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Site-Directed Mutagenesis of Lysine 125 in Malate Dehydrogenase

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Introduction

Malate dehydrogenase is a multimeric enzyme among living organisms that catalyzes the reverse transformation of malate and oxaloacetate using the reduction of NAD+ to NADH. This reaction plays a role in metabolic pathways including the citric acid cycle, gluconeogenesis, and anaerobic metabolism. MDH shares a similar 3-dimensional structure and mechanism with lactate dehydrogenase. Knowing the structure is important when it comes to the redesign of enzyme mutations, which can be a useful method for studying the catalysis of small substrates. Physiological effects of the amino acid sequence alterations are easier to predict when the structure is known. The active site of MDH consists of a hydrophobic vacuole containing binding site for the substrate and nicotinamide ring of the coenzyme. Within the active site there is a loop region containing amino acids 119-137. The active site exhibits an open conformation when the substrate or cofactor is bound and a closed conformation when nothing is bound. The charges within the loop region position the substrate in the correct orientation for efficient catalysis.

It was shown that Lysine125, within the loop region of MDH, made essential interactions with co-factor and nearby residues that may have been involved in catalysis (Shania, 2019). Shown in figure 1, Lys125 and R124 are in close proximity with each other. Since both molecules have a positive charge, they are repelling against each other. We are predicting that the position of Lys125 and R124 are causing G263 to have a less stable hydrogen bond. **We hypothesized that if Alanine replaces Lysine at position 125, then Arg124 will have a better position and be more stably bound to G263 resulting in a better guide for the substrate to the active site.**

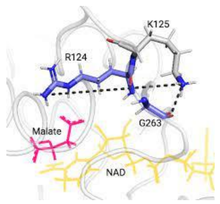

Figure 1. Specific Amino Acids with the Loop Region of MDH. (Shania 2019)

Methods

Expression

A colony of E. coli was added to LB media with the appropriate amount of ampicillin antibiotic. The cells were placed into a large test tube and incubated at 37℃ overnight with shaking. The cells were incubated for 8 hours at 37℃. The cells were induced by adding IPTG. The cells were harvested by centrifuging the culture. The cell pellets were then drained and frozen for lysis and purification.

Lysis

PMSF and Lysozyme were added. The suspension was incubated on ice for 30 minutes. They were sonicated for 1 minute total, 15 seconds on 10 seconds off. The cell lysate suspension was centrifuged. The supernatant was then transferred and placed in the fridge.

Purification

Lysate was added to the top of the drained and prepared column. The lysate ran through the column and was collected. Our lysis buffer was used to wash the column using gravity flow. Our wash buffer was used to wash the column next. Our protein sample was eluted with a buffer.

Bradford Assay

A standard curve was made using various concentrations of BSA. Each concentration was measured on the spectrophotometer at 595 nm. Each fraction of our protein from the purification process and BSA were measured using the spectrophotometer at 595 nm in duplicate.

SDS-PAGE

Lysis buffer and wash buffer were each combined with sample buffer. Our protein fractions 1-5 were each combined with sample buffer. Each sample was vortexed for roughly 20 seconds. The samples were then heated on a heat block, then spun down. Each sample was loaded into the remaining lanes of the gel. The gel ran at 150 V. The gel was stained with Coomassie and rocked for 30 minutes. The gel was then rinsed with water and left in destain overnight.

Enzyme Assay

Na phosphate buffer, DI water, NADH and stock oxaloacetate were added to a cuvette. Various amounts of our mutant MDH enzyme were added to the mix decreasing the amount of water to ensure the total amount of sample stays at 3000 uL. The absorbance of the samples were measured at 340 nm every 10 seconds for 90 seconds. This process was repeated until we achieved a linear line and then repeated 1 more time. After obtaining the specific activity and turnover number, the Km and Vmax values needed to be obtained. Due to running out of our mutated protein, we were unable to determine the Km and Vmax values. The Km and Vmax values were found for the wild type malate dehydrogenase. This was done by taking the absorbance of different concentrations of oxaloacetate. Na phosphate buffer, DI water, enzymes, NADH and oxaloacetate were combined in a cuvette and the absorbance was recorded. The amount of oxaloacetate and water varied depending on the concentration of oxaloacetate used.

PyMol

This program was used along with the MDH dimer with Malate bound to show how the mutation would affect the overall structure. The loop region, 119-137, was changed color and sticks were turned on to differentiate the specific region wanted. Next, the mutation of the specific amino acid being looked into was applied. This mutation being K125 changed to an Alanine.

Results

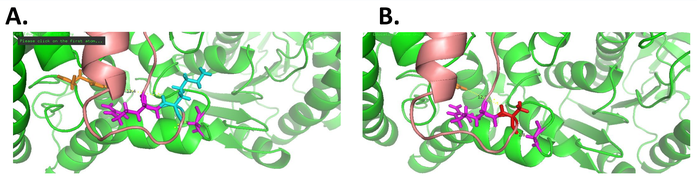

Figure 2. PyMOL of Flexible Loop Region Mutation. The flexible loop region was mutated by changing Lys125 to an Ala. A. Lysine (blue) and B. Alanine (red). Measurements shown from the amino acid at hand to the ligand (12.4Å) and to the neighboring glycine (2.8Å). The measurements shown did not change when the mutation was made.

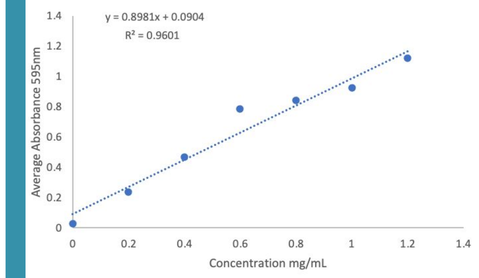

Figure 3: Standard curve with the purpose of finding concentrations of protein. Using a Bradford Assay a standard curve was created with different concentrations of BSA, 0-1.2 mg/ml. All taken without a dilution in a triplet with an absorbance of 595 nm.

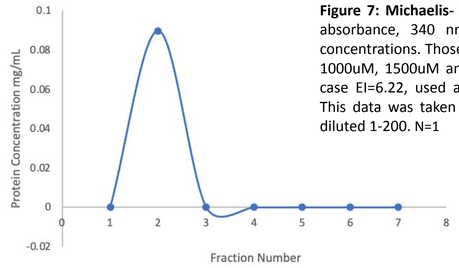

Figure 4: Fraction number vs Protein concentration to determine protein levels. Using the same Bradford assay as in Figure 1, the fractions with unknown amounts of protein were taken. The absorbance was still 595 nm, and the concentrations were found. Fraction 2 was found to have the most amount of protein concentration, this being 0.0897 mg/mL.

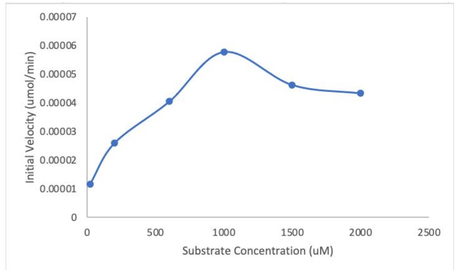

Figure 7: Michaelis- Menten plot of the Wild Type Protein. The absorbance, 340 nm, was read for 30 seconds in different concentrations. Those concentrations were 20uM, 200uM, 600uM, 1000uM, 1500uM and 2000uM. Beer's law, C=A/EI which in this case EI=6.22, used absorbance to determine the initial velocity. This data was taken through two trials of the wild type protein diluted 1-200. N=1

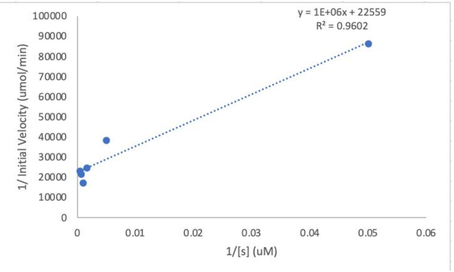

Figure 8: Lineweaver Burk Plot of the Wild type Protein. Using the initial velocity and concentrations as Figure 1 both numbers were taken as the reverse. This gave a R value of 0.9602 and a trend line equation of y=1000000x+ 22559. This data was taken through two trials of the wild type protein diluted 1-200. N=1

Figure 5. Coomassie Stained Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis. Samples were analyzed by adding 30 µl of each sample to 10 µl of 4x SDS buffer sample in order to denature. This was followed by heating the samples for 5 minutes on a 100℃ heat block and then onto a 4-20% polyacrylamide gel alongside a molecular weight marker. The gel was run for 1 hour at 150V and then stained in coomassie stain.

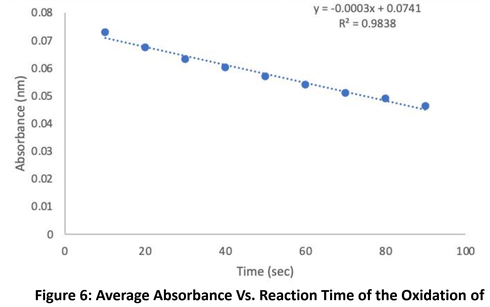

Figure 6: Average Absorbance Vs. Reaction Time of the Oxidation of NADH. A mixture containing NADH, Oxaloacetate, water, Na phosphate buffer and the specific mutated enzyme was used to determine the absorbance in order to see the rate of reaction. The equation of the line was used to determine the specific activity and turnover number. This specific mutation has a specific activity of 0.48 uM/min/ug/mL and a turnover of 17 per minute.

Table 1. Specific activity and Turnover Number of Mutant and Wildtype.

	Wild Type (per minute)	Mutant (uM/min/ug/mL)
Specific activity	29.39	0.48
Turnover Number	1013	17

Conclusions

- Our sample of mutated protein was successfully purified using a column while washing with different buffer solutions.
- After the purification process, we were able to successfully see that we had protein using a Bradford Assay and SDS-PAGE.
- The prediction made by our hypothesis did not prove to be true. We hypothesized that our mutation would cause a better guide for the substrate to the active site. Comparing our specific activity and turnover numbers compared to the wild type, we can conclude that this is not true.

Future Studies

- To repeat the same experiment with the same mutation but making more protein in order to have enough to find Vmax and Km values. This way we could better understand the difference between our mutant and the wild-type.
- To repeat the experiment with a different mutation to find a one that may have a faster enzymatic speed compared to the wild-type.

References

Goward, C. R., Nicholls, D. J. Malate Dehydrogenase: A model for structure, evolution, and catalysis. Protein Sci. 1994 3: 1883-1888.

Shania, Sharon, "Probing the Mobile Loop Region of Malate Dehydrogenase (MDH) for Potential Pathogenic Allosteric Inhibitor Drug Target" (2019). Copley Library Undergraduate Research Awards. 4.