Lab Mini Report 2&3; Sebastian Lechuga; Oct 5th 2023

Introduction

Background

In this lab, we were working with Human Carbonic Anhydrase II (HCAII). It is interesting because it catalyzes a simple but crucial reaction for human homeostatic biological processes (Senes, 2023). The HCAII enzyme is an ideal model for enzyme manipulation through specific mutation because it is simpler to model and insert mutations in comparison to larger and more complex enzymes.

The benefit of utilizing the HCAII enzyme is that it is found in all cell types. Additionally, it is a monomeric protein indicating that it is one gene sequence that encodes for the whole enzyme protein structure. The HCAII enzyme is unique because it utilizes Zinc in the active site but the enzyme structural integrity is conserved even without the presence of Zn because the enzyme remains folded in the apo form (Senes, 2023).

HCAII has been extensively studied with the approximate reaction rate and catalytic efficiency being quantified. It has been found that HCAII is an efficient enzyme with the reaction rate being limited by the diffusion rate of the substrate into the active site (Senes, 2023).

Biologically the HCAII enzyme is important because it has been identified to be involved in the pathogenesis of various human diseases. These illnesses include obesity, glaucoma, and altitude sickness (Vijay M., 2008).

The HCAII catalyzes the reaction between H2O and CO2 to form a bicarbonate ion. There have been two CO2 binding sites identified within the HCAII active site (Merz, 1990). The closest CO2 binding site to the Zinc ion has involvement from Threonine 199.

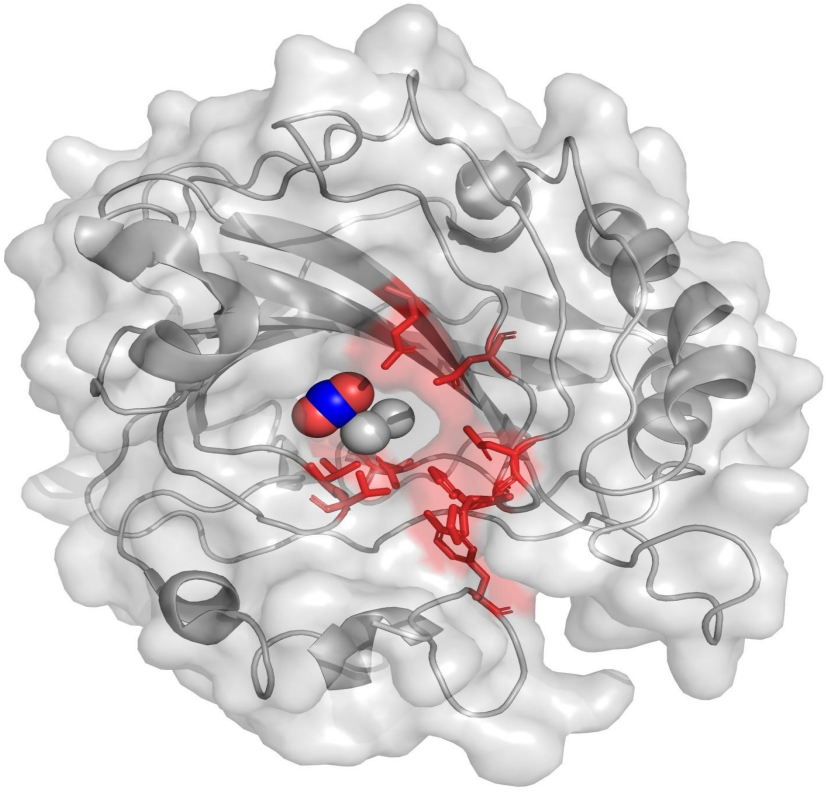

Fig. 1 Pymol Rendering of HCAII Polar Region of Active Site
The three spheres highlighted in red and blue is the CO2 in the active site. The amino acid residues highlighted in red are residues that participate in producing a polar region within the active site. As can be seen the polar region is localized to one half of the active site.

Hypothesis

In this experiment, we have chosen to mutate the Human Carbonic Anhydrase II with a specific single residue mutation. This mutation was mutating Threonine at the 199 residue position to Serine as seen in **Figure 2**. We hypothesize that the mutation will conserve the functionality of the enzyme. However, since T199 is exposed to the active site and involved in several steps of the reaction mechanism.

We hypothesize that the enzyme functionality will be conserved because Threonine and Serine have nearly identical side chains. The only difference is that Serine lacks a methyl group at the beta carbon on the side chain. Regardless, Threonine and Serine both contain amine and hydroxyl groups on their side chains.

Due to the similarity of the wild type and mutated residues, we believe that there will be a deviation in catalytic efficiency due to the slight change in the enzyme's active site structure.

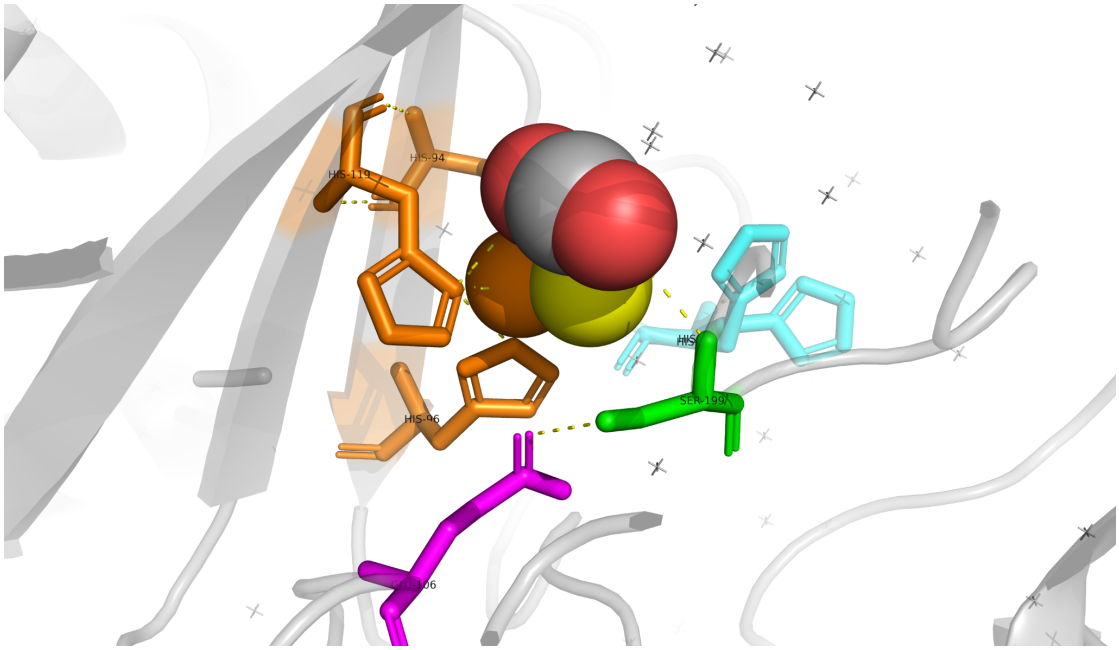

Fig. 2 Pymol Rendering of Mutant HCAII Ligand Binding Structure In this Figure you can see the mutant S199 residue within the ligand binding structure. This figure illustrates the one hydrogen bond between S199 and Glu 106. Also illustrated is the hydrogen bond between S199 and the CO2 reactant within the active site.

General Procedures

We are using PCR reaction to amplify both our wildtype and mutant *Human Carbonic Anhydrase II gene*. The gene is being inserted into the pETblue-2 plasmid via Gibson Assembly PCR.

The pETblue-2 plasmid will then be transformed into E. coli. The purpose of this transformation is to utilize the transcription and translation machinery of E. coli to produce both the WT and mutant HCAII enzymes. After the transformation, we will be testing and measuring the various enzymatic characteristics of both the WT and mutant HCAII enzymes. These measurements will enable us to compare changes in the characteristics and understand the effect of specific mutations within an enzyme's active site.

Methods

Experimental Purpose

In order to amplify the wildtype HCAII gene into a vector plasmid we used several methods including PCR, Enzyme Digestion, Gel electrophoresis, and bacteria transformation to express the desired enzyme. For the initial PCR reaction utilize the Gibson Assembly method to insert our desired HCAII gene into pETBlue-2.

PCR Materials

For the PCR reaction utilizing the Gibson assembly method, we used the lab instructor-provided 5x PCR reaction buffer. For the nucleotides, we used 200 uM dNTP solution in each sample. We added 10ng of DNA template into each sample. For both the forward and reverse primer we used 400nM of each into each sample. For the *Phusion* polymerase, we added 1 uL of 1 U/uL to achieve 1 U of polymerase in each sample.

PCR Procedure

For amplification of the wild-type *HCAII gene* in the pETblue-2 plasmid. We took several precautions to ensure a complete mixture and interaction of the PCR components. It has been found that it is the best practice to pipette large volumes first to then allow for small volumes to be directly pipetted into the large volumes. This precaution is used to ensure that all of the small-volume components are properly incorporated into the PCR reaction. Another precaution taken was incorporating the Phusion polymerase at the last step to prevent denaturing of the enzyme.

The PCR components were combined in a PCR reaction tube fit for the Edison Thermocycler. We began by adding the ddH2O because it was the largest volume component in each sample. Then we added 10 uL of the 5x reaction buffer to achieve a final concentration of 1x PCR reaction buffer. Thirdly, the dNTP mix was added to each sample.

Depending on the sample next a mix of either Primer A & D or Primers B & C. For each mix of primer, we added 2 uL total which was composed of 1 uL of each primer. After the primers, we added 4 uL of DNA template. Lastly, the 1 uL of Phusion polymerase was only added to samples 1 & 2.

Thermocycler Procedure

In order to denature, anneal, and extension phases of the PCR reaction. We used a Thermo Fisher Thermocycler in order to achieve an accurate and consistent temperature phase during the PCR cycles. We are grateful to be able to use this piece of equipment that can hold the samples at the specific temperature for an accurate and specific amount of time.

For the initial cycle, we started at 98 C to begin denaturing the template DNA. For the amplification process, we set up 35 cycles of three separate

temperatures for distinct time frames. The cycles start at 98 C for 10 seconds for denaturing of DNA. This is followed by 50 C for 30 seconds which is meant to promote the annealing state. For the final extension phase, we set the temperature of 72 C for 2 mins. This extension phase is the longest to allow for the polymerase enzyme sufficient time to extend across the whole gene.

After the 35 cycles, the thermocycler finally held the samples at 72 C for 10 mins. Finally at 10 C until the samples were removed from the thermocycler.

Primer Sequences

Table 1. Primer Sequences Used for PCR Reaction

	Forward	Reverse
Insert	Primer A 5’ GTTTAACTTTAAGAAG GAGATATACCATGGCC CATCACTGGGGGTAC	Primer D 5’ CTTAATTAACATTAGT GGTGGTGGTGGTGGT GTTTGAAGGAAGCTT TGATTTGCCTGTTC 3’

	GGCAAAC 3’	
Vector	Primer B 5’ GAACAGGCAAATCAA AGCTTCCTTCAAACAC CACCACCACCACCACT AATGTTAATTAAG 3’	Primer C 5’ GTTTGCCGTACCCCC AGTGATGGGCCATGG TATATCTCCTTCTTAA AGTTAAAC 3’

In **Table 1** it can be seen that the primers are around 54 bps and 60 bps. The primers were set up this way to maintain a similar melting temperature (T_m)to prevent either primer from being heated at an excessive temperature with respect to its T_m.

Negative Control

The negative control had all the PCR reaction components as the mutant sample. However, the only difference was that the Phusion polymerase was not added to the negative control. Since the negative control lacks the polymerase enzyme, there will be no amplification. There are other methods for negative

control but due to the expensive nature of the DpnI enzyme, it makes logical sense to exclude it to reduce costs.

Gel Electrophoresis DNA Visualization

In this experiment, we used Gel Electrophoresis to visualize the concentration of fragment length of the DNA in all three samples. Ethyl Bromide was incorporated into the cooled gel electrophoresis which chemically binds to DNA by intercalation between base pairs. This allows for accurate visualization of the concentration and migration of DNA samples through the gel. The visualization of the fluorescence was done through a machine that displays the gel through UV fluorescence.

The gel used for the gel electrophoresis was 1% agarose. The running buffer was 1x TAE Buffer in order to maintain an equal ionic strength between the running buffer and the gel. For each sample, 6x bromophenol blue dye was used to get a visualization of the nucleic acid migration through the gel. For each sample, 12 uL was loaded into their respective well. The nucleic acid samples were run at 150 V until the ladder migrated approximately halfway through the gel. In the future, we should have let the gel run for a longer period of time to allow for a greater separation of the 1 kB ladder bands.

DpnI Enzyme

In this experiment, we use the DpnI enzyme. For each sample, we added the *DpnI* enzyme. This enzyme is unique because it can distinguish between methylated and unmethylated DNA fragments. During the PCR reaction, the newly synthesized DNA fragments are not methylated. This is crucial because the template DNA is methylated. Due to this, we were able to digest all of the unwanted DNA templates by adding DpnI. The DNA was incubated with DpnI for one hour to allow the enzyme sufficient time to digest the template DNA enough to prevent any template DNA from interfering with future methods.

During the lab, we added 1 uL of DpnI enzyme to each sample. The enzyme was added after the thermocycler phase of the PCR reaction. The enzyme was incubated with the PCR samples for 1 hour at 37 C.

DNA Purification

In order to purify the DNA after the enzyme digestion. A common spin column method was used. The spin column was used to purify all three PCR reaction samples after the DpnI enzyme digestion. All of the samples were purified including the negative control.

Quantifying DNA Concentration

The DNA concentration was measured through NanoDrop laboratory equipment. The NanoDrop was calibrated with the Elution Buffer which was added in the final steps of the DNA spin column purification. The NanoDrop also measures the absorbance at specific wavelengths and was used to determine the 260/280 and 260/230 ratios.

Results

Gel Electrophoresis Results

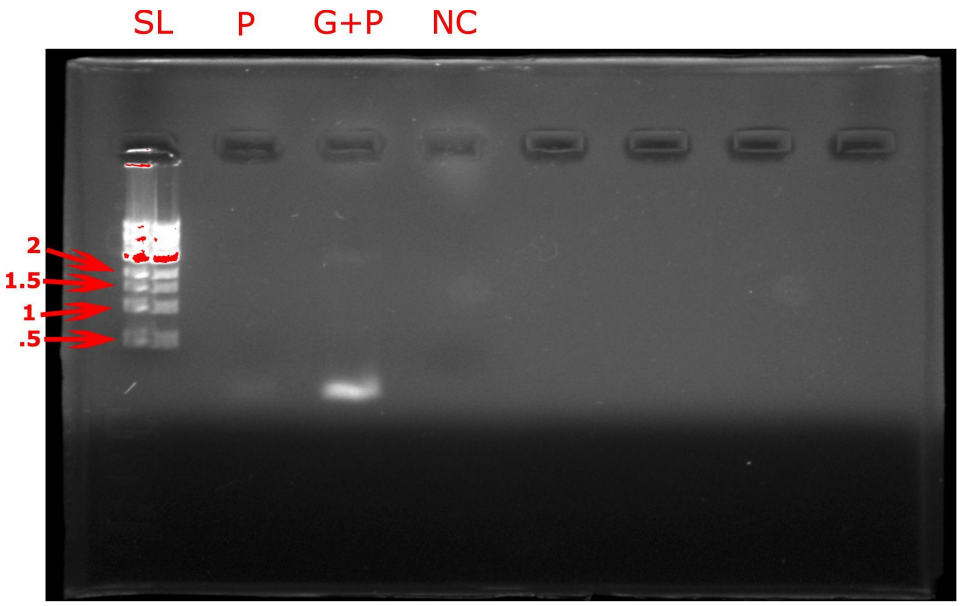

Fig. 3 Gel Electropharesis of HCAII PCR Reaction
Gel is a 1% agarose with Ethyl Bromide. Lanee SL contains the standard Quick-Load® 1kB ladder. Lane P contains the pETblue-2 plasmid. Lane G+P contains both the WT HCAII gene and pETBlue-2 plasmid. Lane NC contains the negative control sample.

As seen in **Figure 3**, we were only able to distinguish four of the bands. This was due to the fact that the larger bands were unable to separate sufficiently to distinguish each one.

The first lane contains the 1 kB ladder. The second well from the left contains the first sample which was the pETBlue-2. The third well from the left contains the second sample with pETBlue-2 plasmid and the *HCAII gene*. The fourth well from the left is the third sample which is the negative control which lacks the Phusion polymerase enzyme.

Our arrows highlight the bands of the ladder that we were able to distinguish. From our gel, we can conclude that the second sample with the pETblue-2 plasmid and the *HCAII gene* yielded the product. However, as can be seen in Figure 3, the nucleic acid band of Sample 2 migrated further than the whole standard ladder. Due to this, we are unable to accurately approximate the size of the product of Sample 2. Due to this irregularity, we believe that the product of Sample 2 is possibly contaminated or contains an impurity.

For the first sample with only the pETblue-2 plasmid, we were unable to identify any bands in the gel. Thus we concluded that there was an error at some point in the PCR and gel electrophoresis process that resulted in no product yield. In Sample 3 we did not expect any product, which was supported by the absence of any bands in the gel electrophoresis.

DNA Concentration Results

Table 2. NanoDrop Measurements of Post-PCR Reaction Samples

	Ideal	Sample 1 pETblue-2 Amplification	Sample 2 HCAII Amplification	Sample 3 Negative Control
DNA Concentration (ng/uL)	N/A	12.6	14.4	3.4
260/280	1.8	1.44	1.85	1.61
260/230	2.0-2.2	0.69	2.06	1.69

In **Table 2** it can be seen that we were able to measure similar DNA concentrations of both Sample 1 with the pETblue-2 amplification and Sample 2 with the HCAII and plasmid amplification. The negative control had a 4-fold decrease in DNA concentration to Samples 1 and 2.

In **Table 2** it can also be seen that Sample 2 with the HCAII amplification had both 260/280 and 260/230 ratios within the ideal range. This ideal range was provided by our lab instructors. This ratio helps us determine the presence of contamination in our samples and due to Sample 2 being within the ideal range, we are confident in the high purity of the Sample.

Since the Sample 1 has ratios that are not reasonably close to the ideal values. We are not confident in the high purity of the sample and instead believe that there could be the presence of an impurity or contaminant.

Sources

Kenneth M. Merz Jr. et al. Carbon dioxide binding to human carbonic anhydrase II Journal of the American Chemical Society 1991 113 (2), 406-411; DOI: 10.1021/ja00002a004

Prost, L. 2022. *Biochemical Methods Laboratory Manual.*

Senes, 2023. Human Carbonic Anhydrase II Lecture

Vijay M. Krishnamurthy, et al. Carbonic Anhydrase as a Model for Biophysical and Physical-Organic Studies of Proteins and Protein–Ligand Binding. Chemical Reviews 2008 108 (3), 946-1051; DOI: 10.1021/cr050262p