Human carbonic anhydrase ii catalyses carbon dioxide biology essay

Human carbonic anhydrase II is one of the fastest studied enzymes known with a variety of roles in reaction catalysis. Its primary function is to catalyze the reversible hydration reaction of carbon dioxide. In addition to carbon dioxide hydration, it is also capable of other latent skills, such as catalyzing esterase activity. The ability of human carbonic anhydrase II to function as a catalyst derives from key residues in and around the active site that play crucial roles in the mechanism. Substitutions to two of those particular key amino acids were performed via Quick-change site directed mutagenesis: H64A and V142D, to investigate the particular role they have in the catalytic active site. Various kinetic experiments and structural analyses were performed on wild-type carbonic anhydrase and the mutants to discern and compare their activity to each other and to literature, including Michaelis-Menten parameters for PNPA hydrolysis, CO2 hydration, and inferring function molecular modelling. Though the same trends can be seen as the literature, individual values were found to be much lower owing to errors in measurement and equipment. Trends were found to coincide with the mutants' known roles in the active site: His64 is the proton shuttle that facilitates proton transfer during the rate limiting step and Val142 participates in the hydrophobic pocket to bind and recruit substrates to interact with the active site. Mutations to both of these sites show that enzyme efficiency and activity strongly decreases.

Introduction

Human carbonic anhydrase II (hCAII) is a zinc metalloenzyme that catalyzes the following reversible reaction: . The enzyme commonly functions to help shuttle carbon dioxide in red blood cells to rid the body of metabolic waste,

and catalyzes the hydrolysis of many aromatic esters [1, 2]. Structurally, a zinc ion is located in the active site, coordinated to 3 histidine residues (H94, H96, H119) and usually a hydroxide ion or water molecule [2].

The mechanism of hCAII proceeds through two major steps: 1) the conversion of carbon dioxide to bicarbonate, and 2) the regeneration of Zn-OH by proton transfer. The active hydroxide that is bound to zinc nucleophilically attacks a nearby carbon dioxide molecule, resulting in a bicarbonate ion binding to zinc [3]. The zinc-oxygen bond breaks to subsequently release a bicarbonate ion, which is replaced with water [3]. The Znâ€'OH bond is regenerated by a proton transfer to the external buffer, which is facilitated by the His64 residue [3]. The proton transfer step is the rate limiting step of the reaction [3]. The diazole side chain on the histidine residue is what gives it the ability to be a proton acceptor and donor. Mutations in that position (His64) usually result in decreased enzyme activity due to a lack of proton transfer; however the reaction does proceed to a lesser degree without an active His residue, possibly due to its extensive water network in the active site forming " secondary proton wires" [4].

Carbonic anhydrase catalyzes one of the most rapid reactions; it is one of the fastest enzymes studied [1]. Its reaction speed is due, in part, by the amphiphilic nature of the active site [1]. The hydrophobic side is used to bind carbon dioxide, while the hydrophilic patch functions to optimally orient the carbon dioxide molecule for the reaction [1]. The hydrophobic wall forms a well-defined pocket near the zinc-hydroxide and is composed of the following amino acids: Val142, Val121, Leu197 and Trp208. The hydrophilic patch consists of Thr198 and Glu106, which form a hydrogen bond network with

the Znâ€’OH to stabilize and orient it for nucleophilic attack on CO2 [2]. Therefore, any modifications to the hydrophobic pocket would change its structure, and consequently, its catalytic efficiency [1].

In this study, the importance and role of His64 and Val142 to the structure and mechanism of hCAII are determined through site-mutagenesis and subsequent characterization of the new mutants, H64A (His64 ƒ Ala) and V142D (Val142 ƒ Asp) via kinetic and structural analysis. The changes that arise from the substitutions may prove to be applicable to drug synthesis because hCAII is known to be involved in a variety of diseases, for example, Marble brain disease, where mutations in the hCAII gene leads to a deficiency in the enzyme which is an autosomal recessive disease [5]. Studies in hCAII mutations can be used to design folding modulators to suppress misfolding which frequently occurs due to hCAII destabilization [5]. Another major disease involved with hCAII gene is osteopetrosis. The hCAII gene's inactivation decreases osteoclast function in bone, and knowledge of hCAII mutations that inactivate the enzyme may lead to better understanding of bone remodelling [6]. Some carbonic anhydrase diseases use inhibitors (CAI) to suppress the hCAII as a therapeutic treatment. Inhibitors prevent hCAII activity by inhibiting either of the reaction steps: the conversion of CO2 which involves V142 in the hydrophobic pocket, or the rate limiting step, proton transfer, in which His64 is crucial.

Experimental Procedure

Site directed mutagenesis via the PCR-based Quick-change method was performed on hCAII as cited in Woolley (2011) for 10 ng and 20 ng wild-type plasmids (hCA2pET24b from Novagen) [7]. Error: Reference source not found

shows the sequence of the primers used in the PCR reactions. Products of PCR mutagenesis reactions were run on 0. 7% agrose gels to determine size. The gels were run at 150 V in 1X TAE buffer. Red safe dye from Intron Biotechnology was used in the agrose gel instead of ethidium bromide for safety reasons [7]. The standard molecular weight ruler used was a 1 kB DNA ladder from Fermentas.

The enzyme, DpnI, was then used to digest methylated DNA (the parent template DNA). The DNA vector that contained the mutation was transformed into supercompetent E. coli turbo cells from New England Biolabs by heat shock [7]. LB-agar plates were prepared to grow the transformed cells containing mutant genes (i. e. H64A and V142D hCAII gene) [7]. Both were injected with Kanamycin to ensure that the culture that grows will have the desired mutation [7]. A miniprep culture was set-up from the LB-agar plate into LB medium to grow one colony for DNA analysis [7].

Restriction enzyme mapping was prepared and XhoI and BglII were chosen, they were used under buffer 3 for optimal efficiency. Plasmid putification was performed using the QIAprep Spin Miniprep Kit, and then the chosen restriction enzymes were carried out and were run on 1% agrose gel [7].

A sample of the purified DNA was sent to an external company (ACGT) for commercial sequencing (Sanger dideoxy type) to verify if the mutagenesis occurred correctly. The sequence was analyzed using the program BioEdit. To determine the level of confidence of the sequencing results, the purified DNA was quantified using UV/Vis absorption via a spectrometer [7]. The concentration was calculated using Ecâ€’1(260) as 50 Î¼g/mL.

Purified plasmid DNA was transformed into E. coli BL2(DE3) cells to initiate protein expression by heat shock, similar to the transformation into turbo cells [7]. The cells were cultured and a single colony was grown. Once sufficiently grown, ITPG and ZnSO4 were added to induce protein expression [7]. SDS-PAGE was used to confirm protein expression and was analyzed against an unstained protein molecular weight marker by Fermentas. The protein and ladder was stained with coomassie blue [7].

Affinity chromatography was used to purify the mutant hCAII proteins [7]. The matrix used was agrose linked to p-(aminomethyl)benzenesulfonamide, exploiting the tight binding that occurs between hCAII and sulphonamides. Once purified, the protein was dialyzed using a 6000-8000 Da dialysis membrane to replace the elution buffer with protein buffer and removes the matrix from the protein [7].

SDS-PAGE is again used to confirm the protein is still present after purification and to check its approximate molecular weight. It was run for two different amounts of protein, 2 Î¼g and 10 Î¼g, and also ran 10Î¼L of wash fractions from affinity chromatography [7]. Protein concentration was determined by UV absorption at 280 nm in a final concentration of 6M guanidine hydrochloride. From the calculated concentrations, purity of the protein could be assessed via SDS-PAGE.

To characterize this purified hCAII protein, a variety of analyses were done. Two types of mass spectrometry (MS) were performed: electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) [7]. The MS analysis was used to confirm the presence of the mutation in hCAII

with intact and digested protein. Protein samples (H64A and V142D hCAII) were not diluted for either of the MS analyses as cited in Woolley (2010). Samples of 10 Î¼L of stock protein concentrations (37. 6 Î¼M H64A and 3. 2 Î¼M V142D hCAII) were used for analysis of the molecular weight of the intact protein by ESI-MS. Both mutants were then digested by Trypsin Gold (MS grade) from Promega and the resulting fragments were evaluated by ESI-MS as well [7]. A 50 Î¼L sample was used for each mutant, 40 Î¼L of the mutant at stock concentration and 10 Î¼L of the Trypsin Gold. A couple Î¼L of the digested mutants were saved for MALDI-MS and the rest was used for ESI-MS. Once the molecular weights for each of the digested fragments were determined by ESI-MS, the products were run through a protein database to confirm the identity of the protein and mutations [7].

The 1 Î¼L of the tryptically digested mutants prepared for ESI-MS, subsequently underwent MALDI-MS. The 1 Î¼L samples were mixed with a matrix consisting of 1 Î¼L Î±-cyano-4-hydroxycinnamic acid (CHCA) and 1 Î¼L of 0. 1% trifluoroacetic acid (TFA) [7]. The entire mixture was pipette onto a MALDI well and was inserted into the mass spectrometer and a MALDI-MS spectra was obtained.

Michaelis-Menten kinetics was used to determine the KM and kcat of the p-nitrophenyl (PNPA) hydrolysis reaction [7]. The ionized product from the hydrolysis, p-nitrophenol (PNP) produces a bright yellow colour that was used to follow the rate of the reaction via the Perkin Elmer Lambda UV/Vis spectrophotometer [7]. Various sample concentrations of PNPA were set up to have a final enzyme concentration of 0. 2 Î¼M in protein buffer [7]. The initial rate measurements of each PNPA concentration were taken for wild-

type enzyme, H64A mutant, V142D mutant, and a blank with no additional enzyme added (refer to data tables in Enzyme Kinetics I [7]). PNP has a molar absorption coefficient (Îµ) of 1. 73Ã-104 Mâ€'1cmâ€'1. This was used to calculate Michaelis-Menten values: Vmax, KM, kcat, and kcat/KM [7].

The ability of hCAII mutants (H64A and V142D) and wild-type hCAII to catalyze the hydration of CO2 was measured. The pH of the solution was measured to track the progress of the reaction because the reaction generates protons. Enzyme solutions were prepared according to table 2 in [7]. The buffer used in the table was 50 mM TRIS buffer (pH 7. 8). Additional enzyme samples were prepared for 25 nM of wild-type hCAII and 100 nM of H64A mutant in a final concentration of 22. 5 and 29. 92 mM imidazole buffer (pH 7. 8) respectively to determine chemical rescue of mutant H64A. The pH of the CO2 hydration assay was measured using a pH probe and pH meter at 5 second increments for a total of 90 seconds starting at the beginning of the reaction [7]. The slope of the initial changes in the first 2 points was considered to be the V0 for each enzyme concentration. From the initial velocity, a kcat value can be calculated for each enzyme using the assumption that [S] >> KM, the Michaelis-Menten equation simplifies to kcat= V0/[E].

The third kinetics experiment used fluorescence to determine the binding constant of dansyl amide (DNSA) and acetazolamide (AZ) (from Sigma-Aldrich) to H64A and wild-type hCAII was performed using the Perkin Elmer Fluorometer [7]. Stocks of 1 mM and 200 Î¼M of DNSA were prepared from a 21. 6 mM DNSA stock by dilution with DMSO. Enzyme stocks were diluted to 0. 25 Î¼M with TRIS buffer to make a 10 mL solution. A 1 mL sample of H64A

from stock made was titrated with DNSA in small increments [7]. The fluorometer emissions were taken at 470 nm. AZ titration in competition with DNSA was not able to be completed.

The last characterization experiment done was molecularly modelling the hCAII wild-type enzyme, as well as the mutants H64A and V142D. The molecular model of hCAII analyzed was derived by x-ray crystallography and found in the Protein Data Bank (PDB) repository. The wild-type and H64A hCAII structures examined had a PDB code of 1CA2 and 1MOO respectively. At present, no crystal structure has been found for V142D hCAII. The Swiss PDB Viewer program was used to visualize the protein structures. Secondary structures of the proteins were able to be observed. Residues around the metal active site and the Ramachandran plot were explored. Homology between hCAII and other carbonic anhydrase isozymes, hCAIV (PDB code 1ZNC) and hCAI (PBD code H1CB), were also studied by performing an iterative magic fit on the Î±-carbons and structure alignment for each pair. The root mean square (RMS) between hCAII and the other isozymes were also analyzed to determine conserved and deviated regions in the structures. The binding of cobalt in the hCAII active site was also investigated (PDB code 3KOI). The structural inhibition of hCAII by AZ was also gleaned by structural analysis (PDB code 3HS4). Its mode of inhibition and binding sites were shown through the crystal structure. Lastly, the Swiss PDB Viewer program was used as a tool to theoretically synthesize mutations and compare it to the actual structure as determined by other scientists, for example, by aligning the virtual and crystallized mutations to determine deviations in structure by performing RMS.

Results

Site-directed mutagenesis PCR. Products from the PCR mutagenesis reactions were examined using 0. 7% agrose gel electrophoresis. Two samples of differing amounts of template DNA (10 ng and 20 ng) were used for each mutant (Error: Reference source not found). Bands were only observed for samples containing 20 ng of the hCA2pET24b DNA template plasmid (Error: Reference source not found). The size of the bands observed coincides with the size of the plasmid used, 6018 bp.

Heat shock transformation and isolation of plasmid. Several colonies were observed after plasmid transformation for both mutants, and 1 colony from each mutant was chosen for restriction enzyme digest with BglII and XhoI.

Quantification of pure plasmid DNA. A 1/20th dilution was carried out on the purified DNA with elution buffer (EB; 0. 1 M Tris, 0. 4 M KSCN, pH 7). The absorption of the diluted DNA at 260 nm and 280 nm was taken by a UV/Vis spectrophotometer and the relative DNA purity was determined (Error: Reference source not found). The assumption that Ecâ€’1 260 = 50 Î¼g/mL for DNA was applied in the calculation of concentrated and diluted concentrations of purified DNA (Error: Reference source not found).

Enzyme restriction digest. Purified plasmid DNA of mutants were digested with XhoI and BglII, separately and together in a single and double digest for both mutants. The digested and undigested samples were run on 1% agrose gel, and 2 bands were observed around the 6000 and 7000 bp marker for all 8 samples (Error: Reference source not found, Error: Reference source not

found). The expected length of the bands in the double digest should be 892 bp and 5126 bp (Error: Reference source not found).

DNA Sequencing. The mutations for both V142D and H64A in the hCAII gene were successful according to the sequenced DNA result obtained from ACGT. Other mutations in the DNA sequence were observed in both mutants, but since the aligned protein sequence was the same, mutations were likely to be silent mutations due to amino acid redundancies. When sequenced in the forward direction by T7 polymerase, a protein mutation was found (K153N) other than the desired mutation of V142D; however, when sequenced in the reverse direction by T7 polymerase terminator (T7TER), K153N was not observed.

Plasmid DNA transformation into E. Coli BL21(DE3) cells. Following transformation into BL21(DE3) cells, colonies were observed for both hCAII mutants (V142D and H64A). A random colony was chosen to be cultured and then was induced to express protein with 270 Î¼M IPTG and 0. 1 mM ZnSO4.

SDS-PAGE for protein expression. Protein expression was tested with SDS-PAGE. The expected molecular weight of V142D hCAII is approximately 29. 2 kDa and the expected molecular weight of H64A hCAII is approximately 29. 1 kDa. SDS-PAGE bands are observed between the ladder markers 25. 0 kDa and 35. 0 kDa for both mutant proteins (Error: Reference source not found, Error: Reference source not found).

Calculation of pure protein concentration and extinction coefficient. Following affinity purification and dialysis, pure protein concentration was calculated from UV absorption measurements at 280 nm and the known

extinction coefficient of hCAII as 50070 Mâ€’1cmâ€’1 (Error: Reference source not found). The final concentration of the samples of V142D and H64A hCAII were 3. 2 Î¼M and 37. 6 Î¼M respectively.

SDS-PAGE to assay purity and check approximate molecular weight. Several samples were loaded into the SDS-PAGE for each mutant protein: lysate and wash fractions (collected from affinity chromatography), 2 Î¼g protein, and 10 Î¼g protein. For H64A, a visible band was only observed for the 10 Î¼g sample (Error: Reference source not found). The band was located between the 35 kDa and 25 kDa markers on the ladder. For V142D, none of the 4 samples resulted in a band on the gel (Error: Reference source not found).

Mass spectrometry. ESI-MS was not successful in analyzing the molecular weight of intact and digested protein of both mutants. A MALDI spectrum was able to be generated for the digested proteins; however, without the digested ESI spectrum to compare to, the peaks from the MALDI spectrum can only be speculatively assigned.

Kinetics: Hydrolysis of PNPA. Using the molar absorption coefficient of PNP (1. 73Ã-104 Mâ€’1cmâ€’1), the rate of each reaction was determined. The predicted rate was calculated using the Michaelis-Menten kinetics: . The plot of predicted rates and actual initial rates vs. PNPA concentration can be seen in Error: Reference source not found, Error: Reference source not found, Error: Reference source not found for wild-type, H64A, and V142D hCAII respectively. The Vmax and KM values for each enzyme were calculated by minimizing the square difference between the predicted and actual reaction

rates, and the kcat was calculated using the equation: (Error: Reference source not found).

Kinetics: CO2 hydration. Initial velocity (V0) values were calculated by measuring the progression of the reaction (via concentration of protons) with time (Error: Reference source not found, Error: Reference source not found, and Error: Reference source not found). kcat values were then calculated using the same equation as in the hydration of PNPA and averaged for the individual enzymes (wildtype, H64A, and V142D hCAII) in a particular buffer (i. e. TRIS or imidazole).

Fluorescence detection of ligand binding. DNSA was titrated with H64A hCAII to determine its affinity for the enzyme. The dissociation constant, KD, for DNSA was determined to be 0. 086 Î¼M when protein concentration was 0. 25 Î¼M. Competitive titration of H64A-DNSA hCAII with AZ was attempted, but was not successful as DNSA binding was too tight, making it difficult to be displaced by AZ.

Molecular modeling. Literature models of wild-type (PDB code 1CA2) and H64A (PDB code 1MOO) hCAII were analyzed. There is no available structure of V142D hCAII at present. The secondary structure of wild-type is composed of 18 Î²-sheets (77 residues) and 10 Î±-helices (42 residues), with the majority of the Î±-helices falling in the domain of right-handed helices, while very few show left-handed helical properties according to the Ramachadran plot. It also seems that the active site is solely composed of Î²-sheets, and no Î±-helices (Error: Reference source not found). Analyzing PDB structure 3HS4 (AZ bound hCAII), the mechanism as to how AZ inhibits hCAII function can be

seen. AZ has 3 binding sites, 2 are novel binding sites and the other provides a mechanism of inhibition. AZ binds the zinc directly at the active site, displacing crucial ligands needed for catalysis. There were some discrepancies found between the crystal structure of H64A [1MOO] as cited on PDB and virtually mutated H64A from wild-type hCAII, resulting in a RMSD (root mean square deviation) of 0. 29 Å (Error: Reference source not found). Since no literature structure of V142D is available, no comparison between virtual and crystal structures could be made.

Discussion

Agrose gel results were only visible for samples that contained 20 ng of the plasmid template DNA, rather than the 10 ng plasmid. This may be a result of more amplification during PCR with the 20 ng plasmid, and so would intensely be more visible. Though the 20 ng samples showed bands at the appropriate 6000 bp mark, there was also a faint band that can be seen near the end of the gel. This may be due to non-specific primer annealing.

Quantification of DNA purity was done by exploiting the peak absorbances of protein and DNA. DNA maximally absorbs at 260 nm, while protein dominantly absorbs at 280 nm. The purity ratio reports the relative amount of DNA compared to protein present in the sample. The purity of both mutants were approximately 1. 8, which is regarded as a relatively pure sample; however, a purity ratio of more than 2. 0 would have been ideal.

The restriction enzyme digest showed 2 bands (7000, 6000 bp) for all samples, which may have been a sign of poor mixing/ pipetting since the volumes of restriction enzyme were extremely small amounts. If this is the

case, only some of the DNA was nicked and some were not, which would result in 2 bands. It was expected that the plasmid sample would have a high band (supercoiled), each of the singly digested samples would have a slightly lower band (nicked), and the doubly digested would show 2 bands that indicated the fragment size of 892 and 5126 bp.

Sequencing results showed that a protein mutation occurred when the sample was sequenced in the forward direction by the T7 polymerase. A lysine at position 153 had mutated to glutamine (K153N). However, this mutation was not observed when the T7 polymerase terminator was used to sequence the sample in the reverse direction. A mutation that occurs in one sequencing direction and not the other is usually attributed to sequencing errors, which may be the reason in this case.

The SDS-PAGE bands for protein expression coincided with the expected molecular weight for both mutants, which could suggest that the correct proteins were expressed; however, there is a possibility that the proteins expressed could be of similar weight, but completely different. Interestingly, the V142D samples that did not include the protein inducer, IPTG, had a more intense band than the faint ones found for the samples that did include IPTG. This may just be a result of mislabelling.

The SDS-PAGE performed to assess purity after the purification process. Mutant V142D had low protein expression as evidenced by its concentration of 3. 2 Î¼M. The V142D mutant should have very low protein expression according to Fierke et al. (1991) because valine at position 142 is uniquely required for maximal expression in E. Coli. It is suggested that by altering

position 142, protein stability decreases [2]. Therefore, the protein that was expressed in the previous SDS-PAGE gel may not be V142D hCAII at all. The sample may have been small fragmented contaminant proteins that would have completely run off the gel altogether. However, the low concentration of V142D after purification may also be a major factor in the lack of gel bands observed as well. Unlike V142D, H64A hCAII concentration should not have affected its lack of bands because it was calculated to have had a reasonable concentration of 37. 6 Î¼M. There were some problems loading the samples into the wells; this could be an explanation as to no observable gel bands.

ESI-MS is dependent on concentration because it affects the size of primary droplets [8]. The unsuccessful determination of molecular weight of V142D hCAII may be attributed to its low concentration. The H64 hCAII mutant was also not able to be successfully analyzed with ESI-MS. A possible reason for the failure was that it was not kept on ice while it was not being used. The enzyme may have become inactive and degraded into smaller fragments. This would explain the ESI-MS output obtained for H64A. No definite molecular mass was determined, but the spectrometer did detect a lot of small protein fragments in the sample, all under 1000 amu.

The kinetic values obtained from PNPA hydrolysis do not follow similar trends found in literature [2]. The kcat/KM for wild-type hCAII (2500±200 Mâ€’1sâ€’1) was found to be significantly larger than V142D hCAII (3±0. 3 Mâ€’1sâ€’1) in literature, more than 800Ã- larger [2]. Experimental calculations yielded kcat/KM for V142D (8. 16 Mâ€’1sâ€’1) to be about 2Ã- larger than wild-type (4. 02 Mâ€’1sâ€’1), which did not follow literature

patterns. The literature trends make more biological sense because during PNPA hydrolysis, the PNPA associates with the hydrophobic pocket, where Val142 is situated, to orient itself to approach the Znâ€'OH [2]. If the hydrophobic pocket is blocked, such as the mutation of valine to a larger aspartic acid (i. e. V142D), PNPA would not be able to approach the zinc in the correct orientation and lead to decreased enzyme efficiency compared to the wild-type. Experimental results may have differed from literature due to the method in which the values were derived. The method in which the initial rate was determined was flawed because possible slow mixing and inconsistent time increments between the addition of PNPA and capturing the reaction by spectrometer could have occurred, and the actual initial rate could have been missed.

CO2 hydration occurs in 2 steps, of which the rate limiting step is proton transfer. The proton transfer is facilitated by residue His64, and thus, changes to position 142 should result in no drastic changes in activity from wild-type hCAII [3]. Mutation to the His64 position, e. g. H64A, significantly decreases activity of the reaction because proton transfer can no longer occur. This trend is seen in experimental data as well, where the kcat for H64A hCAII in TRIS buffer (0. 67 sâ€'1) was much lower than wild-type in TRIS buffer (5. 3 sâ€'1). A chemical rescue of the H64A mutant was also attempted by using substituting the TRIS buffer for imidazole. Since the imidazole can act as a proton acceptor/donor, it is expected that H64A hCAII activity would increase with imidazole. This does indeed occur as seen in experimental values. When rescued with imidazole, the kcat for H64A increases to 3. 03 sâ€'1 from 0. 67sâ€'1 (with TRIS buffer) to enhance the

activity of the reaction by 4-5 fold. This is also seen in literature, Duda et al. (2002) reported an increase in kcat in H64A rescue with imidazole from 105 sâ€’1 to 4Ã-105 sâ€’1, which shows a 4-fold increase in activity [4].

Though wild-type hCAII still has a functional His64 for proton transfer, the addition of imidazole buffer also enhanced its activity during experimentation and similarly found in literature. Experimental data found that wild-type in imidazole increased the kcat from 5. 3 sâ€’1 to 12. 44 sâ€’1, which increased activity by about 2-fold. An et al. (2002) likewise found an increase in wild-type when imidazole was added [1]. The imidazole might enhance the proton transfer step because it is able to approach the Zn active site more closely than His64 does owing to the constraints that His64 is under [9].

AZ dissociation constant was unable to be determined due to the tight binding of DNSA in fluorescence detection. A competition between AZ was set up, where AZ would displace DNSA in the protein complex, hCAII-DNSA, since AZ has a higher affinity for hCAII than DNSA [2]. DNSA may not have dissociated from hCAII during the competition because there was possibly a conformational change which trapped the DNSA to the hCAII active site. Thus, the conformational change would have inhibited the release of DNSA and prevented the binding of AZ.

The structural modeling of wildtype and H64A hCAII allows for the hypothesis of mechanisms of inhibition and catalysis to be inferred and/or supported. Distances between certain residues and water molecules are crucial in determining mechanism, e. g. proton transfer. Thus, studying an accurate

structure/model may reveal mechanisms and functions that cannot be deciphered through experimentation.

Conclusions

Various residues in the hCAII protein have specific and crucial roles that work together to function as an enzyme catalyst. The His64 residue is imperative for proton transfer in CO2 hydration; without the diazole ring, or a functional group that can accept and donate protons in the 64th position, catalysis of the reaction would almost cease and only the background uncatalyzed reaction would be observed. The Val142 residue was found to play a role in PNPA hydrolysis. The hydrophobic pocket at the active site includes Val142, which PNPA first associates to find the optimal orientation before it approaches zinc. Mutations to this site are more tolerant to function as long as the mutations are similar, i. e. substitution of a hydrophobic residue for another.

By investigating individual residues and their structural roles and functions, crucial aspects of the enzyme’s mechanism and properties can be recognized and applied practically. For example, in the pharmaceutical industry, drugs for glaucoma treatment target hCAII in its patients. Learning more about the enzyme can help with inhibitor binding or dissociation, by possibly utilizing site-mutations to enhance or deteriorate binding sites.