EFFECT OF BIOTINYLATION ON THE STRUCTURE AND FUNCTION OF A YEAST tRNA-BINDING PROTEIN

TESIS

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__2018__年__04__月__18__日

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Advisor's Recommendation for Graduate Students

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Abstract

Arc1p is a yeast-specific tRNA-binding protein that has ability to form a ternary complex with glutamyl-tRNA synthetase ($GluRS_c$) and methionyl-tRNA synthetase (MetRS) in the cytoplasm. This complex can significantly enhance the aminoacylation efficiency of these two aaRSs to their respective cognate tRNAs. Recently, it was found that Arc1p can be biotinylated via post-translational modification at Lys86 (K86) in the N-domain. We herein studied the effect of K86 mutation on Arc1p's structure and function. We found that mutation in K86R and K86A dramatically decreased the biotinylation level of Arc1p and altered its secondary structure. However, the mutant Arc1p could effectively rescue the cold-sensitive phenotype of an *ARC1*$^-$ strain, suggesting that biotinylation is dispensable for the rescue activity of Arc1p. Interestingly, K86R was more resistant to protease treatment than the wild-type, while K86A was more sensitive.

Keywords: Arc1p, biotinylation, K86A, K86R, mutation.

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Restu Nugraha

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Abbreviation

aaRS	Aminoacyl-tRNA synthetase
ADH	Alcohol dehydrogenase
Ap	Ampicillin
ATP	Adenosine-5'-triphospate
Arc1p	Aminoacyl-tRNA synthetase cofactor 1 protein
Bp	Base pair
BSA	Bovine serum albumin
GluRS	Glutamyl-tRNA synthetase
MetRS	Methionyl-tRNA synthetase
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetra-acetic acid
GTP	Guanosine 5'-triphosphate
HRP	Horseradish peroxidase
ID	Insertion domain
Kb	Kilobase pair
LB	Luria-Bertani
LiAc	Lithium acetate
ME	Mercaptoethanol
PAGE	Polyacrylamide gel electrophoresis
pBS	pBluescript
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
SDS	Sodium dodecyl sulfate
TE	Tris-HCl-EDTA
TEMED	N,N,N',N', tetramethylethylenediamine
tRNA	Transfer RNA

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YPG Yeast extract-peptone-glycerol

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CHAPTER I

INTRODUCTION

1. 1 Background

Arc1p is a yeast-specific tRNA-binding protein that has the ability to make a ternary complex with glutamyl-tRNA synthetase ($GluRS_c$) and methionyl-tRNA synthetase (MetRS) in the cytoplasm. This complex can significantly enhance the aminoacylation efficiency of these two aaRSs to its cognate tRNAs. Recently, Arc1p can be biotinylated via post-translational modification at Lys86 (K86) in the N-domain. Identification of Arc1p as a novel biotinylated yeast protein is a very interesting find since Arc1p is not known to be involved in any carboxylation reaction (carboxylation, decarboxylation, or transcarboxylation reactions). Examples of biotin-dependent reactions include acetyl-CoA carboxylase, propionyl-CoA carboxylase, pyruvate carboxylase, and 3-methylcrotonyl-CoA carboxylase (10). The attachment of biotin to its cognate apoproteins is catalyzed by specific enzyme: biotin protein ligase (BPL), Bpl1p in yeast, BirA in *E. coli*, or holocarboxylase (HCS) in mammals. Biotin protein ligases can biotinylate different apoenzymes across a wide variety of different organisms (7, 17, 24). This can happen because of the highly conserved similarity of all known biotin binding domains and as a result will affect their primary and tertiary structures (17). Furthermore, in Arc1p a lack of sequence similarity was observed between the biotin binding site and the highly conserved biotin binding consensus sequence of known carboxylases, AM**K**M. Mostly biotinylated lysine residues are positioned within this consensus. However, Arc1p may still be biotinylated in vivo. One interesting thing is that biotinylation of Arc1p was performed by the same enzyme which also catalyzes some yeast carboxylases, Bpl1p. Based on Yao (2016), SS**K**D in Arc1p, may represent a secondary biotinylation site for Bpl1p (39). Because in HCS, biotinylation of Arc1p will only have a small fraction of protein being modified, similar to *E.coli* BirA, which does not have any biotinylation effect when using Arc1p as a substrate (17). From this description, SSKD may become a very important sequence of Arc1p since biotinylation takes place here. In this study, it will be elucidated whether it's changing the conformation or function of Arc1p itself when mutations occur inside of the K86 site within SSKD motifs of Arc1p.

1. 2 Objectives

In this research there are several objectives to be achieved:

1. To characterize the structure functional of Arc1p in *Saccharomyces cerevisiae* wild-type and mutants.
2. To characterize the biological functional of Arc1p in *Saccharomyces cerevisiae* wild-type and mutants.

1. 3 Outlines

The outlines of the study include the followings:

- Chapter 1: Introduction
- Chapter 2: Literature Review
- Chapter 3: Materials and Methods
- Chapter 4: Results
- Chapter 5: Discussions and Conclusions

CHAPTER II

LITERATURE REVIEW

2.1 Arc1p and Its Properties

Arc1p is a yeast-specific tRNA-binding protein that has the ability to make a ternary complex with glutamyl-tRNA synthetase ($GluRS_c$) and methionyl-tRNA synthetase (MetRS) in the cytoplasm (39). This complex's main function is to enhance the aminoacylation efficiency of both, $GluRS_c$ and MetRS, for their cognate tRNAs. Specifically, Arc1p acts as a tRNA-attracting molecule thus enhancing tRNA availability for these two enzymes (13, 36). Terminology of ARC1 came from Aminoacyl-tRNA synthetase Cofactor 1, with a predicted molecular weight 42 kDa, encoding a basic protein of 376 amino acids with pI = 8.99. Arc1p also has a synthetic lethality relationship with *LOS1*, which is related to nucleocytoplasmic exchanges or nuclear pore complexes (NPC) in yeast (12). Simos et al., (1996), reported that *ARC1* gene disruption induced slow growth and reduced MetRS activity. Synthetically lethal $arc1^-$ mutants are complemented by the genes for MetRS and GluRS (35). Arc1p can be divided into three functional domains, N-terminal domain, M domain, and C-terminal domain. The first domain, N-terminal domain, is located in residues 1-131. Most of these residues (1-122) adopt a Glutathione S-Transferase (GST)-like folding. The GST-like fold shared by the N-terminus of Arc1p and the two aaRSs then become a ternary complex. The GST-like fold can also facilitate strong interactions between the N-terminal domain of both aaRSs and Arc1p (34). The second domain is the M domain or central domain in residues 132-200, which have lysine or are alanine-rich. Recently, this domain has displayed a sequence similarity with H1 histones (13). The last domain is C-terminal domain (residues 202-376), which along with M-domain forms a tRNA-binding protein domain (TRBD). In yeast, TRBD is very important for catalytic efficiency of MetRS by increasing affinity for its cognate tRNA (8). The C-terminal domain alone shares homologies with human endothelial-monocyte-activating polypeptide II (EMAP II). EMAP II acts as a potent inhibitor of primary and metastatic tumor growth (2).

One must note that biotin is very essential for activities of Arc1p. Biotin is involved in the transfer of carbon dioxide. It very interesting that while we were dealing with this, Arc1p was not involved in any known biotin-dependent reaction or carboxylation/decarboxylation reaction. Also, Arc1p lacks the AMKM consensus sequence of biotin-binding domains, but this enzyme may still be biotinylated in vivo (17). A recent report exhibits that there is one important motif in Arc1p, SSKD, which can be biotinylated through post-translational modification in vivo (39).

2.2 Role and Function of Arc1p

Arc1p has the ability to form a ternary complex with two aaRSs, glutamyl-tRNA synthetase ($GluRS_c$) and methionyl-tRNA synthetase (MetRS). This complex is known to enhance aminoacylation efficiency in both aaRSs for their cognate tRNAs, One way of enhancement is increasing the binding stability between tRNA-aaRSs interaction. Arc1p itself has many indispensable roles in cellular and transport activity. In fact, free Arc1p has the ability to bind a large variety of tRNAs but binding becomes restricted only to $tRNA^{Met}$ and $tRNA^{Glu}$ upon recruitment of $GluRS_c$ and MetRS into heterotrimeric particle (8). It seems likely that the TRBD of Arc1p works in tandem with the anticodon binding domains in the two aaRSs. This process is similar to aaRSs, which is usually very important for recognition and discrimination of cognate tRNA (16). In other words, Arc1p is a key molecule of the tRNA channel. Arc1p also has a role in stabilizing and delivering tRNAs to aaRSs, by acting like a molecular bridge. In this case, Arc1p helps expose the two tRNAs by changing them into a conformation which is efficient for recognition by their cognates (36).

Arc1p is also known to play a crucial role in adaptation of yeast to face the changing of nutritional carbon source. When under normal condition (fermentation state) yeast uses fermentation to produce energy, just a bit of mitochondrial activity is required, thus most of the GluRS is sequestered by the Arc1p-GluRS-MetRS particle. In contrast, when the yeast is in respiratory state, the expression of Arc1p is drastically decreased, leading to the release of both MetRS and GluRS, which are then redirected to the nucleus and mitochondria, respectively. This is caused by a high demand of mitochondrial activity. In this state, Arc1p's expression will be repressed and allows a large amount of MetRS into

mitochondria to quench the increasing need of organellar translation. Free $GluRS_c$ will be reach into the nucleus upon Arc1p repression (11).

2.3 Aminoacyl-tRNA Synthetases (aaRSs)

Aminoacyl-tRNA Synthetases (aaRSs) are a group of enzymes to catalyzes the attachment of specific amino acids to their cognate tRNAs to produce the aminoacyl tRNAs (also called aa-tRNA or charged tRNA) that are the substrates for translation in ribosome (16). Subsequently, the resulting aminoacyl-tRNAs are then delivered by elongation factor-1 (EF-1) to ribosome and transferred. The charged amino acid is needed for making polypeptide sequences, which then become functional protein, based on the genetic code. Prokaryotes have typically 18-20 aaRSs, one for each amino acid; however, eukaryotes have at least two or more sets of aaRSs, because protein synthesis occurs in both the cytoplasm and organelles (like in mitochondria and chloroplast in plant). For example, in yeast, for each aminoacylation activity, there are two genes that encode distinct sets of protein, one localized in the cytoplasm, and the other one in mitochondria (5). Besides being responsible for producing the raw materials for protein translation, the aaRSs also ensure the fidelity of translation from nucleic acid to amino acid information. This phenomena lead an idea to develop a new therapeutic approach which uses aaRSs as a drug targets. For example, mupirocin for topical treatment infection of *Staphylococcus aureus*. This drug acts through the inhibition of isoleucyl-tRNA synthetase (IleRS) of gram-positive bacteria (22).

AaRSs is divided into 2 classes based on their structure and functional characteristic, class I and class II. Class I consist of 11 families; class II with 13 families. Class I contains two highly conserved sequence motifs, HIGH and KMSKS, while class II contains three highly conserved sequence motifs (22). The main difference between these two classes is that the catalytic domains of all class I aaRSs adopt a Rossman fold, which is located near N-terminus. Class I aaRSs also has a five-stranded parallel of β-sheet and is connected by α-helices. Vice versa, catalytic domains that are commonly found in class II aaRSs are organized instead as a seven-stranded β-sheet flanked by α-helices. The others difference between two classes is that most class I aaRSs are monomers, while class II are homodimers, although other monomeric units, α_4 and $(\alpha\beta)_2$ tetramers are also known (21).

Furthermore, class II aaRSs are divided into 3 subclasses, IIA, IIB, and IIC. Subclass IIA and B, possess subclass anticodon binding domains, while subclass IIC possesses a variety of additional idiosyncratic domains (38).

Some mutations in aaRSs have been known to associate with certain neuropathic diseases. Currently, all known disease-associated mutations in cytoplasmic aaRSs are associated with Charcot-Marie-Tooth (CMT) disease. Mutation in GARS was related with CMT disease type 2D and distal spinal muscular atrophy type V. CMT disease is characterized by progressive degeneration of distal motor and sensory neuron function. There are four aaRSs in human that have correlation with CMT disesase: KARS (lysyl-tRNA synthetase), AARS (alanyl-tRNA synthetase), YARS (tyrosyl-tRNA synthetase), and GARS (glycyl-tRNA synthetase). Besides CMT disease, Labauge et al. (2011) (23), has reported that mutations in two mitochondrial aaRSs, DARS2 (mitochondrial aspartyl-tRNA synthetase) and RARS2 (mitochondrial arginyl-tRNA synthetase), have been implicated in leukoencephalopathy with brain stem and spinal cord involvement, and lactate elevation (LBSL), and infantile encephalopathy (10), respectively (40).

2.4 Multi-aminoacyl-tRNA Synthetase Complex

Multi-aminoacyl-tRNA Synthetase Complex (MARS, also abbreviated MSCs or Multi-Synthetase Complex) is a stable macromolecular complex in which composed by nine aaRSs along with three non-synthetase proteins (11, 26). The highly conserved aaRSs of this complex are MetRS, GluRS, GlnRS, ProRS, IleRS, LysRS, LeuRS, ArgRS, AspRS, and aminoacyl tRNA synthetase complex-interacting multifunctional proteins (AIMP)-1, -2, and -3, also known as p43, p38, and p18, respectively (32). MARS complex have many indispensable roles in cellular activity, such as channeling of aaRSs to increasing the efficiency of translation, act as storage to allow the stimulus-dependent release of constituents with non-canonical functions, and nucleocytoplasmic for transport of tRNAs from the nucleus to the cytoplasm (28, 29, 31). In the beginning, MARS formation was believed to be restricted only in higher eukaryotes, when the 20 aaRSs isolated either from *Saccharomyces cerevisiae* or from *E. coli* were found to be only in a free form (19, 20). But recently, MARS have been identified in low-complexity organisms such as bacteria, archaea, fungi and protozoans (25).

Like we described earlier, Arc1p also has able to form a MARS with MetRS and GluRS. This complex was first identified in the mid 1990's. Arc1p was known as homologous to the AIMP1/p43 assembly factor of the mammalian MARS (18, 36). The real function of MARS in yeast is for enhance the tRNA aminoacylation efficiency (kcat/KM) of MetRS and GluRS. The tRNA binding domain (TRBD) in the M and C-terminal domain of Arc1p has vital role for the low affinity of GluRS and MetRS for their cognate tRNAs (36). As a result, the catalytic efficiency of Arc1p-MetRS is increased up to 500-fold in compare with free enzyme. In other hand, the catalytic efficiency of Arc1p-GluRS also increase by 100-fold for its cognate $tRNA^{Glu}$ (15). Interestingly, another study has been reported that only a 3 to 4-fold increase of the aminoacylation efficiency of MetRS upon binding to Arc1p, (14). Enhancement of tRNA aminoacylation efficiency is mainly because an Arc1p-mediated lowering of the KM of MetRS for its cognate tRNA, which resulting MARS assembled MetRS to be far more efficient at low $tRNA^{Met}$ concentrations (35).

2.5 Biotinylation

Biotin, also known as B_7 (or vitamin H based on the German term "Haut" which mean "skin" or vitamin B_8 according to French nomenclature) was identified in the early 20^{th} century as an essential nutritional component. Biotin has a role as a metabolic cofactor of carboxylases in all domains of life, although its synthesis is restricted to plants, most bacteria, and some fungi (17). Biotin can bind naturally and with high affinity to avidin and streptavidin proteins. Because biotin is small (244 Da), it can be conjugated to many proteins without altering their biological activities. The biotinylated molecule can then be detected in ELISA, dot blot, or Western blot methods (1). Biotin-dependent enzymes includes acetyl-CoA carboxylase which is involved in fatty acid synthesis, and mitochondrial enzymes such as propionyl-CoA carboxylase (involved in the degradation of odd-chain fatty acids, isoleucine, and valine), pyruvate carboxylase (involved in gluconeogenesis), and 3-methylcrotonyl-CoA carboxylase (involved in leucine degradation) (10). Biotin is also attached to histone proteins in animals and is believed to be involved in epigenetic processes and prevention of DNA damage (41). Biotinylation is catalyzed in a two step of reactions; biotin is first activated become biotinyl-5'-AMP in an

ATP dependent manner, and is then transferred onto the ε-amino group of a specific target lysine residue. This reaction has similarities with aminoacyl-tRNA synthetases and lipoyl ligases where the reaction proceeds through the formation of an adenylated intermediate, suggesting that there is a common ancestral relationship between them (30). The involvement of biotinylated proteins in other cellular functions has been suggested. What's more, biotinylation of macromolecules is a common and powerful tool in molecular biology due to the almost irreversible binding of biotin with streptavidin ($K_d = 10^{-14}$ M) (13). This binding is one of the strongest interactions between noncovalent molecules. In Arc1p, biotinylation occurred at Lys86 in N-terminal domain of Arc1p. The finding that a protein like Arc1p can be biotinylated is intriguing because the main targets of this modification are biotin-dependent enzymes used mainly for carboxylation and decarboxylation reactions. Biotinylation of Arc1p may be followed by repression of Arc1p production that could constitute a response for long-term adaptation (3).

CHAPTER III

MATERIALS AND METHODS

3.1 Strain, Culture Medium, and Transformation

a. Strain

i. DH10B (*Escherichia coli* strain) (Gibco)

F- *mcrA (mrr-hsd*RMS-*mcr*BC φ80d*lac*ZM15 Δ*lac*X74 *deoR*

*rec*A1 *end*A1 *ara*Δ139 D (*ara, leu)* 7697 *gal*U *gal*K λ^- *rps*L *nup*G.

ii. INVSc1 (*Saccharomyces cerevisiae* strain)

b. Plasmid Carrier

- pRS315 : (Ap^r, LEU2) (Sikorski and Hieter, 1989)
- pADH1 : (Ap^r, LEU2) (Chang and Wang, 2004)
- pBluescript KS II (+) : Ap^r (Stratagene)

c. Medium

i. LB Medium (Luria-Bertani medium)

1 % Bacto-tryptone (Pronadisa)

0.5 % Yeast extract (Pronadisa)

1 % NaCl (Mallinckrodt)

Agar (Pronadisa)

ii. TB Medium (Teffric-broth medium)

1.2 % Bacto-tryptone

2.4 % Yeast extract

1.25 % K_2HPO_4 (Mallinckrodt)

0.23 % KH_2PO_4 (Riedel de Haen)

0.4 % Glycerol (Mallinckrodt)

iii. SOC medium :

2 % Bacto-tryptone

0.5 % Yeast extract

0.05 % NaCl

2.5 mM KCl (Mallinckrodt)

10 mM $MgCl_2$ (Sigma)

0.4 % Dextrose (Sigma)

pH 7.0 (adjust with 5 M NaOH)

d. Preparation of *E. coli* competent cells

i. Pick a single colony of DH10B strain, place under overnight incubation in 4 ml LB broth at 37 ˚C.

ii. Take 4 ml overnight broth culture, add total culture to 50 ml TB broth, , culture at 37˚C. Culture until 600 nm absorbance value is greater than 0.8 broth (± 4 hours).

iii. Placed the bacteria into a centrifuge tube, ice bath for 30 minutes.

iv. Centrifuge at 4˚C, 10 minutes with speed 4,500 xg.

v. Remove supernatant, and add 30 ml sterile water to wash the cold cell pellet.

vi. Centrifuge at 4˚C, 10 minutes, 4,500 xg. (Repeat step v and vi)

vii. The supernatant with 30 ml of pre-cooled sterile water 10 % glycerol, to re-suspend the cells.

viii. Centrifuge at 4˚C, 10 minutes, 4,500 xg.

ix. Removed the supernatant, and add 1 ml of sterile 10 % glycerol to re-suspend the cell pellet, aliquot in 50 µl tube for each, and then store at -80˚C.

e. Preparation of *S. cerevisiae* competent cells

i. Pick a single yeast strain, culture overnight in 4 ml YPD broth at 30˚C.

ii. Take 4 ml overnight broth cell culture, add to 50 ml of YPD broth, incubate at 4˚C and culture overnight.

iii. Place the yeast culture into a centrifuge tube, centrifuge for 10 minutes, 4,500 xg at room temperature.

iv. Remove supernatant, add 30 ml sterile water to re-suspend the cell pellet.

v. Centrifuge 10 minutes at 4,500 xg, at room temperature.

vi. Mix supernatant in 1.5 ml TE/LiAc/H_2O mixture to break up the cell pellet.

vii. Add glycerol with 10 % final concentration and store in -80˚C freezer.

f. Transformation of *E. coli* competent Cells

i. Add 1 µl plasmid DNA into 50 µl competent *E. coli* cells, placed in cell electroporation small tube, adjust to the current 2.5 KV.

ii. Suction out bacteria using a glass pippette, inject into 1 ml SOC medium, culture with shaker at 37 ˚C for 40 minutes.

iii. Centrifuge for 1 minute at 16,000 xg, removed the supernatant, and mix the remaining liquid with the cell pellet.

iv. Apply the mixed broth onto LB/Amp plate, incubate at 37˚C overnight.

g. Transformation of *S. cerevisiae* Competent Cells

i. Take 1 µg plasmid DNA, 1 µg Salmon sperm DNA (SSD) mix in 1.5 µl eppendorf tube.

ii. Add 100 µl yeast competent cell and 600 µl LiAc/TE/PEG.

iii. Vortex for a short time, and incubate in 30˚C for 30 minutes.

iv. Add 70 µl DMSO, inverse 1-2 times, heat in 45˚C water bath for 10 minutes.

v. Put on ice for 3 minutes, and centrifuge at 1,300 rpm for 30 seconds.

vi. Suction out the supernatant and dilute with 70 µl ddH_2O then spread on YPD/selective medium plate, coated at 30 ˚C for 2-3 days.

3.2 Plasmid Construction

Cloning of wild-type (WT) *ARC1* gene (-300 to + 1128 bp) into pRS315 as a low copy number yeast shuttle vector and using pADH as a high copy number yeast shuttle vector with constitutive *ADH* promoter. As a note, a short sequence encoding a His_6 tag was inserted into the 3’ in the end of multiple cloning sites in these vectors. Open reading frames of these genes were amplified by Polymerase Chain Reaction (PCR) method as an XbaI-SpeI fragment. A pair of gene-specific primers were used in this method. The PCR-amplified fragment was cloned into the XbaI/SpeI sites of pADH after enzyme digestion. Subsequently, to make mutant (K86R and K86A), the WT *ARC1* gene was cloned into pBluescript II KS

(+/-) (Agilent, Santa Clara, CA). The resultant construct was used as a template for mutagenesis with standard protocols by manufacturer (Stratagene, La Jolla, CA).

3.3 Arc1p Purification

Add protease inhibitor cocktail (PIC) to stabilize the protein from degradation. Use cell disruptor to get protein into the solution, protein will be filtered and the particulate cell debris will be removed. After acquiring the protein, add Ni-NTA to catch specific protein that contain 6x-His tag. Every step of protein purification will be described below :

- Pick a single colony and grow in 50 mL of SD/Leu$^-$ in 250 ml flask.
- Shake the culture overnight (O/N) at 30 ˚C.
- Pour 25 ml of O/N culture to 0.9 L of fresh SD/Leu$^-$ (+ biotin 200 µg/L for wild type Arc1p) in a 4 L flask.
- Shake the culture overnight (O/N) at 30 ℃.
- Harvest the cells by centrifugation at 5,000 rpm for 10 min at 4 ℃.
- Start the purification protocol by resuspending the cell pellets in 25 ml of Sonication Buffer (Alternatively, store the cell pellets in –80℃ freezer).
- Add 50 µl protease inhibitor cocktail (without EDTA) to cell suspension.
- Break the cells by passing the cell suspension through a French Press 2X (Alternatively, break the cells with a Beadbeater: beat 30 seconds and rest 30 seconds over a 5 minutes period).
- Spin down cell debris with a centrifuge (12,000 rpm, 30 minutes, 4℃).
- (Pre-equilibrate 8 ml of 50% Ni-NTA suspension with 15 ml Sonication Buffer without 2-ME 2X).
- Collect the supernatant and discard the pellet.
- Mix the supernatant with the equilibrated Ni-NTA resin in a 50 ml tube in cold room for 5 ~ 10 min with constant shaking.
- Spin down (5,000 rpm, 5 minutes, 4℃).
- Discard the supernatant by pipetting.

- Wash the Ni-NTA resin 1X with Wash Buffer I, 2X with Wash Buffer II, and 1X with Wash Buffer I:
  Mix the resin with the buffer by gently shaking for 5 minutes in cold room.
  Spin down (5,000 rpm, 5 minutes, 4℃).
  Discard the supernatant.
- Elute the 6xHis-tagged protein by adding 10 ml Elution Buffer to the Ni-NTA resin.
- Mix for 5 min in cold room with constant shaking.
- Spin down (5,000 rpm, 5 minutes, 4℃).
- Collect the supernatant containing the purified 6xHis-tagged protein.
- Check the purity of the protein prep by 10% SDS-PAGE..
- Concentrate the protein prep to 2.5 ~ 3 ml with PEG4000 in cold room.
- Dialyze the protein prep against 1 L "reused" Dialysis Buffer for 4 ~ 8 hr in cold room.
- Dialyze the protein prep against "fresh" 1 L Dialysis Buffer O/N in cold room.
- Dialyze the protein prep against 1 L Storage Buffer for 8 ~ 12 hr.
- Aliquot 0.5 ml/tube and store at -80 ℃ freezer.

3.4 Western Blotting Method

a. Reagent

i. Transfer buffer

- 30 mM glycine
- 0.037 % SDS
- 20 % methanol
- 48 mM Tris-HCl (pH 8.3)

ii. Staining solution

- 0.1 % amido black (Sigma)
- 10 % methanol
- 2 % acetic acid

iii. Destaining solution

- 50 % methanol

- 7 % acetic acid

iv. 10x Running Buffer (1L)

- 30.3 g Tris base
- 144.0 g Glycin
- 10.0 g SDS

v. Blocking Buffer

- 1 % BSA
- 5 % Skim milk
- TBST

vi. Antibody

- Anti-Streptavidin conjugate with Horseradish Peroxidase/ HRP (Perkin Elmer)
- Anti-His antibody conjugate with Horseradish Peroxidase/ HRP (Invitrogen).

vii. TBST (1 L)

- 20 mM Tris-HCl (pH 7.4)
- 120 mM NaCl
- 0.1 % Tween 20 (J.T. Baker)

viii. 4x sample dye

- 0.2 M Tris-HCl (pH 6.8)
- 8 % SDS
- 0.4 % BPB (Sigma)

ix. ECL (Enhanced chemiluminescence)

- Peroxide : HRP substrate = 1 : 1

b.Experimental Procedure

i. Methanol treated to PVDF membrane immersed in a transfer buffer solution, followed by colloid, PVDF membrane, sequentially stacked on paper electrophoresis.
ii. Transferred bath to 300 mA power-transfer 60 minutes.
iii. Transferred PVDF membrane was immersed in blocking buffer: 5 % non-fat milk, 1 % BSA/TBST, 12 hours at 4 ˚C.
iv. Remove the PVDF membrane, wash with TBST for 10 minutes, repeat three times, add suitably diluted antiserum (anti Streptavidin-HRP antibody or anti His_6 antibody was dissolved blocking buffer), for 1 hour at 4 ˚C, then 10 minutes at 3 TBST times, colorimetric method using chemiluminescence.

3.5 Streptavidin-based Gel Mobility Shift Assay

Measuring the relative biotinylation levels of Arc1p variants from WT and yeast mutants was determined by a streptavidin-based gel mobility shift assay (28). Briefly, 0.1 µg of each purified Arc1p was mixed with 6x SDS loading buffer and heated at 95 °C for 3 minutes. Subsequently, the sample was incubated with 1 g of a tetramer form of streptavidin for 5 min at 25 °C. Sample was loaded into a 10% polyacrylamide gel. After gel electrophoresis, the sample was transferred using a semi-dry transfer instrument to a PVDF membrane in buffer (30 mM glycine, 48 mM Tris base pH 8.3, 0.037% SDS, and 20% methanol). Afterwards, the sample, was probed with a horseradish peroxidase (HRP) anti-His6 tag antibody (Invitrogen) and the results were viewed via x-ray film exposure.

3.6 Circular Dichroism (CD) Spectroscopy

CD Spectroscopy measurements were performed in a Aviv Circular Dichroism Spectrometer Model 410. Buffer in this assay contains 100 mM potassium phosphate (pH 7.4). The results were recorded at 10–80 °C using a 1-mm path length cuvette for far-UV CD (200–260 nm) measurements with a scan speed of 50 nm/min, a time constant of 1 s, and a bandwidth of 1 nm. The average of three independent measurements was the final spectrum. The final protein concentration of Arc1p used in the assay was 1 µM.

3.7 Limited Proteolysis

In order to test whether biotinylation alters the structural flexibility of Arc1p, limited proteolysis with trypsin (Sigma) was determined. The principle of this assay is that a protein is incubated with protease (trypsin), and will cut at specific (recognition) sites throughout the protein. Samples were incubated in a ratio 100:1 at room temperature with various times of incubation, 0, 8, 16 minutes, respectively. Add 4x loading dye 4.6 µl for each sample. Stop the incubation time with heat at 100˚ C in 3-5 minutes, and load 6 µg on SDS-PAGE gel immediately to see the result.

CHAPTER IV

RESULT

4.1 Biotinylation of Yeast Arc1p and Variants

It was previously reported that biotinylation is not a common feature of Arc1p homologues. Sequence alignment analysis revealed that some Arc1p homologues in *Candida albicans* and *Candida tropicalis* also possessed a sequence similar to SSKD i.e. TSKD (**Fig. 7**). Even so, they were not biotinylated in vivo (39). Sequences surrounding SSKD may also contribute to this specific modification at the corresponding position. Based on western blotting analysis in **Fig. 10**, it was revealed that only Arc1p^{B+}was biotinylated in vivo, while these two mutants, Arc1p^{K86R} and Arc1p^{K86A} were not. Western blot assays used anti-HRP-streptavidin, because of the binding strength with biotin. Mutation will completely erase the biotinylation activity. This result indicates that, mutation in SSKD motif, especially in K86, will impair biotinylation ability.

4.2 Biotinylation of Arc1p's is not Required for the Rescue Activity

Arc1p has a key role in some cellular activities, such as in multiple cellular pathways, tRNA channel, and nucleocytoplasmic transport (10, 17). Despite having very vital functions, *arc1Δ* mutants are not lethal. This is caused by the operation of a second, exportin Los1p which is required by aminoacylation-independent nuclear tRNA export pathway in yeast (37). Lethality in yeast only occurred when inactivation of both *ARC1* and *LOS1* occured simultaneously, resulting in no additional tRNA export pathway existing in yeast (17, 36). In order to investigate whether biotinylation of Arc1p will affect the activity in vivo, genes encoding the WT, K86R, and K86A mutant of Arc1p were respectively cloned in pADH vector (high-copy number yeast shuttle vector with a constitutive *ADH* promoter and a *LEU2* marker). Subsequently the resulting constructs were tested in an *arc1*$^{-}$ yeast strain at 20ºC, 30ºC, and 37ºC respectively in SD/-Leu plates. As we can see in **Fig. 8**, in 20ºC the knockout strain exhibited normal growth despite needing a longer time if compared with 30ºC and 37ºC. The WT, K86R, and K86A mutant successfully rescued the knockout strain even in low temperature. In the absence of a

functional *ARC1* gene, the knockout strain still retained a near-normal growth phenotype. This finding is consistent with previous reports, mutants of Arc1p still showed growth-supporting activity on par with WT (17, 39). This result indicates that biotinylation is dispensable for the rescue activity of Arc1p.

4.3 Effect of Biotinylation on the Secondary Structure in Vitro of Arc1p

To explore whether biotinylation affects the secondary structure of Arc1p, purified Arc1p variants were subjected to CD spectroscopy. This assay is used to study and analyze the secondary structure or conformation changes of macromolecules, in which sensitive to its environment, temperature, pH, or interaction with other molecules. An important concept of CD spectroscopy was that this is a result of the interaction of polarized light with chiral molecules, since most biological molecules are chiral. The best example is 19 of the 20 common amino acids that form proteins are themselves chiral, along with the higher structures of proteins, like DNA and RNA (9). The secondary structures of Arc1p can be determined by CD-spectroscopy under far-UV exposure. An α-helix can be detected on negative bands at 222 and 208 nm, while β-sheet may be detected at 218 nm. As we can see in **Fig. 12**, Arc1p^{K86R} and Arc1p^{K86A} have slight differences if we compared them with Arc1p^{B+}. These two mutants, possess different protein structures and verify conformation changes. Mutation in these two sites may cause the secondary structure to become relatively unstable. This finding is consistent with previous results in which mutation within the SSKD site will be affect the stability of Arc1p (39).

Subsequently, in order to get a more clear result, we determined melting curves of Arc1p^{K86R}, Arc1p^{K86A}, and also Arc1p^{B+}. This assay was carried out using CD spectroscopy at 10-80˚C. As shown in **Fig. 12**, both Arc1p^{K86R} and Arc1p^{K86A} start to lose their secondary structure at temperatures above 40˚C and had a melting temperature at ~50˚C. It is very interesting because these two mutants have similar trends in thermal stability of secondary structures. This finding is consistent with previous data (39), in which biotinylation will impact the thermal stability of Arc1p.

4.4 Purification and Characterization of Arc1p Variant

Purifying proteins of WT Arc1p (designated herein as $Arc1p^{B+}$) from yeast transformant was carried out in a yeast-defined medium rich of biotin or containing 200µg/l, while normal condition medium contains ~2µg/l of biotin. Meanwhile, these two mutants were grown in normal medium condition. In order to determine relative biotinylation levels of Arc1p and mutants, streptavidin-based gel mobility shift assay was conducted. Like we reported earlier, streptavidin-biotin is the strongest binding interaction between two noncovalent molecules to date with $K_d = 10^{-14}$ M. So from this assay, we can measure the relative level of biotinylation. As shown in **Fig. 11**, $Arc1p^{K86R}$ and $Arc1p^{K86A}$ completely lost biotinylation activity. If we compare with $Arc1p^{B+}$, the biotinylation activity was totally lost. This finding suggests that mutation in K86 of Arc1p will erase the biotinylation activity completely. Note that in previous studies, the biotinylation level of Arc1p does not increase in higher levels of biotin in growth medium (39).

To elucidate whether biotinylation changes Arc1p's structural flexibility, limited proteolysis was conducted. Proteolysis of a protein substrate specifically can occur only if the polypeptide chain has the ability to bind and adapt to the specific stereochemistry of the protease's active site (33). In this assay, trypsin was used in a ratio of 100:1 to perform limited proteolysis at 30˚C. As shown in **Fig. 13**, $Arc1p^{B+}$was much more resistant to the protease than the mutant. Interestingly, in two mutants, there is some difference between them, whereas $Arc1p^{K86R}$ looks more resistant than $Arc1p^{K86A.}$ In $Arc1p^{K86A}$, when protease was carried out after 8 min or more, the protein was completely hydrolyzed. In contrast, in $Arc1p^{B+}$ and $Arc1p^{K86R}$, these two proteins still remained even after 16 minutes of treatment. These results suggested that there are differences in structural flexibility between $Arc1p^{K86R}$ and $Arc1p^{K86A}$.

CHAPTER V

DISCUSSION AND CONCLUSION

5.1 Discussion

Arc1p has become one of the most intriguing research topics in recent years. Arc1p is an auxiliary protein of the MARS complex, which also consists of two aaRSs, GluRS and MetRS. This protein is also known as a homologue of human p43. Although Arc1p lacks a highly conserve biotinylation consensus and does not engage in any biotin-dependent carboxylases reaction, Arc1p still has biotinylation activity, even when modified by Bpl1p in yeast (17, 39). One another interesting thing is that Arc1p is only biotinylated by Bpl1p, while other biotin protein ligases, such as HCS in mammals and BirA in *E. coli*, do not act on Arc1p as a substrate. It has recently been known that a high degree of similarity among biotin binding domains make broad substrate specificity. Since it is known that biotin protein ligase has the ability not only to biotinylate different in the apoenzyme in the same organism, but even came from different organisms, this finding is remarkable. Like in previous reports, the SSKD motif may represent a secondary biotinylation site for yeast Bpl1p (39). Arc1p that had been grown under biotin-rich medium possessed ~15 % biotinylation, in comparison with biotin-free medium (28), two biotinylation sites maybe the cause of this phenomena.

K86 is known as a biotinylation site of Arc1p. This site is located in the N-terminal domain of Arc1p (**Fig. 2**). Lysines are often found in active proteins or binding sites. Lysine is a member of the positively charged amino acid group. Sometimes lysine side chains are involved in forming hydrogen bonds with negatively-charged non-protein atoms. Take note that, lysine also frequently plays an important role in structures. Meanwhile, arginine is also a positively charged amino acid, one that allows interaction with negatively-charged non-protein atoms (e.g. anions or carboxylate groups). This amino acid will be the most preferred substitute for other positively-charged amino acids, like lysine in this case. Besides being positively charged amino acids, both of these amino acids are also frequently involved in salt-bridges where they pair with a negatively charged aspartate or glutamate to create stabilizing hydrogen bonds that can be important for protein stability. Arginine is also frequently found in active proteins and binding sites (4). This reason may explain why some assays exhibit no a huge difference between WT and K86R mutants, like in limited

proteolysis (**Fig. 11**). Nonetheless, although lysine and arginine may both interact with phosphate, arginine has the ability to make multiple hydrogen bonds, while lysine contains only a single amino group, so it means it's more limited in the number of hydrogen bonds it can form. It is because arginine contains a complex guanidinium group on its side chain which has geometry and charge distribution that it's great for binding negatively-charged groups on phosphates. Based on Copley and Barton's research (1994), a substitution from arginine to lysine in some contexts cause great damage (4). In this view, it may explain why mutation in K86 always deleted biotinylation activity (**Fig. 8** and **Fig. 9**).

Substitution of K86A is based on the fact that alanine is not particularly hydrophobic and is nonpolar. This is further corroborated by the fact that alanine can be substituted by other small amino acids. However, alanine plays an important role in substrate recognition or specificity. So in a way it is related with mutation in K86A, because this site is very important for biotin binding. Still, alanine is rarely directly involved in protein function because its side chain is very non-reactive (4).

Biotinylation was not required for rescue activity in various temperatures (**Fig. 6**). So this finding indicates that protein biotinylation is not required for the physiological activity of Arc1p. K86R was capable of restoring viability of *arc1Δ los1Δ* double mutants and with no difference from WT (15). K86R mutant of Arc1p, this mutation impaired biotinylation and also the structural stability of Arc1p (CD wl). This effect was that mutants failed to act as effective cofactors at high temperatures (39). Finally, all the results obtained indicate that there are slight differences in characteristics and structures of protein between K86R and K86A, although functionally it is still consistent with previous reports. More research, like *in silico* modelling, is needed to confirm and explore these results.

5.2 Conclusion

In this study it is revealed that mutation in K86R and K86A dramatically decreased biotinylation activity of Arc1p. Also, these two mutants changed the secondary structure in comparison with $Arc1p^{B+}$. However, K86R looks more resistant under protease treatment, while K86A was very sensitive. In addition, K86 in the N-domain of Arc1p was very important, especially for biotinylation activity.

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Appendix

Table 1. Main Differences between Class I and II of aaRSs

Characteristic	Class I	Class II
Conserved motifs	Motif 1, Motif 2, and Motif 3	HIGH and KMSKS
Active-site fold	Parallel β-sheet	Antiparallel β-sheet
Aminoacylation site	2'-OH	3'OH
Catalytic domain	Rossmann fold	Seven-stranded β-sheet flanked by α-helices
Structure	Mostly monomers	Mostly homodimers
Orientation of tRNA restricted	V loop surface far from enzyme	V loop surface near from enzyme
Example	Met, Val, Ile, Leu, Cys, Glu, Gln, Arg, Trp, Tyr	Ala, His, Pro, Thr, Ser, Gly, Asp, Asn, Lys, Phe

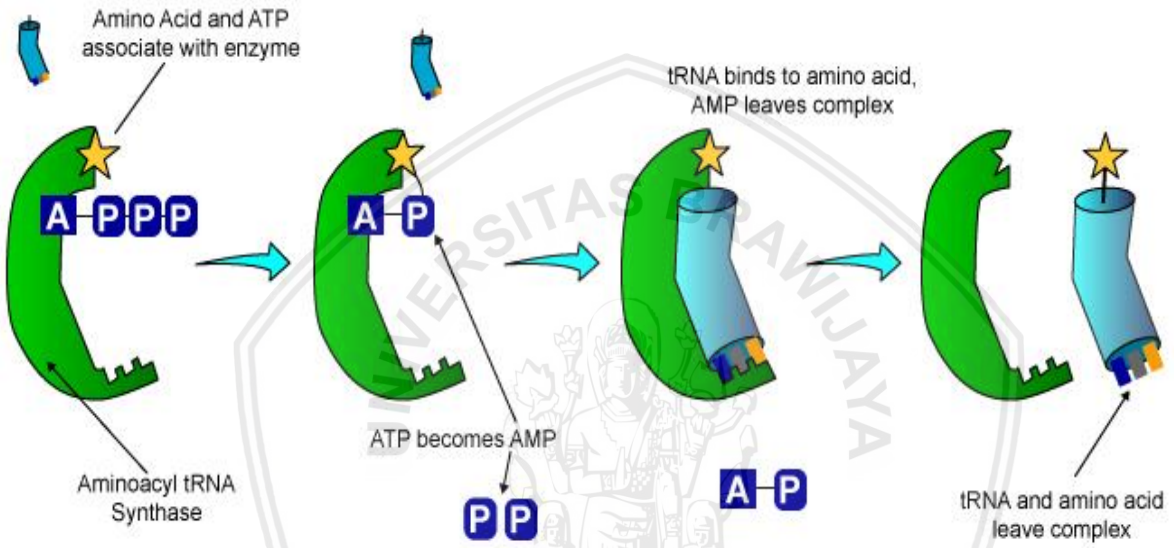

Figure 1. Aminoacylation process (The Pennsylvania State University, 2018).

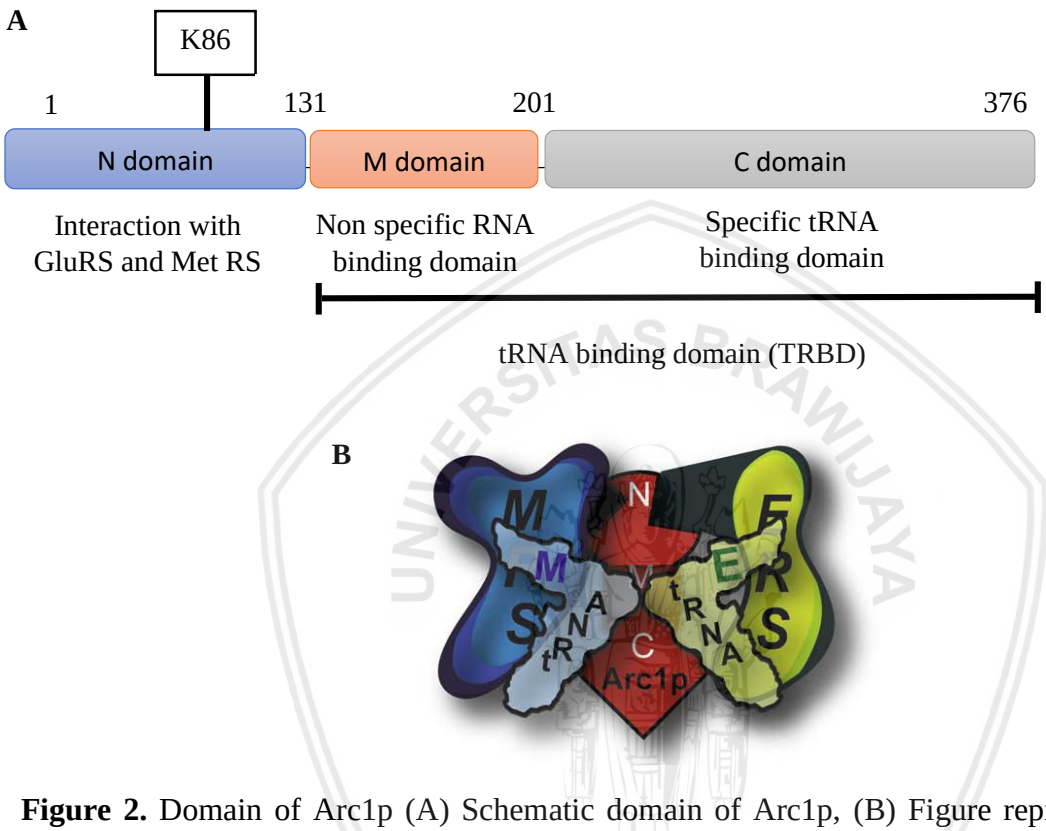

Figure 2. Domain of Arc1p (A) Schematic domain of Arc1p, (B) Figure representing the full Arc1p, GluRS, and MetRS complex together with the cognate tRNAs (11).

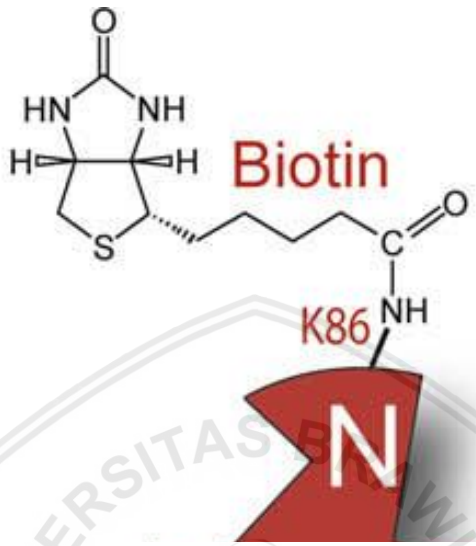

Figure 3. Interaction between N-terminal domain of Arc1p with biotin (11).

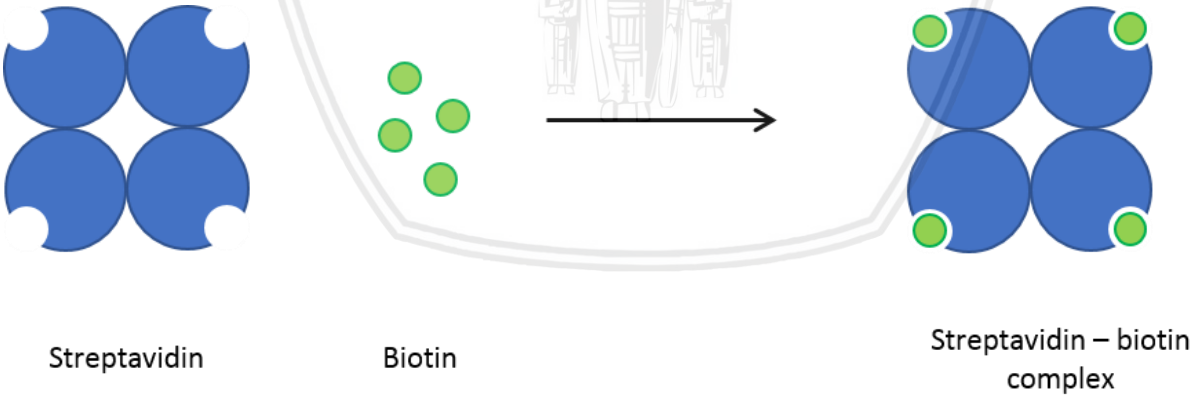

Figure 4. Interaction between streptavidin and biotin (https://www.expedeon.com, 2017)

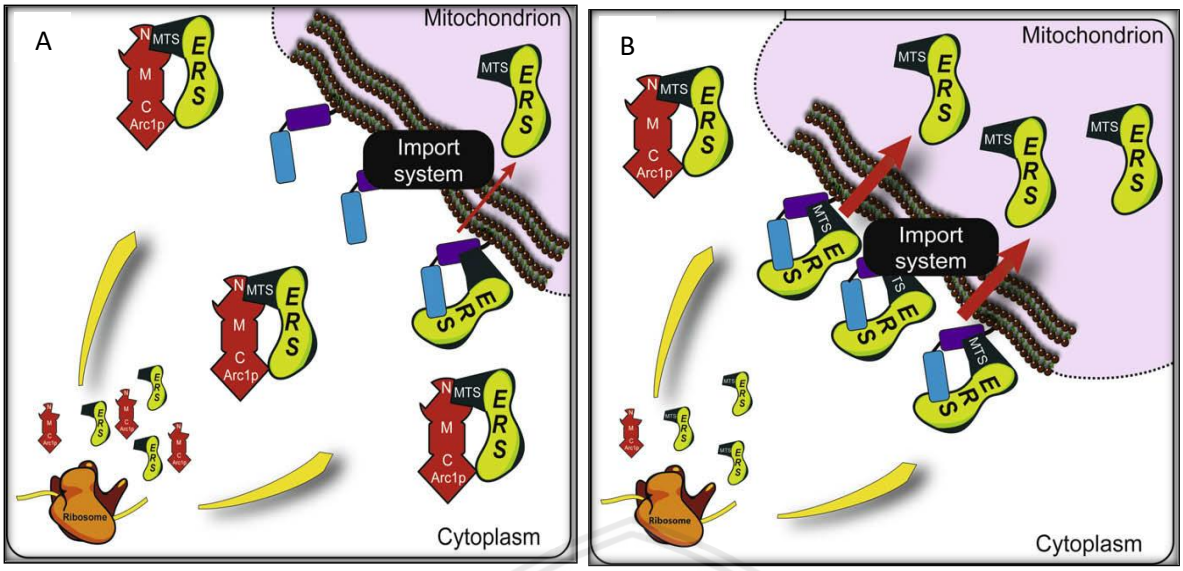

Figure 5. Models for the role of Arc1p during fermentation and respiration. **(A) Fermentation**. When yeast was growing under fermentative conditions for example on glucose media, Arc1p is numerous and cERS is separated from mitochondria in the cytosol. This condition is maintained in order to keep minimal mitochondrial activity. **(B) Respiration**. In contrast, when yeast grown under respiratory conditions (has able to release the energy) the cells are in demand of high organellar activity inside the mitochondria. As a result, Arc1p's expression had to be repressed to allow import of large amounts of cERS into the mitochondria to answer the augmented need of organellar translation. **Note:** cMRS is not represented here, but when Arc1p repression, free cMRS is reach to the nucleus (13)**.**

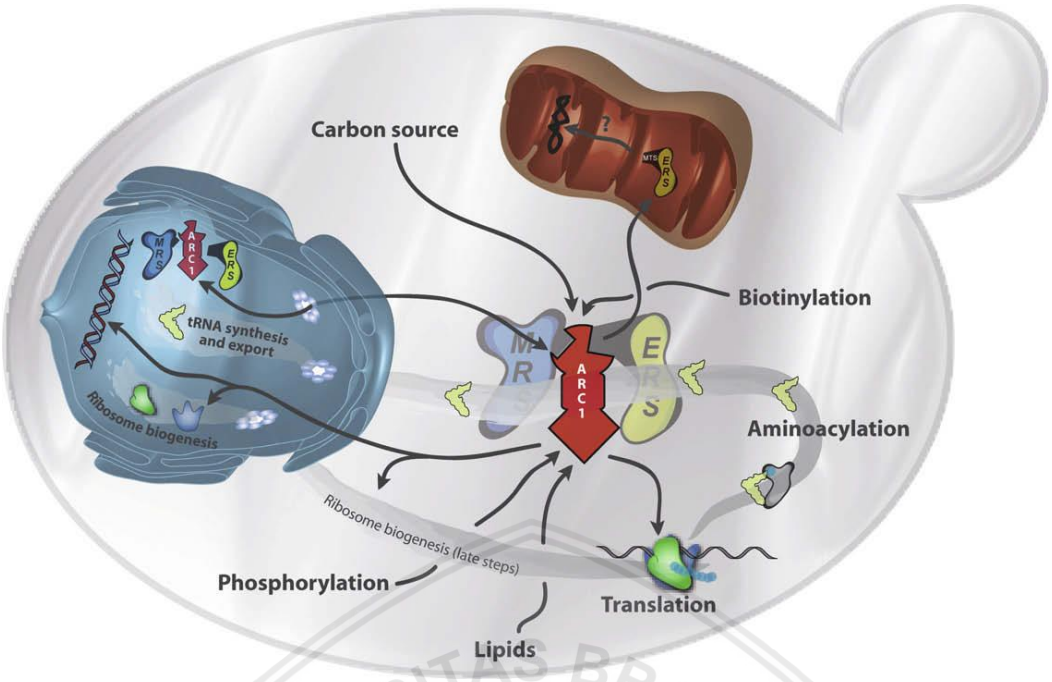

Figure 6. Arc1p is involved in many pathways. Like we know, Arc1p can be phosphorylated or biotinylated. This causes dissociation complex and allow Arc1p, cERS, cMRS, or all individual components to reach the nucleus. Besides have a function in tRNA export, upon in there, it may bind to genomic DNA lesions, like cERS upon import inside the mitochondria. Arc1p may acts as a chaperone and influence the synthesis of ribosomes. Expression of Arc1p also regulated by the carbon source and makes the relocation of cERS and cMRS. And then, Arc1p is engaged in the lipid binding capacity (13).

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S. cerevisiae 76  PLIKDLVASSKDVKST-YTTYRHILRWIDYMQN—LLEVSS  133
V. polyspora  72  PIIRELLASSKDVSNN-SAQYRHIIRWVDYLQQ—LLQLPA  109
C. albicans   82  S-----LASQWTSKDD-VAKYRHILRWADLVQN--TLVN-V 113
C. tropicalis 82  P-----LASKWTSKDD-IAKFRHILRWVDLVQN--TLVT-V 113
Y. lipolytica 90  ----ALYASLKEWKEDQVKAHRHIIRWADLIQNIGLFDLAA 127
Sz. pombe     157 SYICGLSAKEGYKLNN-------VCRWFDFIQHQESVMEAA 190
```

Figure 7. Alignment of the potential biotinylation sites of Arc1p homologues from various yeast species (grey background stands for SSKD motif, black background stand for K86).

	Construct	Protein	Vector	Prom
①	**pCHT449**	**Sc** Arc1p^{B+}	**pYY1**	**ADH**
②	**pRIC246**	**Sc** Arc1p^{K86R}	**pYY1**	**ADH**
③	**pVIV27**	**Sc** Arc1p^{K86A}	**pYY1**	**ADH**

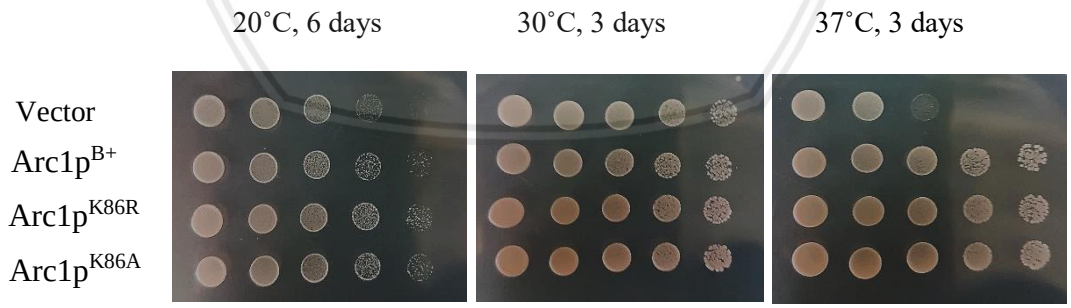

Figure 8. Complementation assay an *arc1*$^{-}$ strain by wild-type (WT) and mutated *ARC1* genes in various temperature

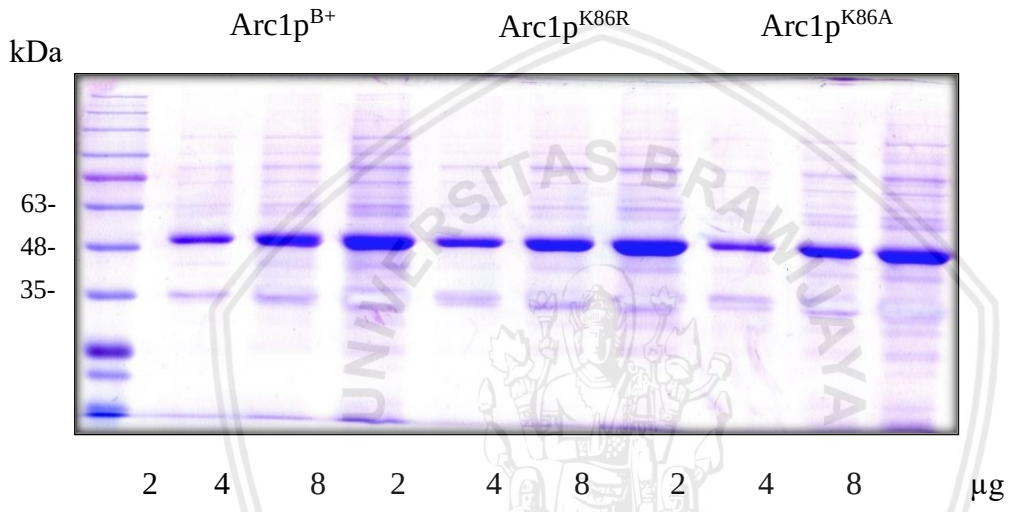

Figure 9. Protein purification of Arc1p^{B+}, Arc1p^{K86R}, and Arc1p^{K86A}, respectively.

Figure 10. Western blotting analysis using HRP-anti-Strep and HRP-anti-His_6.

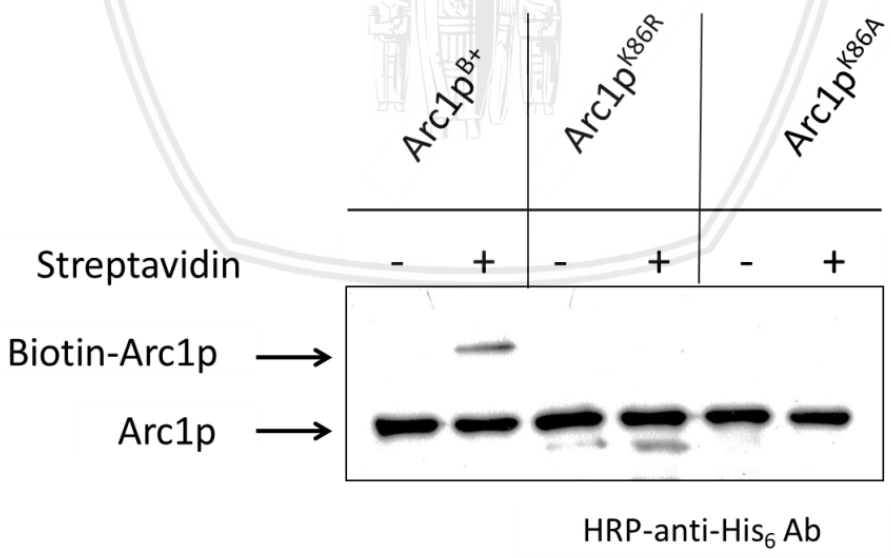

Figure 11. Streptavidin-based gel mobility shift assay.

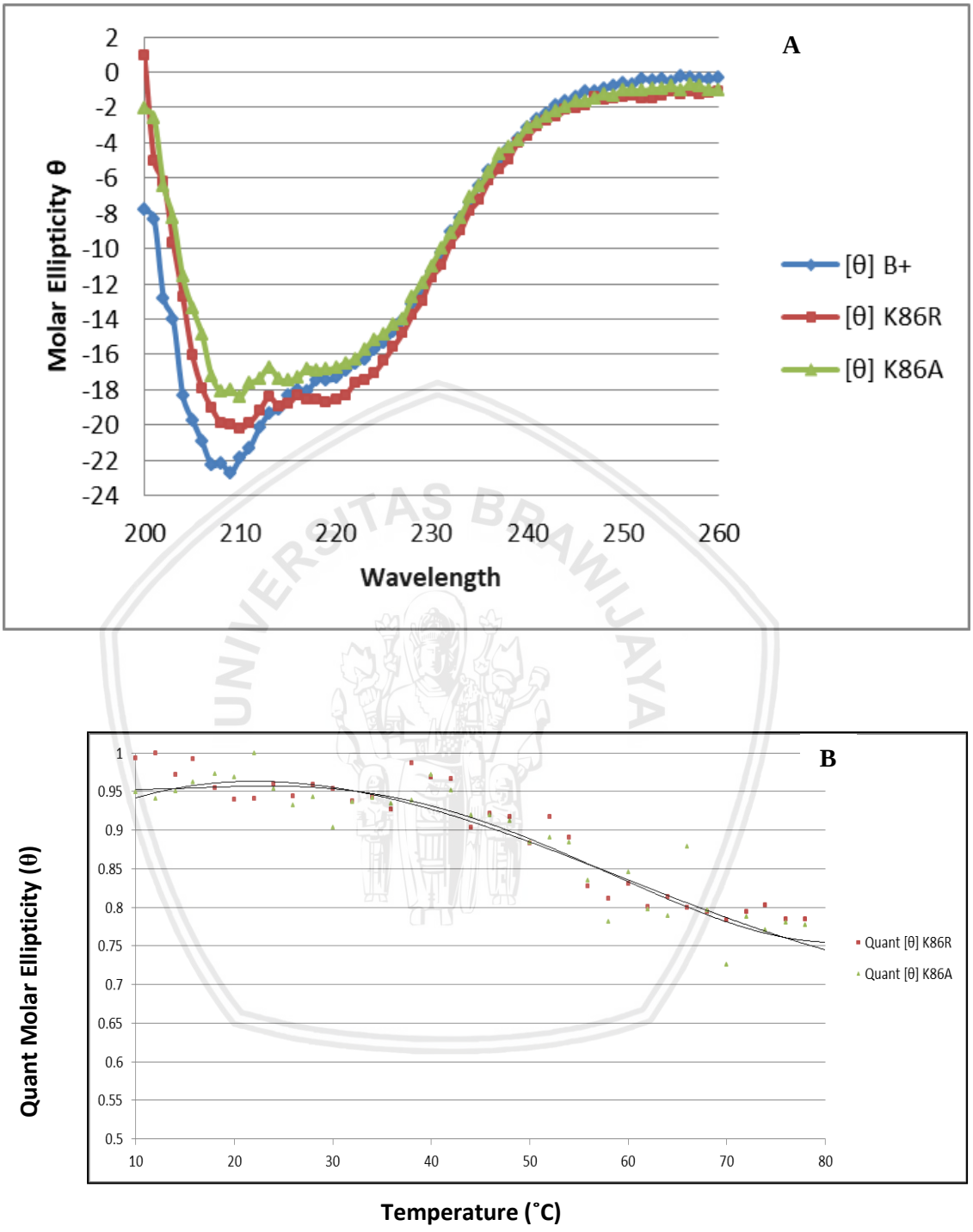

Figure 12. CD-spectroscopy Analysis (A) Comparison CD spectra of Arc1p^{B+}, Arc1p^{K86R}, and Arc1p^{K86A}.(B) Melting curves of two mutants Arc1p^{K86R}, and Arc1p^{K86A}.

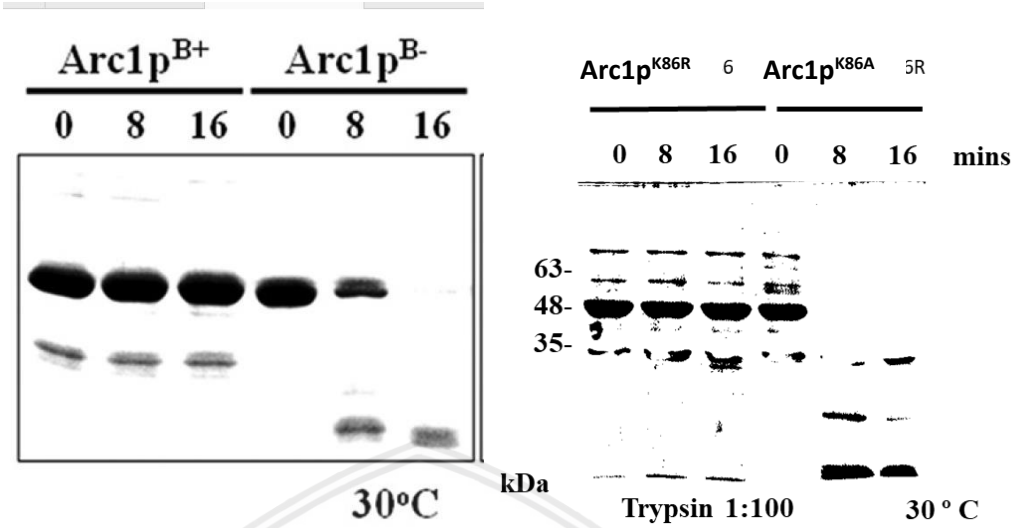

Figure 13. Limited proteolysis of Arc1p^{B+} and Arc1p^{B-} (39) in comparison with Arc1p^{K86R}, and Arc1p^{K86A}, incubated with trypsin for various time at 30 °C and the resultant mixture was loaded into each well for analysis by SDS-PAGE and Coomassie Brilliant Blue staining.

Figure 14. C-terminal domain of yeast Arc1p (202-376) and the probably cleavage site of trypsin (K and R amino acid residues).