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Studying the Phosphorylation of Isocitrate Dehydrogenase in Humans

An Honors Thesis submitted in partial fulfillment of the requirements for Honors Studies in Chemistry

By
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Spring 2023

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Abstract

Isocitrate dehydrogenase is an important enzyme in the citric acid cycle where it catalyzes the oxidative decarboxylation of isocitrate to alpha-ketoglutarate. While there are three isoforms of isocitrate dehydrogenase (IDH1, IDH2, and IDH3), this research will focus on IDH1. The phosphorylation of isocitrate dehydrogenase is a process that has been linked to the formation of both luminal-like and basal-like breast cancer. Despite these correlations, the mechanisms that cause breast cancer development are unknown. To examine this, an enzyme activity assay for each phosphorylation variant and crystallization were conducted. The results of these indicate that phosphorylation at each site (IDH1-T77, IDH1-S188, and IDH1-S237) leads to a decrease in isocitrate dehydrogenase activity when compared to the wild type.

Introduction

Isocitrate dehydrogenase (IDH) is an enzyme that plays an important role in the citric acid cycle in humans. In the citric acid cycle, isocitrate dehydrogenase catalyzes the conversion of isocitrate to alpha-ketoglutarate. In addition to this conversion, isocitrate dehydrogenase also plays a role in the reduction of nicotinamide adenine dinucleotide (NADP⁺) to nicotinamide adenine dinucleotide phosphate (NADPH) and formation of carbon dioxide (Huang et al., 2019). There are three isoforms of isocitrate dehydrogenase: IDH1, IDH2, and IDH3. While all isoforms of isocitrate dehydrogenase have an important role in cell metabolism and cancer development, this research will focus on IDH1. This isoform is a NADP⁺-dependent enzyme that is found in the peroxisomes and cytosol (Fujii et al., 2016). In the cell, IDH1 plays an essential role in cell metabolism and maintenance.

Mutations in IDH1 have been linked to various cancers due to its role in the cell. When a mutation is present at IDH1-R132, the enzyme catalyzes the reduction of alpha-ketoglutarate to the oncometabolite 2-hydroxyglutarate (Figure 1) (Fathi et al., 2014). The most common mutation at this site is the replacement of arginine with either cysteine or histidine (Medeiros et al., 2017). High concentrations of 2-hydroxyglutarate have been linked to cancer development. The presence of 2-hydroxyglutarate in high concentrations leads to the hypermethylation of promoter sequences, leading to abnormal cell differentiation (Dang & Su, 2017; Fathi et al., 2014). The buildup of 2-hydroxyglutarate also causes changes in gene expression that can lead to the activation of oncogenes and deactivation of tumor suppressor genes (Gavel et al., 2017). This decrease in differentiation and changes in normal gene expression has been linked to many types of cancers, including breast cancer.

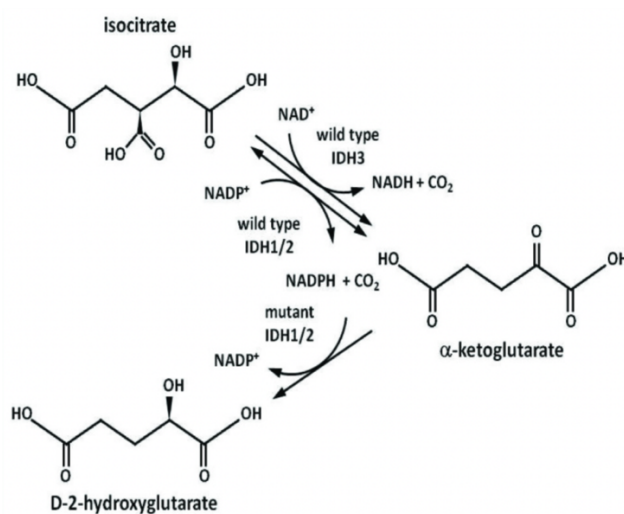


Figure 1. The mechanism of mutant isocitrate dehydrogenase conversion of alpha-ketoglutarate to D-2-hydroxyglutarate (Gavel et al., 2017).

Decreased levels of IDH1 are also associated with disease progression. The expression of IDH1 can be decreased either through micro RNAs miR-32-5p and miR-92b-3p or the formation of heterodimers (Liu et al., 2018; Zhao et al., 2009). This decrease in IDH1 activity leads to an increase in the migration and invasion ability of breast cancer cells, contributing to the progression of cancer (Liu et al., 2018). In addition, when IDH1 activity is decreased so is the level of alpha-ketoglutarate. The decrease alpha-ketoglutarate is linked to an increase in the expression and stabilization of hypoxia-inducible factor 1-alpha (HIF-1 α) (Fujii et al., 2016). Hypoxia-inducible factor 1-alpha is associated with cancer development. When present in high concentrations, HIF-1 α promotes tumor growth through increased angiogenesis (Zhao et al., 2009).

Posttranslational modifications of isocitrate dehydrogenase, such as phosphorylation, have also been found in cancer cells. For the IDH1 isoform, multiple phosphorylation sites are linked to the development of cancers (Chen et al., 2019). When looking specifically at breast cancers, certain phosphorylation sites on IDH1 are correlated with both luminal-like and basal-like breast cancer development. Phosphorylation of the T77, S188, and S237 sites have been linked to breast cancer development (Figure 2) (Mertins et al., 2016; Mertins et al., 2014). Of these phosphorylation sites, the T77 site is correlated with luminal-like breast cancer development while the S188 and S237 sites are linked to basal-like development (Mertins et al., 2016; Mertins et al., 2014). This implies that there are site-specific impacts of phosphorylation on breast cancer development.

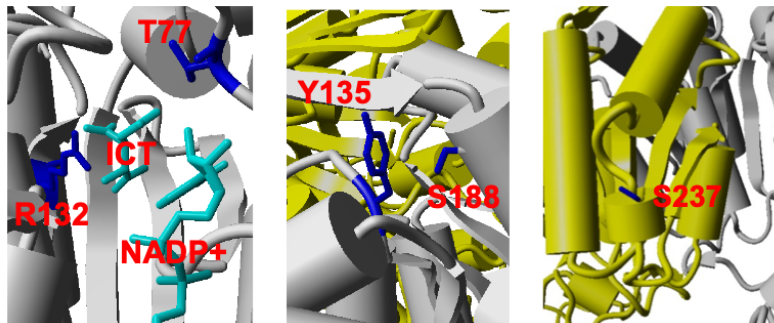


Figure 2. Phosphorylation sites on IDH1, represented in dark blue (PDB ID: 1t0l).

Despite the association between phosphorylation at these sites and breast cancer development, it is still unclear why this happens. The relevant phosphorylation sites occur at different places on IDH1. The T77 site is located at the active site, S188 is located close to the dimer interface, and S234 is not located at either the active site or dimer interface (Ma et al.,

2017; Xu et al., 2004). These differences in phosphorylation site location indicate that there are different mechanisms impacting IDH1 function.

Approach

To determine these impacts on function, IDH1 with each of the phosphorylation modifications and the wild type was purified. These purified protein samples were then used to conduct an enzyme activity assay and crystallization. The enzyme activity assay provides insight into how phosphorylation at each site impacts the ability of IDH1 to convert isocitrate to alpha-ketoglutarate. Crystallization of the phosphorylated IDH1 variants using the hanging drop method allows for examination of the structural changes that occur with phosphorylation at each site.

Understanding the hanging-drop crystallization technique and microseeding

The hanging-drop method works by suspending a drop of sample and reagent above pure reagent of higher concentration (Figure 3). Water then diffuses out of the drop until equilibrium between the drop and the pure reagent are reached, promoting nucleation (Dessau & Modis, 2011). Crystallization occurs when the drop is supersaturated, a state that is achieved by water diffusing out of the drop.

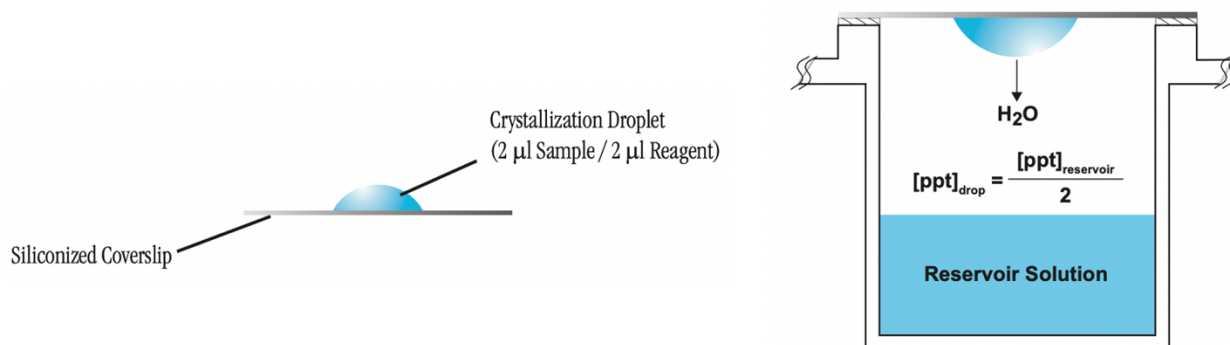


Figure 3. Representation of hanging drop crystallization ("Hanging Drop Vapor Diffusion Crystallization," 2017).

While having a high level of supersaturation promotes nucleation, lower levels of supersaturation promote ordered growth of large crystals (Bergfors, 2003). This type of growth can be achieved through microseeding. In microseeding, the nuclei formed are crushed and added to new drops to continue growth (Zhu et al., 2005). These seeds should be added when the drop has metastable zone conditions. When in the metastable zone, crystal growth will occur without forming new nuclei (Figure 4) (Chayen, 1998). Growing crystals using this technique allows growth conditions to be optimized with less uncertainty than spontaneous nucleation, making successful crystal growth more likely (Stura, 1999). These crystals, if large enough, can be used to determine protein structure through x-ray diffraction.

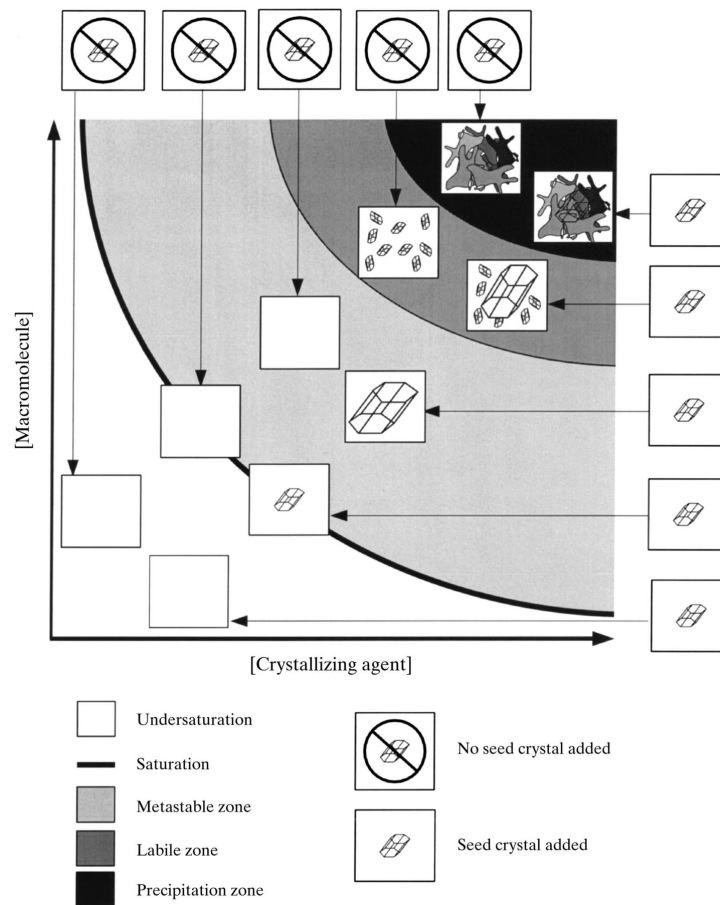


Figure 4. Representation of crystal formation with and without a seed crystal (Luft & DeTitta, 1999).

Methodology

The purification of isocitrate dehydrogenase and phosphorylated variants

The isocitrate dehydrogenase and phosphorylated variants were purified using affinity chromatography. To prepare the sample, imidazole lysis buffer (20 mM), half of an ULTRA protease inhibitor pill, and a small portion of nuclease were added. Once the nuclease is added, the sample was kept on ice as much as possible. The solution was then vortexed to resuspend the

pellet and sonicated with pulses every 15 seconds for 3 minutes at 70%. Once sonication was complete, the sample was centrifuged at 19,000 x g and 4°C for 15 minutes.

Once the sample was prepared, it was filtered through a syringe using a 0.45µm filter into a column with 50% volume nickel resin. The columns used were 10mL Econo-Pac Disposable Chromatography Columns distributed by Bio-Rad. The liquid was collected, and the column was washed with imidazole (50mM). Sample fractions were then collected using an imidazole elution buffer (200mM). These fractions are then loaded into a SDS-PAGE gel to confirm protein purification.

The isocitrate dehydrogenase enzyme activity assay

This enzyme activity assay was conducted using the QuantiChrom Isocitrate Dehydrogenase Assay Kit (DIDH-100) by BioAssay Systems. The enzyme assay determines activity by measuring the reduced form of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). When isocitrate is converted into alpha-ketoglutarate, NADPH is produced. The reduction of MTT is dependent on the NADPH generated by this reaction, making the enzyme activity directly proportional to the amount of reduced MTT observed (Grela et al., 2018).

To perform the assay, water, calibrator solution, and sample were added to the clear flat bottom well plate. Then, the working reagent solution was prepared by mixing 9µL of substrate, 9µm of NADP/MTT solution, 1µL of diaphorase, and 70µL of assay buffer per well being used. The working reagent solution was then added, and the wells mixed. The plate was then incubated at 37 °C. The optical density of the plate was read at 565nm after 10 minutes and

again after 30 minutes. These values were then used to calculate isocitrate dehydrogenase activity.

The crystallization of isocitrate dehydrogenase

The crystallization of isocitrate dehydrogenase was conducted using the hanging drop method. This procedure was done using indexes 67 and 75 from the Hampton Research index screen. To create the coverslip, 2 μ L of reagent and 2 μ L of the sample were added to form a drop. The drop was then mixed. The prepared coverslip was then flipped so that the drop is hanging and placed above a grease-rimmed well of a VDX plate containing 1mL of reagent. The coverslip was then pressed lightly to create a seal.

Once nucleation had occurred on the hanging-drop plate, microseeding was performed. The crystals were added to a microcentrifuge tube containing reagent and a Seed Bead. This tube was vortexed for 3 minutes, crushing all crystals present. Another coverslip was then prepared by creating a drop with the solution from the microcentrifuge tube. This coverslip was also placed over a grease-rimmed well containing reagent and sealed. The seeded wells were later examined under a polarizing microscope to determine the size of the crystals that formed.

Results & Discussion

Data and analysis

Before conducting the enzyme activity assay or crystallization procedure, the purification of the IDH variants and wild type had to be confirmed. To do this, an SDS-PAGE gel was run using the fractions collected from the nickel resin chromatography column. An SDS-PAGE gel

separates the proteins based on size, with larger proteins traveling a shorter distance and smaller proteins traveling a longer distance. Since this method separates proteins based on size, bands that are aligned horizontally on a gel indicate proteins of the same size. Analysis of the SDS-PAGE gel for IDH1 and its phosphorylation variants showed aligned horizontal bands (Figure 5). This alignment indicates that the proteins were purified, and further experiments could be conducted.

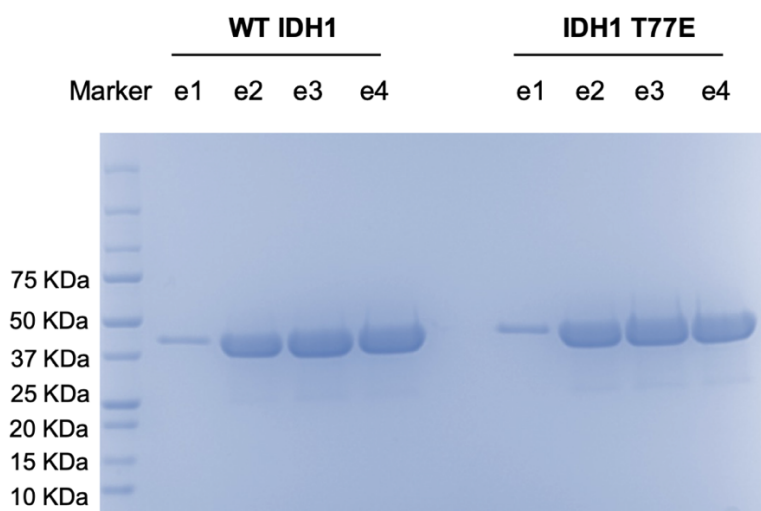


Figure 5. The SDS-PAGE gel obtained for IDH1 wild type and T77 phosphorylation variant.

Once the purity of the IDH1 and phosphorylation variants was confirmed, an enzyme activity assay was conducted. This enzyme activity assay indicated significant decreases in IDH1 activity for all phosphorylation variants (Figure 6). As noted, decreases in isocitrate dehydrogenase activity have been linked to the development of cancers. Decrease in the activity of IDH1 present in breast cancer cells has been showing to increase cancer migration and

invasion (Liu et al., 2018). When the activity of isocitrate dehydrogenase is decreased, so is the level of alpha-ketoglutarate present in the cell. This decrease in alpha-ketoglutarate has been shown to increase the stability of HIF-1 α , promoting tumor growth (Fujii et al., 2016; Zhao et al., 2009). This enzyme activity assay implies that the phosphorylation variants of IDH1 promote breast cancer development through similar mechanisms.

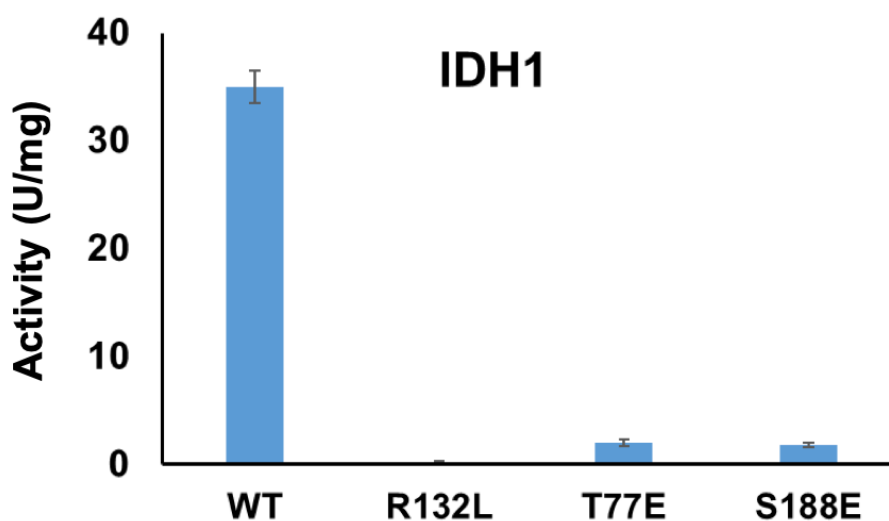


Figure 6. Graphical representation of enzyme activity assay comparing the activity of the wild type, mutant R132, and two phosphorylation variants.

While the enzyme activity assay provides insight into how these phosphorylation variants promote cancer development, it does not examine why enzyme activity is affected in this way. To examine this, crystallization was done to determine how phosphorylation impacts the structure of IDH1. The impacts on the structure provide insight into why the enzyme activity is decreased.

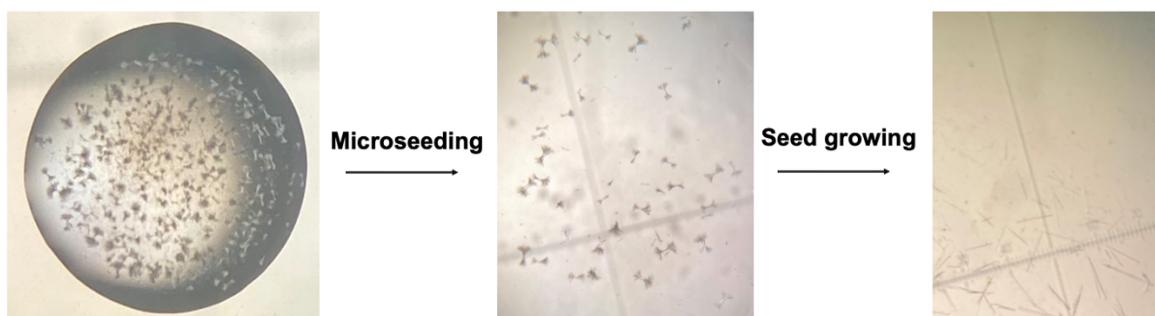


Figure 7. Crystallization and microseeding process for IDH1 and phosphorylation variants.

While some crystals did grow using indexes 67 and 75 from Hampton Research Index Screen, none were large enough to use in determining the structure of the IDH1 phosphorylation variants. Despite this, it is thought that phosphorylation at the T77 site decreases isocitrate binding due to the addition of a negative charge and phosphorylation of S188 is thought to affect dimer formation (Ma et al., 2017; Thorsness & Koshland, 1987). However, the structure of the phosphorylation variants must be determined to confirm these theories.

Implications

Isocitrate dehydrogenase is an important enzyme in the citric acid cycle that catalyzes the conversion of isocitrate to alpha-ketoglutarate, generating NADPH and carbon dioxide in the process. Mutations, decreased activity level, and posttranslational modifications of isocitrate dehydrogenase have been found in many types of cancers. Understanding the mechanisms behind these cancer-forming processes can aid in the development of cancer treatments. Currently, many new medications that inhibit mutant isocitrate dehydrogenase activity are being developed to treat various cancers (Golub et al., 2019). Since the phosphorylation variants

IDH1-T77, IDH1-S188, and IDH1-S237 are linked to breast cancer, understanding their role in cancer formation can be utilized in future drug development.

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