รายงานวิจัยฉบับสมบูรณ์

โครงการ: การศึกษาความจำเพาะในการจับกับทีอาร์เอ็นเอ ของเอนไซม์แอสปาร์ทิลทีอาร์เอ็นเอซินทิเทสจากแบคทีเรีย *Helicobacter pylori* ที่ทำให้เกิดโรคในมนุษย์

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บทคัดย่อ

รหัสโครงการ : MRG5180073

ชื่อโครงการ : การศึกษาความจำเพาะในการจับกับทีอาร์เอ็นเอ ของเอนไซม์แอสปาร์ทิลทีอาร์เอ็นเอซินทิเทสจากแบคทีเรีย *Helicobacter pylori* ที่ทำให้เกิดโรคในมนุษย์

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บทคัดย่อ:

การเชื่อมต่อของกรดอะมิโนและทีอาร์เอ็นเอถือเป็นขั้นตอนที่สำคัญมากในการรักษาระดับความถูกต้องของการสังเคราะห์โปรตีนในเซลล์ ข้อมูลที่ถูกต้องซึ่งแฝงอยู่ในรูปของรหัสเบสสามตัวจะถูกถ่ายทอดไปได้ก็ต่อเมื่อโมเลกุลของทีอาร์เอ็นเอ ได้รับการต่อเชื่อมเข้ากับกรดอะมิโนที่ถูกต้อง นับถึงปัจจุบันนี้ ได้มีการค้นพบกระบวนการเชื่อมต่อของกรดอะมิโนในแบบไม่ตรงไปตรงมาหลายกระบวนการแล้ว ซึ่งหนึ่งในขั้นตอนที่พบบ่อยที่สุด คือการสังเคราะห์โมเลกุลของ Gln-tRNA^{Gln} และ Asn-tRNA^{Asn} โดยเอนไซม์กลูแทมินิลทีอาร์เอ็นเอซินทิเทส แบบไม่จำเพาะ และ แอสปาร์ทิลทีอาร์เอ็นเอแบบไม่จำเพาะ และ Asp/Glu-ADT ได้มีความพยายามเป็นจำนวนมากในการทำความเข้าใจการเกิดความไม่จำเพาะในการจับกับสับสเตรทของเอนไซม์ในกลุ่มนี้ ในกรณีของแอสปาร์ทิลทีอาร์เอ็นเอแบบไม่จำเพาะนั้น เอนไซม์นี้จะทำการต่อกรดอะมิโนแอสปาร์ทิก เข้ากับทีอาร์เอ็นเอแอสปาร์ทิก และทีอาร์เอ็นเอแอสปาราจีน จากนั้น โมเลกุลของทีอาร์เอ็นเอที่มีกรดอะมิโนที่ไม่ถูกต้อง (Asp-tRNA^{Asn}) ก็จะถูกแก้ไขโดยเอนไซม์ Asp/Glu-ADT ซึ่งมีอยู่ในสิ่งมีชีวิตเดียวกันนั้น โดยจุดกำเนิดของความไม่จำเพาะของเอนไซม์นั้น น่าจะมาจากบริเวณที่เอนไซม์จับกับทีอาร์เอ็นเอสับสเตรทนั่นเอง ซึ่งบริเวนโดเมนที่ใช้จับกับแอนติโคดอนนั้น จัดว่าเป็นบริเวณที่สำคัญที่สุดบริเวณหนึ่ง ในการวิจัยครั้งนี้ เราได้รายงานการสลับโดเมนแอนติโคดอนของเอนไซม์แอสปาร์ทิลทีอาร์เอ็นเอแบบไม่จำเพาะของแบคทีเรีย *Helicobacter pylori* ที่ทำให้เกิดโรคในมนุษย์ กับโดเมนแอนติโคดอนของแอสปาร์ทิลทีอาร์เอ็นเอซินทิเทส และแอสปาราจินิลทีอาร์เอ็นเอซินทิเทส จาก *Eschericia coli* โดยโปรตีนที่ได้นั้น (เรียกว่าไคเมอราดี และไคเมอราเอ็น) มีปริมาณโครงสร้างแบบทุติยภูมิที่คล้ายคลึงกับในเอนไซม์แอสปาร์ทิลทีอาร์เอ็นเอแบบไม่จำเพาะซึ่งเป็นตัวต้นแบบ โดยความสามารถในการเร่งปฏิกิริยาของทั้งสองไคเมอริคเอนไซม์นี้ พบว่าน้อยกว่าของเอนไซม์ต้นแบบ อย่างไรก็ตาม ความชอบจับกับทีอาร์เอ็นเอแอสปาร์ทิคของไคเมอราดีนั้นมีมากกว่าของเอนไซม์ต้นแบบ ซึ่งแสดงให้เห็นถึงความสำคัญของโดเมนแอนติโคดอนที่มีต่อความจำเพาะในการจับกับทีอาร์เอ็นเอสับสเตรท เมื่อนำโปรตีนทั้งสองไปผลิตใน *E. coli* โฮส พบว่าความเป็นพิษที่มีต่อการเจริญเติบโตของโฮสเซลล์มีความสอดคล้องกันกับประสิทธิภาพในการเร่งปฏิกิริยาและความจำเพาะในการจับกับทีอาร์เอ็นเอสับสเตรทของเอนไซม์ไคเมอราดี และไคเมอราเอ็น

คำหลัก : แอสปาร์ทิลทีอาร์เอ็นเอซินทิเทส, *Helicobacter pylori*, ทีอาร์เอ็นเอ

Abstract

Project Code : MRG5180073

Project Title : Investigation of tRNA Specificity for the Non-discriminating Aspartyl-tRNA Synthetase from the Human Pathogen *Helicobacter pylori*

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Project Period : May 15th, 2008 to May 14th, 2010

Abstract:

Aminoacylation of tRNAs is the crucial biochemical process that ensures the fidelity of protein translation. The correct message, embedded as a three letter code, in the DNA will only be translated correctly when the adaptor molecule, the tRNA, is correctly charged. To date, several indirect aminoacylation processes have been discovered. The most common ones are the synthesis of Gln-$tRNA^{Gln}$ and Asn-$tRNA^{Asn}$ via activities of the non-discriminating glutaminyl-tRNA synthetase (ND-GluRS), non-discriminating aspartyl-tRNA synthetase (ND-AspRS), and the Asp/Glu-ADT. Several efforts have been made in order to unlock the peculiar tRNA specificity of the non-discriminating enzymes. In the case of ND-AspRS, the enzyme aspartylates both $tRNA^{Asp}$ and $tRNA^{Asn}$ and the mischarged Asp-$tRNA^{Asn}$ is then converted to Asn-$tRNA^{Asn}$ by the action of Asp/Glu-ADT, which coexists in the same organism containing ND-AspRS. The relaxed tRNA specificity was speculated to reside within the interface between the enzyme and tRNA substrate. Among those areas, the anticodon binding domain presents the most crucial interaction of all. Here we report the anticodon binding domain swapping between the ND-AspRS from the human pathogen *Helicobacter pylori* and those of the discriminating AspRS and AsnRS from *E. coli*. The resulting chimeras (Chimera-D for the ND-AspRS enzyme with *E. coli* AspRS anticodon binding domain and Chimera-N for the one with *E. coli* AsnRS anticodon binding domain) maintain the same secondary structure content compare to the wild-type enzyme. The catalytic activity of these chimeras is lower than the wild-type enzyme as expected. However, the preference for $tRNA^{Asp}$ in Chimera-D is a lot higher than those of wild-type enzyme, indicating the significant contribution of the anticodon binding domain toward tRNA specificity. The heterologous toxicity, when overexpressed in *E. coli* host cells, of these chimeras also correlates well with their tRNA specificity and catalytic activity.

Keywords : Aspartyl-tRNA Synthetase, *Helicobacter pylori*, tRNA

Executive summary

Aminoacylation of transfer RNAs (tRNAs) is at the heart of protein biosynthesis, a cellular process shared by all form of life on this planet.[1-3] This transformation can be accomplished by a group of enzymes called aminoacyl-tRNA synthetases (AARSs). These enzymes have to accomplish a very complicated task as they have to selectively aminoacylate tRNA isoacceptor(s) with a cognate amino acid. This process represents two types of interaction in molecular biology, namely, protein (AARSs)/small molecule (amino acid) and protein (AARSs)/tRNAs interactions. Therefore, Investigation of the mechanistic details and evolutionary pattern of this group of enzymes will provide us a complete picture of the central process shared by every organism.

It had been hypothesized that there are 20 AARSs, one for each standard amino acid. However, several investigations of complete genomes of various organisms reveal a very intriguing piece of information. Some organisms, including human mitochondria, all archaea, and many pathogenic bacteria, are often missing one or two aminoacyl-tRNA synthetases, namely glutaminyl- and asparaginyl-tRNA synthetases (GlnRS and AsnRS, respectively).[3,4] The genes encoding for GlnRS and/or AsnRS are often missing in most bacteria, archaea, and organelles. In these cases, glutaminyl-$tRNA^{Gln}$ and/or asparaginyl-$tRNA^{Asn}$ must be made indirectly. These processes arise from the activity of a non-discriminating glutamyl and aspartyl-tRNA synthetase (ND-GluRS and/or ND-AspRS), which will charge glutamic acid onto both $tRNA^{Glu}$ and $tRNA^{Gln}$ and charge aspartic acid onto both $tRNA^{Asp}$ and $tRNA^{Asn}$ respectively. These observations and several other well characterized indirect aminoacylation pathways underline the importance of characterizing unexpected genetic variations in AARS as well as the novel indirect aminoacylation mechanisms.[4-32]

Not only do the AARSs catalyzed aminoacylation of the tRNAs, several alternative functions of AARSs have been reported, especially the ones with involvement in diseases and therapeutics. Some of these examples are:

1) The human lysyl-tRNA synthetase (LysRS) has been shown to interact with HIV-1 GAG protein and play a significant role in HIV-1 viral particle assembly.[33,34]

2) The non catalytic factors AIMP2/p38 and AIMP3/18, which are part of the mammalian tRNA synthetase macromolecular protein complex, are found to be potent tumor suppressors and play a critical role in the signaling pathways that are involved in cell proliferation and cell death.[35-37]

3) Recently, leucyl-tRNA synthetase (LeuRS) has been shown to be a target of an antifungal agent.[38,39] The compound AN2690, a molecule in development for the

treatment of onychomycosis, inhibits yeast LeuRS by forming a stable $tRNA^{Leu}$-AN2690 adduct in the editing site of LeuRS and consequently prevents catalytic turnover. The scientific reports mentioned here truly emphasize the significance of AARSs in medical and pharmaceutical fields.

Helicobacter pylori (*H. pylori*) was first isolated and successfully cultured in 1984 by Barry Marshall. This bacteriam is the causative agent behind stomach ulcers, chronic gastritis, and stomach cancer. It is worth noting that Barry J. Marshall and J. Robin Warren shared the Nobel Prize in Physiology/medicine in 2005 for their discovery of the bacterium *H. pylori* and its role in gastritis and peptic ulcer disease.[40] The genome of *H. pylori* was completely sequenced in 1997.[41] Like some other bacteria, *H. pylori* genome lacks genes encoding for AsnRS and GlnRS. (This was the first example of a bacterium that lacked direct aminoacylation pathways for both Asn-$tRNA^{Asn}$ and Gln-$tRNA^{Gln}$.) Interestingly, the genome of this bacterium contains 2 copies of gene encoding for GluRS1 and GluRS2 and one copy of the non-discriminating AspRS (ND-AspRS). GluRS1 is a canonical, discriminating GluRS that charges $tRNA^{Glu}$ with glutamic acid. However, the role of GluRS2 is to specifically aminoacylate $tRNA^{Gln}$ with glutamic acid.[15,42,43] The mischarged Glu-$tRNA^{Gln}$ is then converted to the correctly charged Gln-$tRNA^{Gln}$ by the Asp-$tRNA^{Asn}$/Glu-$tRNA^{Gln}$ amidotransferase (Asp/Gly-Adt).[23,28,44-46] This unexpected specificity of GluRS2 in *H. pylori* leads us to further characterize the specificity and evolutionary pattern of the ND-AspRS in this pathogenic bacterium. Such information can also be used in medical and pharmaceutical sciences. Specifically, if significant structural differences can be identified between the human AspRS and the ND-AspRS from *H. pylori*, the ND-AspRS from this pathogenic bacterium could be a potential target for antibacterial drug development.

วัตถุประสงค์

1. To construct mutants of the non-discriminating aspartyl-tRNA synthetase (ND-AspRS) from *Helicobacter pylori* for the investigation of tRNA specificity.
2. To construct the ND-AspRS chimeras by swapping anticodon binding domain of *H. pylori* ND-AspRS with that of *E. coli* discriminating AspRS (D-AspRS) and asparaginyl-tRNA synthetase (AsnRS).
3 To evaluate a correlation between tRNA specificity and heterologous toxicity of these mutants and chimeras *in vivo*.
4. To determine the kinetic parameters of these mutants and chimeras.

วิธีทดลอง

Materials. All reagents and chemicals were purchased from Sigma Aldrich or Acros Organics (Thermo Fisher Scientific). Aspartic acid [2,3-^{3}H] was purchased from American Radiolabeled Chemicals Inc. (ARC). Oligonucleotides were purchased from Pacific Science. Plasmids were purified using plasmid purification kit from Qiagen. The chemical (calcium chloride) competent cells (DH5α) were purchased from Invitrogen. DNA sequencing was performed by MACROGEN (Macrogen Inc., Seoul, Korea). The entire reading frame was fully sequenced for every construct.

Isolation of Genomic DNA (E. coli K12). An overnight culture of *E. coli* K12 (DH5α) was used to inoculate 20 mL of LB broth. The culture was rigorously shaken at 37 ^{o}C until the OD_{600} reached 0.4. The cells were harvested by centrifugation at 5,000 rpm for 15 minutes. The cell pellets were then resuspended in 0.75 mL 1X TES buffer (10 mM Tris-HCl, 10 mM EDTA pH 8.0, 0.2% SDS) and incubated at 75 ^{o}C for 5 minutes. The cells were then extracted by phenol:chloroform (3:1 V/V) twice. After centrifugation at 3,000 rpm for 10 minutes, a clear aqueous layer was transferred to a new eppendorf tube and the genomic DNA was then precipitated by adding 1/10 of the volume of 3 M sodium acetate followed by 3X of the volume of ice-cold ethanol and the mixture was left overnight at -20 ^{o}C. The genomic DNA was then recovered after centrifugation at 14,000 rpm for 30 minutes followed by a wash using 70% ice-cold ethanol. The agarose gel electrophoresis showed plenty amount of *Ec.* genomic DNA. The resulting genomic DNA was used as a template for gene amplification without any further purification.

Construction of A105G mutant. The A105G mutation (C464G in *aspS* gene) was introduced into plasmid pPTC001 (A plasmid containing *aspS* gene from *H. pylori*) using QuickChange Site-Directed Mutagenesis (Stratagene) according to the manufacturer's instruction. The resulting plasmid (pPC001) was generated using Pt#005 and Pt#006 (See supporting information for primer sequences). The correct introduction of C464G point mutation was confirmed by DNA sequencing of the entire open reading frame.

Construction of Chimera-D and Chimera-N of H. pylori ND-AspRS. The genes encoding the N-terminal domains of the discriminating AspRS and the AsnRS from *E. coli* were amplified from the *E. coli* (DH5α) genomic DNA. The *E. coli* genomic DNA sample was obtained according to the general protocol. The gene encoding the N-terminal domains of the *E. coli* AspRS was generated using primers Pt#007 and Pt#008 (See supporting information for primer sequences). These primers introduced flanking *BamHI* and *KpnI* sites onto 5' and 3' ends of the gene. The primers Pt#009 and Pt#010 were used in the amplification of the gene encoding the N-terminal domain of *E. coli* AsnRS. These primers also introduced flanking *BamHI* and *KpnI* restriction sites in order to facilitate the subsequent cloning into pPC001 plasmid. The PCR product generated from primers Pt#007 and Pt#008 was then cloned into the shuttle vector pGEM-T Easy according to the manufacturer's protocol (Promega) to generate pPC005, a plasmid containing the N-terminal anticodon binding domain AspRS gene from *E. coli*. Similarly, the PCR product generated from primers Pt#009 and Pt#010 was also cloned into pGEM-T Easy shuttle vector to generate pPC006, a plasmid containing the N-terminal anticodon binding domain AsnRS gene from *E. coli.*

The gene encoding the N-terminal anticodon binding domain of *E. coli* AspRS was obtained from the *BamHI* and *KpnI* digestion reaction of pPC005. The gene was then inserted into pPC001 to generate pPC007, a plasmid encoding *H. pylori* ND-AspRS Chimera-D. Correspondingly, the plasmid encoding chimera-N of *H. pylori* ND-AspRS, pPC008, was generated by insertion of the gene from *BamHI* and *KpnI* digestion reaction of pPC006 into pPC001. The identity of every construct was confirmed by DNA sequencing of the entire open reading frame.

Expression and purification of the wild type H. pylori ND-AspRS, Chimera-D, and Chimera-N. The wild type *Hp* ND-AspRS, Chimera-D, and Chimera-N were expressed and purified according to the protocol previously reported. Briefly, *E. coli* (DH5α) calcium chloride chemical competent cells were transformed with an appropriate

plasmid (pPTC001 for wild type ND-AspRS, pPC007 for Chimera-D, and pPC008 for Chimera-N) and selected on Luria-Broth (LB) agar plates containing 100 μg/mL ampicillin. The ND-AspRS, Chimera-D, and Chimera-N were expressed for 30 minutes in order to avoid toxicity effects. The crude lysate was purified by Ni-NTA affinity chromatography as described previously. The purity of expressed proteins was confirmed to be >95% homogeneity by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified proteins were kept at -20 ^{o}C in protein storage buffer containing 33 mM phosphate buffer (pH 7.4), 3 mM Tris-Cl, 1.5 mM β-mercaptoethanol (BME), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 50% glycerol. Final protein concentrations were determined in triplicate by the Bradford Protein Assay (Bio-Rad) according to the manufacturer's instruction.

In vivo transcription and purification of Hp tRNA^{Asp} and tRNA^{Asn}. Hp tRNA^{Asp} and tRNA^{Asn} were each transcribed *in vivo* according to the protocol previously reported. Total tRNA was isolated by Nucleobond Kit (CloneTech) according to the manufacturer's instruction. This total tRNA contains a mixture of *E. coli* tRNAs enriched with overtranscribed *Hp* tRNA. Since the post-transcriptional modification of tRNA^{Asp} is crucial for tRNA recognition of AspRS, this approach seems to be a reasonable method of choice for obtaining *Hp* tRNA^{Asp} and tRNA^{Asn} as the post-transcriptional modification of tRNA is conserved in different bacteria. The amount of tRNA^{Asp} and tRNA^{Asn}, obtained from the *in vivo* overtranscription mentioned above, were quantified in triplicate using the plateau aminoacylation assays previously described.

Aminoacylation Assays. Aminoacylation assays were performed in 100 mM Hepes (pH 7.5), 2 mM ATP, 4 mM $MgCl_2$, 10 μM aspartic acid and 80 μCi/mL L-[2,3-^{3}H] aspartic acid. The ND-AspRS, Chimera-D, and Chimera-N were each added to a final concentration of 0.2 μM. Each tRNA was denatured at 75 ^{o}C for 5 minutes and then refolded by addition of 8 mM $MgCl_2$ at 65 ^{0}C and cool to room temperature. Each tRNA was assayed at the concentration of 0.2 μM.

Native polyacrylamide gel electrophoresis (Native-PAGE). Each protein was analyzed by Native-PAGE in order to estimate its ability to form dimer under native and slightly denatured conditions. The native-PAGE was run using 7% polyacrylamide gel with running buffer (without SDS in every component). The slightly denatured condition

was obtained by preparing 7% polyacrylamide gel (without SDS) and run the gel in 0.01% SDS running buffer. In all experiments, the protein samples were prepared using loading dye without SDS, and bovine serum albumin (BSA, ~67 kD) and bovine gamma globulin (BGG, ~140 kD) were used as protein molecular weight markers.

Growth Study. Toxicity and cell viability profiles of *E. coli* cultures overexpressing the wild type *Hp* ND-AspRS, Chimera-D, and Chimera-N were evaluated according to the general procedure previously described. Briefly, a single colony was grown overnight in a 5 mL LB-ampicillin. This culture was used to inoculate 200 mL culture of the same medium to an OD_{600} of 0.05. The culture was incubated at 37 ^{0}C with agitation. The protein expression was induced by adding isopropyl-β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Growth was monitored over the period of 7 hours. The reported data represents the average of experiments conducted in triplicate.

ผลการทดลอง

Swapping the N-terminal anticodon binding domain of Hp. ND-AspRS with those of E. coli AspRS and AsnRS does not drastically alter the secondary structure content of the enzyme. The AspRS and AsnRS belong to class IIb of all synthetases and exist as homodimeric proteins in nature. In order to investigate the contribution of the anticodon binding domain toward the tRNA specificity in *Hp* ND-AspRS, we decided to replace the N-terminal anticodon binding domain of the wild type *Hp* ND-AspRS with those of *E. coli* AspRS (Discriminating AspRS) and AsnRS, generating the Chimera-D and Chimera-N respectively. A closer look at the sequence similarity in the anticodon binding domain (104 amino acids long) revealed that the one from *E. coli* AspRS share a high percent similarity to those of *Hp* ND-AspRS (approximately 36%) where relatively lower similarity (approximately 14%) was observed between *E. coli* AsnRS and *Hp* ND-AspRS (Figure 1A and B). We hypothesized that the introduction of *E. coli* AspRS anticodon binding domain into *Hp.* ND-AspRS should yield a more stable and presumably more catalytically active enzyme due to the sequence similarity. In both cases of domain swapping, a unique restriction site must be introduced around the flexible loop that connects the anticodon binding domain and the catalytic domain (the hinge region). As it has been shown that this area of AspRS also interact with the tRNA substrate and, undeniably, contributes to its tRNA specificity, the restriction site must be introduced at the position where the amino acid is less conserved in order to avoid the interruption of substrate binding. In response to the aforementioned criteria, the consurf

analysis was performed for the *Hp* ND-AspRS in order to find the best position for unique restriction site introduction. Since, to date, the crystal structure of the bacterial type ND-AspRS is not available, the analysis started with generation of homology model of *Hp* ND-AspRS using the Geno3D. This automatic web server performs homology modeling for a given protein sequence by searching for homologous proteins with known crystal structures. The server then extracts geometrical restraints and builds a 3D model of the protein. The obtained 3D model was then used as a template for the consurf analysis. This online tool makes it possible to visualize functionally important region on a given protein structure by extracting the protein sequence from a given PDB file followed by sequence alignment analysis. The color coded consurf score calculated from previous step is then mapped onto the surface of the protein. Figure 1C shows results from consurf analysis of *Hp* ND-AspRS homology model. The expansion of flexible loop area revealed the less conserved alanine105 with the consurf score of 2 (The range is from 1 to 9, where 1 is the least conserved and 9 is the most conserved). This position was selected also because the introduction of *KpnI* restriction site will only require a single base mutation (C314G, *Hp* ND-AspRS numbering). The newly introduced *KpnI* site, along with pre-existing *BamHI* site in pPTC001, will facilitate the domain swapping for *Hp* ND-AspRS.

The plasmid pPC001 possessing *KpnI* was generated by means of site-directed mutagenesis. Then, the genes encoding N-terminal anticodon binding domain of *E. coli* AspRS and AsnRS were introduced into pPC001 generating pPC007 and pPC008 respectively. A brief overexpression profile analysis for Chimera-D (pPC007) and Chimera-N (pPC008) indicated the presence of soluble proteins with the molecular weight around 67 kD. Since it has been reported that the heterologous overexpression of *Hp* ND-AspRS is toxic to *E. coli* host cells, the expression of Chimera-D and Chimera-N was also conducted for 30 minutes after IPTG induction. Purified Chimera-D and Chimera-N were then subjected to the CD spectroscopic analysis in order to determine their secondary structure content. The parallel experiment was also conducted with the wild type *Hp* ND-AspRS in order to compare the CD profile between the wild type enzyme and its chimeras. The CD spectra for each protein were shown in figure 2A. In general, Chimera-D and Chimera-N provide relatively similar CD spectra compared with the wild type enzyme. The calculated secondary structure content was shown in figure 2B for each protein. These values are the average from the results generated by three separate programs, SELCON3, CDSSTR, and CONTINLL. The results also indicate only small discrepancies in the secondary structure content of the

wild type ND-AspRS, Chimera-D, and Chimera-N. Therefore, introducing the N-terminal domain from *E. coli* AspRS and AsnRS into those of *Hp* ND-AspRS gives soluble chimeric proteins with small discrepancies in secondary structure content.

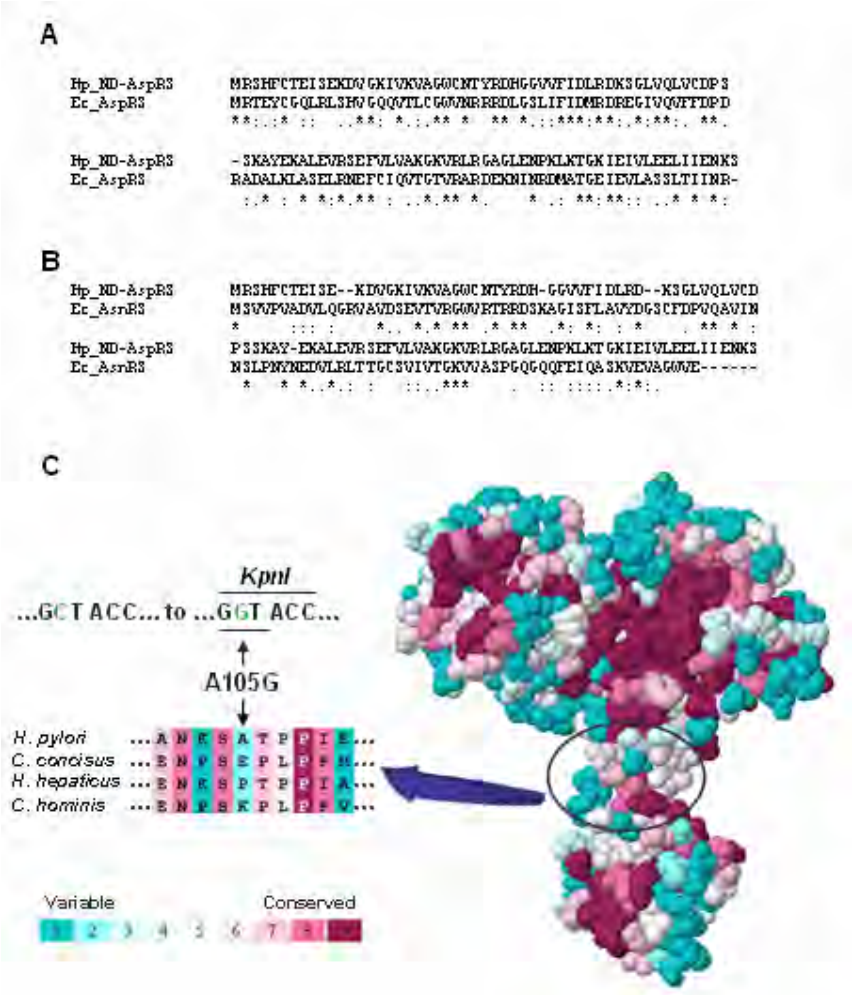
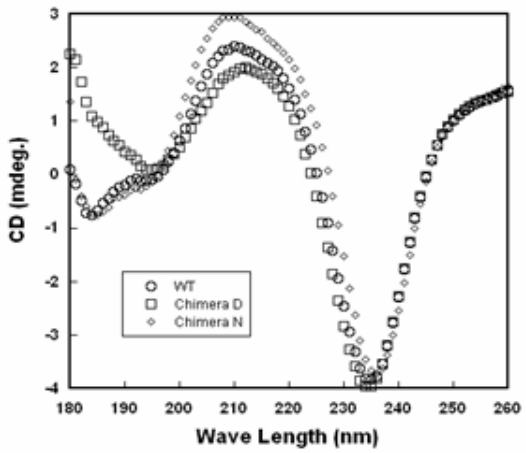


Figure 1. Rationalization for the construction of Chimera-D and Chimera-N. A) The N-terminal domain alignment for *Hp* ND-AspRS and *E. coli* AspRS. B) The N-terminal domain alignment for *Hp* ND-AspRS and *E. coli* AsnRS. C) Consurf result showing less conserved region in the hinge domain of *Hp* ND-AspRS. The alanine105 receives turquoise color code, thus represent variable position in the protein. The single base mutation was introduced in order to generate the *KpnI* restriction site. This site facilitates the introduction of N-terminal anticodon binding domain from *E. coli* AspRS and AsnRS.

A



B

	Helix	Sheet	Turns	PP2	Unordered
WT	8.10	24.73	18.17	10.07	34.97
D	20.83	13.87	19.20	18.67	26.23
N	12.00	24.47	17.37	14.07	29.67

Figure 2. Circular Dichroism (CD) Analysis of Wild-type *Hp* ND-AspRS, Chimera-D, and Chimera-N. A) CD spectra overlay of Wild-type ND-AspRS, Chimera-D, and Chimera-N. B) Secondary structure content of Wild-type ND-AspRS, Chimera-D (as D in the table), and Chimera-N (as N in the table) calculated from three programs; CONTINLL, SELCON3, and CDSSTR. Values shown here are average numbers from the aforementioned programs.

The ability to form dimmer is decreased in Chimera-D, and the effect is even more pronounced in Chimera-N compare to the wild type enzyme.

Introduction of the N-terminal domain from enzymes of different organism clearly affect the ability to form homodimer of the wild-type ND-AspRS. When native-PAGE was performed under slightly denatured condition (in the presence of 0.01% SDS in running buffer), the distribution of monomer and homodimer can be observed (Figure 3.). The ability of form dimer of Chimera-D is clearly lower than the wild-type ND-AspRS. The dimerization degree in Chimera-N is the worst among three proteins analyzed. This might be the result from the incompatibility around surface area between the anticodon binding domain and the catalytic domain of the enzyme. Once can see that, introducing foreign N-terminal domain into the wild-type enzyme will also introduce the whole new set of amino acids around the contact surface of the chimeras. Therefore, it is conceivable to see such discrepancies for dimerization.

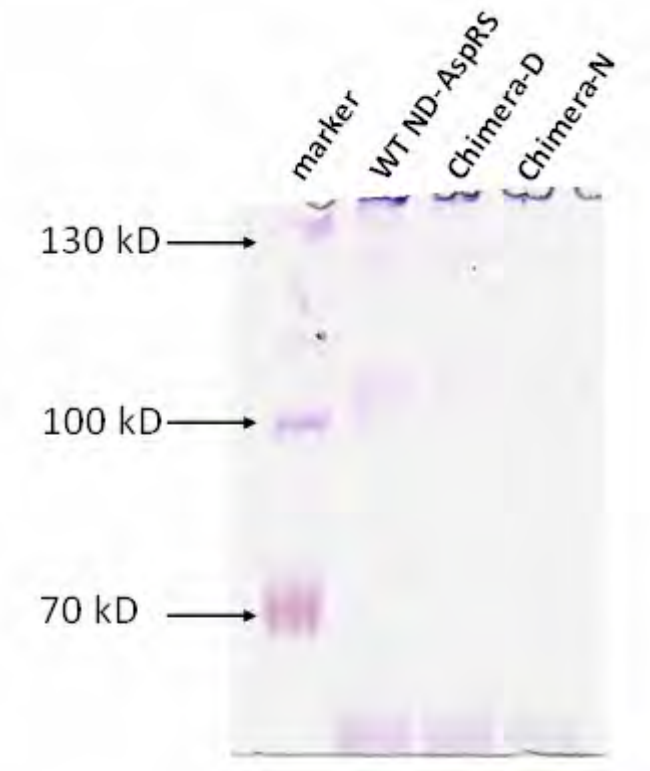


Figure 3. The Native PAGE Analysis of Wild-type *Hp* ND-AspRS, Chimera-D, and Chimera-N. The native PAGE was run in running buffer containing 0.01% SDS. Under this condition, both homodimer and monomer of the wild-type enzyme are clearly visible. The amount of dimer and monomer in each sample is significantly different. The portion of homodimer in wild-type enzyme is the highest among the three, and the lowest homodimer content is observed in Chimera-N. These results correlate well with the ability to form dimers in each protein.

The $tRNA^{Asp}$ specificity is enhanced in Chimera-D where the Chimera-N exhibit very low catalytic activity for both $tRNA^{Asp}$ and $tRNA^{Asn}$.

It has been shown that the wild-type ND-AspRS prefers $tRNA^{Asp}$ as a substrate over $tRNA^{Asn}$. The preference for $tRNA^{Asp}$ over $tRNA^{Asn}$ was evaluated using the aminoacylation assays as well as the Michaelis-Menten kinetic analysis. Then, we set forth to study tRNA specificity for our newly constructed Chimera-D and Chimera-N. As expected, both chimeras exhibit very low catalytic activity compare to the wild-type enzyme, especially in the case of Chimera-N where the aminoacylation efficiency is only slightly above the no enzyme control experiments. This observation reveals the conformational changes in Chimera-D and Chimera-N compare to the wild-type enzyme. Despite the fact that the secondary structure content in these two chimeras is approximately the same as wild-type enzyme, the conformation of these two chimeras may be different in the way that does not provide the ability to catalyze aminoacylation reaction to both tRNAs. However, the preference for $tRNA^{Asp}$ can still be observed in the case of Chimera-D. Based on the aminoacylation assay shown in Figure 4a and b, the preference for $tRNA^{Asp}$ in Chimera-D is clearly higher than the wild-type enzyme. Therefore, anticodon binding domain of ND-AspRS strongly contributes to its tRNA specificity. In order to quantify the contribution of this domain toward tRNA specificity, the Michaelis-Menten kinetic experiments were conducted. Unfortunately, neither of the chimeras behave well in MM kinetic experiments despite the fact that several aminoacylation conditions were explored.

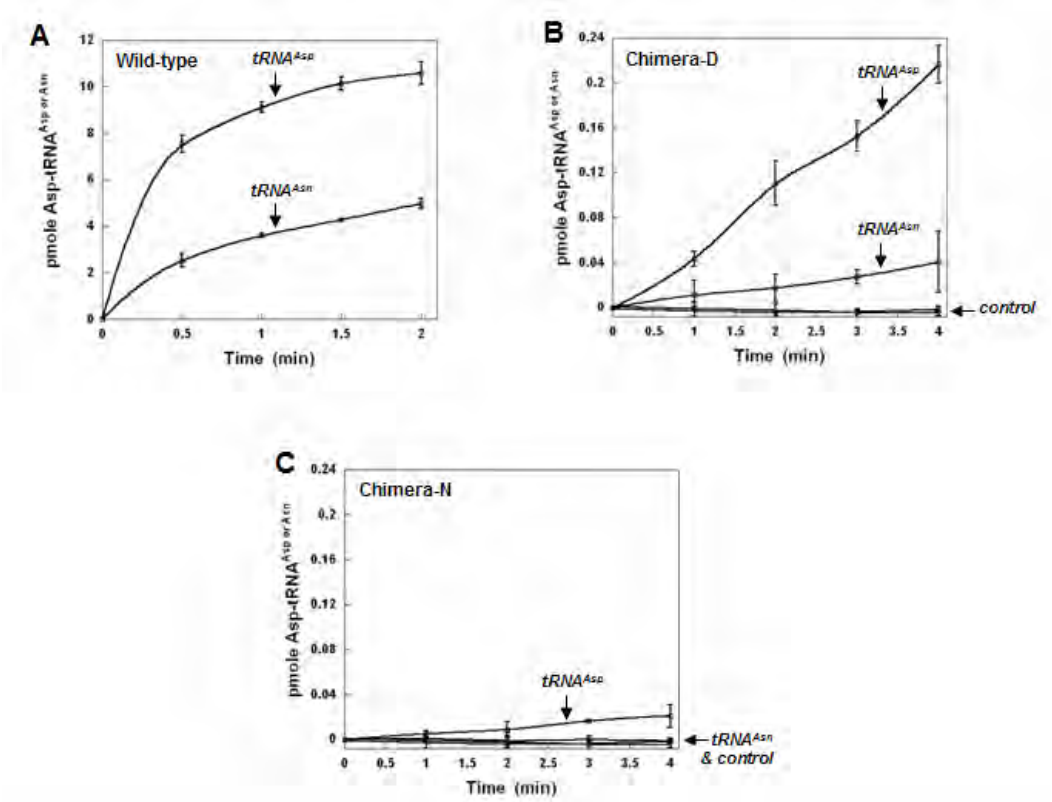


Figure 4. Catalytic Activity of Wild-type ND-AspRS, Chimera-D, and Chimera-N toward $tRNA^{Asp}$ and $tRNA^{Asn}$. The aminoacylation of $tRNA^{Asp}$ and $tRNA^{Asn}$ with wild-type ND-AspRS A), Chimera-D B), and Chimera-N C). These assays are run under identical condition (2 μM $tRNA^{Asp}$, 2 μM $tRNA^{Asn}$, and 0.2 μM enzyme)

The differences in catalytic activity and tRNA specificity of Chimera-D and Chimera-N were observed through heterologous expression of these proteins in E. coli.

Due to the fact that both chimeras are a lot less catalytically active compare to the wild-type enzyme, *in vivo* heterologous expression of these proteins in *E. coli* host cells is less toxic than those of wild-type ND-AspRS. The results in Figure 5 represent an average of experiments conducted more than 6 times in order to ensure the small difference in cell viability between those expressing Chimera-D and Chimera-N. The Chimera-N appears to be less toxic to *E. coli* host cell than Chimera-D due to its low catalytic activity compare to Chimera-D.

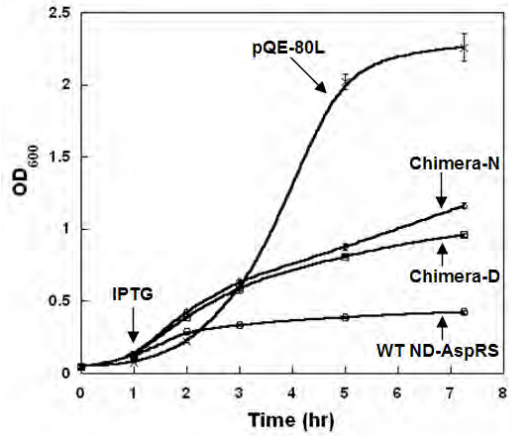


Figure 5. Heterologous Expression of Chimera-D and Chimera-N is less Toxic Than That of Wild-type ND-AspRS.

สรุปและวิจารณ์ผลการทดลอง และข้อเสนอแนะสำหรับงานวิจัยในอนาคต

The relaxed tRNA specificity of the non-discriminating Aspartyl-tRNA synthetase has been the center of attention for many years. Despite the fact that several mutations in the anticodon binding domain were studied, our work presented here is the first example of the complete introduction of foreign domain from different organism into the non-discriminating enzyme. The introduction of the N-terminal anticodon binding domains of the discriminating AspRS and AsnRS from *E. coli* revealed a contribution of the anticodon binding domain toward tRNA specificity. Although not quantitatively, this is the first example of domain swapping in any aminoacyl-tRNA synthetase that provides catalytically active enzyme (Chimera-D). Chimera-D and Chimera-N have similar secondary structure content to the wild-type enzyme as observed in CD spectra and the calculated secondary structure content. In theory, these two chimeras should preserve the structural architecture of class IIb of aminoacyl-tRNA synthetase and exist as homodimer in their catalytically active form. The native-PAGE in the presence of 0.01% denaturant confirmed the existence of both homodimer and monomer of the wild-type enzyme. The degree of dimerization is significantly lower in Chimera-D and the lowest in Chimera-N. This observation could be the result from the differences in amino acid content around the surface of the N-terminal anticodon binding domain and the catalytic domain. The ability to form dimmers for these chimeras also correlates well with their catalytic activity in which Chimera-N is nearly catalytically inactive. (Only slightly active with $tRNA^{Asp}$) Despite the fact that both chimeras are less active than the wild-type for aspartylation reaction, the Chimera-D prefers $tRNA^{Asp}$ over $tRNA^{Asn}$ as a substrate. In deed, the preference for $tRNA^{Asp}$ is significantly higher than those of wild-type enzyme, indicating that our hypothesis regarding the contribution of N-terminal domain of the ND-AspRS toward tRNA specificity is valid and verified. The catalytic activities of these chimeras also correlate nicely with the heterologous toxicity when expressed in *E. coli* host cells. Since the chimera-N is nearly catalytically inactive toward both tRNAs, its overexpression is slightly less toxic to the host compare to Chimera-D. Both chimeras are a lot less active than the wild-type enzyme, and therefore, show less toxic phenotype compare to the wild-type AspRS.

The future investigation will concern a closer look at the conformational change within the anticodon binding domain upon binding to substrates (ATP, Asp, $tRNA^{Asp}$, and $tRNA^{As}$). These experiments will complete the overall changes in the anticodon binding domain and will also provide more information regarding the interface between the anticodon binding domain and the catalytic domain. The study will employ solution

dynamic NMR experiments. To do this, the anticodon binding domain (approximately 104 amino acids) needs to be labeled, independently from the rest of the enzyme. This domain will be subjected to a series of ligand titration experiments. The independent overexpression of the anticodon binding domain is now completed. The next step for us is overexpression of N-15 and C-13 labeled protein. Once the experiments are completed, the information obtained will yield a complete mechanism of binding between the Non-discriminating AspRS to its tRNA substrates.

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Appendix : Cloning primer sequences

Primer	Sequence (5'→ 3')
Pt#001	ggtgtaacacttatagagaccttggaggcgtggtttttattgatttaaggg
Pt#002	cccttaaatcaat aaaaaccacgcctccaaggtctctataagtgttacacc
Pt#003	ggcgctgggttagaaaaccctaaacGaaaaacgggtaaaattgaaatcg
Pt#004	cgatttcaattttacccgtttttCgtttagggttttctaacccagcgcc
Pt#005	gttaattattgaaaataaaagcGGTACCccaccgattgaaattggcaac
Pt#006	gttgccaatttcaatcggtggGGTACCgcttttattttcaataattaac
Pt#007	CGCggatccATGCGTACAGAATATTG
Pt#008	cggGGTACCgcggttgatg
Pt#009	cgcGGATCCatgagcgttgtgcc
Pt#010	cggGGTACCatcttcaaccc

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

Chuawong, P., Likittrakulwong, W., Fuengfuloy, P., Svasti, J., “The Anticodon-Binding Domain Swapping in the Non-discriminating Aspartyl-tRNA Synthetase from *Helicobacter pylori*: The contribution to the tRNA Specificity and Catalytic Activity”, *Biochemistry,* Submitted

2. การนำผลงานวิจัยไปใช้ประโยชน์

-เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)

งานวิจัยชิ้นนี้ ได้ก่อให้เกิดความร่วมมือทางวิชาการ กับ The department of chemistry, Wayne State University ประเทศสหรัฐอเมริกา

-เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่)

งานวิจัยชิ้นนี้ เป็นส่วนหนึ่งในการพัฒนาการเรียนการสอนในระดับปริญญาโท และปริญญาเอก ของนิสิต จำนวน 2 ท่าน (นายวิโรจน์ ลิขิตตระกูลวงศ์ ระดับปริญญาเอก และนางสาวพิชญดา เฟื่องฟูลอย ระดับปริญญาโท)

3. อื่น ๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

งานวิจัยชิ้นนี้ ได้ถูกนำเสนอ ณ การประชุมประจำปีของนิสิตนักศึกษาผู้ได้รับทุนในโครงการเครือข่ายเชิงกลยุทธ์ ของสำนักงานการอุดมศึกษาแห่งชาติ (นายวิโรจน์ ลิขิตตระกูลวงศ์)