of cold adaptation in the Antarctic archaeon, Methanococcoides burtonii.* Mol Microbiol, 2004. 53(1): p. 309-21.
12. Boonyaratanakornkit, B.B., et al., *Transcriptional profiling of the hyperthermophilic methanarchaeon Methanococcus jannaschii in response to lethal heat and non-lethal cold shock.* Environ Microbiol, 2005. 7(6): p. 789-97.
13. Suzuki, Y., et al., *Possible involvement of an FKBP family member protein from a psychrotrophic bacterium Shewanella sp. SIB1 in cold-adaptation.* Eur J Biochem, 2004. 271(7): p. 1372-81.
14. Graumann, P., et al., *Cold shock stress-induced proteins in Bacillus subtilis.* J Bacteriol, 1996. 178(15): p. 4611-9.
15. Kandror, O. and A.L. Goldberg, *Trigger factor is induced upon cold shock and enhances viability of Escherichia coli at low temperatures.* Proc Natl Acad Sci U S A, 1997. 94(10): p. 4978-81.
16. Deuerling, E., et al., *Trigger factor and DnaK cooperate in folding of newly synthesized proteins.* Nature, 1999. 400(6745): p. 693-6.
17. Deuerling, E., et al., *Trigger Factor and DnaK possess overlapping substrate pools and binding specificities.* Mol Microbiol, 2003. 47(5): p. 1317-28.
18. Teter, S.A., et al., *Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains.* Cell, 1999. 97(6): p. 755-65.

19. Ferbitz, L., et al., *Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins.* Nature, 2004. 431(7008): p. 590-6.

20. Kramer, G., et al., *L23 protein functions as a chaperone docking site on the ribosome.* Nature, 2002. 419(6903): p. 171-4.

21. Kaiser, C.M., et al., *Real-time observation of trigger factor function on translating ribosomes.* Nature, 2006. 444(7118): p. 455-60.

22. Patzelt, H., et al., *Three-state equilibrium of Escherichia coli trigger factor.* Biol Chem, 2002. 383(10): p. 1611-9.

23. Lill, R., et al., *The "trigger factor cycle" includes ribosomes, presecretory proteins, and the plasma membrane.* Cell, 1988. 54(7): p. 1013-8.

24. Morino, Y. and E.E. Snell, *A kinetic study of the reaction mechanism of tryptophanase-catalyzed reactions.* J Biol Chem, 1967. 242(12): p. 2793-9.

25. Hogberg-Raibaud, A., O. Raibaud, and M.E. Goldberg, *Kinetic and equilibrium studies on the activation of Escherichia coli K12 tryptophanase by pyridoxal 5'-phosphate and monovalent cations.* J Biol Chem, 1975. 250(9): p. 3352-8.

26. Morino, Y. and E.E. Snell, *The relation of spectral changes and tritium exchange reactions to the mechanism of tryptophanase-catalyzed reactions.* J Biol Chem, 1967. 242(12): p. 2800-9.

27. Morino, Y. and E.E. Snell, *The subunit structure of tryptophanase. I. The effect of pyridoxal phosphate on the subunit structure and physical properties of tryptophanase.* J Biol Chem, 1967. 242(23): p. 5591-601.

28. Raibaud, O. and M.E. Goldberg, *Structural and functional interdependence of the*

protomers of Escherichia coli K 12 tryptophanase during binding of pyridoxal 5'-phosphate. J Biol Chem, 1976. 251(9): p. 2814-9.

29. Morino, Y. and E.E. Snell, *The subunit structure of tryptophanase. II. A correlation of ultracentrifugal and chemical studies.* J Biol Chem, 1967. 242(23): p. 5602-10.

30. Mizobata, T., et al., *Effects of the chaperonin GroE on the refolding of tryptophanase from Escherichia coli. Refolding is enhanced in the presence of ADP.* J Biol Chem, 1992. 267(25): p. 17773-9.

31. van der Vies, S.M., et al., *Conformational states of ribulosebisphosphate carboxylase and their interaction with chaperonin 60.* Biochemistry, 1992. 31(14): p. 3635-44.

32. Rabilloud, T., *Mechanisms of protein silver staining in polyacrylamide gels: a 10-year synthesis.* Electrophoresis, 1990. 11(10): p. 785-94.

33. Lim, H., et al., *Identification of 2D-gel proteins: a comparison of MALDI/TOF peptide mass mapping to mu LC-ESI tandem mass spectrometry.* J Am Soc Mass Spectrom, 2003. 14(9): p. 957-70.

34. Pappin, D.J., P. Hojrup, and A.J. Bleasby, *Rapid identification of proteins by peptide-mass fingerprinting.* Curr Biol, 1993. 3(6): p. 327-32.

35. Nishihara, J.C. and K.M. Champion, *Quantitative evaluation of proteins in one- and two-dimensional polyacrylamide gels using a fluorescent stain.* Electrophoresis, 2002. 23(14): p. 2203-15.

36. Gothel, S.F., et al., *Cyclophilin and trigger factor from Bacillus subtilis catalyze in vitro protein folding and are necessary for viability under starvation conditions.*

Biochemistry, 1998. 37(38): p. 13392-9.

37. Rahfeld, J.U., et al., *Isolation and amino acid sequence of a new 22-kDa FKBP-like peptidyl-prolyl cis/trans-isomerase of Escherichia coli. Similarity to Mip-like proteins of pathogenic bacteria.* J Biol Chem, 1996. 271(36): p. 22130-8.

38. Blankenhorn, D., J. Phillips, and J.L. Slonczewski, *Acid- and base-induced proteins during aerobic and anaerobic growth of Escherichia coli revealed by two-dimensional gel electrophoresis.* J Bacteriol, 1999. 181(7): p. 2209-16.

39. Yamagata, A., et al., *Mapping of phosphorylated proteins on two-dimensional polyacrylamide gels using protein phosphatase.* Proteomics, 2002. 2(9): p. 1267-76.

40. Combet C., B.C., Geourjon C. and Deléage G., *Network Protein Sequence Analysis.* TIBS 2000. 25(291): p. 147-150.

41. Halligan, B.D., et al., *ProMoST (Protein Modification Screening Tool): a web-based tool for mapping protein modifications on two-dimensional gels.* Nucleic Acids Res, 2004. 32(Web Server issue): p. W638-44.

42. Raibaud, O. and M.E. Goldberg, *The dissociated tryptophanase subunit is inactive.* J Biol Chem, 1976. 251(9): p. 2820-4.

43. Metzler, C.M., R. Viswanath, and D.E. Metzler, *Equilibria and absorption spectra of tryptophanase*. J Biol Chem, 1991. 266(15): p. 9374-81.

44. Raibaud, O. and M.E. Goldberg, *The reactivity of one essential cysteine as a conformational probe in Escherichia coli tryptophanase. Application to the study of the structural influence of subunit interactions.* Eur J Biochem, 1977. 73(2): p. 591-9.

45. Chen, S.S. and P.C. Engel, *Dogfish M4 lactate dehydrogenase: reversible inactivation by pyridoxal 5'-phosphate and complete protection in complexes that mimic the active ternary complex.* Biochem J, 1975. 151(2): p. 447-9.

46. Raetz, C.R. and D.S. Auld, *Schiff bases of pyridoxal phosphate with active center lysines of ribonuclease A.* Biochemistry, 1972. 11(12): p. 2229-36.

47. Valinger, Z., P.C. Engel, and D.E. Metzler, *Is pyridoxal 5'-phosphate an affinity label for phosphate-binding sites in proteins?: The case of bovine glutamate dehydrogenase.* Biochem J, 1993. 294 (Pt 3): p. 835-9.

48. Caro M Metzler, A.C., and David E. Metzler, *Equilibria and Absorption Spectra of Schiff Bases*. J. Am. Chem. SOC, 1980. 102: p. 6075-6082.

49. ENGEL, S.-S.C.a.P.C., *The Equilibrium Position of the Reaction of Bovine Liver Glutamate Dehydrogenase with Pyridoxal 5'-Phosphate.* Biochem. J., 1975. 147: p. 351-358.

50. French, T.C., D.S. Auld, and T.C. Bruice, *Catalytic Reactions Involving Azomethines. V. Rates and Equilibria of Imine Formation with 3-Hydroxypyridine-4-Aldehyde and Amino Acids.* Biochemistry, 1965. 4: p. 77-84.

51. Piszkiewicz, D. and E.L. Smith, *Bovine liver glutamate dehydrogenase. Equilibria and kinetics of inactivation by pyridoxal.* Biochemistry, 1971. 10(24): p. 4538-44.

52. Phillips, R.S., et al., *Replacement of lysine 269 by arginine in Escherichia coli tryptophan indole-lyase affects the formation and breakdown of quinonoid complexes.* J Biol Chem, 1991. 266(28): p. 18642-8.

53. Ku, S.Y., P. Yip, and P.L. Howell, *Structure of Escherichia coli tryptophanase.* Acta

Crystallogr D Biol Crystallogr, 2006. 62(Pt 7): p. 814-23.

54. Bennion, B.J. and V. Daggett, *The molecular basis for the chemical denaturation of proteins by urea*. Proc Natl Acad Sci U S A, 2003. 100(9): p. 5142-7.

55. Gopinathan, K.P. and R.D. DeMoss, *Reversible dissociation of tryptophanase into protein subunits*. Proc Natl Acad Sci U S A, 1966. 56(4): p. 1223-5.

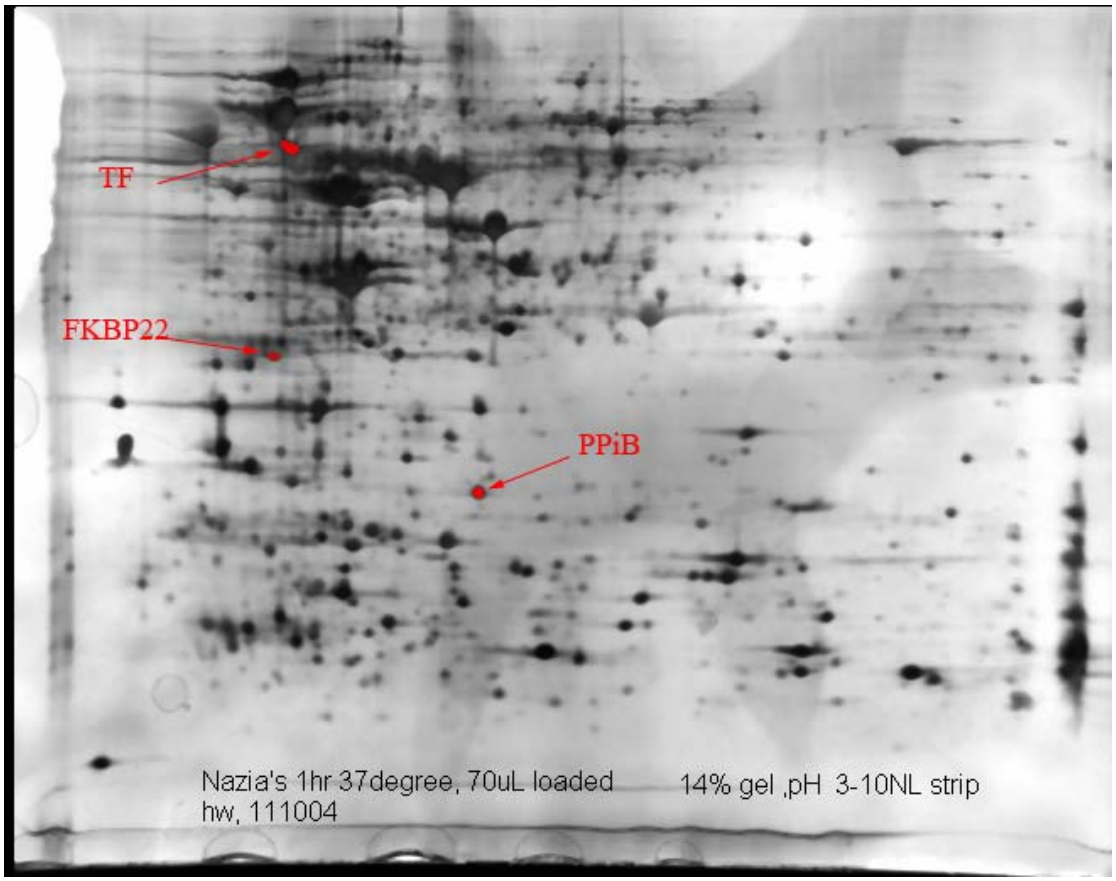
56. London, J., C. Skrzynia, and M.E. Goldberg, *Renaturation of Escherichia coli tryptophanase after exposure to 8 M urea. Evidence for the existence of nucleation centers.* Eur J Biochem, 1974. 47(2): p. 409-15.

57. Mizobata, T. and Y. Kawata, *The folding characteristics of tryptophanase from Escherichia coli.* J Biochem (Tokyo), 1995. 117(2): p. 384-91.

58. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. 72: p. 248-54.

59. Tomoyasu, T., et al., *Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the Escherichia coli cytosol*. Mol Microbiol, 2001. 40(2): p. 397-413.

Figure 2.1



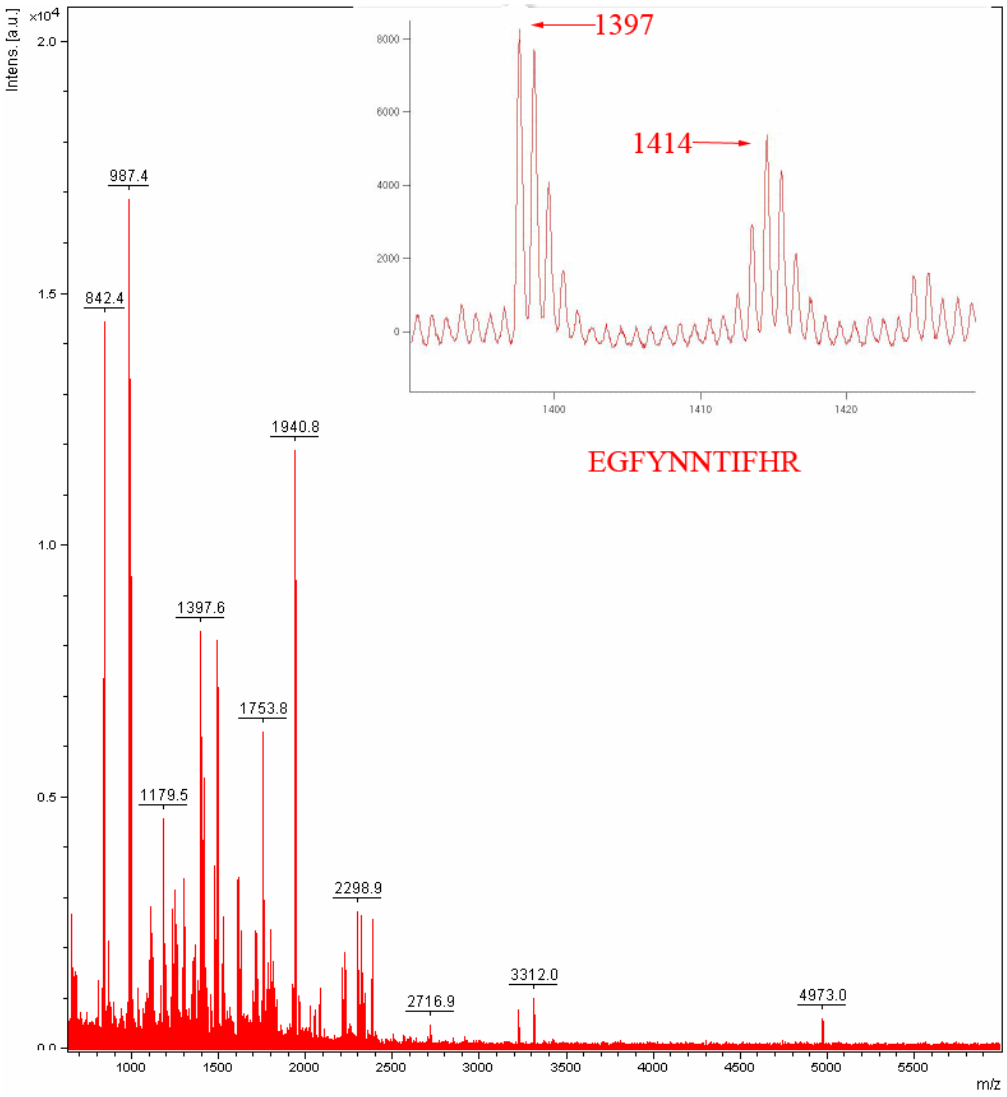
14% 24-cm 2D acrylamide gel for proteome from wild type *E. coli* cells grown at 37 ℃. 1 mg total cells extract was loaded. Spots for *E. coli* PPIases are shown in red.

Table II

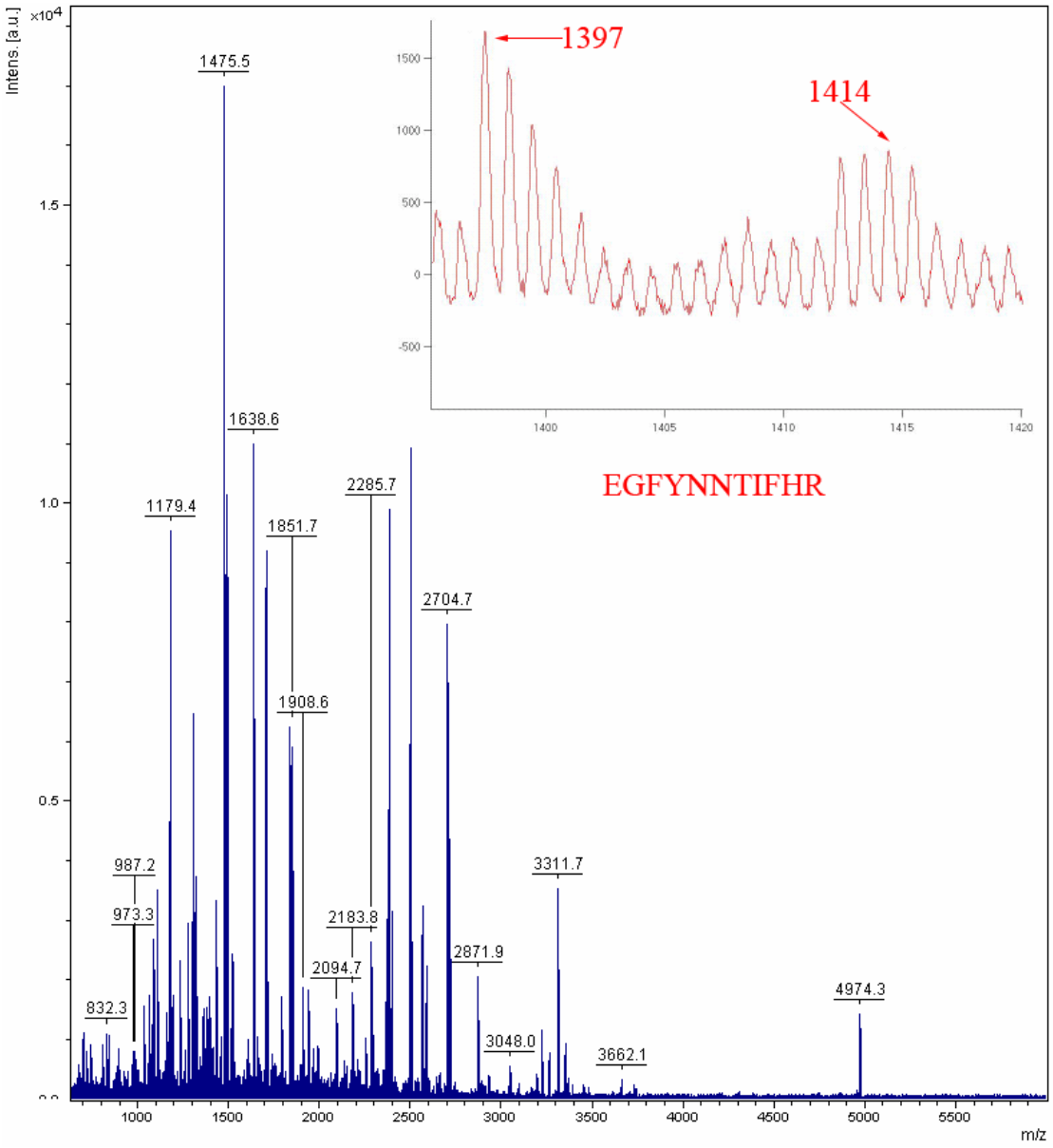
Three spots were identified by Peptide mass fingerprint of Mascot

Identified protein	Mascot score	molecular weight	theoretical peptides	matched peptides	Sequence coverage %
Trigger factor	264	47994	32	23	48
Fkbp22	71	22202	13	6	32
PpiB	50	18256	11	4	32

Figure 2.2

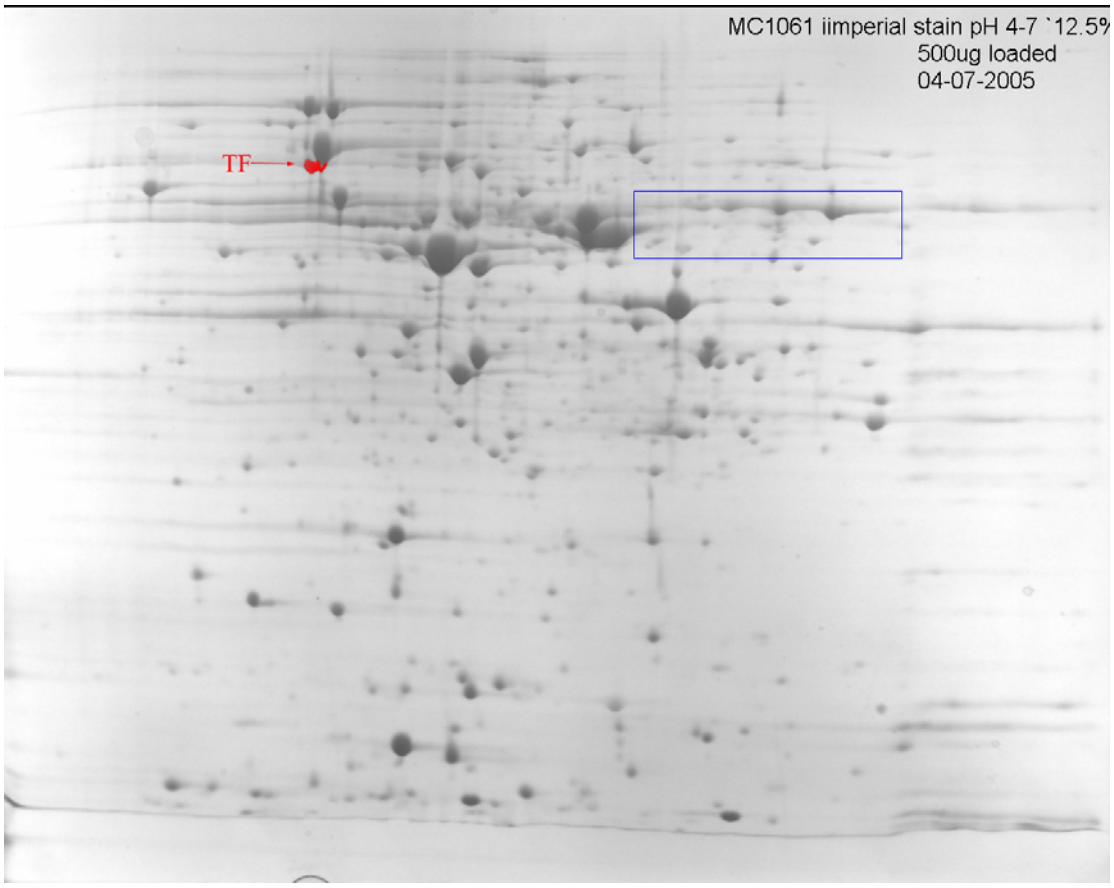


(A) MALDI-MS spectra for tryptic peptides from PPiB derived from MC1061 and *Δtig::kan* MC1061 grown at 37 °C. Six matched peptide peaks were found from the spot for PPiB. The inset shows the ratio of unlabeled peak and ^{15}N-labeled peak. Peak at 1397 is from growth with natural ammonium chloride. The peak at 1414 shows the isotopic peak. The shape difference between two peak clusters is due to different abundance of the major isotope. The mass difference between the peak clusters is 17. There are 18 Ns in peptide sequence. Degradation of amino group at N-terminus could cause the discrepancy.

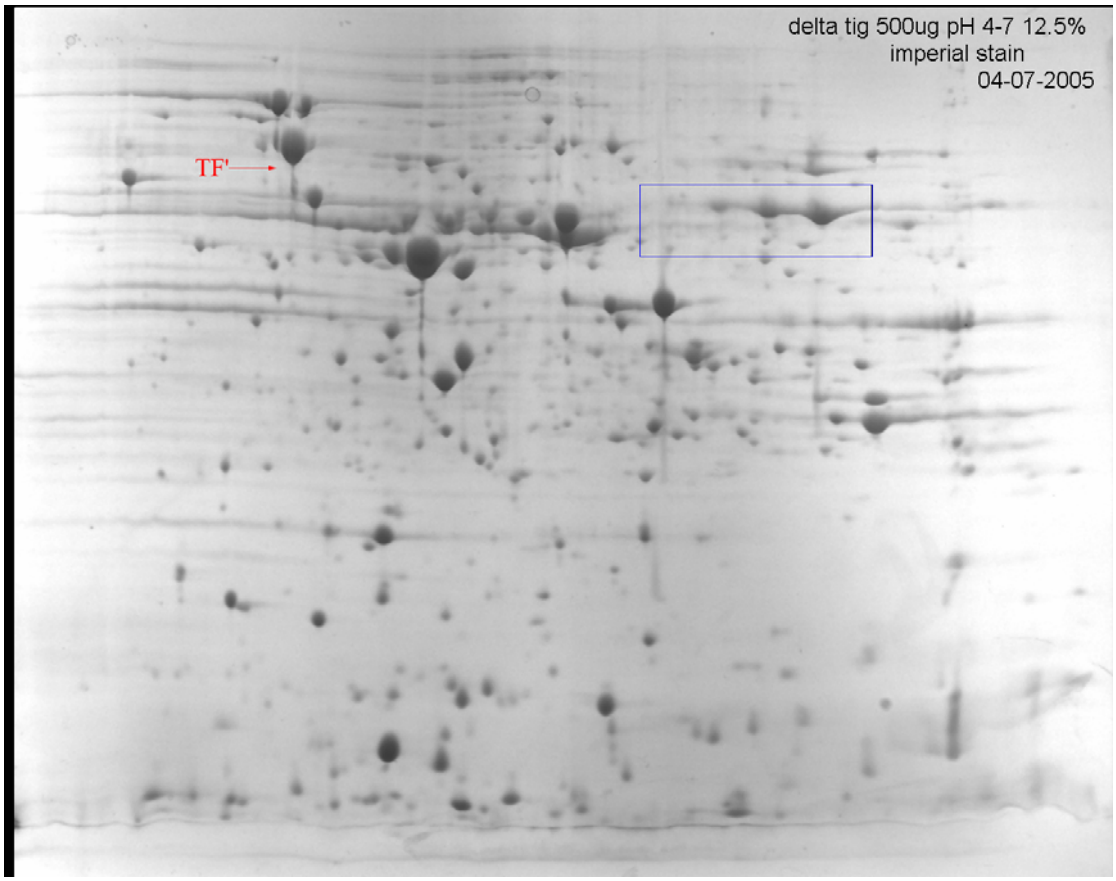


(B). MALDI-MS spectra for tryptic peptides from PPiB derived from MC1061 and Δtig:kan MC1061 grown under 4 °C.

Figure 2.3 (A)

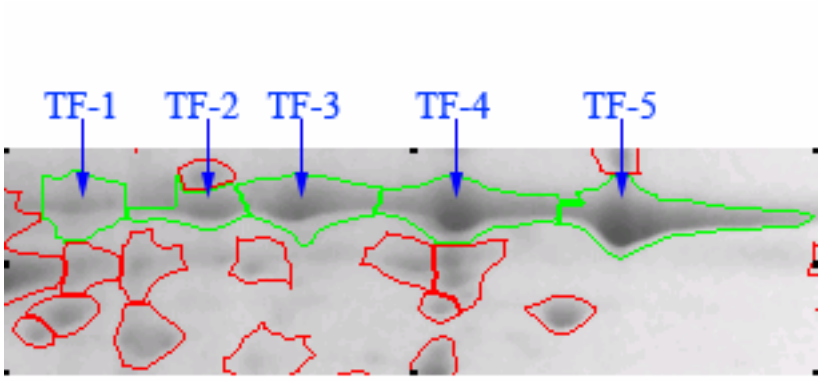


(B)

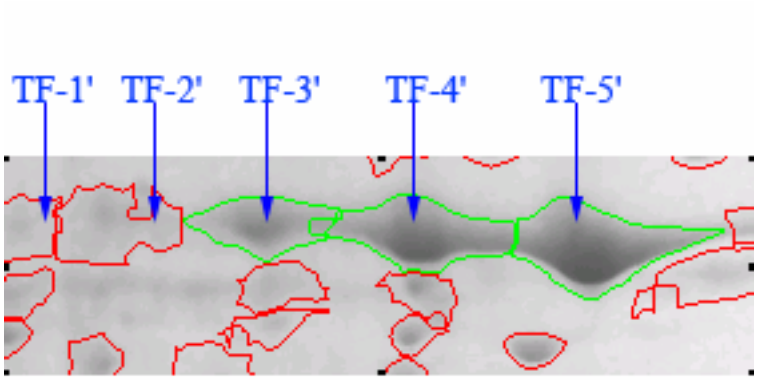


Proteins with pI between 4-7 expressed from wild type MC1061 strain and *Δtig::kan* MC1061. (A) 2D map for proteome from wild type MC1061. Spots from trigger factor are colored in red. (B) 2D map for proteome from *Δtig::kan* MC1061. The relative position of trigger factor on 2D map for wild type was indicated.

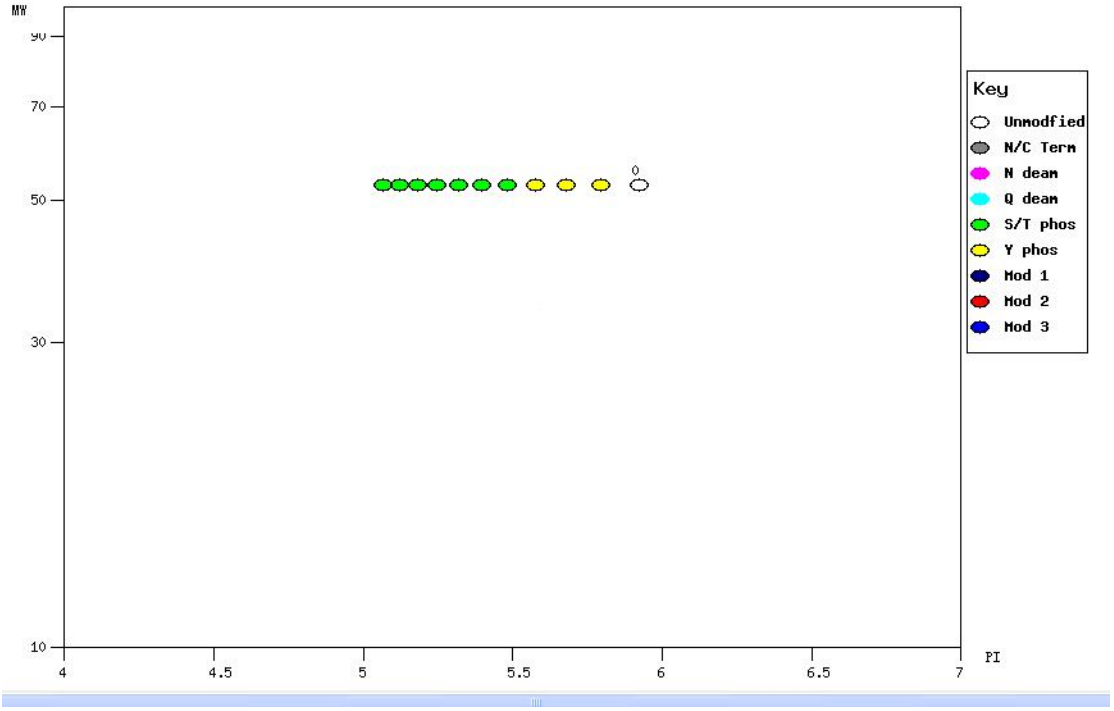
Figure 2.4.



(A) The blue region indicated in **Figure 2.3**(A). Five spots, TF 1-5, were identified as tryptophanase by mass spectrometry.



(B) The blue region indicated in Figure 2.3(B). Three spots, TF 1'-3', were identified as tryptophanase by mass spectrometry.



(C) Prediction of spot pattern for tryptophanase with ten hypothetical phosphorylation sites on serine, threonine, or three on tyrosine by ProMoST.

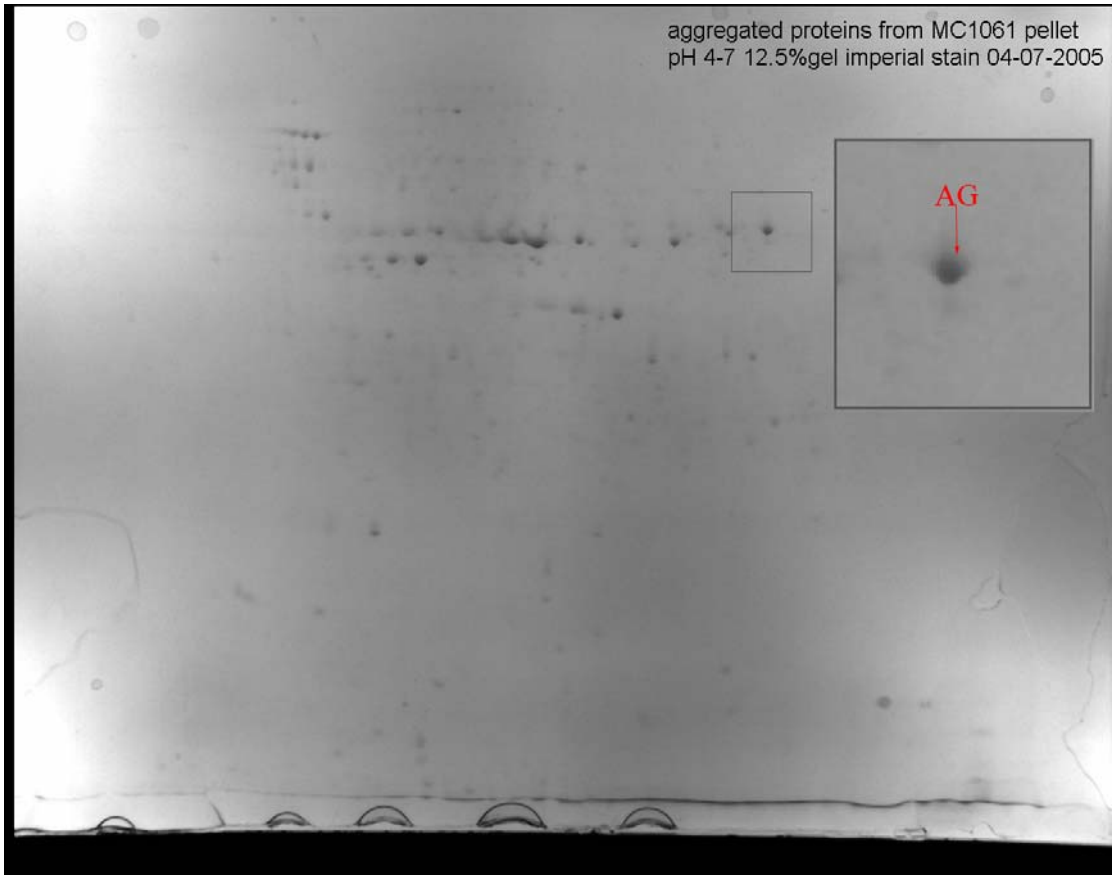
TF-1	KDWTIEQITR ETYKYADMLA MSAKKDAMVP MGGLLCMKDD SFFDVYTECR
TF-2	KDWTIEQITR ETYKYADMLA MSAKKDAMVP MGGLLCMKDD SFFDVYTECR
TF-3	KDWTIEQITR ETYKYADMLA MSAKKDAMVP MGGLLCMKDD SFFDVYTECR
TF-4	KDWTIEQITR ETYKYADMLA MSAKKDAMVP MGGLLCMKDD SFFDVYTECR
TF-5	KDWTIEQITR ETYKYADMLA MSAKKDAMVP MGGLLCMKDD SFFDVYTECR
TF-3'	KDWTIEQITR ETYKYADMLA MSAKKDAMVP MGGLLCMKDD SFFDVYTECR
TF-4'	KDWTIEQITR ETYKYADMLA MSAKKDAMVP MGGLLCMKDD SFFDVYTECR
TF-5'	KDWTIEQITR ETYKYADMLA MSAKKDAMVP MGGLLCMKDD SFFDVYTECR
	251 270 300

(D) Sequence coverage between 251 and 300 for eight spots identified as tryptophanase. Lys270 is the PLP binding site. Sequences in red color were polypeptides identified by mass spectrometry after trypsin digestion

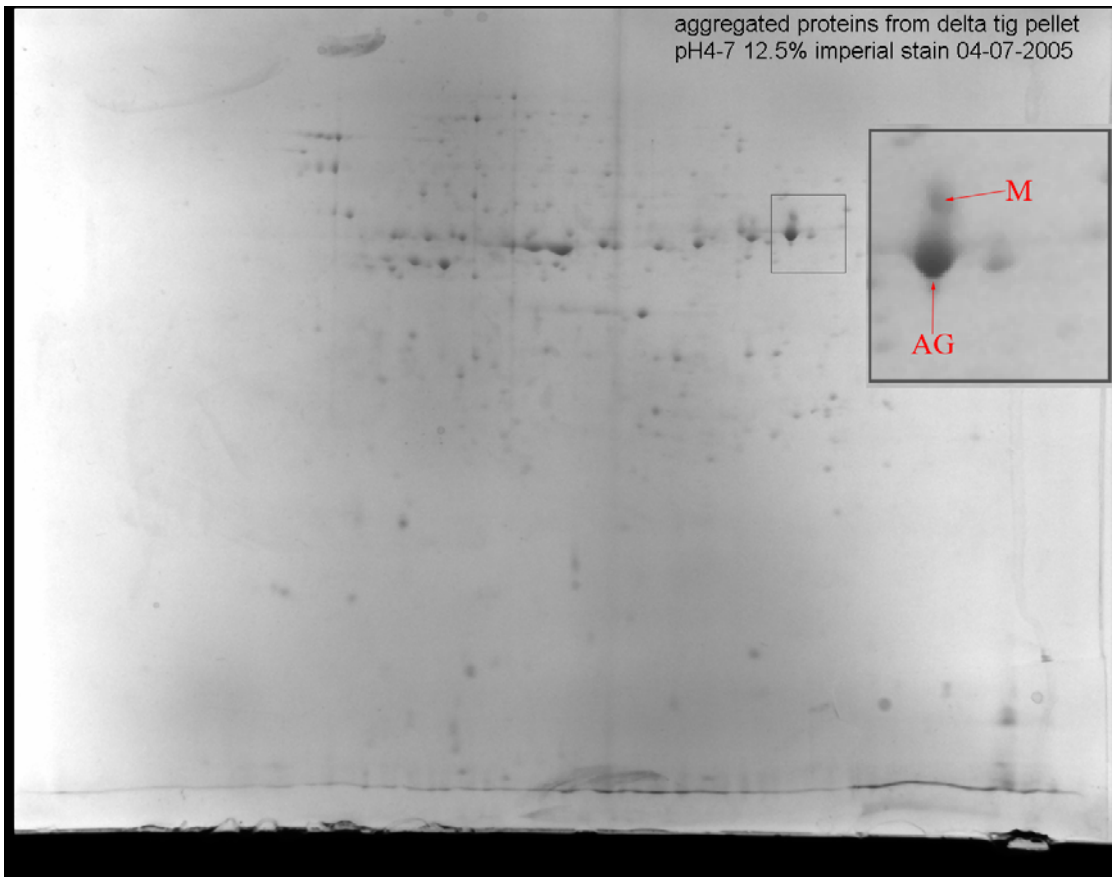
Gel	SpotID	X	Y	Intensity	Area	Vol	%Vol
w. t.	T-1	784	236	45	71.3405	1268.65	7
	T-2	832	238	62	52.6452	1611.93	8.8
	T-3	866	237	74	98.638	2774.02	15.2
	T-4	924	240	96	120.659	4714.67	25.8
	T-5	991	244	121	149.104	7880.37	43.2
mutant	T-3'	846	239	61	100.358	2168.89	11.2
	T-4'	904	245	97	146.81	6346.44	33
	T-5'	967	252	119	174.108	10740.1	55.8

(E) Quantitative analysis of spots from two gels by ImageMaster. "Volume" indicates the relative amount of trytpophanase in each spot.

Figure 2.5 (A)



(B)



Aggregated proteins with pI between 4-7 expressed from wild type MC1061 strain and *Δtig::kan* MC1061. The big spot (AG) on both gels was identified as tryptophanase. The small spot (M) on tig deletion gel was missing on the wild type gel. (A) 2D map for aggregated proteins from MC1061. (B) 2D map for aggregated proteins from *Δtig:: kan* MC1061

Chapter III:

Effect of Trigger Factor in Refolding of Tryptophanase from *Escherichia coli*

INTRODUCTION

Trigger factor is a ribosome-associated chaperone in bacteria. It is the first protein to interact with newly synthesized polypeptides and assist co-translational protein folding.[1-3] The binding pocket of trigger factor faces towards the tunnel exit of the 50S ribosome, which forms a protected "cradle" for small proteins to fold. For multi-domain proteins, it has been postulated that trigger factor dissociates from the 50S ribosome and binds to the folding nascent chains until the binding site of substrate is buried after further conformational change of polypeptides. As trigger factor may associate with nascent chains for prolonged periods, additional trigger factor may occupy the vacated ribosome and bind to the elongating polypeptide.[1, 3] Trigger factor monomer binds to ribosome in a 1:1 stoichiometry.[2, 4] But the ratio of trigger factor to ribosome in the cytosol is 2.6:1.[5]

The effective molecular mass for the trigger factor peak on size exclusion chromatography (SEC) was 54-80 kDa, indicating a fast monomer/dimer equilibrium. This equilibrium is assumed to protect the inner hydrophobic binding site from the crowded environment in the cytosol.[3] Ribosome binding would induce the conformational change of trigger factor to expose the binding pocket, and substrate binding would stabilize this conformation.[1, 3] Unbound trigger factor is predicted to serve as a "pool" to provide monomeric trigger factor for dynamic ribosome binding.[3] *In vitro* trigger factor shows chaperone activity and peptidyl-prolyl cis/trans isomerase (PPIase) activity.

Tryptophanase catalyzes the *in vivo* degradation of L-tryptophan to indole and

ammonium pyruvate.[6] Active tryptophanase is a tetramer with coenzyme, pyridoxal-5'-phosphate (PLP) binding to each subunit. Potassium ion is also necessary for activating trytophanase.[7-10] Both holo- and apo-tryptophanases are found in cell extracts, but apo-tryptophanase dominates.[9] Holo-tryptophanase is much more stable than the apo-form, which exists in equilibrium between monomer and dimer. Refolding of chemically denatured tryptophanase results in heavy aggregation. Large amounts of chaperonin GroE depresses the aggregation and improve recovery of active trytophanase up to 80%.[12]

In general, for multi-domain proteins, folding of the monomer is fast, but quaternary structure formation varies over a wide time range. Thus, aggregation could happen before the hydrophobic core forms. For some proteins, chaperones can prevent misassembly by affecting the kinetic partition between folding and aggregation.[13] It is unclear if chaperones function in the assembly of oligomers after monomer folding. We report here the discovery of an unknown function of trigger factor to assist the assembly of the tetrameric structure of trytpophanase.

EXPERIMENT PROCEDURE

Molecular cloning

Trigger factor and TF fragments (N-terminus, FKBP domain, FKBP domain+C-terminus) were cloned into the TOPO vector for overexpression by other Eidsness group members.

TF variants TFF198A and TFF233Y, which are deficient in PPIase activity, were generated using STRATAGENE QuikChange II site-directed mutagenesis kit. Forward and reverse primers are detailed below:

TFF186A-for: GATCCCGGGCGCTGAAGACGGTATCAAAGG
rev: TACCGTCTTCAGCGCCCGGGATCATACGAC
TFF233Y-for: GTAAAGCAGCGAAATACGCTATCAACCTGAAG
rev: TTCAGGTTGATAGCGTATTTCGCTGCTTTACCT

Overexpression of trigger factor, TF mutants and TF fragments

Plasmids were transformed into Invitrogen BL-21(DE3) competent cells by heat shock (42 °C). To express each protein, one liter of cell culture was grown in LB media at 37 °C to OD_{600nm} of 1.0. In order to induce expression, IPTG was added to a final concentration of 1 mM. Growth was continued for another five hours. Cells were harvested by centrifugation at 5000 rpm for 15 min in a Beckman Avanti J-25I centrifuge.

Sample preparation for NMR analysis

After transformation, cells were plated onto M9+supplements plate as in Chapter 2. ^{15}N-labeled ammonium chloride was used in the growth medium. The plate was incubated at 37 ℃ until colonies were observed. 100 mL M9+supplements medium was inoculated with a single colony and grown overnight at 37 ℃ with shaking (250 rpm). Then I carried out a 20-fold dilution of cell culture into 1 L M9 + "55-2" (20 mL per liter) + trace metals (1 mL per liter) (details of solutions below). ^{13}C-labeled glycerol was used in this step. Cells were grown for 5 hours at 37 ℃, then switched to 20 ℃ and continued to grow overnight (12 h). Cells were harvested and stored following standard procedure.

50X carbon source,55-2:

0.5 % glycerol, 0.05 % glucose and 0.2 % lactose

1000X trace metal:

50 mL of 0.1 M $FeCl_3$ prepared in 0.1 M HCl, 2 mL of 1 M $CaCl_2$, 1 mL of 1 M $MnCl_2$, 1 mL of 1 M $ZnSO_4$, 1 mL of 0.2 M $CoCl_2$, 2 mL of 0.1 M $CuSO_4$, 1 mL of 0.2 M $NiCl_2$, 2

mL of 0.1 M Na_2MoO_4 and 2 mL of 0.1 M H_3BO_3. Bring volume to 100 mL with Nanopure water.

Purification of trigger factor and TF fragments

The cell pellets were resuspended in buffer A (20 mM Hepes, 150 mM NaCl, pH 7.0) and lysed by 4x5-s sonication pulses (Fisher 550 Sonic Dismembrator). Cell debris was removed by centrifugation at 17,000 rpm for 30 min. The supernatant was applied to a nickel-chelating Sepharose column (GE Healthcare, 5 mL) and bound proteins were eluted by a linear gradient from 0 to 0.5 M imidazole in buffer A. Protein samples were buffer-exchanged to 50 mM Tris-HCl, pH 7.5, with Millipore Amicon centrifugal filter. TF proteins were further purified by anion-exchange chromatography on a 6 mL Resource Q column (GE Healthcare). Fractions were collected and analyzed by SDS PAGE.

Purification of TF variants, F198A and F233Y

A lot of contaminating proteins and polypeptides were found in F198A and F233Y samples after following the purification procedure above. F198A and F233Y variants were purified again with 1 M ammonium sulfate in FPLC buffer A and B on a nickel-chelating column. A minor precipitate was removed by vacuum filtration, and ammonium sulfate was removed by buffer-exchange.

Mass spectrometry

Selected spots were cut out of gels and chopped into small pieces. The gel pieces were destained with ammonium bicarbonate in 50% acetonitrile for 10 min. This step was repeated four times to completely dehydrate gel sample. Gel particles were reswelled in 50 μL of 10 mM DTT in 40 mM NH_4HCO_3 at 55 ℃ for one hour. After cooling to room

temperature, sample was spun down and solvent was drawn out. 50 μL 0f 55 mM iodoacetamide in 40 mM NH_4HCO_3 was added to the gel sample to methylate newly formed S-H groups. The sample was incubated at room temperature for 45 min. Then the gel pieces were washed with 40 mM NH_4HCO_3 and 100% CH_3CN twice. 10 ng/μL trypsin was added to re-swell the gel particles on ice for 45 min. After washing with 40 mM NH_4HCO_3, the digestion reaction was incubated at 37 ℃ overnight. Digested products were extracted with 5% formic acid/50% CH_3CN. The resulting peptides were washed with Nutip (Glygen Corp.) and loaded on a MALDI target, which was submitted to the Chemical and Biological Mass Spectrometry Facility for analysis.

PPIase activity assay

A chymotrypsin-coupled PPIase activity assay was used to measure PPIase activity of trigger factor, TF fragments and TF variants. Trigger factor proteins were added to final concentration from 0 to 20 uM. PPIase activity was monitored at 10 °C for 4 min after addition of 5 μL 5 mM peptide substrate, suc-Ala-Phe-Pro-Phe-4-nitroanilide. The progress curves were analyzed with Igor Pro 5.0 (Wavemetrics). $k_{c/t}$ was calculated from k_{obs} at various trigger factor concentrations.

GAPDH Refolding Assay

GAPDH was denatured in 4 M Gdn·HCl overnight at 4 °C. The unfolded GAPDH was diluted 100-fold to final concentration of 1.5 μM in dilution buffer (0.1 M potassium phosphate, pH 7.5, containing 1 mM EDTA, 5 mM DTT and various concentrations of trigger factor). The solution was kept on ice for 30 min. The reaction mixture was incubated at 25 °C for 3 h. The GAPDH activity of refolded protein was characterized by

the method described on http://www.worthington-biochem.com/GAPD/. The slope of the initial linear part of the progress curve (0.1-0.8 min) was used to determine the activity of GAPDH.

Tryptophanase activity assay

Tryptophanase , PLP, and SOPC were kindly provided by Professor Robert Phillips, University of Georgia. Refolding assays of tryptophanase were performed at 25 °C with a Varian Cary-100 spectrophotometer equipped with a temperature controller. 100 μM tryptophanase was mixed with equal volume of 8 M Gdn-HCl. Tryptophanase was denatured for one hour at room temperature and diluted 100-fold with prechilled dilution buffer (50 mM Hepes, pH 7.8, 5 mM DTT, 200 μM PLP). The diluted solution was kept at 4 °C for 30 min , then transferred to a 25 °C water bath. 20 μL aliquots were taken at 30-min intervals and assayed for activity. Trigger factor proteins were included in the dilution buffer at different concentrations. The enzymatic activity of tryptophanase was assayed by measuring the decrease of absorbance at 360 nm for 5 min.

Native gel

Coomassie blue (G250) was added to individual samples to a final concentration of 0.02 %. Protein samples were loaded onto a Criterion Tris-HCl 4-20% gel (Bio-Rad). Components for the cathode buffer were 25 mM Tris, 192 mM glycine and 0.02 % Coomassie blue G250. The anode buffer included 25 mM Tris and 192 mM glycine. Gels were run at a constant 200 V for 55 min. After running, the gel was rinsed with water 3 times and incubated in Colloidal Coomassie Staining (10 % ammonium sulfate, 0.1 % Coomassie G250, 2 % phosphoric acid and 20 % methanol) overnight.

RESULTS AND DISCUSSION

Purification of Trigger Factor and Trigger Factor Variants

The wild type trigger factor and its truncated expression products (FKBP and PC domains) were soluble and purified as His-tag constructs by nickel affinity chromatography followed by anion exchange. There was little contamination due to unspecific binding to the affinity column. Further purification was done with anion exchange column. Proteins were checked for purity with Tris-HCl SDS PAGE. All proteins showed correct size on gels, consistent with previous reports,[14] showed PPIase activity.

Trigger Factor variants F198A and F233Y, could not be purified in the same way as wild type. After anion exchange, a lot of contamination remained (Figure 3.1.A). We hypothesized that contaminants were binding to F198A or F233Y by hydrophobic interaction, and tried to purify them away with detergent (0.1% Triton X-100), but the result was not satisfactory. Then we tried purification with ammonium sulfate. High concentrations of ammonium sulfate (20-50%) would precipitate proteins without denaturing them. Low concentrations of ammonium sulfate would interrupt the hydrophobic interaction. So we purified F198A and F233Y in 1M ammonium sulfate (about 13%). The purity of both proteins was improved greatly.

The difficulties of purifying trigger factor variants brought up the importance of the PPIase activity of trigger factor, since both variants lack PPIase activity.[15] The role of the PPIase domain in trigger factor is still mysterious. It seems that without PPIase activity, trigger factor purification is difficult, probably because of binding of small proteins or polypeptides through hydrophobic interaction. According to the most recently proposed

mechanism of trigger factor chaperone activity, the functional unit is the monomer.[3] Trigger factor dimerizes just to stabilize free trigger factor in solution by occluding the hydrophobic active site. The monomer exists in solution with a closed conformation and in equilibrium with trigger factor dimer. Without PPIase activity, F198A or F233Y monomer seemed unstable. On native gels, wild type trigger factor shows monomer and dimer bands around 50 and 75 kDa. (Figure 3.1.B) The dimer band was dominant for F198A or F233Y. The monomer of wild type trigger factor might be more stable in solution than variants without PPIase activity. So the function of the PPIase domain could be related to stabilizing the hydrophobic site of trigger factor in aqueous solution.

Stability of Trigger factor and its FKBP domain

In 2002, there was no structure of trigger factor available. Since the PPIase activity of trigger factor towards protein substrates is much higher than other PPIases, we were interested in the structure of the TF-FKBP domain which might be special compared to other PPIases of FKBP family. The molecular weight of purified TF-FKBP was identified as 15402±5 kDa by mass spectral analysis. NMR spectroscopy is normally suitable for a full solution structural analysis of small proteins. For ^{15}N-HSQC measurement, the protein was dissolved in buffer of 10% D_2O, 50 mM KPi, pH 6.5, and 200 mM NaCl. The data collection temperature was 25 °C. The ^{15}N-HSQC spectrum for TF-FKBP domain showed good dispersion, which indicates a folded 3D structure (Figure 3.2.A). But after 71 h of data collection in an HNCACB experiment, some shift of amide resonances chemical shifts was observed in the ^{15}N-HSQC spectrum. (Figure 3.2.B).[16] Signals overlapped heavily in the spectral range of 7.5-8.7 ppm. There were still some proteins in folded conformation in

the solution. Old buffer was exchanged to fresh buffer of 50 mM HEPES, pH 7.5 and 300 mM NaCl. Dispersion of peaks on HSQC spectrum for the protein sample after buffer-exchange indicated that TF-FKBP recovered its folded conformation (Figure 3.2.C).

TF-FKBP may be more stable in HEPES buffer or at high pH. We divided the refolded TF-FKBP into two parts. One part was frozen at -20 °C for a week. The other part was kept at room temperature for a week. ^{15}N-HSQC spectrum for the frozen sample was the same as the spectrum at the beginning of the experiment (Figure 3.2.D). However, a ^{15}N-HSQC spectrum typical of that for random coil was observed for the sample at room temperature (Figure 3.2.E). We exchanged the old buffer for fresh HEPES buffer with different pH. But this time, unfolded TF-FKBP could not be refolded again. So temperature is the most important factor to affect the stability of TF-FKBP.

The positions of TF-FKBP on Tricine gel before and after buffer exchange are different from the position of native TF-FKBP (Figure 3.3.A). This difference may be the consequence of cleavage of the N-terminal histidine tag (35 amino acids) or conformational change. Since HSQC spectra showed no conformational difference between the fresh TF-FKBP and refolded protein, the size difference of TF-FKBP on gels may be the result of losing the N-terminal tag. N-terminal amino acid sequencing confirmed our hypothesis (Figure 3.3.B). After refolding, TF-FKBP lost the whole histidine tag. Even the fresh TF-FKBP sample after purification lost part of the tag. Thus, the N-terminal tag may stabilize the FKBP domain of trigger factor in solution.

Next, we tested the stability of full-length trigger factor. The folding status of full-length trigger factor could not be measured by NMR spectroscopy because of its large

size. So we chose electrophoresis to monitor the stability of trigger factor under different conditions. Normally, we can observe a minor band below the major band for trigger factor on proteins gels, which may be the product after losing the histidine tag. We confirmed that part of trigger factors lost their His-tag by N-terminal amino acid sequencing. The trigger factor sample was in buffer of 50 mM Tris, pH 8.0, 300 mM NaCl after purification through anion exchange column. A preliminary study showed that there was some degradation after 1.9 mM trigger factor was incubated at room temperature for three days. There was no apparent degradation after heating the sample at 95 °C for one hour (Figure 3.4.A). The degradation of trigger factor may be a slow procedure and high temperature seems not to accelerate this procedure.

There are four factors, buffer, time, pH and temperature that could affect the stability of trigger factor. We performed more systematic experiments to understand what makes trigger factor degrade. We studied the buffer effect first. Some trigger factor sample was "buffer-exchanged" into phosphate buffer of 50 mM KPi, pH 6.5, 200 mM NaCl. Samples in either Tris buffer or phosphate buffer were kept at room temperature for 10 d (Figure 3.4.B). The trigger factor band for the sample in Tris buffer completely disappeared on gel after ten days. Bands below 50 kDa were observed for trigger factor in phosphate buffer. It seems that phosphate buffer delays degradation of trigger factor. Later, when we analyzed degradation products from degraded trigger factor with mass spectrometry, peptide peaks from both ends of the TF sequence could not be detected any more (Figure 3.4.C). Thus, the degradation of trigger factor could start from either end or both ends of its sequence.

When we kept trigger factor in phosphate buffer at room temperature for a period of

five days, we found the intensity of the lower MW band increased as time passed (Figure 3.4.D). Increasing or decreasing the pH of sample solutions did not change the pattern of gel bands for trigger factor, which suggested that pH did not affect the integrity of trigger factor (Figure 3.4.E). One-day incubation of trigger factor at certain high temperatures resulted in multiple lower bands under 50 kDa and made a higher band around 100 kDa clearer. The gel band around 100 kDa seems to be from the cross-linked dimer of trigger factor (Figure 3.4.F).

At even higher temperatures (39.7 and 45.4 °C), a second extra band around 200 kDa became clear. The molecular weight is consistent with the size of trigger factor tetramer. Our experiments showed that the degradation of trigger factor is a slow process, even at high temperature. The pH of the buffer does not affect degradation of trigger factor. Since pH is not an important factor for stability of trigger factor, the significant difference of degradation products in Tris or phosphate buffer might be the result of interaction of trigger factor molecule with Tris or phosphate molecules. High temperature may accelerate degradation and formation of trigger factor dimers or tetramers. The temperature effect may be related to the fact that overexpression of trigger factor reduces viability of *E. coli* cells at 50 °C.[17] Formation of oligomers at high temperature may inactivate trigger factor *in vivo*. On the other hand, this change could be necessary for trigger factor to avoid self-digestion.

Trigger Factor enhances the recovery of tryptophanase activity upon renaturation.

A tryptophanase refolding assay was carried out to investigate the effect of trigger factor on the recovery of tryptophanase activity. Tryptophanase can regain approx. 18 % of

its native activity after chemical denaturation and removal of denaturant without any chaperone or other reagents to assist protein folding. In the presence of trigger factor at a 14:1 molar ratio (trigger factor monomer to tryptophanase monomer), nearly 60 % of tryptophanase activity can be recovered. The same concentration of bovine serum albumin (BSA) had no effect on tryptophanase refolding, which indicated that our result was not artificial (Figure 3.5.A).

We found that temperature is very important for this assay. If the whole experiment is carried out at room temperature, trigger factor did not improve the refolding of tryptophanase. If the denatured tryptophanase is diluted into pre-chilled buffer (with trigger factor) and incubated at room temperature, about 40% of tryptophanase activity can be recovered. Extending the cold pre-incubation time up to 10 h did not have a significant effect on tryptophanase refolding (Figure 3.5.C). According to the folding study, refolding and aggregation of trypotophanase starts right away upon dilution.[18] Refolding intermediates tend to form nonspecific aggregates through hydrophobic interaction to protect hydrophobic regions from exposure to solvent. At low temperature, such interactions may be slowed. Under these conditions, trigger factor has more time to bind polypeptides and save them from forming irreversible aggregates.

As reported, the recovery activity of refolded tryptophanase can be improved up to 80 % with 15-fold excess of GroE 21-mer.[12] The assay condition was different from ours: the denaturation time was 10 sec in 4 M Gdn-HCl and we incubated the denaturation reaction for one hour. The initial concentration of tryptophanase in the refolding assay with GroE was about 1 mg/mL. We denature 5 mg/mL tryptophanase in 4 M Gdn-HCl. The

recovered yield of tryptophanase depends on initial concentration as previously reported.[18] We started the refolding assay with about four times more concentrated tryptophanase and much longer denaturation times, but still recovered 60 % activity. So the effect of trigger factor on tryptophanase was significant.

On the other hand, GroE suppressed the aggregation of tryptophanase by binding to the folding intermediates right away. No cold incubation was included in the refolding procedure. This indicates that GroE binding intermediates happened before the formation of aggregates. The binding of trigger factor to substrate is far slower than aggregation under room temperature. In the crowded environment *in vivo*, trigger factor may not be an efficient chaperone to save unfolded proteins and polypeptides from misfolding at room temperature or heat shock temperatures. This could explain why the deletion of *tig* gene is not lethal for cell growth at ambient or high temperature. On the other hand, trigger factor overexpression can improve the viability of *E. coli.* at low temperatures.[17] Given more time to bind substrate, trigger factor apparently could play a more important role in protecting unfolded protein against unspecific aggregation at low temperature or during cold shock.

It is surprising to find that TF-double (PC) did not have any effect on activity recovery of tryptophanase (Figure 3.5.A). Increasing PC concentration to 28 μM or 42 μM did not improve activity recovery. This is inconsistent with recently published results, which showed that the domain combination of PC has substantial chaperone activity in vitro.[19] The activity of PC domain on refolding of denatured GAPDH or RNase T1 was shown to be extremely similar to full-length trigger factor. To assist refolding of tryptophanase might

require all three domains of trigger factor. Substrate binding sites were found on all three domains of trigger factor during translation.[20] Therefore tryptophanase might be the first specific substrate of trigger factor to be reported (Figure 3.5.A).

The PPIase activity of trigger factor is not essential for its chaperone activity as previously reported by Kramer, G., et al. To understand the role of PPIase activity in refolding of denatured tryptophanase, we made two trigger factor variants which lack PPIase activity as reported before.[15] Trigger factor variants lacking PPIase activity can still assist the refolding of tryptophanase (Figure 3.5.B). However, the isolated PPIase functional domain of trigger factor has no effect on tryptophanase refolding. The chaperone activity of TF-F233Y is a little lower than wild type trigger factor. Surprisingly, TF-F198A is more efficient than wild type trigger factor in assisting unfolded tryptophanase to regain its activity. A similar result was observed in refolding GAPDH with trigger factor and TF-F198A. [15] The mutation in the FKBP domain would not change the binding specificity. So TF-F198A might be slightly more active than wild type trigger factor. The mutation of Phe198 could make the binding or releasing of substrate a little more efficient due to a minor change in the binding pocket.

Refolding process of tryptophanase monitored by Native Gel experiments

We followed the refolding process of tryptophanase by running blue native gels.[21-23] With addition of the anionic triphenlymethane dye CBB G-250 into samples before running the gel, proteins and protein complexes travel to the anode due to the negative charges from attached dye molecules and are separated mainly according to the molecular weight and shape of different proteins. This method can preserve the oligomeric structure of native proteins and avoid aggregation problems during electrophoresis. Blue

native gel is an ideal technique to detect formation of tetramers or other oligomers during tryptophanase refolding.

We first investigated the dependence of recovered tryptophanase activity on the concentration of trigger factor or its variants (Figure 3.6). There are no significant differences in gel band intensities upon increasing the concentration of trigger factor from 3.5 to 28 μM (Figure 3.6.A), but the activity assay result showed that the recovered tryptophanase activity was improved from 32% to 62% with trigger factor or from 40% to 70% with TF-F198A when increasing the concentration (Figure 3.6.B).

Tetramerization and conformational transformation from apo-enzyme to holo-enzyme were shown as two rate-limiting steps in the activation of tryptophanase.[24, 25] Our results suggested that reassembly of the tetrameric structure may not need trigger factor, but the conformational change to native (active) state requires trigger factor to reach the maximal recovered activity. This is consistent with the previous result that the holo-tryptophanase is in an energetically unfavorable conformation.[24] Trigger factor may catalyze this transformation or stabilize the unstable conformation. Since TF-F198A functioned as well as wild type trigger factor (Figure 3.6.B), the PPIase activity of trigger factor may not be involved in such a transformation. Multiple bands on the native gel for trigger factor were not observed on SDS PAGE. The lower band is near the position of 50 kDa and the upper band is above 70 kDa (Figure 3.1.B). The range is consistent with absolute masses (54 kDa to 80 kDa) obtained from SEC and static light scattering. Multiple bands between could result from a reversible monomer/dimer equilibrium.[4] This pattern was not observed in TF-F198A and TF-F233Y, which could be the consequence of

depletion of PPIase activity. Further research could compare differences of equilibria between wild type TF and TF mutants (Figure 3.1.B).

Next, we followed the refolding process of unfolded tryptophanase using TF-F198A (Figure 3.6.C). The estimated pI (6.1) of tryptophanase is within the pI binding range (pI > 5.4) of G-250 dye. G-250 dye was added to each sample immediately at each time point. The binding of negatively charged dye could prevent the oligomer from aggregation and "freeze" the assembly of oligomer due to electric repulsion,[21-23] such that the oligomeric states of tryptophanase at different refolding phases were trapped after adding the dye. The gel band intensity does not vary much after 30 min cold incubation, which suggests that the assembly of tetramer was finished during cold incubation. But almost no trytophanase activity was recovered at this time. After 10 min, about 40% of tryptophanase activity was achieved. It took about 30 min for unfolded trytpophanase to reach the maximum recovery of activity upon dilution with chaperone.

Apo-form or other protomers of tryptophanase could be regained quickly after dilution of a denatured sample, but refolding to its holo conformation might be a slow process. It was shown that the binding of the first co-enzyme to apo-tryptophanase made the binding of second PLP 35 times slower. Binding of PLP may induce a change in conformation, which makes it difficult for other PLPs to approach the binding site.[10] This might make the full recovery of tryptophanase activity very slow compared to tetramer assembly. It was shown that unfolded tryptophanase bound to GroEL first. Then GroEL released the substrate upon binding of ATP or ADP.[12] Unlike the GroE system, trigger factor does not bind nucleotides. It is not clear how trigger factor regulates its chaperone activity. A new

mechanism was proposed that ribosome docking induces activation of trigger factor monomer and exposes its hydrophobic binding site for incoming polypeptides.[3] Unbound trigger factor monomers are stabilized by dimerization. The equilibrium would be between trigger factor dimer in solution and ribosome-bound monomer. Trigger factor would be released from polypeptide due to its folding. If we apply this mechanism to its *in vitro* chaperone function, the cycle would be between trigger factor dimer and monomer associated with substrate. Without ribosome, trigger factor might need more time to bind unfolded polypeptide and incubation of reaction at cold temperature becomes necessary. As mentioned above, PLP binding is a slow procedure and would cause a change of tryptophanase conformation. Trigger factor then could be released after formation of fully functional holo-tryptophanase.

PPI activity and chaperone activity of apo- and zinc(Ⅱ)-binding trigger factor

It was found that the folding arrest activity of trigger factor from *Thermus thermophilus* is dependent on Zn^{2+} ion.[26] This protein showed significant difference in inhibition of spontaneous folding of denatured proteins with or without Zn^{2+}. Our collaborator, Dr. Michael Crowder group, at Miami University, is trying to determine whether trigger factor from *E. coli* is also a Zn^{2+}-dependent chaperone. We carried out PPIase activity and chaperone activity assays for their apo- and Zn^{2+} -binding trigger factor. The sample was purified from cell extracts with a Q-Sepharose anion exchange column, which is different from our purification method. Purified trigger factor was treated with five equivalents of Zn^{2+} to obtain holo-trigger factor. Apo-trigger factor was prepared by treatment with five equivalents of EDTA. Zn-trigger factor contains one equivalent of Zn^{2+}.

PPIase activity was performed with polypeptide, Suc-Ala-Phe-Pro-Phe-4-nitroanilde, as substrate. The k_{cat}/K_m value we obtained for apo-trigger factor was 0.77 $\mu M^{-1}s^{-1}$, compared to 0.40 $\mu M^{-1}s^{-1}$ for Zn-trigger factor, suggesting that there is some difference in PPIase activity between apo- and Zn-binding TF. There are several k_{cat}/K_m values published for TF with the same substrate: 0.88, 0.74 and 1.3 $\mu M^{-1}s^{-1}$).[14, 27, 28] Our k_{cat}/K_m value for apo-trigger factor is consistent with the values above. And k_{cat}/K_m value of the Zn-binding TF is a little lower. But I do not think Zn(II) inhibited PPIase activity completely, since it is apparent that Zn-TF accelerated the hydrolysis a lot compared to control.

We also compared chaperone activity of both apo- and holo-trigger factor using the GAPDH refolding assay. The refolding of GAPDH reached a maximum with about three-fold excess of apo-trigger factor or two-fold of Zn-binding trigger factor. Since the result of GAPDH assay varies a lot day to day, the result did not show much difference between two samples (Figure 3.7.A)/ The ratio of trigger factor to substrate with the highest recovery percentage is close to the value reported before.[29] There are few papers about the chaperone activity of “free” trigger factor. Most studies were focused on the function of ribosome-bound TF.

The GAPDH refolding assay is used as a standard method to measure the chaperone activity of TF. The GAPDH refolding assay seems to measure the folding arrest activity. The activity of denatured GAPDH can be recovered up to 60% of native protein with certain TF/GAPDH ratio. If one adds too much trigger factor, this recovery percentage decreases.[29] The suggested explanation involves formation of dimeric TF as the concentration of TF increases. The dimeric TF would not release GAPDH after binding,

which causes the recovered activity to decrease at some point with increasing TF concentration. They also found that adding DnaK-J-GrpE with ATP could restart the folding of GAPDH. This observation is similar to the result for the GFP refolding assay used to measure the folding arrest activity of trigger factor.[26] So our result from GAPDH refolding assay could indicate that in *E. coli*, trigger factor may not need Zn^{2+} to activate its chaperone activity.

We also carried out tryptophanase refolding assays on both samples, because according to our earlier result, this assay may check different refolding activity from the folding arrest activity measured by GAPDH assay. The activity recovery of denatured tryptophanase would reach maximum as a function of TF concentration, but not decrease with further increase in trigger factor concentration. So trigger factor may have a more specific chaperone activity to tryptophanase as discussed above. Tryptohanase denatured by 4M Gdn-HCl recovered about 65% of its native activity with 14-fold of apo- or Zn-binding trigger factor (Figure 3.7.B). The recovery yield is similar to that found using our trigger factor sample. No significant difference in chaperone activity between apo- and Zn-binding trigger factor was observed in the tryptophananse refolding experiments. On the other hand, this result also validated our discovery about the function of trigger factor in reactivating tryptophanase. Trigger factor samples prepared in different laboratories both enhanced the refolding yield of tryptophanase by the same amount. Our observation about improved refolding yield of tryptophanase was also not the result of the engineered histidine tag. The tryptophanase refolding assay may serve as a chaperone activity assay for trigger factor or its mutants in the future.

REFERENCES

1. Crooke, E., et al., *ProOmpA is stabilized for membrane translocation by either purified E. coli trigger factor or canine signal recognition particle.* Cell, 1988. **54**(7): p. 1003-11.
2. Crooke, E., et al., *ProOmpA spontaneously folds in a membrane assembly competent state which trigger factor stabilizes.* Embo J, 1988. **7**(6): p. 1831-5.
3. Crooke, E. and W. Wickner, *Trigger factor: a soluble protein that folds pro-OmpA into a membrane-assembly-competent form.* Proc Natl Acad Sci U S A, 1987. **84**(15): p. 5216-20.
4. Stoller, G., et al., *A ribosome-associated peptidyl-prolyl cis/trans isomerase identified as the trigger factor.* Embo J, 1995. **14**(20): p. 4939-48.
5. Kandror, O., et al., *Trigger factor is involved in GroEL-dependent protein degradation in Escherichia coli and promotes binding of GroEL to unfolded proteins.* Embo J, 1995. **14**(23): p. 6021-7.
6. Deuerling, E., et al., *Trigger factor and DnaK cooperate in folding of newly synthesized proteins.* Nature, 1999. **400**(6745): p. 693-6.
7. Valent, Q.A., et al., *Early events in preprotein recognition in E. coli: interaction of SRP and trigger factor with nascent polypeptides.* Embo J, 1995. **14**(22): p. 5494-505.
8. Kramer, G., et al., *L23 protein functions as a chaperone docking site on the ribosome.* Nature, 2002. **419**(6903): p. 171-4.
9. Zarnt, T., et al., *Modular structure of the trigger factor required for high activity in protein folding.* J Mol Biol, 1997. **271**(5): p. 827-37.

10. Van Duyne, G.D., et al., *Atomic structure of FKBP-FK506, an immunophilin-immunosuppressant complex.* Science, 1991. **252**(5007): p. 839-42.

11. Michnick, S.W., et al., *Solution structure of FKBP, a rotamase enzyme and receptor for FK506 and rapamycin.* Science, 1991. **252**(5007): p. 836-9.

12. Kramer, G., et al., *Trigger factor peptidyl-prolyl cis/trans isomerase activity is not essential for the folding of cytosolic proteins in Escherichia coli.* J Biol Chem, 2004. **279**(14): p. 14165-70.

13. Vogtherr, M., et al., *NMR solution structure and dynamics of the peptidyl-prolyl cis-trans isomerase domain of the trigger factor from Mycoplasma genitalium compared to FK506-binding protein.* J Mol Biol, 2002. **318**(4): p. 1097-115.

14. Ludlam, A.V., B.A. Moore, and Z. Xu, *The crystal structure of ribosomal chaperone trigger factor from Vibrio cholerae.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13436-41.

15. Ferbitz, L., et al., *Trigger factor in complex with the ribosome forms a molecular cradle for nascent proteins.* Nature, 2004. **431**(7008): p. 590-6.

16. Merz, F., et al., *The C-terminal domain of Escherichia coli trigger factor represents the central module of its chaperone activity.* J Biol Chem, 2006. **281**(42): p. 31963-71.

17. Martinez-Hackert, E. and W.A. Hendrickson, *Structures of and interactions between domains of trigger factor from Thermotoga maritima.* Acta Crystallogr D Biol Crystallogr, 2007. **63**(Pt 4): p. 536-47.

18. Lakshmipathy, S.K., et al., *Identification of nascent chain interaction sites on trigger factor.* J Biol Chem, 2007.

19. Schlunzen, F., et al., *The binding mode of the trigger factor on the ribosome: implications for protein folding and SRP interaction.* Structure, 2005. **13**(11): p. 1685-94.

20. Baram, D., et al., *Structure of trigger factor binding domain in biologically homologous complex with eubacterial ribosome reveals its chaperone action.* Proc Natl Acad Sci U S A, 2005. **102**(34): p. 12017-22.

21. Kristensen, O. and M. Gajhede, *Chaperone binding at the ribosomal exit tunnel.* Structure, 2003. **11**(12): p. 1547-56.

22. Blaha, G., et al., *Localization of the trigger factor binding site on the ribosomal 50S subunit.* J Mol Biol, 2003. **326**(3): p. 887-97.

23. Kaiser, C.M., et al., *Real-time observation of trigger factor function on translating ribosomes.* Nature, 2006. **444**(7118): p. 455-60.

24. Patzelt, H., et al., *Three-state equilibrium of Escherichia coli trigger factor.* Biol Chem, 2002. **383**(10): p. 1611-9.

25. Stewart, D.E., A. Sarkar, and J.E. Wampler, *Occurrence and role of cis peptide bonds in protein structures.* J Mol Biol, 1990. **214**(1): p. 253-60.

26. Kiefhaber, T., et al., *Folding of ribonuclease T1. 2. Kinetic models for the folding and unfolding reactions.* Biochemistry, 1990. **29**(12): p. 3061-70.

27. Kofron, J.L., et al., *Determination of kinetic constants for peptidyl prolyl cis-trans isomerases by an improved spectrophotometric assay.* Biochemistry, 1991. **30**(25): p. 6127-34.

28. Zhao, Y. and H. Ke, *Crystal structure implies that cyclophilin predominantly catalyzes*

the trans to cis isomerization. Biochemistry, 1996. **35**(23): p. 7356-61.

29. Harding, M.W., et al., *A receptor for the immunosuppressant FK506 is a cis-trans peptidyl-prolyl isomerase.* Nature, 1989. **341**(6244): p. 758-60.

30. Ranganathan, R., et al., *Structural and functional analysis of the mitotic rotamase Pin1 suggests substrate recognition is phosphorylation dependent.* Cell, 1997. **89**(6): p. 875-86.

31. Pahl, A., K. Brune, and H. Bang, *Fit for life? Evolution of chaperones and folding catalysts parallels the development of complex organisms.* Cell Stress Chaperones, 1997. **2**(2): p. 78-86.

32. Liu, J., C.M. Chen, and C.T. Walsh, *Human and Escherichia coli cyclophilins: sensitivity to inhibition by the immunosuppressant cyclosporin A correlates with a specific tryptophan residue.* Biochemistry, 1991. **30**(9): p. 2306-10.

33. Bierer, B.E., et al., *Two distinct signal transmission pathways in T lymphocytes are inhibited by complexes formed between an immunophilin and either FK506 or rapamycin.* Proc Natl Acad Sci U S A, 1990. **87**(23): p. 9231-5.

34. Clardy, J., *The chemistry of signal transduction.* Proc Natl Acad Sci U S A, 1995. **92**(1): p. 56-61.

35. Schreiber, S.L. and G.R. Crabtree, *The mechanism of action of cyclosporin A and FK506.* Immunol Today, 1992. **13**(4): p. 136-42.

36. Lu, K.P., S.D. Hanes, and T. Hunter, *A human peptidyl-prolyl isomerase essential for regulation of mitosis.* Nature, 1996. **380**(6574): p. 544-7.

37. Lazar, S.W. and R. Kolter, *SurA assists the folding of Escherichia coli outer*

membrane proteins. J Bacteriol, 1996. **178**(6): p. 1770-3.

38. Dartigalongue, C. and S. Raina, *A new heat-shock gene, ppiD, encodes a peptidyl-prolyl isomerase required for folding of outer membrane proteins in Escherichia coli.* Embo J, 1998. **17**(14): p. 3968-80.

39. Kleerebezem, M., M. Heutink, and J. Tommassen, *Characterization of an Escherichia coli rotA mutant, affected in periplasmic peptidyl-prolyl cis/trans isomerase.* Mol Microbiol, 1995. **18**(2): p. 313-20.

40. Kok, R.G., et al., *A gene of Acinetobacter calcoaceticus BD413 encodes a periplasmic peptidyl-prolyl cis-trans isomerase of the cyclophilin sub-class that is not essential for growth.* Biochim Biophys Acta, 1994. **1219**(3): p. 601-6.

41. Gothel, S.F., et al., *Cyclophilin and trigger factor from Bacillus subtilis catalyze in vitro protein folding and are necessary for viability under starvation conditions.* Biochemistry, 1998. **37**(38): p. 13392-9.

42. Dolinski, K., et al., *All cyclophilins and FK506 binding proteins are, individually and collectively, dispensable for viability in Saccharomyces cerevisiae.* Proc Natl Acad Sci U S A, 1997. **94**(24): p. 13093-8.

43. Stoller, G., et al., *An 11.8 kDa proteolytic fragment of the E. coli trigger factor represents the domain carrying the peptidyl-prolyl cis/trans isomerase activity.* FEBS Lett, 1996. **384**(2): p. 117-22.

44. Hesterkamp, T. and B. Bukau, *Identification of the prolyl isomerase domain of Escherichia coli trigger factor.* FEBS Lett, 1996. **385**(1-2): p. 67-71.

45. Patzelt, H., et al., *Binding specificity of Escherichia coli trigger factor.* Proc Natl Acad

Sci U S A, 2001. **98**(25): p. 14244-9.

46. Schiene-Fischer, C., et al., *Evaluation of similarities in the cis/trans isomerase function of trigger factor and DnaK.* Biol Chem, 2002. **383**(12): p. 1865-73.

47. Teter, S.A., et al., *Polypeptide flux through bacterial Hsp70: DnaK cooperates with trigger factor in chaperoning nascent chains.* Cell, 1999. **97**(6): p. 755-65.

48. Agashe, V.R., et al., *Function of trigger factor and DnaK in multidomain protein folding: increase in yield at the expense of folding speed.* Cell, 2004. **117**(2): p. 199-209.

49. Vorderwulbecke, S., et al., *Low temperature or GroEL/ES overproduction permits growth of Escherichia coli cells lacking trigger factor and DnaK.* FEBS Lett, 2004. **559**(1-3): p. 181-7.

50. Genevaux, P., et al., *In vivo analysis of the overlapping functions of DnaK and trigger factor.* EMBO Rep, 2004. **5**(2): p. 195-200.

51. Deuerling, E., et al., *Trigger Factor and DnaK possess overlapping substrate pools and binding specificities.* Mol Microbiol, 2003. **47**(5): p. 1317-28.

52. Suno, R., et al., *Trigger factor from Thermus thermophilus is a Zn2+-dependent chaperone.* J Biol Chem, 2004. **279**(8): p. 6380-4.

53. Huang, G.C., et al., *Chaperone and antichaperone activities of trigger factor.* Eur J Biochem, 2002. **269**(18): p. 4516-23.

54. Huang, G.C., et al., *Assisted folding of D-glyceraldehyde-3-phosphate dehydrogenase by trigger factor.* Protein Sci, 2000. **9**(6): p. 1254-61.

55. Liu, C.P., S. Perrett, and J.M. Zhou, *Dimeric trigger factor stably binds*

folding-competent intermediates and cooperates with the DnaK-DnaJ-GrpE chaperone system to allow refolding. J Biol Chem, 2005. **280**(14): p. 13315-20.

56. Fanghanel, J., *Enzymatic catalysis of the peptidyl-prolyl bond rotation: are transition state formation and enzyme dynamics directly linked?* Angew Chem Int Ed Engl, 2003. **42**(5): p. 490-2.

57. W. A. Wood, I.C.G., and W. W. Umbreit, *FUNCTION OF PYRIDOXAL PHOSPHATE: RESOLUTION AND PURIFICATION OF THE TRYPTOPHANASE ENZYME OF ESCHERICHIA COLI.* J. Biol. Chem., 1947. **170**(1): p. 313.

58. Morino, Y. and E.E. Snell, *The subunit structure of tryptophanase. I. The effect of pyridoxal phosphate on the subunit structure and physical properties of tryptophanase.* J Biol Chem, 1967. **242**(23): p. 5591-601.

59. Newton, W.A., Y. Morino, and E.E. Snell, *Properties of Crystalline Tryptophanase.* J Biol Chem, 1965. **240**: p. 1211-8.

60. Newton, W.A. and E.E. Snell, *Catalytic Properties of Tryptophanase, a Multifunctional Pyridoxal Phosphate Enzyme.* Proc Natl Acad Sci U S A, 1964. **51**: p. 382-9.

61. Morino, Y. and E.E. Snell, *The relation of spectral changes and tritium exchange reactions to the mechanism of tryptophanase-catalyzed reactions.* J Biol Chem, 1967. **242**(12): p. 2800-9.

62. Phillips, R.S., T.V. Demidkina, and N.G. Faleev, *Structure and mechanism of tryptophan indole-lyase and tyrosine phenol-lyase.* Biochim Biophys Acta, 2003. **1647**(1-2): p. 167-72.

63. Phillips, R.S., N. Johnson, and A.V. Kamath, *Formation in vitro of hybrid dimers of H463F and Y74F mutant Escherichia coli tryptophan indole-lyase rescues activity with L-tryptophan.* Biochemistry, 2002. **41**(12): p. 4012-9.

64. Kiick, D.M. and R.S. Phillips, *Mechanistic deductions from multiple kinetic and solvent deuterium isotope effects and pH studies of pyridoxal phosphate dependent carbon-carbon lyases: Escherichia coli tryptophan indole-lyase.* Biochemistry, 1988. **27**(19): p. 7339-44.

65. Kiick, D.M. and R.S. Phillips, *Mechanistic deductions from kinetic isotope effects and pH studies of pyridoxal phosphate dependent carbon-carbon lyases: Erwinia herbicola and Citrobacter freundii tyrosine phenol-lyase.* Biochemistry, 1988. **27**(19): p. 7333-8.

66. Phillips, R.S., E.W. Miles, and L.A. Cohen, *Differential inhibition of tryptophan synthase and of tryptophanase by the two diastereoisomers of 2,3-dihydro-L-tryptophan. Implications for the stereochemistry of the reaction intermediates.* J Biol Chem, 1985. **260**(27): p. 14665-70.

67. Phillips, R.S., E.W. Miles, and L.A. Cohen, *Interactions of tryptophan synthase, tryptophanase, and pyridoxal phosphate with oxindolyl-L-alanine and 2,3-dihydro-L-tryptophan: support for an indolenine intermediate in tryptophan metabolism.* Biochemistry, 1984. **23**(25): p. 6228-34.

68. Gopinathan, K.P. and R.D. DeMoss, *Reversible dissociation of tryptophanase into protein subunits.* Proc Natl Acad Sci U S A, 1966. **56**(4): p. 1223-5.

69. Cowell, J.L. and R.D. DeMoss, *Trytophanase from Aeromonas liquefaciens. Subunit*

structure and aggregation of the enzyme into enzymatically active polymeric species. J Biol Chem, 1973. **248**(18): p. 6262-9.

70. Raibaud, O. and M.E. Goldberg, *The dissociated tryptophanase subunit is inactive.* J Biol Chem, 1976. **251**(9): p. 2820-4.

71. Morino, Y. and E.E. Snell, *The subunit structure of tryptophanase. II. A correlation of ultracentrifugal and chemical studies.* J Biol Chem, 1967. **242**(23): p. 5602-10.

72. Skrzynia, C., J. London, and M.E. Goldberg, *The tryptophanase from Escherichia coli K12. 3. Further characterization of hybrids between the apo- and holoenzyme.* J Biol Chem, 1974. **249**(7): p. 2325-6.

73. Raibaud, O. and M.E. Goldberg, *Structural and functional interdependence of the protomers of Escherichia coli K 12 tryptophanase during binding of pyridoxal 5'-phosphate.* J Biol Chem, 1976. **251**(9): p. 2814-9.

74. Raibaud, O. and M.E. Goldberg, *The reactivity of one essential cysteine as a conformational probe in Escherichia coli tryptophanase. Application to the study of the structural influence of subunit interactions.* Eur J Biochem, 1977. **73**(2): p. 591-9.

75. Dunathan, H.C., *Conformation and reaction specificity in pyridoxal phosphate enzymes.* Proc Natl Acad Sci U S A, 1966. **55**(4): p. 712-6.

76. Isupov, M.N., et al., *Crystal structure of tryptophanase.* J Mol Biol, 1998. **276**(3): p. 603-23.

77. Hogberg-Raibaud, A., O. Raibaud, and M.E. Goldberg, *Kinetic and equilibrium studies on the activation of Escherichia coli K12 tryptophanase by pyridoxal 5'-phosphate and monovalent cations.* J Biol Chem, 1975. **250**(9): p. 3352-8.

78. Phillips, R.S. and P.D. Gollnick, *Evidence that cysteine 298 is in the active site of tryptophan indole-lyase.* J Biol Chem, 1989. **264**(18): p. 10627-32.

79. Honda, T., et al., *Characterization of the reactivity of sulphydryl groups in tryptophanase by a dual-monitoring high-performance liquid chromatographic system with a site-directed fluorescent reagent.* J Chromatogr, 1986. **371**: p. 353-60.

80. Ku, S.Y., P. Yip, and P.L. Howell, *Structure of Escherichia coli tryptophanase.* Acta Crystallogr D Biol Crystallogr, 2006. **62**(Pt 7): p. 814-23.

81. Phillips, R.S. and K.J. Doshi, *Cleavage of Escherichia coli tryptophan indole-lyase by trypsin at Lys406 affects the transmission of conformational changes associated with monovalent cation activation.* Eur J Biochem, 1998. **255**(2): p. 508-15.

82. Shaw, M.K., A.G. Marr, and J.L. Ingraham, *Determination of the minimal temperature for growth of Escherichia coli.* J Bacteriol, 1971. **105**(2): p. 683-4.

83. Kandror, O., A. DeLeon, and A.L. Goldberg, *Trehalose synthesis is induced upon exposure of Escherichia coli to cold and is essential for viability at low temperatures.* Proc Natl Acad Sci U S A, 2002. **99**(15): p. 9727-32.

84. Strocchi, M., et al., *Low temperature-induced systems failure in Escherichia coli: insights from rescue by cold-adapted chaperones.* Proteomics, 2006. **6**(1): p. 193-206.

85. Privalov, P.L., et al., *Cold denaturation of myoglobin.* J Mol Biol, 1986. **190**(3): p. 487-98.

86. Graziano, G., et al., *A reassessment of the molecular origin of cold denaturation.* J Biochem (Tokyo), 1997. **122**(2): p. 395-401.

87. Jones, P.G., R.A. VanBogelen, and F.C. Neidhardt, *Induction of proteins in response to*

low temperature in Escherichia coli. J Bacteriol, 1987. **169**(5): p. 2092-5.

88. Jones, P.G., et al., *DNA gyrase, CS7.4, and the cold shock response in Escherichia coli.* J Bacteriol, 1992. **174**(18): p. 5798-802.

89. Perrot, F., et al., *Protein synthesis in Escherichia coli at 4 degrees C.* Electrophoresis, 2000. **21**(8): p. 1625-9.

90. Goldstein, J., N.S. Pollitt, and M. Inouye, *Major cold shock protein of Escherichia coli.* Proc Natl Acad Sci U S A, 1990. **87**(1): p. 283-7.

91. Ideno, A., et al., *FK506-binding protein of the hyperthermophilic archaeum, Thermococcus sp. KS-1, a cold-shock-inducible peptidyl-prolyl cis-trans isomerase with activities to trap and refold denatured proteins.* Biochem J, 2001. **357**(Pt 2): p. 465-71.

92. Goodchild, A., et al., *A proteomic determination of cold adaptation in the Antarctic archaeon, Methanococcoides burtonii.* Mol Microbiol, 2004. **53**(1): p. 309-21.

93. Boonyaratanakornkit, B.B., et al., *Transcriptional profiling of the hyperthermophilic methanarchaeon Methanococcus jannaschii in response to lethal heat and non-lethal cold shock.* Environ Microbiol, 2005. **7**(6): p. 789-97.

94. Suzuki, Y., et al., *Possible involvement of an FKBP family member protein from a psychrotrophic bacterium Shewanella sp. SIB1 in cold-adaptation.* Eur J Biochem, 2004. **271**(7): p. 1372-81.

95. Graumann, P., et al., *Cold shock stress-induced proteins in Bacillus subtilis.* J Bacteriol, 1996. **178**(15): p. 4611-9.

96. Kandror, O. and A.L. Goldberg, *Trigger factor is induced upon cold shock and*

enhances viability of Escherichia coli at low temperatures. Proc Natl Acad Sci U S A, 1997. **94**(10): p. 4978-81.

97. Bradford, M.M., *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding.* Anal Biochem, 1976. **72**: p. 248-54.

98. Tomoyasu, T., et al., *Genetic dissection of the roles of chaperones and proteases in protein folding and degradation in the Escherichia coli cytosol.* Mol Microbiol, 2001. **40**(2): p. 397-413.

99. Rabilloud, T., *Mechanisms of protein silver staining in polyacrylamide gels: a 10-year synthesis.* Electrophoresis, 1990. **11**(10): p. 785-94.

100. Lim, H., et al., *Identification of 2D-gel proteins: a comparison of MALDI/TOF peptide mass mapping to mu LC-ESI tandem mass spectrometry.* J Am Soc Mass Spectrom, 2003. **14**(9): p. 957-70.

101. Pappin, D.J., P. Hojrup, and A.J. Bleasby, *Rapid identification of proteins by peptide-mass fingerprinting.* Curr Biol, 1993. **3**(6): p. 327-32.

102. Nishihara, J.C. and K.M. Champion, *Quantitative evaluation of proteins in one- and two-dimensional polyacrylamide gels using a fluorescent stain.* Electrophoresis, 2002. **23**(14): p. 2203-15.

103. Rahfeld, J.U., et al., *Isolation and amino acid sequence of a new 22-kDa FKBP-like peptidyl-prolyl cis/trans-isomerase of Escherichia coli. Similarity to Mip-like proteins of pathogenic bacteria.* J Biol Chem, 1996. **271**(36): p. 22130-8.

104. Blankenhorn, D., J. Phillips, and J.L. Slonczewski, *Acid- and base-induced proteins*

during aerobic and anaerobic growth of Escherichia coli revealed by two-dimensional gel electrophoresis. J Bacteriol, 1999. **181**(7): p. 2209-16.

105. Yamagata, A., et al., *Mapping of phosphorylated proteins on two-dimensional polyacrylamide gels using protein phosphatase.* Proteomics, 2002. **2**(9): p. 1267-76.

106. Combet C., B.C., Geourjon C. and Deléage G., *Network Protein Sequence Analysis.* TIBS 2000. **25**(291): p. 147-150.

107. Halligan, B.D., et al., *ProMoST (Protein Modification Screening Tool): a web-based tool for mapping protein modifications on two-dimensional gels.* Nucleic Acids Res, 2004. **32**(Web Server issue): p. W638-44.

108. Metzler, C.M., R. Viswanath, and D.E. Metzler, *Equilibria and absorption spectra of tryptophanase.* J Biol Chem, 1991. **266**(15): p. 9374-81.

109. Chen, S.S. and P.C. Engel, *Dogfish M4 lactate dehydrogenase: reversible inactivation by pyridoxal 5'-phosphate and complete protection in complexes that mimic the active ternary complex.* Biochem J, 1975. **151**(2): p. 447-9.

110. Raetz, C.R. and D.S. Auld, *Schiff bases of pyridoxal phosphate with active center lysines of ribonuclease A.* Biochemistry, 1972. **11**(12): p. 2229-36.

111. Valinger, Z., P.C. Engel, and D.E. Metzler, *Is pyridoxal 5'-phosphate an affinity label for phosphate-binding sites in proteins?: The case of bovine glutamate dehydrogenase.* Biochem J, 1993. **294 (Pt 3)**: p. 835-9.

112. Caro M Metzler, A.C., and David E. Metzler, *Equilibria and Absorption Spectra of Schiff Bases.* J. Am. Chem. SOC, 1980. **102**: p. 6075-6082.

113. ENGEL, S.-S.C.a.P.C., *The Equilibrium Position of the Reaction of Bovine Liver*

Glutamate Dehydrogenase with Pyridoxal 5'-Phosphate. Biochem. J., 1975. **147**: p. 351-358.

114. French, T.C., D.S. Auld, and T.C. Bruice, *Catalytic Reactions Involving Azomethines. V. Rates and Equilibria of Imine Formation with 3-Hydroxypyridine-4-Aldehyde and Amino Acids.* Biochemistry, 1965. **4**: p. 77-84.

115. Piszkiewicz, D. and E.L. Smith, *Bovine liver glutamate dehydrogenase. Equilibria and kinetics of inactivation by pyridoxal.* Biochemistry, 1971. **10**(24): p. 4538-44.

116. Phillips, R.S., et al., *Replacement of lysine 269 by arginine in Escherichia coli tryptophan indole-lyase affects the formation and breakdown of quinonoid complexes.* J Biol Chem, 1991. **266**(28): p. 18642-8.

117. Bennion, B.J. and V. Daggett, *The molecular basis for the chemical denaturation of proteins by urea.* Proc Natl Acad Sci U S A, 2003. **100**(9): p. 5142-7.

118. London, J., C. Skrzynia, and M.E. Goldberg, *Renaturation of Escherichia coli tryptophanase after exposure to 8 M urea. Evidence for the existence of nucleation centers.* Eur J Biochem, 1974. **47**(2): p. 409-15.

119. Mizobata, T. and Y. Kawata, *The folding characteristics of tryptophanase from Escherichia coli.* J Biochem (Tokyo), 1995. **117**(2): p. 384-91.

120. Mizobata, T., et al., *Effects of the chaperonin GroE on the refolding of tryptophanase from Escherichia coli. Refolding is enhanced in the presence of ADP.* J Biol Chem, 1992. **267**(25): p. 17773-9.

121. Lill, R., et al., *The "trigger factor cycle" includes ribosomes, presecretory proteins, and the plasma membrane.* Cell, 1988. **54**(7): p. 1013-8.

122. Morino, Y. and E.E. Snell, *A kinetic study of the reaction mechanism of tryptophanase-catalyzed reactions.* J Biol Chem, 1967. **242**(12): p. 2793-9.

123. van der Vies, S.M., et al., *Conformational states of ribulosebisphosphate carboxylase and their interaction with chaperonin 60.* Biochemistry, 1992. **31**(14): p. 3635-44.

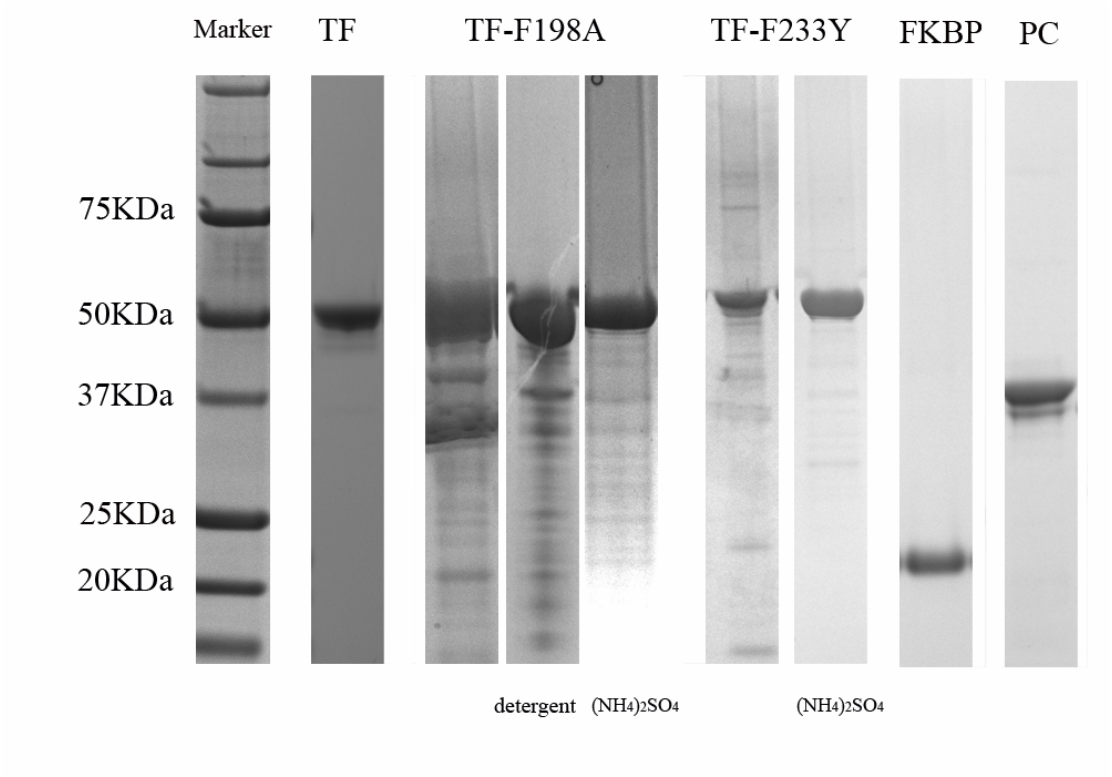
124. Schwarzinger, S., et al., *Random coil chemical shifts in acidic 8 M urea: implementation of random coil shift data in NMRView.* J Biomol NMR, 2000. **18**(1): p. 43-8.

125. Krause, F., *Detection and analysis of protein-protein interactions in organellar and prokaryotic proteomes by native gel electrophoresis: (Membrane) protein complexes and supercomplexes.* Electrophoresis, 2006. **27**(13): p. 2759-81.

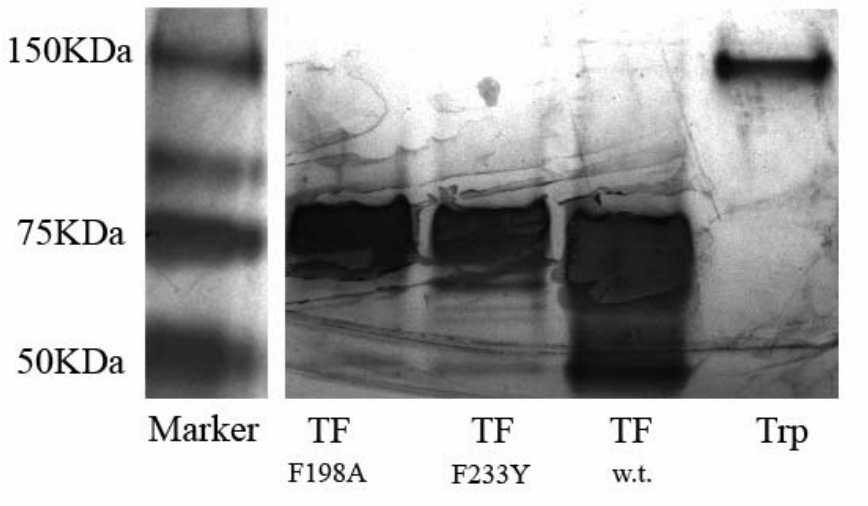
126. Schagger, H., W.A. Cramer, and G. von Jagow, *Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis.* Anal Biochem, 1994. **217**(2): p. 220-30.

127. Schagger, H. and G. von Jagow, *Blue native electrophoresis for isolation of membrane protein complexes in enzymatically active form.* Anal Biochem, 1991. **199**(2): p. 223-31.

Figure 3.1

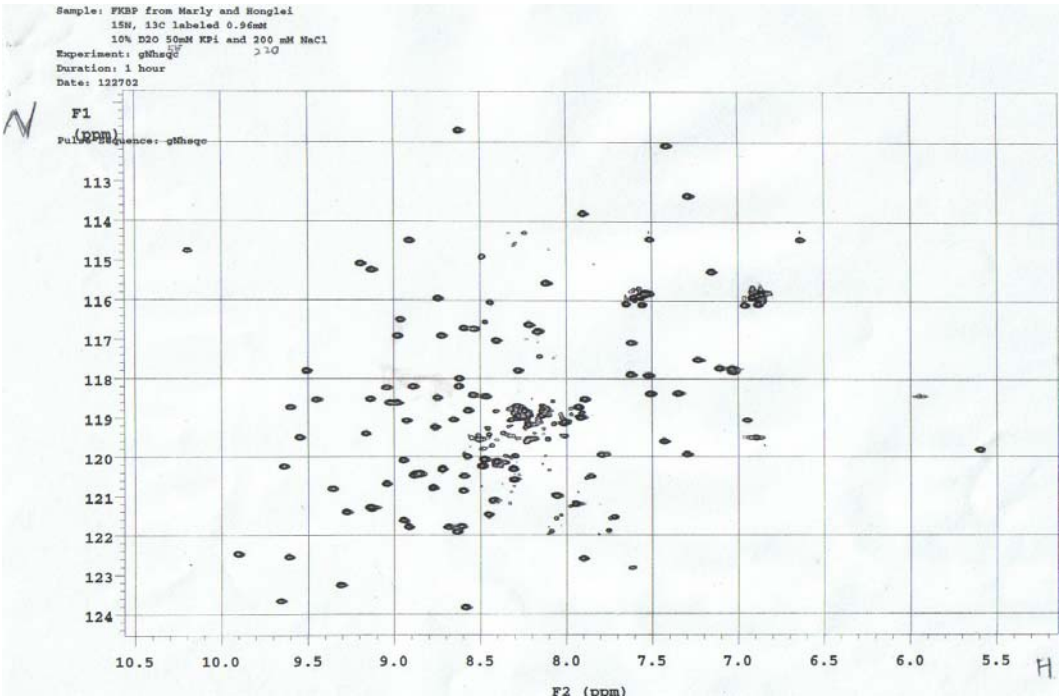


(A) Purified trigger factor, its fragments (FKBP domain and FKBP+C-terminal double domain) and mutants (F198A and F233Y). Purification results for TF-F198A with standard method, with 0.1% Triton X-100 and with 1M ammonium sulfate. Purification results for TF-F233Y with standard method and with 1M ammonium sulfate.

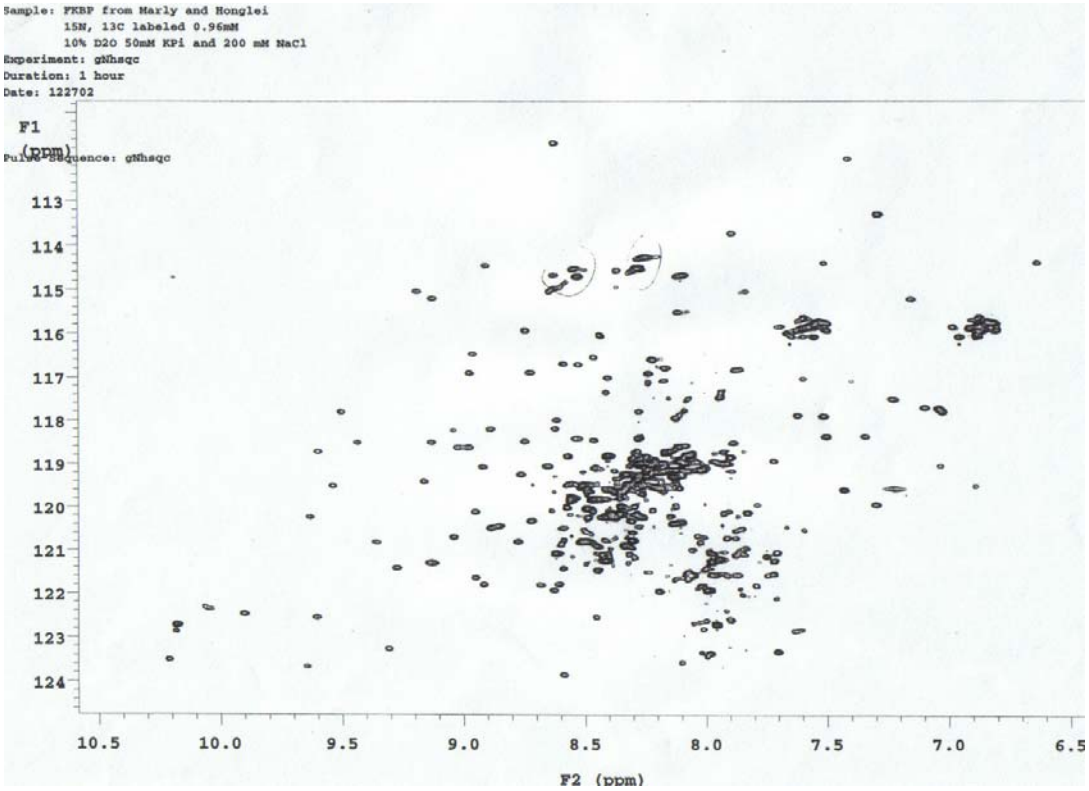


(B) Wild type trigger factor, TF-F233Y and TF-F198A on native gel.

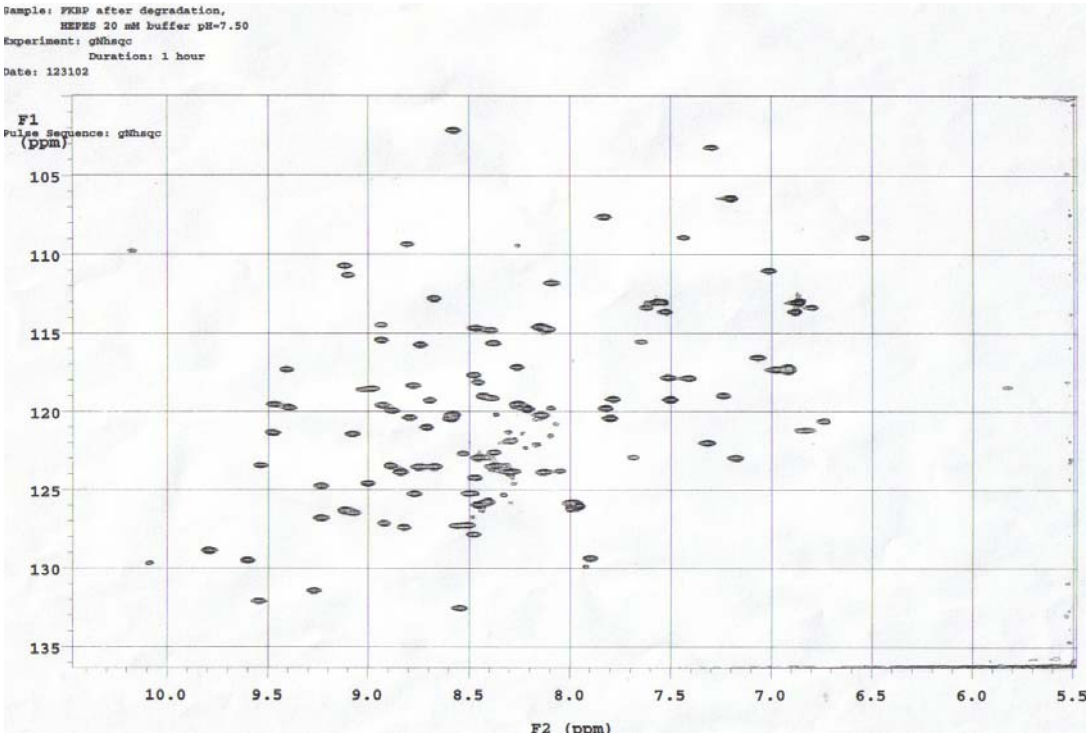
Figure 3.2



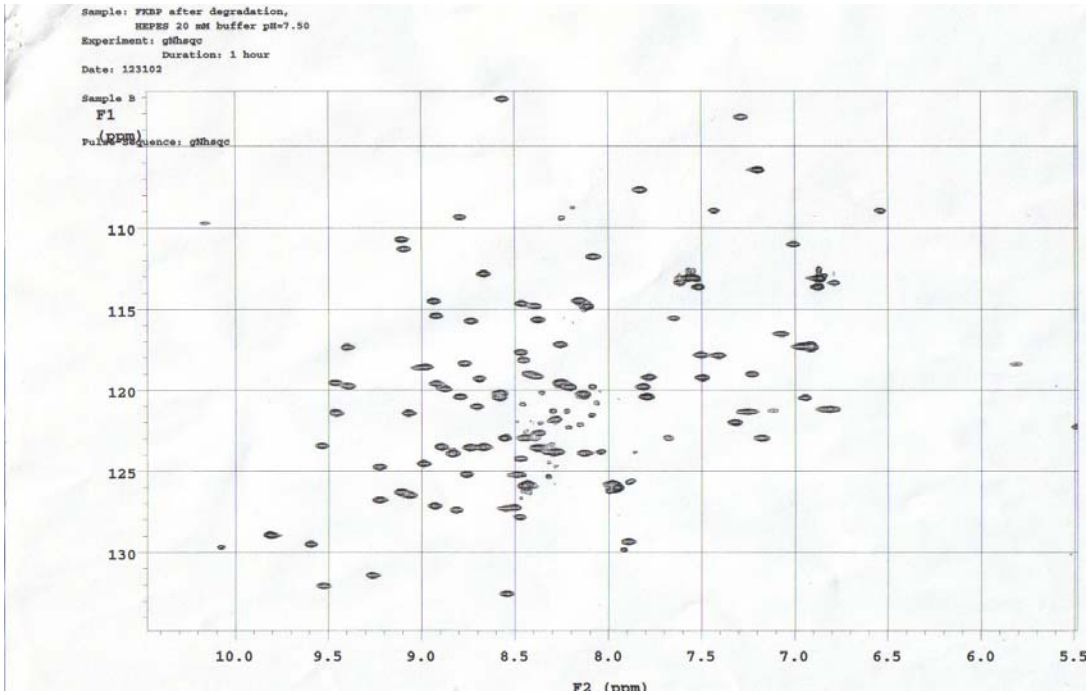
(A) ^{15}N-HSQC spectrum of 1mM TF-FKBP domain sample collected at 298K with a ^{1}H resonance frequency of 600MHz.



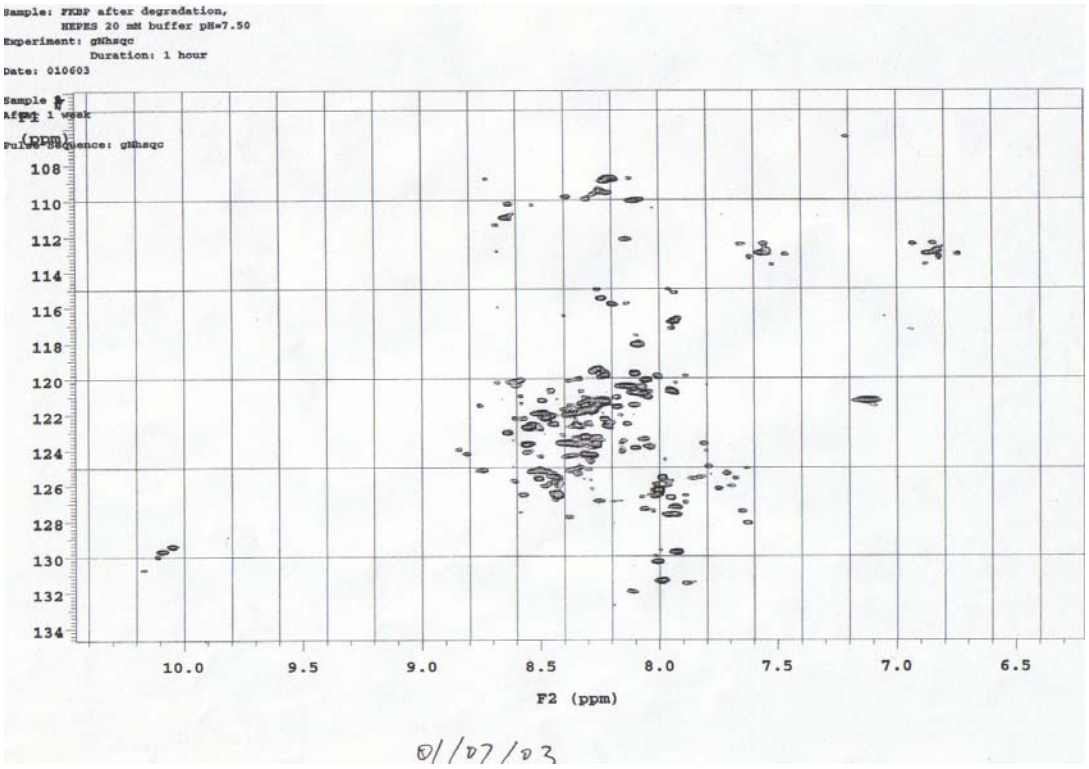
(B) ^{15}N-HSQC spectrum of 1mM TF-FKBP domain sample after incubation at 298K for 71 hours



(C) ^{15}N-HSQC spectrum of refolded TF-FKBP domain sample in fresh HEPES buffer

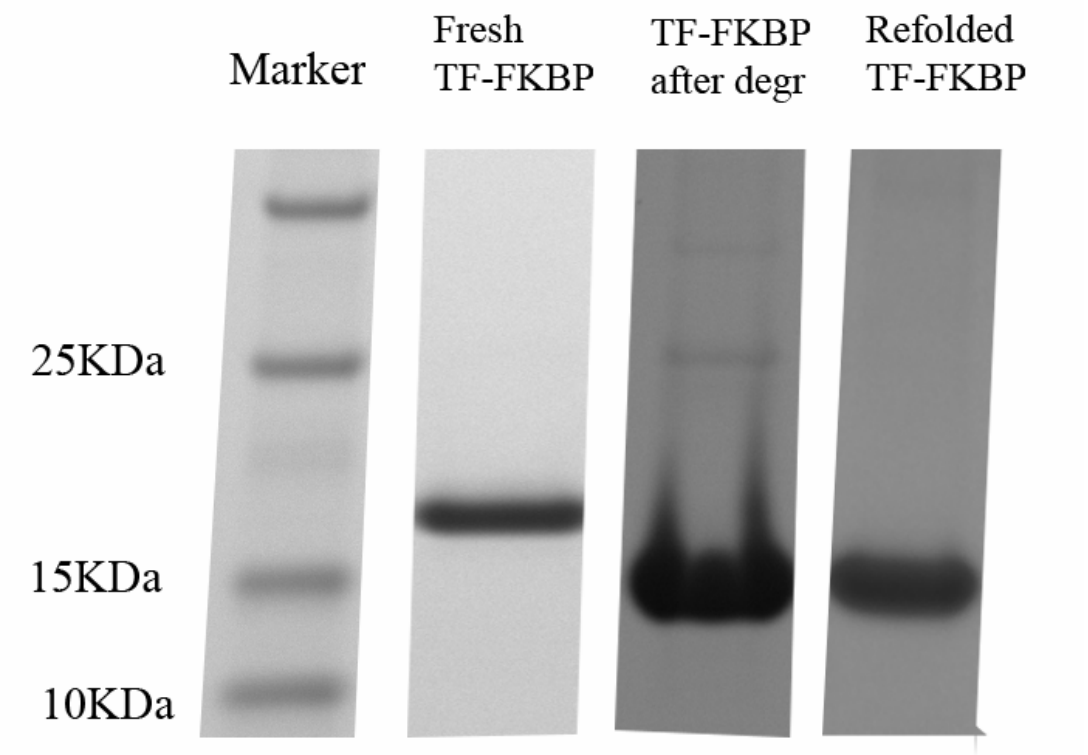


(D) ^{15}N-HSQC spectrum of refolded TF-FKBP domain sample frozen for a week

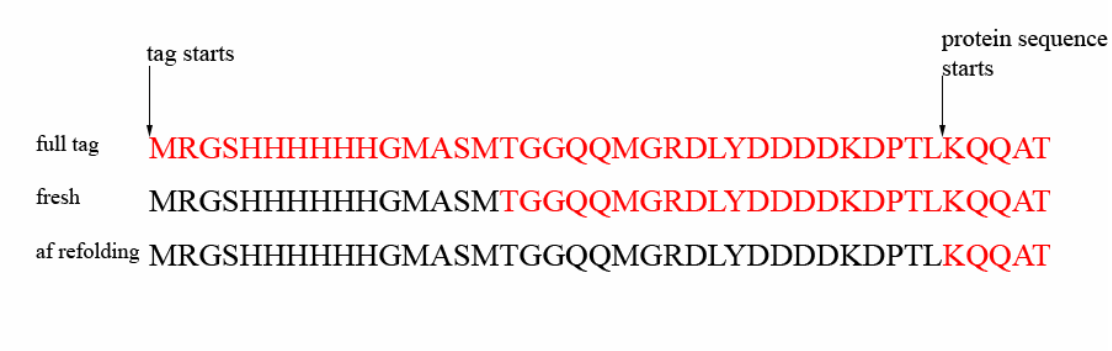


(E) ^{15}N-HSQC spectrum of refolded TF-FKBP domain sample kept at room temperature for a week

Figure 3.3

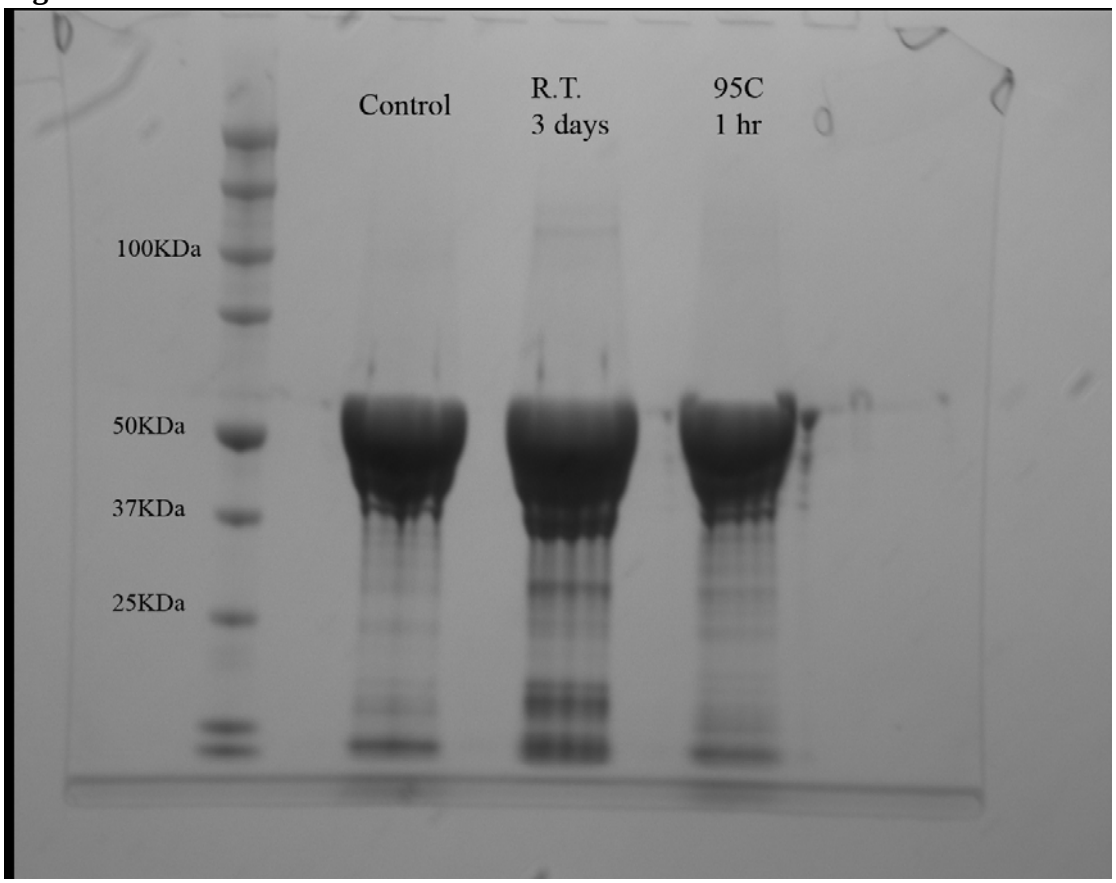


(A) position of TF-FKBP domain on 10-20% Tricine gels before and after degradation. The band on the third lane was from the TF-FKBP after refolding

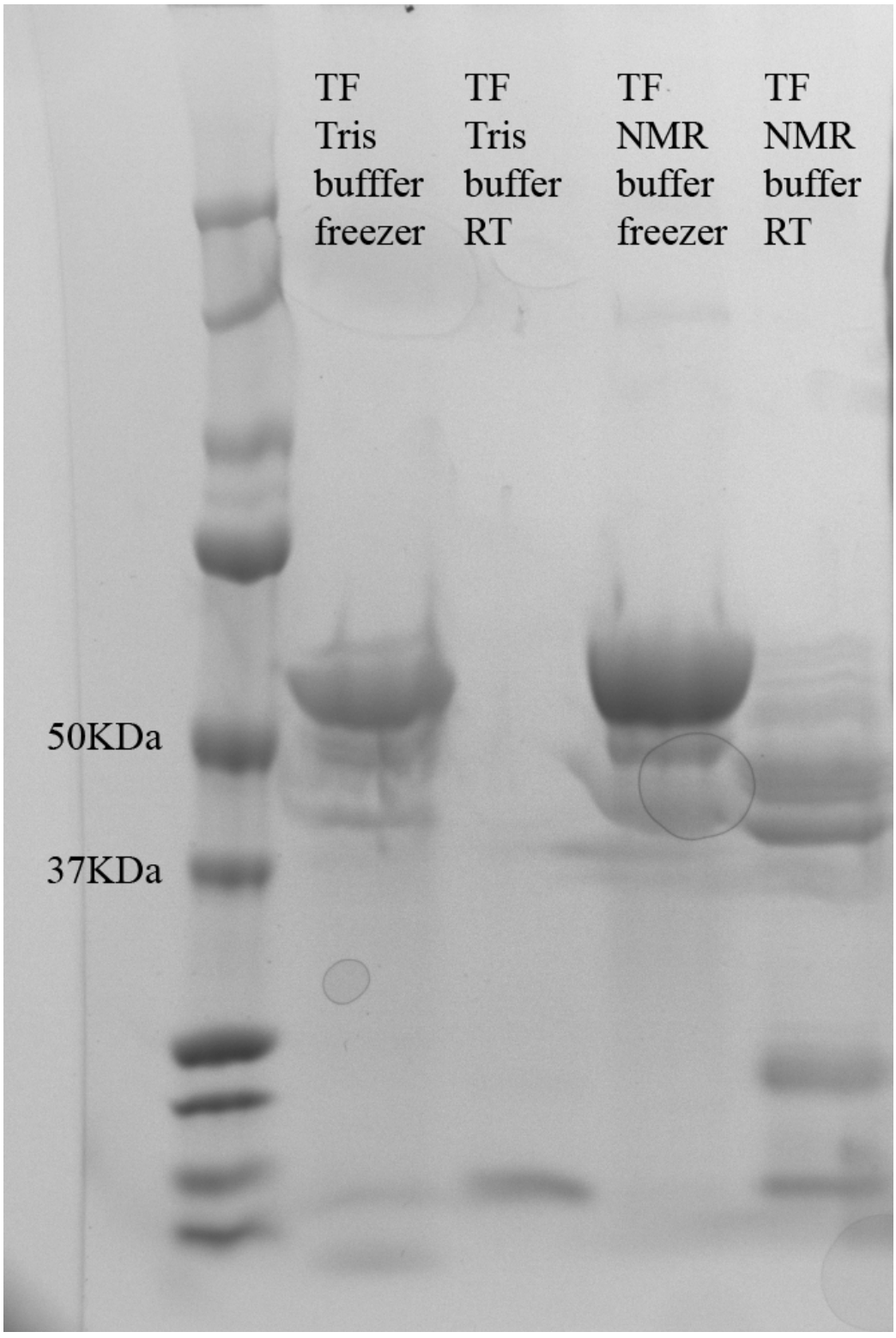


(B) N-terminal sequence (in red) for fresh and refolded TF-FKBP domain sample in **Fig.3.3**.A

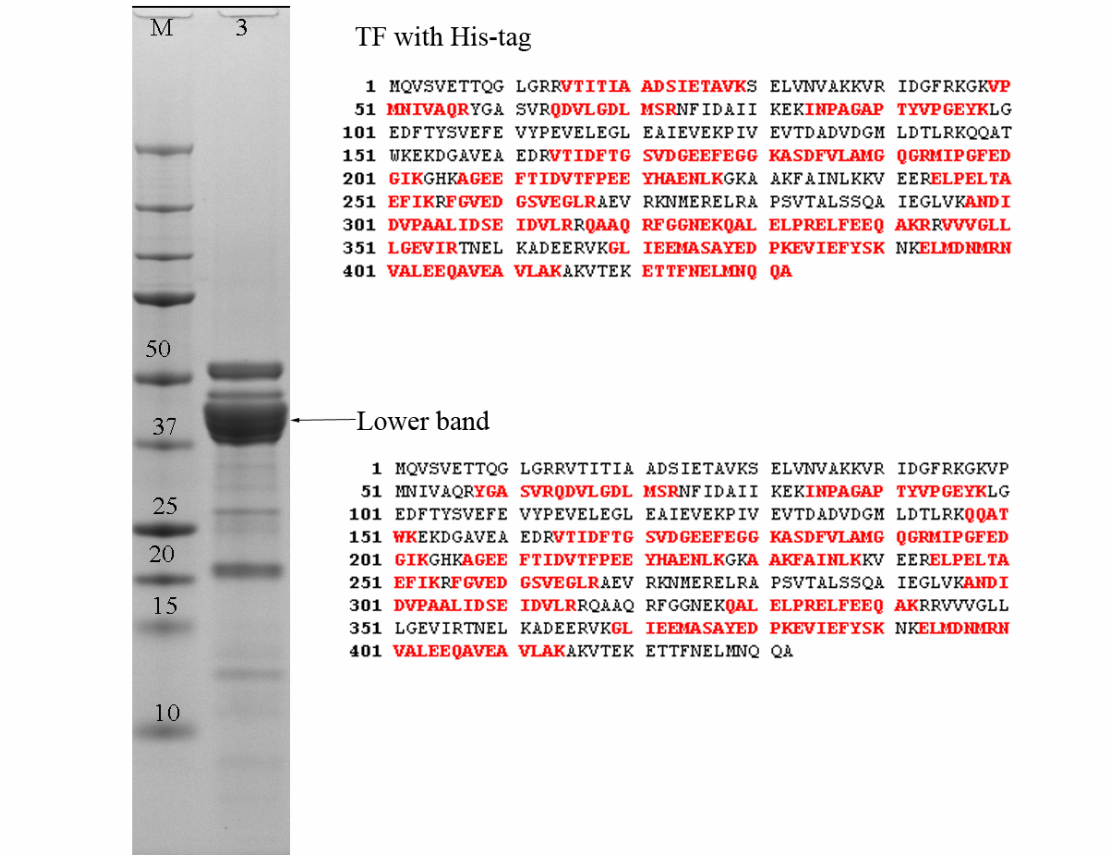
Figure 3.4



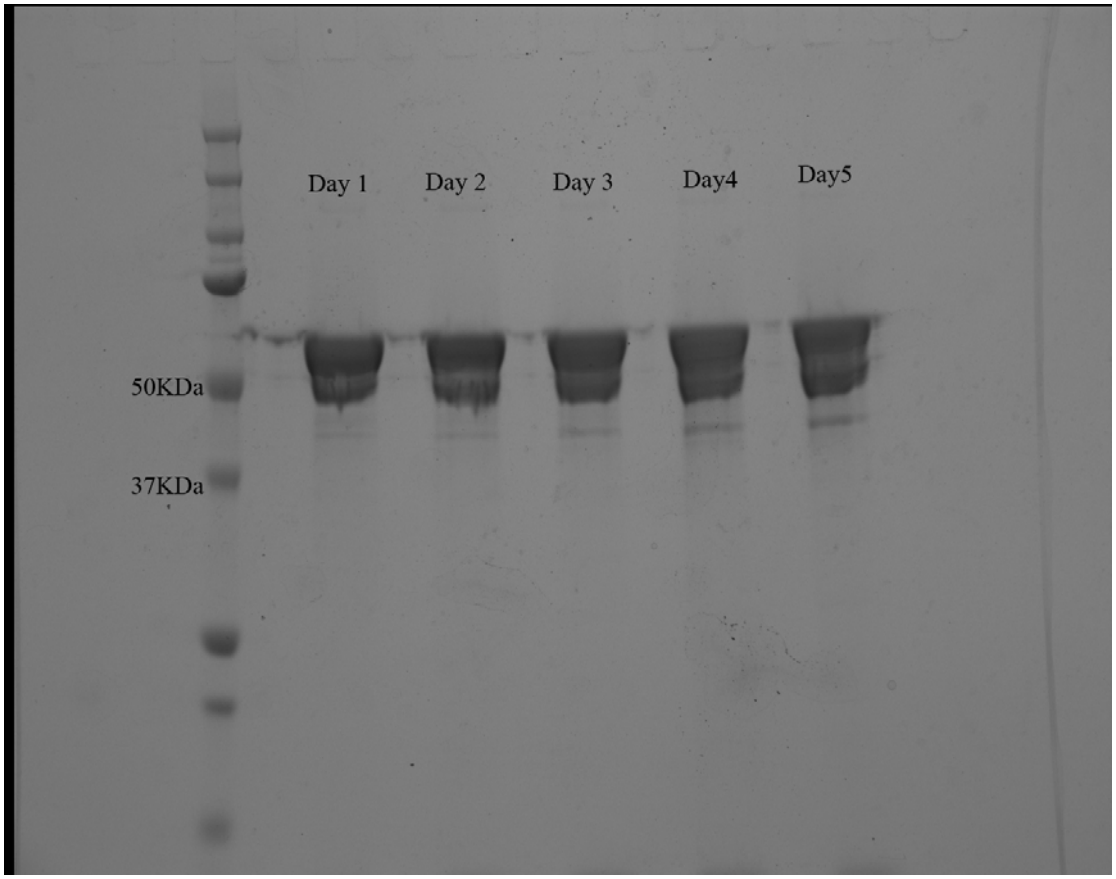
(A) 4-12% Bis-Tris gel for Stock TF, sample after incubation at room temperature for 3 days and sample boiled at 95℃ for one hour.



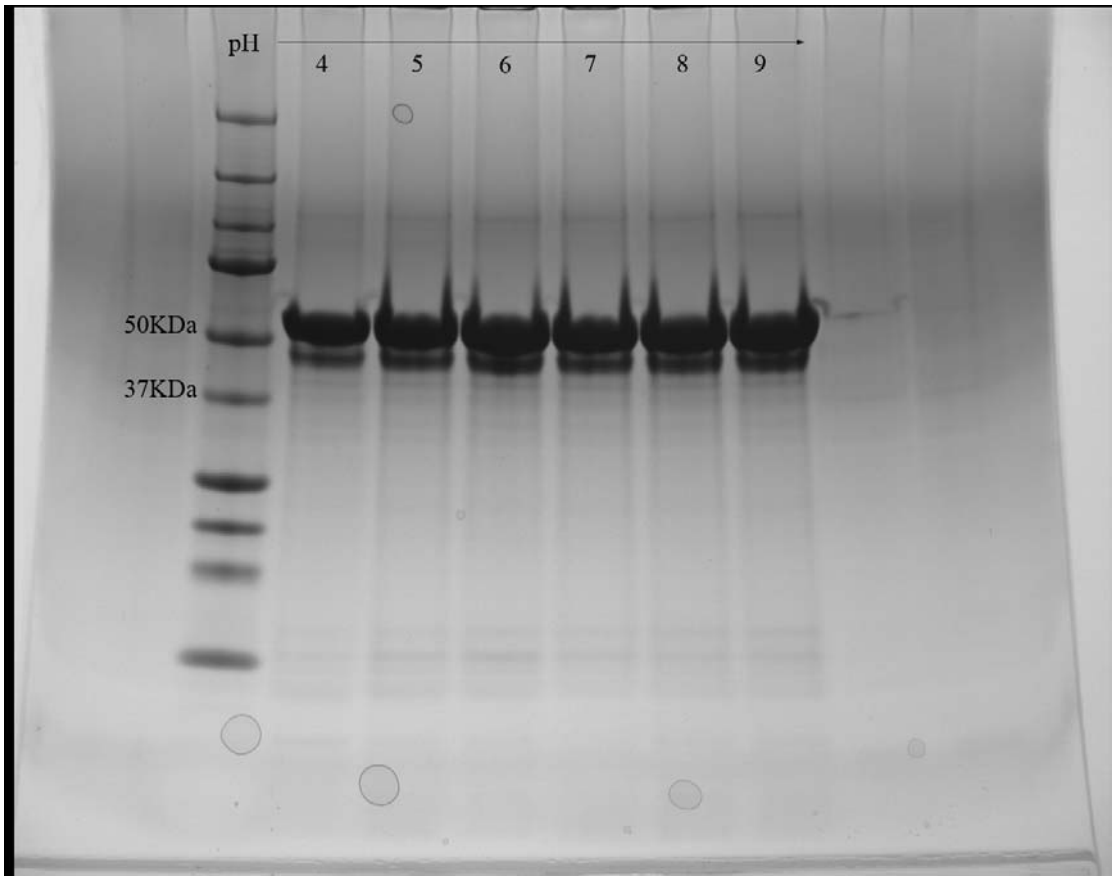
(B) 4-12% Bis-Tris gel for TF sample in Tris and phosphate buffer incubated at room temperature for 10 days



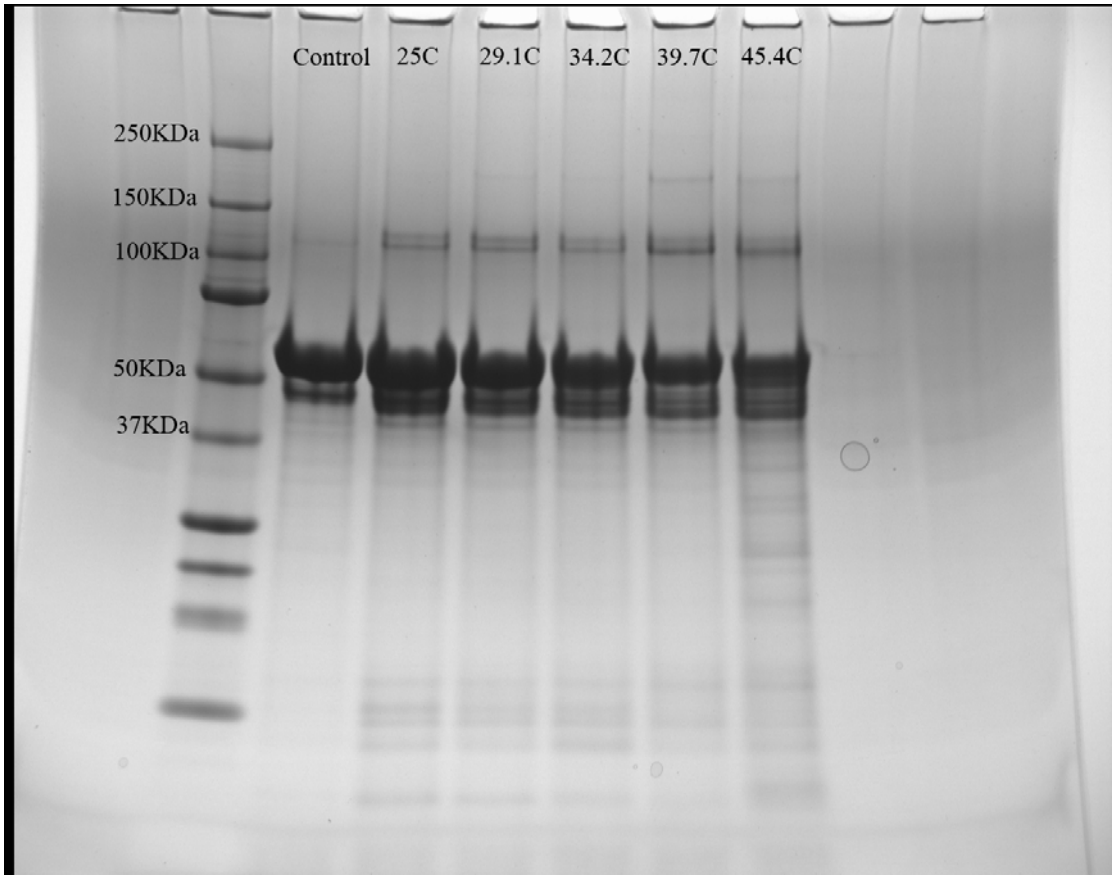
(C) Peptide peaks (in red) identified by MS for degraded TF, compared with full length TF



(D) 4-12% Bis-Tris gel for TF sample incubated at room temperature for a period of 5 days

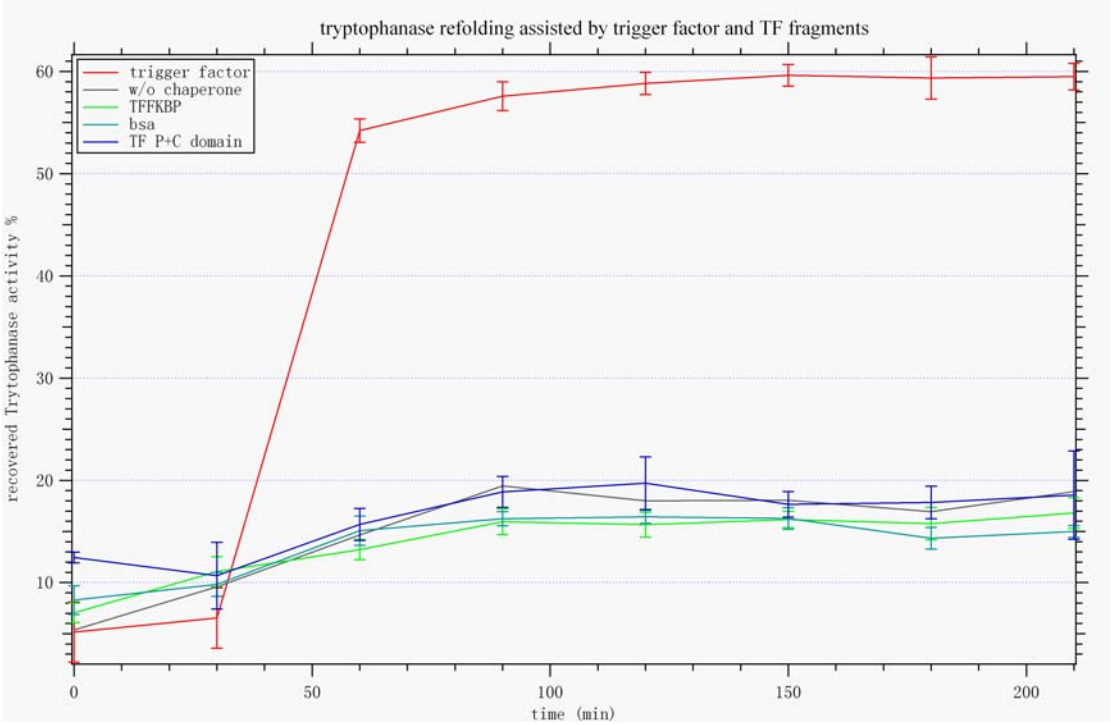


(E) 4-12% Bis-Tris gel for TF in phosphate buffers with different pH

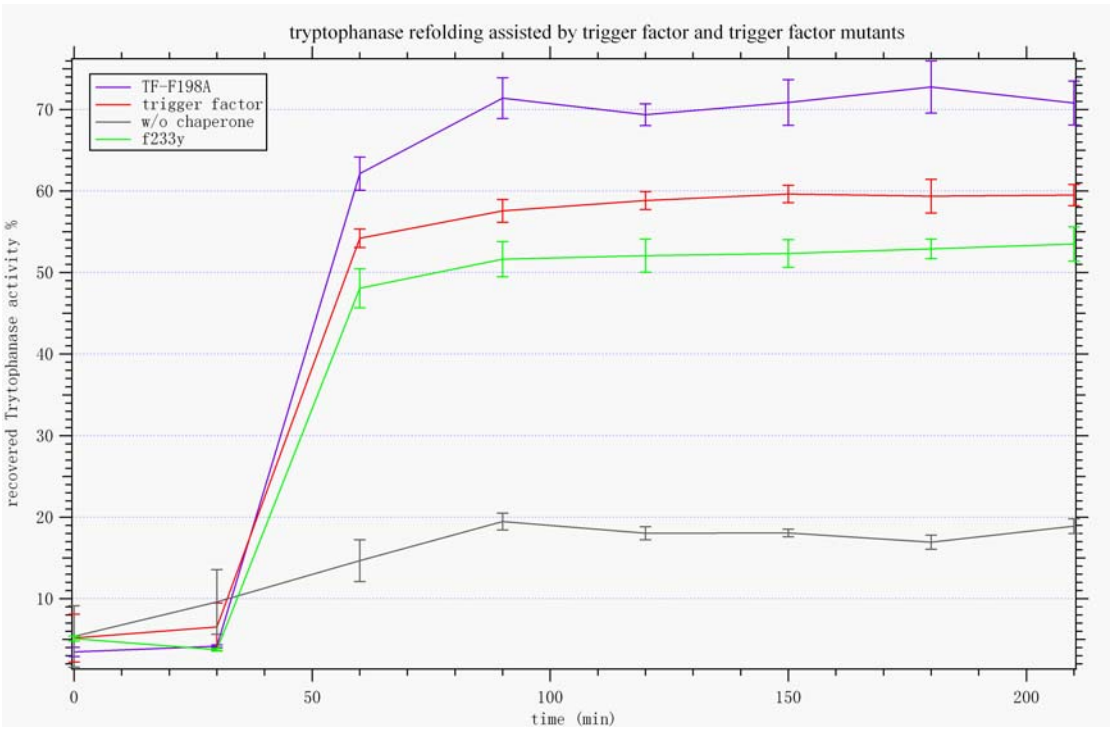


(F) Gel for TF in phosphate buffers incubated at different temperatures for 24 hours

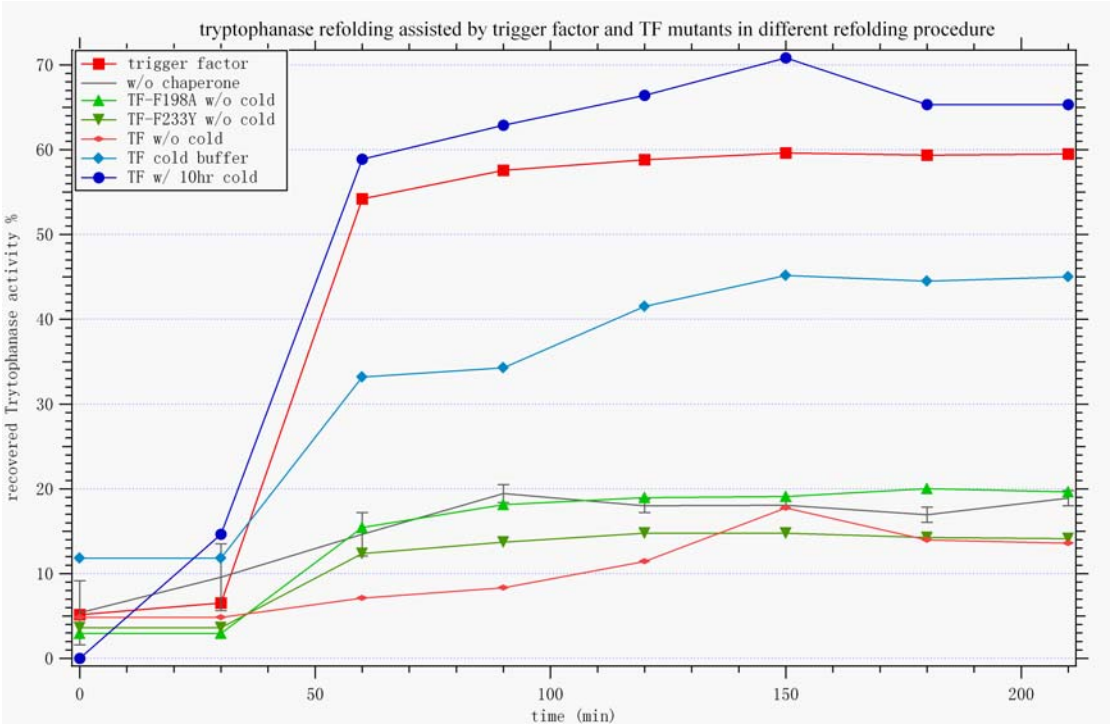
Figure 3.5



(A) Recovered activity of tryptophanase in buffer only, with 14 fold of trigger factor, TF-FKBP, TF-FKBP+C-terminal double domain and BSA.

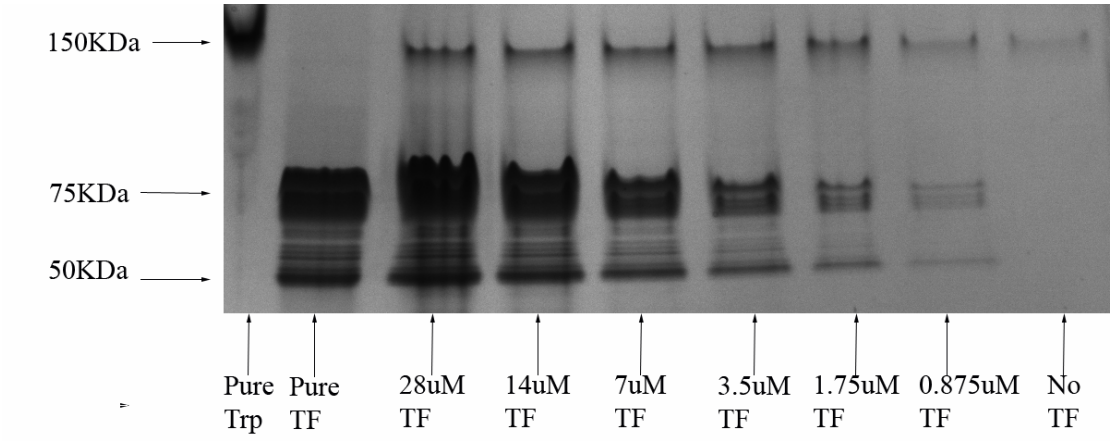


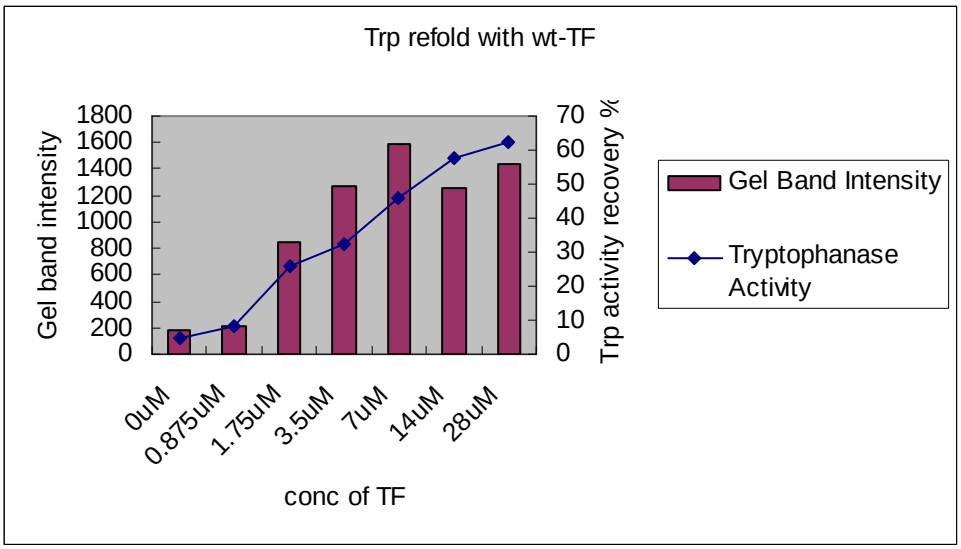
(B) Recovered activity of tryptophanase in buffer only, with 14 fold of trigger factor, TF-F198A and TF-F233Y.



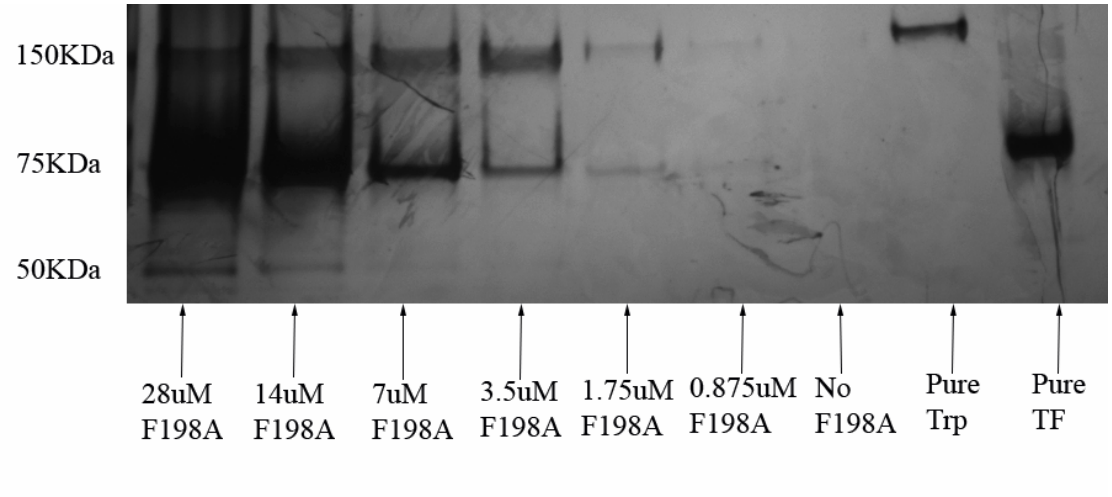
(C) Recovered activity of tryptophanase with different duration of cold temperature.

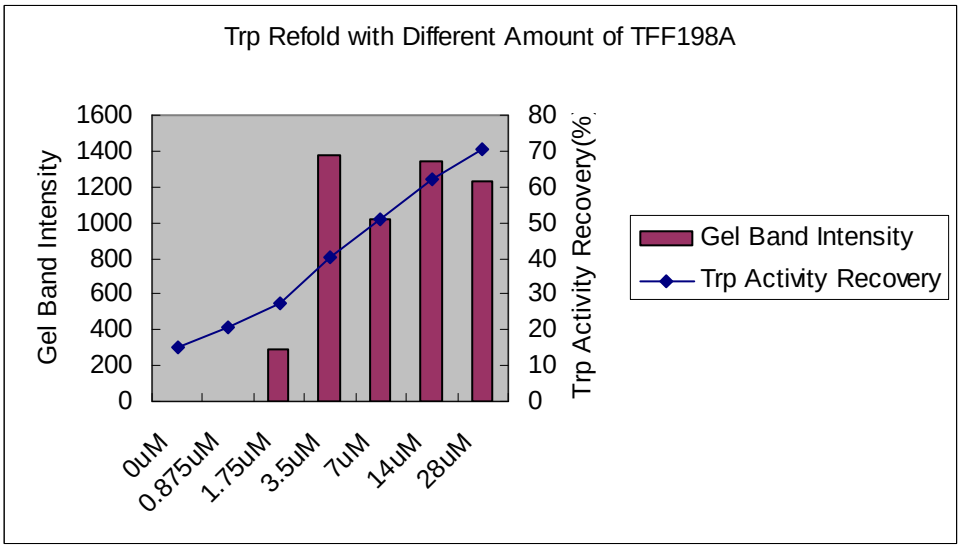
Figure 3.6



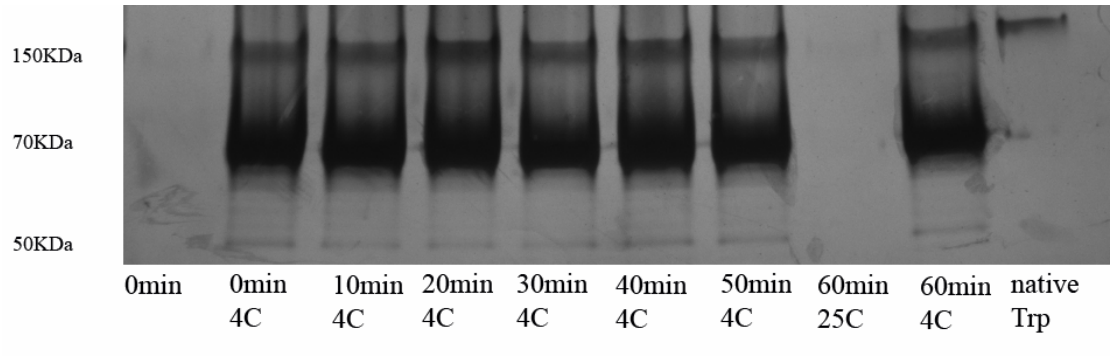


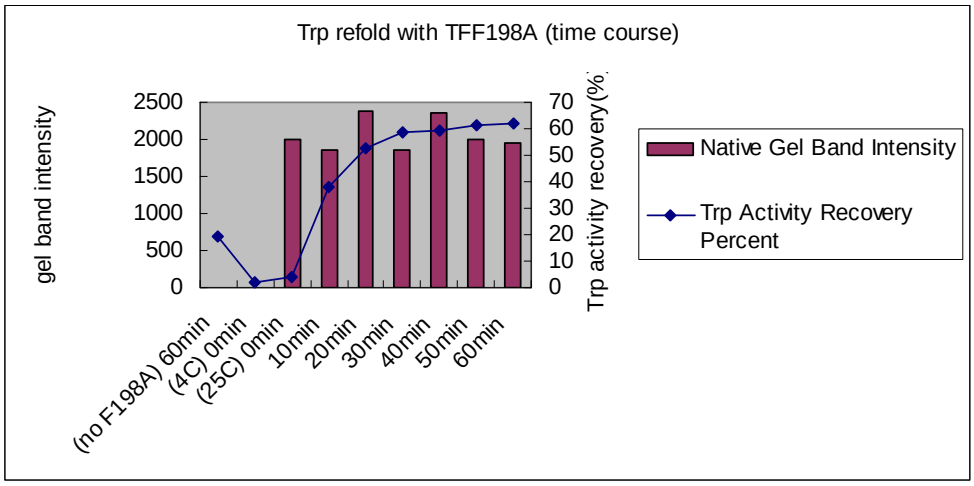
(A) Refolded tryptophanase with different concentrations of trigger factor on native gel. And plot with tryptophanase spots intensities and recovered activity.





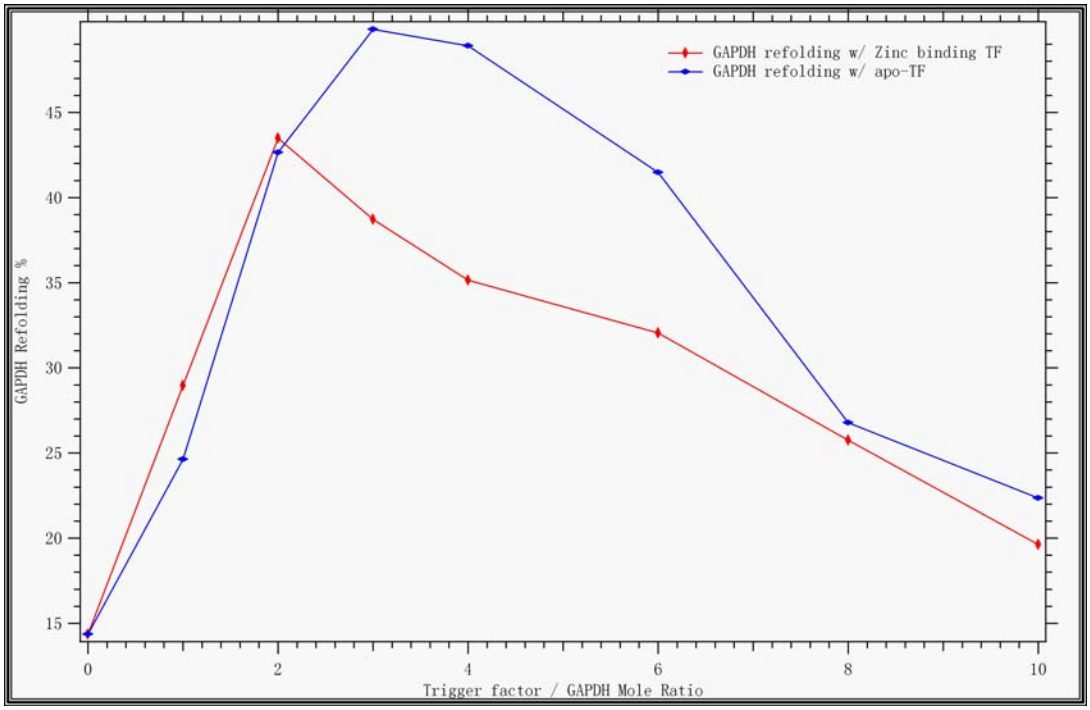
(B) Refolded tryptophanase with different concentrations of TF-F198A on native gel. And plot with tryptophanase spots intensities and recovered activity.



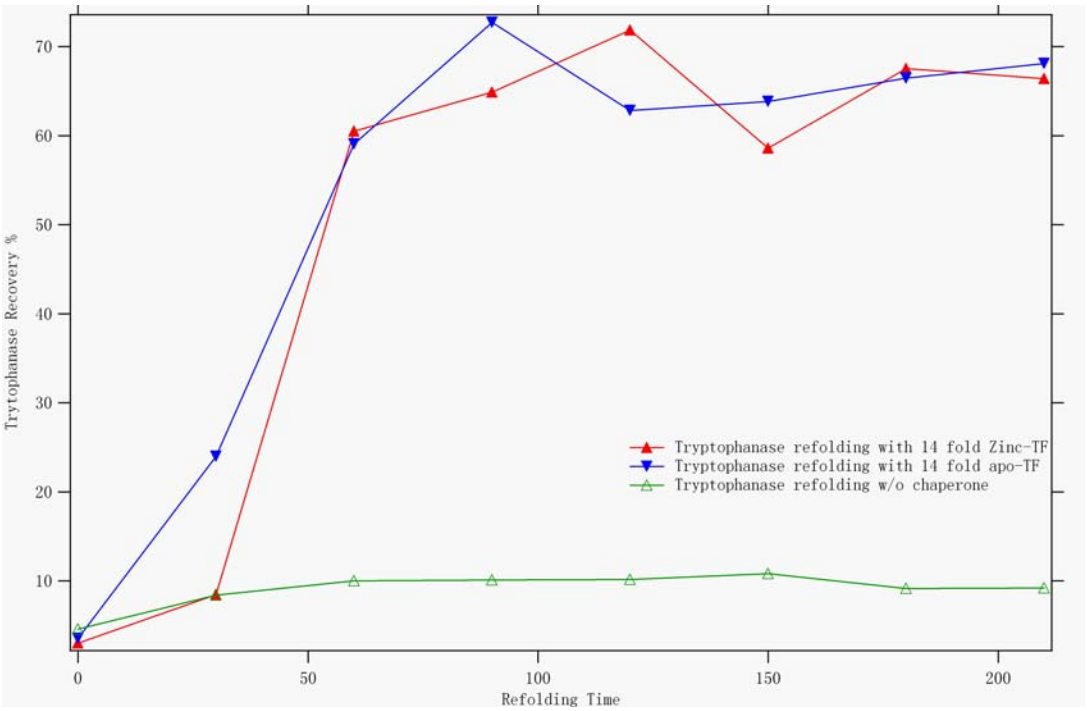


(C) Refolded tryptophanase at different time point during refolding with TF-F198A on native gel. And plot with tryptophanase spots intensities and recovered activity.

Figure 3.7



(A) GAPDH refolding assay for apo- and Zinc binding TF



(B) Tryptophanase refolding assay for apo- and Zinc binding TF

Chapter IV:

Conclusions and Future Works

The original aim of this project is to understand the role of PPIase in protein folding under cold stress. As a cold shock protein and efficient PPIase, trigger factor drew our attention at very beginning. If trigger factor, as a PPIase, is essential for proline-limited protein folding at low temperature, deletion of *tig*-gene might induce overproduction of other PPIases. We investigated the relationship of trigger factor and the only other cytosolic PPIase, PPiB using two dimensional electrophoresis and mass spectrometry. The data we obtained through this method did not show apparent evidence that cold stress (4℃) had caused significant change of PPiB expression in *tig*-deletion *E. coli* strain comparing to its expression in the same strain grown at 37℃. But the quality of mass spectrum for PPiB grown at 4℃ was low due to growth difficuty at low temperature. High background surpressed the isotope labeling pattern of peptide peaks of PPiB. Further modifications to experiment method, such as increasing the sample loading amount or applying higher cold shock temperature-8℃ or 12℃ , may be necessary to improve the signal/noise ratio of peptide peaks. Our observation still needs to be confirmed with more convincing data. On the other hand, three cytosolic PPIases in *E. coli* have not been identified on 2DE gels. It is essential to study the level of these enzymes in cells upon deletion of trigger factor at low temepratures.

Some spots of tryptophanase were missing on 2DE gels for proteome of Δtig strain, although most tryptophanase is preserved in both strains. More tryptophanase was found aggregated in tig-deletion strain than in wild-type *E. coli.* Trigger factor may prevent

aggregation of tryptophanase in unstable conformation. To investigate different conformations of trytophanase, it is necessary to understand the cause of pI shift observed in the gel spot pattern for tryptophanase. pI difference of the missing spots from the preserved spots suggested post-translational modification. PLP binding is one probable way to cause the multi-spots pattern of tryptophanase on 2DE gels. Various experiments could be carried out to confirm the hypothesis. The whole protein could be extracted from gel matrix and analyzed by mass spectrometry. Another option is to fix PLP to apo-tryptophanase by reduction with reducing agent, so the peptide mass shift caused by modification could be detected in mass spectrometer.

The *in vitro* refolding experiments indicate the significant effect of trigger factor on denatured tryptophanase refolding. The recovery yield of tryptophanase was improved 3 times with trigger factor compared to spontaneous refolding. The result from native gel shows that trigger factor may play a role in oligomeric assembly and conformational transition from apo to holo-tryptophanase. But no direct interaction between trigger factor and tryptophanase can be detected on native gels, so other teckniques, such as crosslinking, could be applied to exhibit more detail of the activity of trigger factor on typtophanase refolding. Better understanding of additional function of trigger factor could be achieved in the future.