Phosphomimetics of Malate Dehydrogenase in Saccharomyces cerevisiae

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Abstract

Malate dehydrogenase is a key cellular protein involved in cellular metabolism and respiration. In *Saccharomyces cerevisiae,* three types of MDH exist: mitochondrial, cytoplasmic, and peroxisomal. Work was done with MDH plasmids *in vitro* in *E. coli* to create phosphomimics, which are site-directed point mutations that mimic a phosphorylated residue by installing an aspartic or glutamic acid into the location of a normal phosphorylatable residue. Work was done to successfully install these mutations onto their respective plasmid, followed by protein induction and isolation. Additional work needs to be done to further purify the protein and determine kinetics and enzyme activity. This summer's work laid a foundation for future work with these modified MDH proteins.

Background Information

Malate Dehydrogenase

The malate dehydrogenase proteins are responsible for several important cellular functions, particularly regarding cellular respiration and metabolism.[1-4] Malate dehydrogenase, or MDH, exists in the mitochondrial matrix and freely in the cytoplasm, with an additional type within the peroxisomes in *Saccharomyces cerevisiae*.[1] In yeast, mitochondrial MDH is called MDH1, cytosolic MDH is called MDH2, and peroxisomal MDH is called MDH3.[2] MDH is an essential protein in several reactions within the cell. Still, it is perhaps most importantly involved in the Krebs cycle. The conversion of oxaloacetate to malate by MDH in the final step of the Krebs cycle restarts the vital series of chemical reactions that reduce FAD and NAD^+ into the proper cofactors to be used in oxidative phosphorylation.[3] MDH1 is primarily involved in the Krebs cycle functions of MDH with its location in the mitochondria. Cytoplasmic MDH2 is mainly responsible for controlling the malate/aspartate shuttle that translocates electrons into the mitochondria following glycolysis.[1] MDH3 plays an important role in transforming glyoxylate into malate following the glyoxylate cycle that metabolizes fatty acids into glucose.[2]

Saccharomyces cerevisiae and *Escherichia coli* Work

This work was done as part of a larger study supervised by Dr. Joseph Provost of San Diego State University as part of the MDH course-based undergraduate research experience community, also called MDH CUREs. Researchers from several institutions have been studying MDH in various organisms; for our experimentation, the three types of MDH located within *Saccharomyces cerevisiae* were studied. *Saccharomyces cerevisiae* is a standard, laboratory-grade yeast that shares many core cellular functions with that of mammalian cells, allowing for an applicable comparison to human cells. The ability to more easily manipulate the genome of *Saccharomyces cerevisiae* also makes them a more suitable candidate for experimentation. All work done regarding yeast MDH was performed *in vitro* in competent *Escherichia coli*. To do so, specifically designed plasmids were utilized and transformed into the *E. coli*; these plasmids contained the respective MDH gene coding region, resistance to the antibiotic kanamycin, an inducible T7 promoter, and a 6-his tag for protein isolation. Instead of doing *in vivo* work on yeast, work was done with *E. coli* because of their ease of duplicating and taking up mutations, as well as having a solely isolated version of the respective MDH gene to modify. The plasmids used were pET28a MDHM_YEAST, pET28a MDHC_YEAST, and MDHP_YEAST, representing MDH1, MDH2, and MDH3 respectively. Each plasmid is between 6.2-6.4 kbp in length.

Phosphorylation and Phosphomimetics

A useful method of protein-protein interaction and protein signaling involves phosphorylation and dephosphorylation. On their own, most proteins are usually inactive unless they become phosphorylated, which is simply the addition of a phosphate group to a protein. Proteins called kinases can act to phosphorylate other proteins, creating a shape change in the now phosphorylated protein, allowing it to either phosphorylate another protein in a signaling cascade or to serve another specific function within the cell.[4] Malate dehydrogenase, in this same sense, has to initially become phosphorylated to carry out its multitude of functions; without it, MDH would not be able to function properly. During phosphorylation, the large, negatively charged phosphate group selectively binds to serine, threonine, and tyrosine residues, usually located within the active site of the particular protein.[4] A few phosphorylatable sites within the active site of yeast MDH1, MDH2, and MDH3 have been identified experimentally, but the list is not exhaustive.

To determine additional phosphorylation sites in MDH, further work needs to be done to test whether any additional phosphorylatable residues can be successfully phosphorylated during signaling.To determine any additional phosphorylation sites and to measure their effect on MDH function, a technique known as phosphomimetics was employed. This procedure involves inducing a site-directed point mutation within the genome of a particular protein coding sequence so that a phosphorylatable serine, threonine, or tyrosine is instead transcribed and translated into an aspartic acid or glutamic acid.[5] By doing so, the phosphorylatable residues are replaced with a negatively charged amino acid like aspartic or glutamic acid, which mimics the negative charge found when the protein is phosphorylated in that site while also potentially inducing a shape change. This mimicry allows for the observance of any changes to function without having to induce phosphorylation within the protein, essentially leaving the protein "on".[5] A downfall of this method is that normal proteins do not exist in a continuously phosphorylated state in a healthy cell, meaning that there is no way to stop the mimicry of phosphorylation in the mutated cell types; as well, phosphorylation in a normal protein may involve several different phosphorylatable residues, which would not be able to be represented with a single point mutation.[4] Nonetheless, the use of phosphomimics is still a useful technique in determining the effect of a mutated, continuously active protein.

Importance

MDH, if dysregulated, would cause a change in the rate of metabolism. The dysregulation of metabolism is seen in many different cancer types, neurogenerative disorders, and diabetes, so having a better understanding of how MDH is phosphorylated and how a continuously active MDH would affect metabolism would be important to understand.[4] With this knowledge, various treatment plans could be developed for those who suffer from a mutated MDH gene. As well, understanding MDH phosphorylation and activity could be important when engineering cells for valuable compounds like pharmaceuticals and biofuels, since metabolism could be optimized to yield higher production.[4] Finally, the increased understanding of the complexity of post-translational modifications and regulatory mechanisms would overall add to our comprehension of the intricate inner workings of the cell.

Materials and Methods

Datasheets for each respective *Saccharomyces cerevisiae* MDH mutant were found on the MDH CUREs Community website. The protein code of each mutant was extracted and compared to their human equivalent using NCBI's protein BLAST function. The Agilent QuikChange II site-directed mutagenesis kit protocol was used initially, but little success was noted; NEB's Q5 Site-Directed Mutagenesis kit was used primarily for experimentation after this. *E. coli* already transformed with each MDH plasmid was grown overnight at 37°C in liquid broth and kanamycin at 100 µg /mL; plasmid was extracted using the QIAprep Spin Miniprep Kit and quantified using a spectrophotometer. PCR was done next by Q5 Site-Directed Mutagenesis and QuikChange II protocol. PCR product from NEB kit was transformed into BL21 competent cells following New England Biolab's C2527H protocol and plated onto kanamycin plates at a concentration of 100 µg /mL; PCR product from Agilent kit was transformed into XL-1 Blue super competent cells. Plasmid was extracted from successful transformants for sequencing using the QIAprep Spin Miniprep protocol; upstream and downstream primers were designed for Sanger sequencing of samples, which was done by Eurofins Genomics.

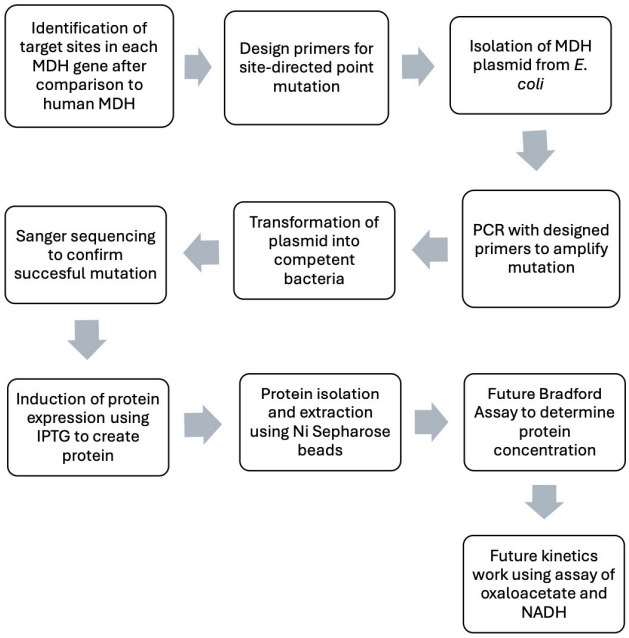

Figure 4: Flowchart of Experimental Design

Overnights of each sample were added to a solution of 90mL Terrific Broth, made from the Cold Spring Harbor Laboratory Terrific Broth Medium recipe, 10mL of 100mM potassium phosphate buffer (pH 8.0), and kanamycin at a concentration of 100 µg/mL. The solution was incubated at 37°C until optical density measured at 600 nm on a spectrophotometer was around 1.0. The solution was chilled on ice for 5 minutes, to which a 0.5mM solution

of isopropyl β-d-1-thiogalactopyranoside was added; the solution was then incubated at 16°C for 48 hours. OD 600 was measured after this period, and cells were harvested via centrifugation at 4°C.

Harvested cells were resuspended using 10mL of lysis buffer (50mM Tris-Cl pH 8.0, 300mM NaCl), followed by 0.5mg/mL lysozyme, 10mg/mL DNase, and 1mL of a 100mMsolution of phenylmethylsulfonyl fluoride, (PMSF), in dry ethanol. The solution was incubated on ice for 30 minutes with occasional stirring. Cells were broken apart using liquid nitrogen and a mortar and pestle, and the resulting product was centrifuged for 30 minutes at 14,000 rpm and 4°C. The supernatant was transferred into a conical tube, with 100µL being saved for SDS-PAGE along with a sample of the pellet. To prepare the flow column, 1 mL of a 50% slurry of Ni Sepharose 6 Fast Flow Resin was washed three times with H_2O and once with a His-binding buffer (50mM Tris-Cl pH 8.0, 300mM NaCl, 5mM imidazole), allowing solution to flow through after each wash and ensure the beads remained submerged throughout; all work was done at 4°C. The contents of the conical tube were transferred into a flow column, mixed well, and placed on a cell rotator for 20 minutes. The solution was allowed to flow through, and 100 µL was saved for SDS-PAGE. The flow column was then washed with a His-wash buffer (50mM Tris-Cl pH 8.0, 300mM NaCl, 15mM imidazole) three times. 0.5 mL of a His-elution buffer (50mM Tris-Cl pH 8.0, 300mM NaCl, 300mM imidazole) and 1mM of PMSF in dry ethanol was added to the solution, and flow through was captured in an Eppendorf tube to be used later; this was repeated a total of three times. 50µL of each eluent was saved for SDS-PAGE. An SDS-PAGE was conducted on samples to determine the presence of purified protein.

Results and Discussion

Sites for phosphomimicry were chosen upon examination of a protein BLAST, which yielded shared regions of protein between yeast MDH and its respective human counterpart; pre-identified regions of potential phosphorylation had also been identified and yeast, so these regions were also taken into consideration. Table 1 shows the comprehensive list of created phosphomimics, showing what residue was changed, its location within the protein, and who created it. Phosphomimics created by Dorian Green ‘24, Connor Eickelman ‘24, and Nathan Cabrera ’24 in the spring of 2024 were also used during experimentation. It is important to note that some of the created point mutations were not necessarily phosphomimics; instead, the original protein was changed into a phosphorylatable residue. These changes were made because a comparison of yeast MDH and its respective human MDH showed a phosphorylation site in the human MDH where there was none in the yeast MDH, so our point mutation was changed to match that of human MDH. In another example, Connor Eickelman’s change of threonine to alanine was done to create a
ring-shaped structure in the active site to increase the binding of a substrate potentially. Figure 1 shows a plasmid map from the plasmids used during experimentation with the noted important regions of the particular plasmid.

Table 1: Phosphomimics Used During Experimentation. All MDH1 and MDH2 mutants were made by Ethan Currin, whereas another mutation on the 179th residue of MDH2 by Dorian Green ’24 was also used; Nathan Cabrera ’24 and Connor Eickelman ’24 made threonine to aspartic acid and threonine to alanine mimics, respectively in MDH3.

MDH1	MDH2	MDH3
158 R →T	179 T→D	146 T→D
180 S→D	188 T→D	146 T→A
183 T→D	221 I→Y	

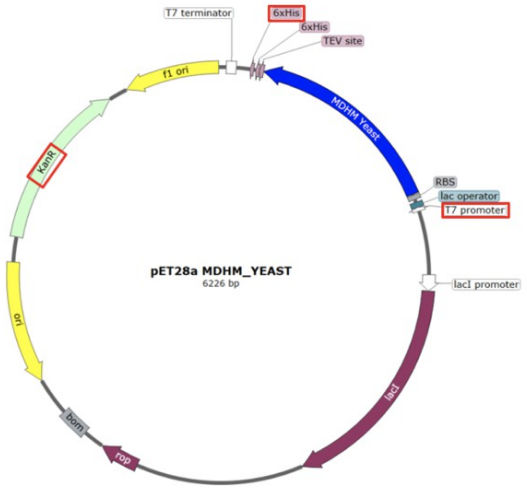

Figure 1: Plasmid Map of MDH1, with a notice on the kanamycin resistance gene, the 6-his tag, and the inducible T7 promoter

Initially, Agilent’s QuikChange II site-directed mutagenesis kit was used to generate our desired phosphomimics, but this kit yielded no successful transformations at first. Eventually, NEB’s Q5 site-directed mutagenesis kit was used, which did yield transformations in all desired phosphomimics except for the 221st residue in MDH2; a last retry of the QuikChange II kit yielded a successful phosphomimic of the 221st residue and the 179th residue of MDH2. We had also wished to create a phosphomimic for the 216th

residue of MDH2 to change from serine to aspartic acid, but no successful transformation was noted in either mutagenesis kit. Nonetheless, it is important to note the presence of the kanamycin resistance gene in the plasmids; with this gene, we were able to select for bacteria that had successfully taken up the plasmid, since untransformed bacteria would have been killed by kanamycin, meaning that only successful transformations would grow on the plates. Figure 2 shows the outcome of all of the transformation efforts, with a representative result shown in Figure 3. Sanger sequencing of transformation colonies of each phosphomimic further confirmed that the appropriate mutation had been made.

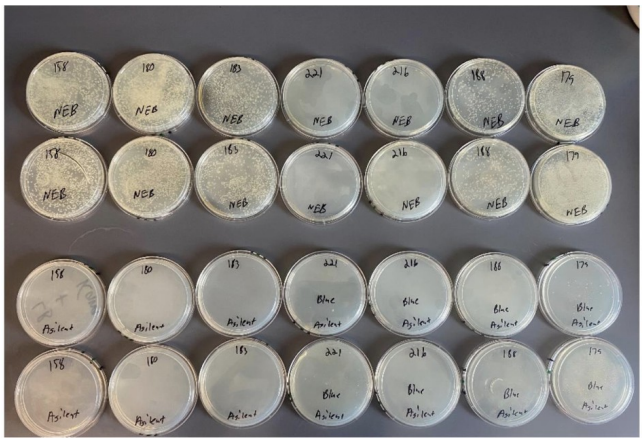

Figure 2: Growth of successful transformants on kanamycin plates following successful transformations

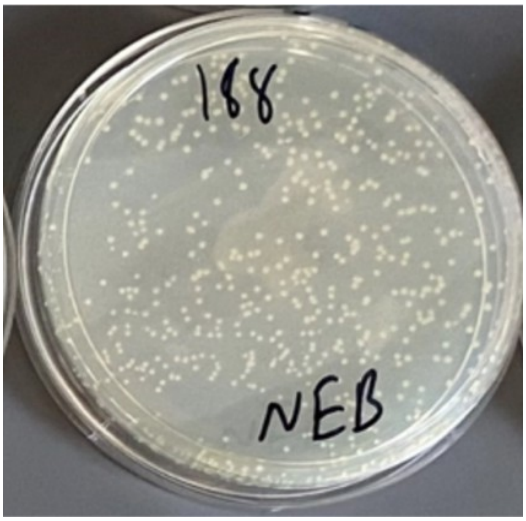

Figure 3: Expected result after transformation. Each individual bacterial growth represents a colony of a successful transformation

Throughout experimentation, several problems arose that caused some troubleshooting and hindered our progress during the summer. Initially, work was being done to install point mutations using CRISPR-Cas9 gene editing, but this proved to be unsuccessful after several attempts at transformation. The plasmid we were using for the CRISPR experiments, PML-104, conferred resistance to ampicillin, which unfortunately caused confusion with our pET28a plasmids and led to us believing that the pET28a plasmids also conferred ampicillin resistance, although this was not the case. We would have recognized this earlier, but the ampicillin plates we plated our first round of transformations on were expired, causing no selection among transformed bacteria, and the new plates we made following this were made with stock ampicillin that had also expired, also causing no selection. It was only when we made fresh ampicillin and fresh ampicillin plates that we realized that we were using the wrong antibiotic since there was no growth on any of the plates. Following this, plates were made from a stock solution of kanamycin that also had expired, causing no selection again. Thankfully, a new solution of kanamycin and fresh plates allowed us to successfully continue our experiments, although this put us several weeks behind.

Following transformation, the successful transformants were grown in a mixture of Terrific broth and an 8.0 pH potassium phosphate buffer to induce logarithmic growth. The addition of isopropyl β-d-1-thiogalactopyranoside to this solution acted to induce the T7 promoter, encoded within the plasmid; this promoter causes unregulated expression of the respective MDH gene found on the plasmid, essentially creating a bulk supply of protein within the cell. After a 48-hour incubation period at 16°C, the OD 600 of each sample was measured, as shown in Table 2. Extrication of the protein from the cells was done with a lysis buffer to lyse the cellular membrane, DNase to destroy any DNA in the cell, lysozyme to further lyse the cells, and phenylmethylsulfonyl fluoride to act as a protease inhibitor to protect our protein. Liquid nitrogen was used to break apart the cells, with the product of this being centrifuged to remove organelles and cellular membrane particulates. Following this separation, the protein mixture was allowed to flow through Sepharose beads containing nickel ions; these nickel ions have a high affinity to bind to the 6-his tag found on the plasmid at the end of the respective MDH protein. Because of this, the beads trap the protein as it flows through the column. After this, the protein was simply removed from the beads using a buffer with a high concentration of imidazole.

Table 2: OD 600

Sample	OD600 after 48 hrs/16℃
Wild Type MDH1	2.50
Wild Type MDH2	2.50
Wild Type MDH3	2.50
CE	2.50
NC	2.44
DG	2.43
MDH1 158	2.36
MDH1 180	2.28
MDH1 183	2.41
MDH2 179	2.33
MDH2 188	2.26
MDH2 221	2.48

To complete this project, the kinetics aspect of experimentation needs to be completed. With the setbacks experienced during this summer, we were not able to finish doing the remainder of the protein work with our phosphomimics. All of the protein has been successfully isolated, as confirmed by SDS-PAGE, but an enzymatic assay needs to be done on the isolates. First, a Bradford assay would need to be conducted to determine the relative protein concentration of the isolated proteins; this would ensure that the same amount of protein was being used across each sample during the protein kinetics experiments.[6] After that, a series of kinematic experiments would be conducted to determine the rate at which MDH can convert oxaloacetate into malate in the presence of NADH. These experiments would be conducted in a UV-vis spectrometer that would allow us to solve Beer's law equation and, from that knowledge, be able to solve for enzyme activity and specific activity.[6,7] These protocols are listed on the MDH CUREs Community website.

Conclusion and Future Goals

In conclusion, a total of six different phosphomimics were created via site-directed mutations. From there, protein expression was induced, and protein was isolated from the cells. If this project were to be continued in the future, kinetics work would need to be performed on the protein isolates, which are currently stored in a -20°C freezer. If the stored protein had lost its enzymatic ability, then the protein induction and isolation experiments could be conducted again using cryogenically stored samples of each of the phosphomimics. Apart from this work, it would be interesting to see if the CRISPR-Cas9 site-directed mutagenesis experiments we conducted could be refined to yield a successful transformation. Additionally, doing *in vivo* work on yeast to create a phosphomimic in the MDH gene would also be interesting. Overall, the experimentation done this summer created a groundwork for further experiments by classes at Hampden-Sydney or for others wishing to do work in the MDH CUREs community.

Acknowledgments

I would like to thank Dr. Wolyniak for advising me throughout this project. I also would like to thank Dr. Wolyniak and the Hampden-Sydney Office of Undergraduate Research for providing the funds to pursue this project, as well as the Hurt Scholar program for providing me with additional funding. I would like to thank the MDH CUREs Community for their inspiration and help with this project. I would like to thank Joshua Ferriera and Robert Frazier, who I shared a lab with this summer, as well as Mrs. Jenkins and Mrs. Matthews for providing me with all the appropriate chemicals and glassware I needed to complete this project.

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