Biomolecular architecture: Why some proteins have additional domains?

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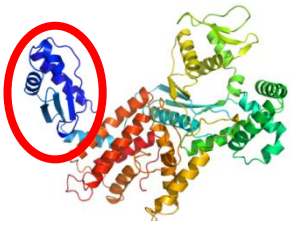

Figure 1. Structure of investigated protein with emphasized N-terminal domain.

In this project we worked with a protein called arginyl-tRNA synthetase from archaeon *Methanothermobacter thermautotrophicus* (MtArgRS) which can bind amino acid arginine with particular tRNA, and the product of reaction then takes part in the translation process. The accent of the research was placed on its N-terminal domain (*Figure 1*). In general, domains can be thought of as protein building blocks and they can be combined in different arrangements to build proteins with different functions.

In earlier research it was discovered that MtArgRS uses this particular domain for seryl-tRNA synthetase (MtSerRS) binding, but it is not known if this domain plays the role in binding $tRNA^{Arg}$ while MtArgRS is not in complex with MtSerRS.[1] Therefore, during this project we produced MtArgRS in large quantities and investigated if this particular domain plays role in binding tRNA.

I. INTRODUCTION

It is well known that proteins play a big role in our organism. They can act like enzymes (make chemical reactions faster), antibodies (make us resistant), structural components of cells, etc. There is a set of 20 amino acids which serve as protein building blocks. Proteins are not found as straight chains in cells, but rather they fold; protein domain is a compact part of protein 3D structure and often can be stable and folded independently. Many proteins consist of several structural domains, while certain domain may appear in a variety of different proteins.

Sequence of amino acids in every protein is dictated by mRNA sequence and the process of translation occurs at ribosomes. tRNA molecules also participate in translation as adaptors: if anticodon of tRNA is complementary to mRNA codon, incorporation of amino acid attached to the opposite side of tRNA into growing chain happens. The rules by which this occurs are given in genetic code table (*Figure 2*). Proteins which are responsible for amino acid and cognate tRNA binding are called aminoacyl-tRNA synthetases and arginyl-tRNA synthetase is the one which binds $tRNA^{Arg}$ with amino acid arginine.

The subject of this research was arginyl-tRNA synthetase from *M. thermautotrophicus* (MtArgRS). This protein can bind MtSerRS by employing its N-terminal domain (emphasized in figure 1). However, it is not known what is the role of this domain (if there is one) when MtArgRS is not bound to MtSerRS. It can be assumed that this domain has significant role in:

1. cognate tRNA binding (based on structure of homologous PhArgRS in complex with tRNA)[2]
2. and/or in aminoacylation

If assumption 1) is valid, we should see the difference in tRNA binding affinity when using wild type MtArgRS and protein with deleted N-terminal domain.

If assumption 2) is valid, there should be difference in kinetic parameters of aminoacylation between these two enzymes (wild type and mutant). These experiments require handling with radioactivity, so we are not going perform them during S3, but respective parameters have previously been determined.

In this project, we aimed to quantify the affinity of wild type and mutant MtArgRS towards cognate $MttRNA^{Arg}$. In other words, our goal is to determine K_d values that going to tell if the abovementioned assumption 1) is correct.

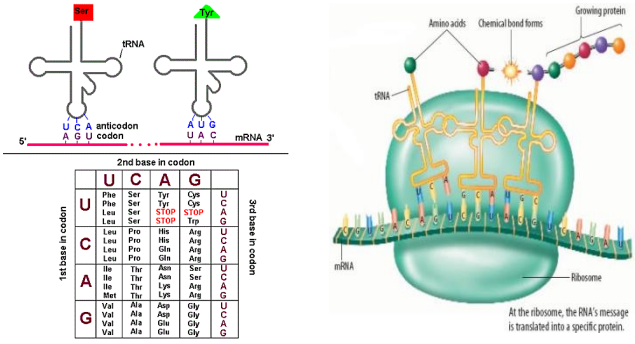

Figure 2. (left) Genetic code table. (right) Scheme of ribosomal protein synthesis in which tRNA and mRNA take part.

II. MATERIALS AND METHODS

Growing bacteria

Special kind of *E. coli* bacteria, prepared in a way that they contain small circular DNA (plasmid) with gene for MtArgRS, were grown. This allows them to produce wanted protein in large quantities. Bacteria were grown in LB media to which two antibiotics were added. We grew them in LB media because it is the most optimal way of growing bacteria and we used antibiotics so that other bacteria and microorganism cannot contaminate our samples.

IPTG addition

After we grew bacteria until optical density of 0,54 was achieved (determined spectrophotometrically at wavelength of 600 nm), we added IPTG substance to final concentration of 50 mmol dm^{-3} in order to remove repressor from plasmid so the over expression of MtArgRS can start. Before that, repressor protein inhibited expression of MtArgRS. In other words, if we did not put IPTG, bacteria would not be able to produce MtArgRS. After 4 h of overexpression, bacterial culture was centrifuged, pellets were collected and storaged in the fridge until the next day. On the next day, bacterial cells were resuspended and pooled into two tubes.

Protein purification

In order to destroy the cell membrane of the bacteria and release the protein, we added 1 mL of small glass beads (approximately of the same size as bacterial cells) to bacteria and shook the in an Eppendorf tube using Vortex. We separated supernatant (soluble part of bacterial cells, including MtArgRS and some bacterial cell components) from insoluble part by centrifugation and we used only supernatant in further procedure.

Affinity chromatography

Affinity chromatography is a method used for isolation of a specific molecule from biochemical mixtures based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. In our case, affinity chromatography was used in order to purify MtArgRS (*Figure 3*).

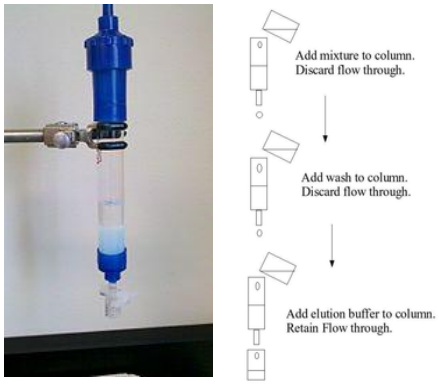

Figure 3. Affinity chromatography column and scheme of procedure.[3]

The stationary phase beads contained nickel(II) ions which can strongly bind hexahistidine tag added to MtArgRS. After binding, we put buffer D whose role was to push the protein off the column because this buffer contains imidazole (similar to histidine) in high concentration (300 mmol dm^{-3}).

SDS-PAGE

Electrophoresis is a commonly used analytical method in biochemistry. The principle of electrophoresis: mixture of molecules is applied to gel, electric current is then passed through the gel and the molecules separate because they migrate at different rates because of the difference in size and charge.

SDS-PAGE is a specific type of electrophoresis, where SDS stands for sodium dodecyl sulfate and PAGE for polyacrylamide gel electrophoresis. SDS-PAGE is used for analysis of proteins under denaturating conditions. SDS is a detergent molecule which causes proteins to denaturate, i.e. their structure is disrupted. Negatively charged detergent molecules bind to denaturated proteins. Because of this, all proteins in sample end up with the approximately same ratio of mass and charge and eventually they migrate according to their mass (small proteins migrate faster than large proteins; *Figure 4*). Mass of a protein can therefore be estimated from the resulting SDS gel. Since protein molecules are not visible, the gel must also be dyed after the electrophoresis. We dyed the gel with Coomassie Brilliant Blue so we can see our proteins, and after dying we boiled the gel in order to remove extra colour.

We used this type of analysis in order to examine if overexpression and purification of MtArgRS by affinity chromatography was successful.

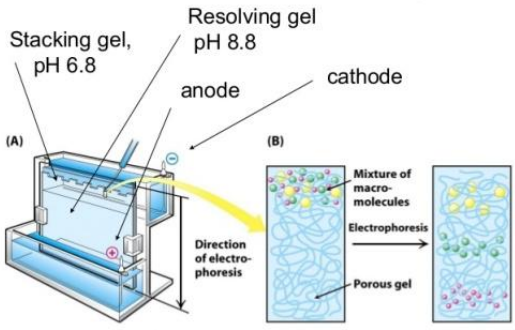

Figure 4. SDS PAGE[4]

Gel-retardation electrophoresis

This method is often used in investigation of protein and nucleic acid complexes because of the difference in migration between protein, nucleic acid and complex. Nucleic acids are highly negatively charged and smaller than protein – because of this, nucleic acid alone migrates fast. Proteins are usually quite larger than nucleic acids and are not as highly charged – because of this, proteins migrate significantly slower than nucleic acids. However, when nucleic acid binds to the protein, the complex has more negative charge than the protein and it migrates faster than protein.

At the same time, since protein is larger than nucleic acid, despite the additional charge, complex will be slower than the nucleic acid alone. Because of this, bands belonging to protein, nucleic acid and complex can be easily identified on gel.

The method itself is called gel-retardation because protein slows down the migration of nucleic acid. If mixtures containing the same amount of MtArgRS, but varying amount of tRNA, are added to the gel, from the amount of formed complex K_d can be determined using the following equation:

$$\vartheta = \frac{c(\text{tRNA})}{K_d + c(\text{tRNA})}$$

The amount of complex is denoted by ϑ and dissociation constant is given as K_d. Hence, from the ϑ dependence on tRNA concentration, K_d can be calculated.

III. DISCUSSION AND RESULTS

SDS gel

Although we encountered some problems during SDS-PAGE (diluted buffer, heating), in the end we managed to obtain the wanted information. On figure 5 it can be seen that MtArgRS was purified successfully. In the first column molecular weight marker (mixture of a few proteins with known molar masses) was added. Upper thicker line in first column tells us that everything on that height has molar mass equal to 75 000 g mol^{-1}, while second thicker line says the analogous thing for proteins of molar mass equal to 50 000 g mol^{-1}. It can be seen that the thick line originating from eluate sample is placed between the aforementioned two lines regarding the height on gel. Since the molar mass of MtArgRS is approximately 65 000 g mol^{-1}, we can conclude that this is in fact MtArgRS. Hence, we managed to purify it from bacterial cell content. In the last column, supernatant after opening bacteria was added and it can be seen that almost everything is coloured. This is because in this column we can find lots of proteins which since bacteria needs proteins to survive, i.e. these are bacterial proteins.

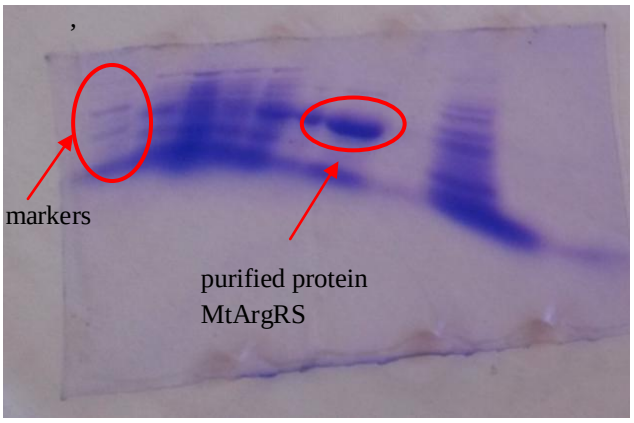

Figure 5. Results of SDS-PAGE. Samples originating from MtArgRS purification by affinity chromatography were analyzed.

Native gels – K_d determination

While in SDS-PAGE the electrophoretic mobility of proteins depends primarily on their molecular mass, in native PAGE the mobility depends on both the protein's charge and its hydrodynamic size.

In total we made two native gels, one with wild type of our protein, and another with mutant type of our protein (purified before the S3). In the first well, sample of protein was added. In the last one, only tRNA was added. Wells 2 – 9 contained constant amount of protein, but varying amount of tRNA. First and last column therefore serve as negative controls (bands belonging to complexes must not be found in these two columns).

The gel with mutant type of MtArgRS has the same bands in every column. We concluded that we probably by mistake added only tRNA in samples placed in wells 2 – 9. We concluded this because we put the sample of pure tRNA in the last column and all other columns are the same as that one. Also, nothing can be seen in the first column, so probably the wrong tube was used during sample preparations and protein was not added.

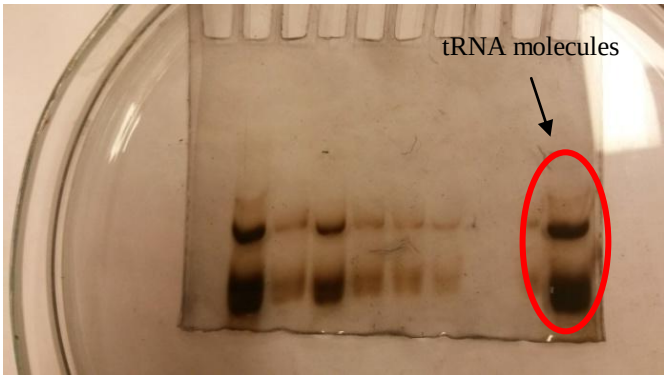

Figure 6. Gel-retardation assay with mutant type MtArgRS.

On gel with wild type MtArgRS bands belonging to proteins and tRNA molecules can be seen, but also we can see the complexes of tRNA and protein on a height between proteins and tRNA. As was mentioned before, proteins are bigger so they travel slower, so on our gel they are on the top. On contrary, tRNA which is smaller and travels faster is on the bottom of the gel.

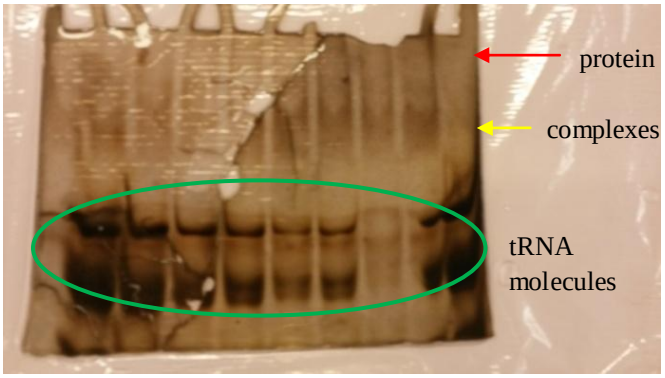

Figure 7. Gel-retardation assay with wild type MtArgRS.

However, since the gel broke during its separation from the glasses, it was dyed unequally. Because of that, the quantification of the amount of formed complex was not good enough for K_d calculation. Quantification was performed using program ImageJ the way it is shown on *Figure 8*, but instead of hyperbolic curve, the obtained data look like it is represented on *Figure 9*.

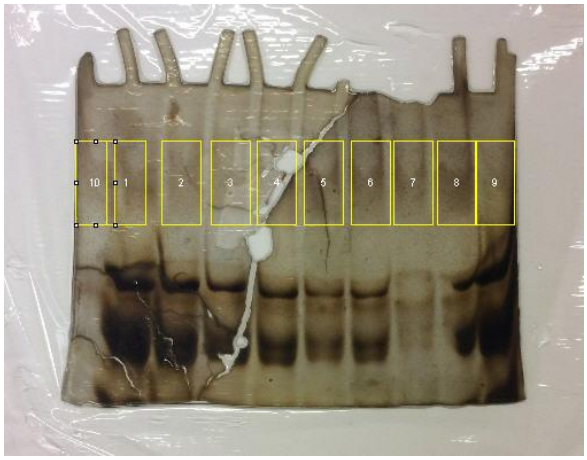

Figure 8. Calculation of complex amount from band colour using ImageJ.

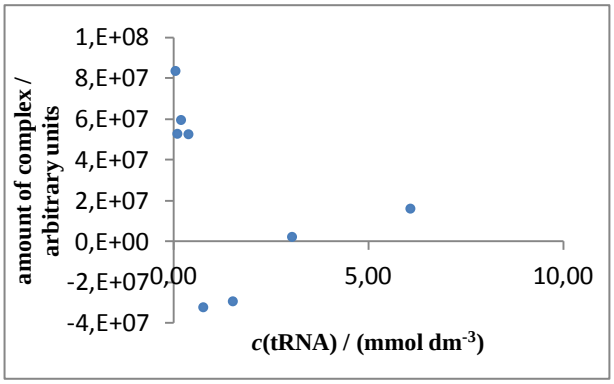

Figure 9. Amounts of complex calculated for different amounts of tRNA added to wells of gel represented on figure 7.

IV. CONCLUSION

During this project, we successfully overexpressed wild type MtArgRS in *E. coli* and purified it using affinity chromatography based on interactions between nickel(II) ions and hexahistidine tag added to protein. We managed to see our purified protein using SDS-PAGE. However, we did not manage to find out if N-terminal domain of MtArgRS plays the role in binding tRNA. We did not manage to do it because a mistake was made in preparing the samples for native gel electrophoresis of mutant protein so complex bands were not formed, while gel with wild type protein was broken and K_d determination was not possible. However, the fact that the complex bands did form on gel with wild type protein means that the tested conditions for native gel electrophoresis are good for this system. Therefore, dissociation constant determination is possible in these conditions and the role of investigated domain would be determined if there was more time to repeat the experiment.

V. ACKNOWLEDGMENTS

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VI. REFERENCES

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[2] http://www.rcsb.org/pdb/explore.do?structureId=2zue (05 August 2015)
[3] https://en.wikipedia.org/wiki/Affinity_chromatography (05 August 2015)
[4] http://www.slideshare.net/ouopened/sds-page-lecture (05 August 2015)