

T.R.
GEBZE TECHNICAL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

**EFFECT OF ACTIVE SITE MUTATIONS IN NAD⁺-
DEPENDENT CHAETOMIUM THERMOPHILUM FORMATE
DEHYDROGENASE ON CONVERSION OF CARBON DIOXIDE
TO FORMATE**

BERİN YELMAZER
A THESIS SUBMITTED FOR THE DEGREE OF
MASTER OF SCIENCE
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THESIS SUPERVISOR
ASSOC. PROF. DR. BARIŞ BİNAY

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T.C.
GEBZE TEKNİK ÜNİVERSİTESİ
FEN BİLİMLERİ ENSTİTÜSÜ

NAD⁺ BAĞIMLI *Chaetomium thermophilum*
FORMAT DEHİDROGENAZIN AKTİF
BÖLGESİNDEKİ MUTASYONLARIN
KARBONDİOKSİTİ FORMATA
DÖNÜŞTÜRMESİNDEKİ ETKİSİ

BERİN YELMAZER
YÜKSEK LİSANS TEZİ
MOLEKÜLER BİYOLOJİ VE GENETİK ANABİLİM DALI

DANIŞMANI
DOÇ. DR. BARIŞ BİNAY

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SUMMARY

Formate dehydrogenases (FDHs) are a set of oxidoreductases that catalyze the oxidation of formate to carbon dioxide with the simultaneous reduction of NAD^+ to NADH. Therefore, FDHs have been utilized in coenzyme regeneration systems that produce NADH which would be expensive to manufacture by conventional chemical synthesis. Another promising application of FDHs is carbon fixation from the atmosphere and the oceans using the reverse reaction, the reduction of carbon dioxide to formate. It has been recently discovered that the *Chaetomium thermophilum* formate dehydrogenase (CtFDH) enzymes also have high potential in the reverse direction, the reduction of aqueous CO_2 to formate.

Studying the enzymatic fixing of CO_2 in model systems is needed to reveal how this reaction happens in the enzyme. In this study, it is aimed to analyze especially how specific mutations can affect the reverse reaction from carbon dioxide to formate. The active site of NAD^+ -dependent *Chaetomium thermophilum* formate dehydrogenases was studied by three mutations. The kinetic values of the enzymes with the designed mutations were compared with the wild type CtFDH enzyme, respectively.

In CtFDH, Asn120Cys and Asn256Asp mutations that were close to the active site inactivated the enzyme for formate oxidation and retained the activity for hydrogen carbonate. Also, another mutation made the enzyme lose its activity in both directions. The most interesting mutant structure was further studied with the molecular dynamic calculations. The simulations also revealed a surprising possibility that the mutation Asn120Cys in CtFDH allows the local structure to change so that more space is created into the active site and then two hydrogen carbonates can fit there filling the open place and allowing the reaction to happen.

The obtained results will generate new understanding about the structure-function relationships in the FDH active site. Lastly, this work will provide a helpful theoretical reference for the in vitro evolution of an enzyme active on CO_2 .

Key Words: NAD^+ -dependent formate dehydrogenase, enzyme engineering, reduction of CO_2 , catalytic mechanism, molecular modelling.

ÖZET

Format dehidroegenazlar (FDH'ler), format iyonunu (HCOO^-), karbondioksit (CO_2) yükseltgerken, NAD^+ iyonunun da NADH ' e indirgenmesini katalize eden bir oksidoredüktaz grubu enzimleridir. NADH geleneksel kimyasal yöntemlerle üretilmesi pahalıdır. Bu nedenle FDH'ler, NADH 'yi elde etmek amacıyla koenzim rejenerasyon sistemlerinde kullanılmaktadır. Ayrıca FDH'lerin ters reaksiyon ile karbondioksiti formata indirgeyip, atmosferde ve okyanuslarda karbon fiksasyonu yapması umut verici başka bir uygulamadır. Bununla birlikte, yakın zamanda *Chaetomium thermophilum* format dehidrogenaz (CtFDH) enziminin CO_2 'nin formata indirgenmesi için yüksek aktiviteye sahip olduğu keşfedilmiştir.

Bu çalışmada, NAD^+ bağımlı FDH enziminin katalitik bölgesine uygulanan spesifik mutasyonların, CO_2 'nin formata indirgendiği ters reaksiyon üzerindeki etkisini analiz etmek amaçlanmıştır. NAD^+ bağımlı CtFDH'in aktif bölgesi üç mutasyon ile incelenmiştir. Tasarlanan mutasyonların enzim kinetik değerleri sırasıyla, yabanıl tip CtFDH ile karşılaştırılmıştır. En ilginç mutant enzim için ise moleküler dinamik hesaplamaları ile daha kapsamlı analizler yapılmıştır.

CtFDH'in, aktif bölgesinde ve bu bölgeye yakın pozisyonlarda gerçekleştirilen Asn120Cys ve Asn256Asp mutasyonları enzimin format yükseltgeme aktivitesi kaybolmasına sebep olurken, CO_2 indirgeme aktivitesini korunmuştur. Ayrıca, diğer bir mutasyon (Asp283Asn) ise enzimin aktivitesinin her iki yönde de kaybolmasına neden olmuştur. Moleküler dinamik simülasyonlar, Asn120Cys mutasyonunun, lokal yapının değişmesine neden olduğunu göstermiştir. Böylece aktif bölgede daha fazla alanın oluşturulmasına olanak tanıyıp, hidrojen karbonatların açılan alanı doldurmasına izin vererek, reaksiyonun gerçekleşmesini sağladığı ortaya konmuştur.

Elde edilen sonuçlar FDH'lerin aktif bölgesindeki yapı-fonksiyon ilişkileri hakkında yeni bir anlayış yaratacaktır. Son olarak, bu çalışma, CO_2 üzerinde aktif olan bir enzimin in vitro evrimi için yararlı bir teorik referans olacaktır.

Anahtar Kelimeler: NAD^+ -Bağımlı Format Dehidrogenaz, enzim mühendisliği, CO_2 indirgemesi, katalitik mekanizma, homoloji modelleme.

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LIST of ABBREVIATIONS and ACRONYMS

<u>Abbreviations</u>	<u>Explanations</u>
<u>and Acronyms</u>	
°C	: Celsius Degree
μ	: Micro
Arg	: Arginine
Asn	: Asparagine
Asp	: Aspartic acid
BCA	: Bicinchoninic Acid
CO ₂	: Carbon Dioxide
Cys	: Cysteine
FDH	: Formate Dehydrogenase
Gly	: Glycine
HCl	: Hydrochloric Acid
His	: Histidine
Ile	: Isoleucine
IPTG	: Isopropyl β-D-1-thiogalactopyranoside
kDa	: KiloDalton
L	: Liter
LB	: Luria-Bertani
mg	: Milligram
min	: Minute
mM	: Millimolar
NAD ⁺	: Nicotinamide Adenine Dinucleotide
NADP	: Nicotinamide Adenine Dinucleotide Phosphate
NaOH	: Sodium Hydroxide
NaPi	: Sodium Phosphate
OD	: Optical Density
PCR	: Polymerase Chain Reaction
Phe	: Phenylalanine
SDS-PAGE	: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
sec	: Second

Ser	: Serine
SOC	: Super Optimal Broth with Catabolite Repression
Thr	: Threonine
Tyr	: Tyrosine
Val	: Valine
Wt	: Wild Type

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1. INTRODUCTION

1.1. Aim and Contribution

Formate dehydrogenases were discovered in the early 1950s from green pea (*Pisum sativum*) and at that time they were treated as another new dehydrogenases, with no obvious applications [1]. But it was not until the 1970s when the scientific community realized their underlying potential. First off, formate dehydrogenases were used as model enzymes for hydride transfer reactions. The reaction itself is fairly simple. Formate donates a hydride to NAD^+ which yields carbon dioxide and NADH. Producing NADH by traditional chemical synthesis is an expensive and laborious process. The idea of NADH regeneration systems utilizing formate dehydrogenases was introduced soon after the first kinetic studies [2], [3].

Meanwhile, the global warming has been progressing at an alarming rate. Atmospheric carbon dioxide is one of the most significant greenhouse gases, and it contributes to the ever-increasing ocean acidification as well. Thus, many technic has emerged to solve this problem. The most promising idea of enzymatic carbon fixation has emerged during the last decade. This is where formate dehydrogenases come into question. For example, pure enzymes or environmentally harmless microorganisms genetically engineered to produce large amounts of formate dehydrogenase could be placed into the oceans around the world to convert dissolved carbon dioxide and bicarbonate into formate, thus reducing atmospheric carbon and consequently decelerating the global warming.

Carbon fixation with formate dehydrogenases is plausible because the conversion from formate to carbon dioxide and from NAD^+ to NADH is essentially reversible, as the major part of all enzymatic reactions. The ability of certain members of this class, such as FDH from *Chaetomium thermophilum* in particular, to efficiently catalyze the reversible oxidation of formate to carbon dioxide [4]. Although, having evolved to formate oxidation, formate dehydrogenases should be studied further and carefully engineered to favor the reverse reaction before using them in the field.

Most previous studies have reported that NAD^+/NADH -dependent FDHs show low catalytic efficiency for the reduction of CO_2 . This is a major restricting factor impeding the biotechnical applications of FDHs in fixing CO_2 .

To our knowledge, the exact role of the FDH active site of neighbouring amino acid residues in the reverse reaction, from HCO_3^- to formate, has not been previously studied. Also, the research of the conversion of CO_2 or HCO_3^- to formate in general has been very limited [4].

Thus, the aim of this thesis is to clarify the structure-function relationship for the reverse reaction, mutations were created into the active site of the FDHs from *Chaetomium thermophilum*.

2. LITERATURE REVIEW

2.1. Enzymes as Catalysts

Enzymes are biocatalysts that consist of a proteinic backbone with N- and C-terminus and an active site, which can be a metal ion form for example Zn or Mg. Enzymes increase the biochemical reactions rate by decreasing the activation energy. In general enzymes are divided into six major groups according to the type of reaction they catalyse [5].

Table 2.1: Classification of enzyme.

Class	Enzyme	Reaction Type	Enzyme Code
1	Oxidoreductases	Transfer of electrons	EC 1.X.X.X
2	Transferases	Transfer of functional groups	EC 2.X.X.X
3	Hidrolases	Hidrolysis reactions	EC 3.X.X.X
4	Lyases	Cleavage of C-C, C-O, C-N and other bonds, often forming a double bond	EC 4.X.X.X
5	Isomerases	Transfer of groups within a molecule	EC 5.X.X.X
6	Ligases	Bond formation coupled to ATP hydrolysis	EC 6.X.X.X

The Enzyme Commission number is a numerical classification scheme based on chemical reactions catalysed by enzymes. Enzyme code numbers consist of four digits separated by dots and the first number indicates which of the six main enzyme groups the enzyme has entered, the second number indicates the chemical structure or functional group the enzyme affects, the third digit indicates the acceptor, and the fourth digit indicates the order number the enzyme has received in a certain class [6].

Catalytic activities of enzymes depend on their essential protein conformation. Also, some groups of enzymes require sacrificial co-enzymes as energy donors. Possible co-factors are either inorganic ions or complex organic molecules. Those cofactors can be a prosthetic group which is tightly bound to the enzyme or a coenzyme which is released from the enzyme's active site during the reaction. Coenzymes transport chemical groups or rather charges, substrates and formed products. For example, dehydrogenases use the coenzyme NADH, Nicotinamide adenine dinucleotide, as electron acceptor (Figure 2.1.).

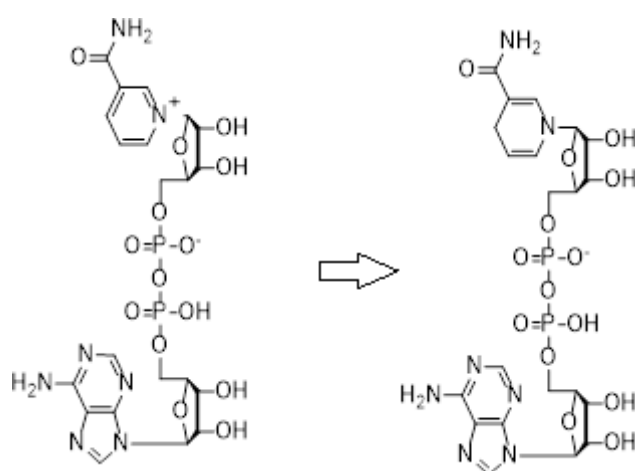


Figure 2.1. Structure of NAD⁺ (reduced form) and NADH oxidized form.

The active part of NADH is the nicotinamide heterocyclic ring. At the beginning of the reaction NADH is bound non-covalently by the enzymes and is released as its oxidized form NAD⁺ after the reaction is finished. To be reusable for a reduction process NAD⁺ has to be regenerated to NADH [7].

For an overall enzyme catalysed process, a simplified schematic can be written as;

$E + S \rightleftharpoons [ES] \rightarrow E + P$ where E is the enzyme, S is the substrate and P is the product. These stepwise reactions were proposed in 1913 by Leonor Michaelis and Maud Menten in their general theory of enzyme action [8].

The first step of an enzymatic reaction is that the enzyme binds reversibly with the substrate to form an enzyme-substrate complex. This step is relatively fast. In a slower second step the [ES] complex breaks down and the product is formed. The enzyme is unchanged and able to bind the next molecule of substrate. According to

this model, the second step limits the rate of the overall reaction. Michaelis and Menten derived an equation which describes the relationship between substrate concentration and reaction rate for a one-substrate enzyme catalyzed reaction [9].

Enzymes show a very high specificity for their substrate. Two models have been investigated to explain the binding of a substrate to an enzyme. In the lock-and-key model the substrate and the active site of the enzyme are thought to fit together like a key into its lock. The two shapes are considered as rigid and fixed, and perfectly complement to each other. In the induced-fit model the binding of the substrate induces a conformational change in the active site of the enzyme [7].

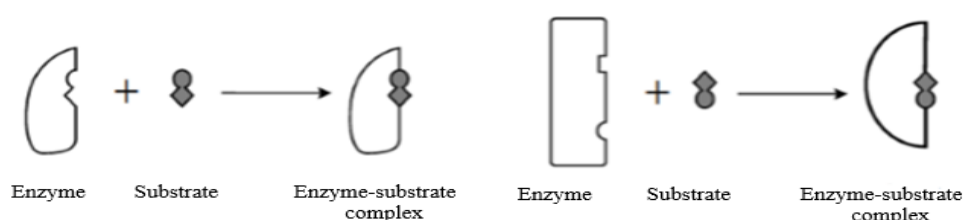


Figure 2.2. Binding methods of a substrate to an enzyme.

For the utilization of enzymes, it is essential to know that the three-dimensional conformation of the enzyme has an important effect on the catalytic activity. The catalytic activity of the enzyme is lost if the enzymes is denatured or broken down into its component amino acids [7]. Not only the conformation but also the active center plays an important role in enzyme catalysis. The active site of an enzyme is the part where the enzyme interacts with the substrate to form a substrate-enzyme complex. The active center consists of two sites which are lined with amino acid residues whose substituent groups binds the substrate and catalyze its chemical formation [10].

2.2. Fundamentals of Formate Dehydrogenases

Formate dehydrogenase is a member of oxidoreductase class of enzymes. NAD^+ -dependent formate dehydrogenase is a member of the superfamily of D-specific 2-hydroxy acid dehydrogenases and catalyse the oxidation of formate anion to CO_2 while reducing the NAD^+ to NADH. (Figure 2.3.)

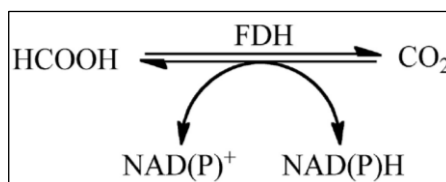


Figure 2.3: The reaction catalyzed by FDH.

Formate dehydrogenase was firstly found in a plant *Phaseolus vulgaris* at 1921. The first study of FDH was done by Davidson for the formation of NADH by ethanol, succinate and glutamate [11].

NAD⁺-dependent formate dehydrogenases (FDHs) have been the favourite models of enzymologists since the mid-1970s [12]. Although the reaction they catalyse is rather simple, chemical and pharmaceutical industries have already utilized formate dehydrogenases in NADH regeneration systems [13]. NADH regeneration systems are used in the syntheses of chirally active compounds, such as certain drugs and amino acids, in which the main synthesis reaction consumes NADH. Without constant regeneration, the high-priced NADH required would have to be acquired commercially [12]. Another yet hypothetical but promising use for formate dehydrogenases is in climate change mitigation. With enzyme engineering, formate dehydrogenases could be made more efficient in utilizing atmospheric CO₂ and bicarbonate dissolved in the oceans [14].

Formate ions are oxidized to carbon dioxide and simultaneously NAD⁺ is reduced to NADH in a simple hydride transfer reaction where the carbon-hydrogen bond in formate is broken and a new one is formed between the released hydride and C₄N of the nicotinamide ring of NAD⁺ [15].

The most extensively studied FDH enzyme to date is that of *Pseudomonas* sp., strain 101 (*Pse*FDH), whose residue numbering and structural properties are often used as a reference when studying FDHs from other species. This is because FDHs from bacteria are the best stable known FDHs in general, making them easier to analyze. Another well-known stable FDH is the one found in yeast *Candida boindii* [16], [17].

2.3. Structure and Localization of Formate Dehydrogenases

NAD⁺-dependent FDHs are highly specific for both formate and NAD⁺ contains two identical subunits which range from 70 kDa to 100 kDa [18].

All known formate dehydrogenases found in organisms exist as homodimers. The whole molecule is somewhat globular in shape and each subunit which range from 70 kDa to 100 kDa also has two domains: a coenzyme-binding domain and a catalytic domain [2]. The coenzyme-binding domain specifically recognizes and binds NAD⁺ and the catalytic domain is responsible for catalysis. The two catalytic domains are located on the opposite ends of the FDH enzyme complex, as seen in Figure 2.4. Catalytic domains are shown in red and purple, coenzyme binding domains in blue and cyan. Image made with PyMOL (The PyMOL Molecular Graphics System, Version 1.3).

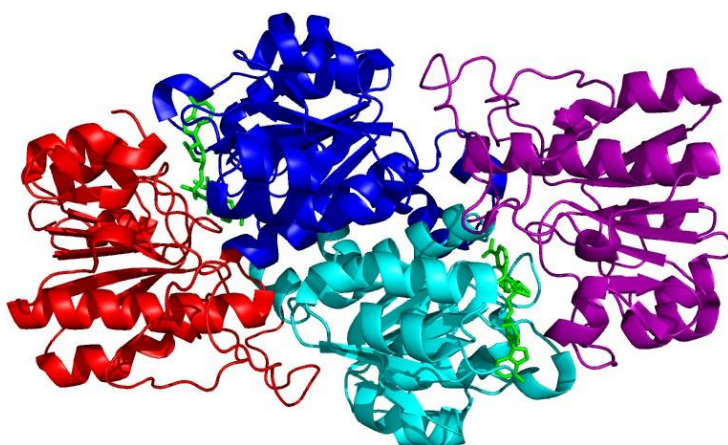


Figure 2.4: The FDH complex with two bound NAD⁺ molecules (green).

NAD⁺-dependent formate dehydrogenases have been identified from a diverse set of microorganisms and higher plants. Key characteristics of the most well-known FDHs have been listed in Table 2.2. Data adapted from Popov & Lamzin (1994) [2].

Table 2.2. Physical and chemical properties of certain NAD⁺-dependent FDH.

Source	Subunits × molecular mass (kDa)	Activity (u/mg)	K _M (mM)
<i>Pseudomonas</i> sp. 101	2 × 44	16.0	15
<i>Pseudomonas oxalaticus</i>	2 × 100, 2 × 59	-	0.14
<i>Moxarella</i> sp. C-1	2 × 48	6.0	13
<i>Candida boindii</i>	2 × 36	2.4	13
<i>Candida methylica</i>	2 × 46	10.0	13
<i>Candida methanolica</i>	2 × 43	7.5	3
<i>Pichia pastoris</i> NRRL-Y-7556	2 × 47	8.2	16
<i>Pichia pastoris</i> IFP 206	2 × 34	2.8	15
<i>Pisum sativum</i>	2 × 42	3.7	1.7

FDHs from different organisms have strong similarities between the amino acid sequences. Especially the amino acid residues where the substrate and coenzyme bound, and the catalytic activity occur are highly conserved in evolutionary period. Popov [3] has shown that the residues Phenylalanine 98, Isoleucine 122, Asparagine 146, Alanine/Glycine 198, Glycine 200, Glycine 203, Arginine 284, Glutamine 313, and Histidine 332 are catalytically important and can be seen in Figure 2.5. Figure 2.5. modified scheme originally adapted from Torres et al. (1999) [19].

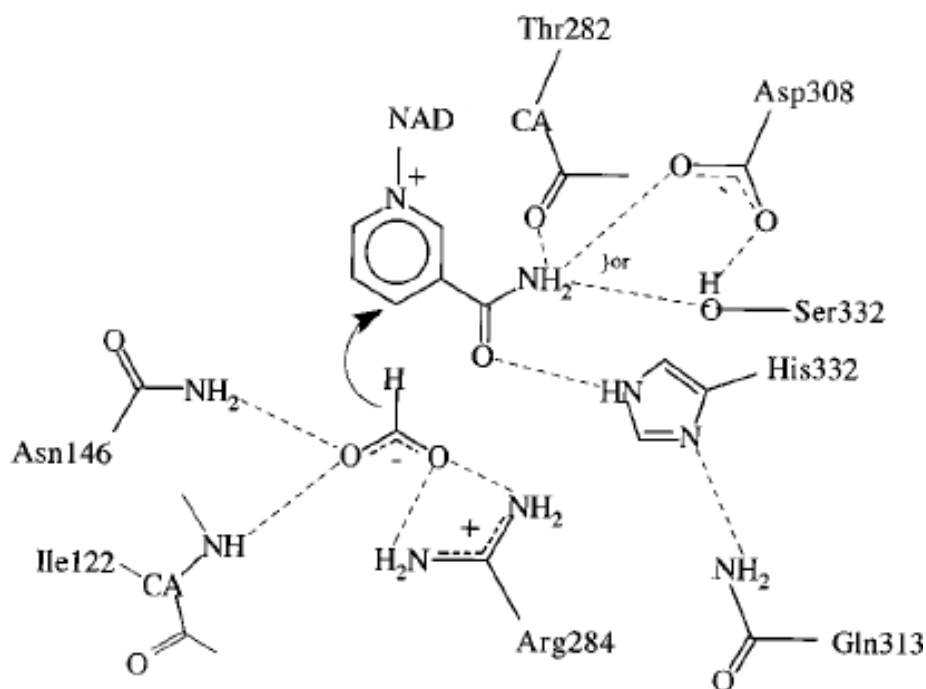


Figure 2.5: Catalytically important residues in the active site of *PseFDH*.

2.4. CO₂ Reduction with FDHs

Enzymes show catalytic CO₂ reduction ability at high yields under moderate reaction conditions. Further, biocatalysts possess favorable properties such as biodegradability and selectivity. Already in 1976, the group of Ruschig et al. presented the reduction of CO₂ to formate with formate dehydrogenase and the co-enzyme NADH [20]. Later, Kuwabata et al. show the electrochemical reduction of CO₂ using dehydrogenases as well but substituting the coenzyme NADH by chemical mediators [21].

Also, it has been found that FDHs from acetogens catalyze preferably the reduction of CO₂. Wood-Ljungdahl pathway uses the CO₂ as a carbon source and as a result of this, FDH is naturally reducing the CO₂ [22]. The condition determining the catalysis direction is that the enzyme has low affinity against formate. Aslan et. al. demonstrated that therefore, FDH is an attractive candidate for using for biotechnological applications intend to produce formic acid and chemical feedstocks from CO₂ [4], [23].

2.5. Protein engineering methods and applications

Protein engineering aims at modifying the structure and function of a protein through mutagenesis by various means. Methods are conventionally grouped into two main categories: rational design and directed evolution. Also, a combination of these two methods are also semi rational design method. [24]. In rational design; detailed knowledge of the structure and function of the residues of protein are needed for creating desired function. Being of well-developed various site-directed mutagenesis techniques makes rational design inexpensive and easy. The main disadvantage of rational design is that 3-D structural knowledge of a protein can be unavailable and be very difficult to predict the effects of each amino acid mutations. In directed evolution, random mutagenesis technique is applied to a protein, and selected screening method enable to detect variants of protein having the desired qualities. Further rounds of random mutagenesis can be applied to each mutant protein obtained from the previous one in order to produce better results.

2.5.1. Directed Evolution

This method is based on the construction of the enzyme library and the experimental screening of the desired phenotype by screening methods. Directed evolution techniques have many big advantages. It does not neither depend on any structural knowledge of a protein nor need to predict what effect a given mutation will have. But it needs mutated large amounts of protein that is screened for desired properties. Existing high-throughput screening- selection methods cannot be applicable for all proteins and desired activities. The most common method of directed evolution is error prone PCR, saturation mutagenesis and DNA shuffling. More recently, a technique is called CASTing (combinatorial active-site saturation), focussing into residues of the active site of a protein for pair-wise saturation mutagenesis. [24].

2.5.2. Semi-Rational Design

Rational design allows certain residues of proteins to be manipulated. Directed evolution allows protein engineering by mimicking the natural selection mechanism and does not require the detailed structure of the protein. Although both approaches are strong, both have limitations. Hence, a semi-rational approach has been developed as a combination of rational design and directed evolution. In this method, knowledge is needed as much as rational design about protein structure. Directed mutagenesis is applied to the region of interest. However, the primers used in this technique are degenerative primers that vary for some amino acids. Thus, as in directed evolution, a library containing more than one mutation of the protein of interest is created.

Iterative Saturation Mutagenesis (ISM) is named as accelerated directed evolution method by group of Manfred T. Reetz. ISM combines rational design and directed evolution [25]. ISM is used in producing novel biocatalysts by using producing data based on rational design and directed evolution. So ISM can be considered as a semi-rational design technique. With this property, ISM makes a decrease in the amount of laboratory work that is necessary in mutagenesis. In the application of ISM, having effective expression and screening method are essential. Because of occurrence the high number colonies depends on used degenerative codon, screening all colonies effectively in a short time the most important part of this semi-rational design. This technique basically is based on selecting candidate amino acids which are located primarily in the active site area. The amino acids which are located in the active site pocket of the enzyme and therefore make contact with the substrate for the enzyme, or not located in the active site but play a crucial role in the function of the enzyme (for example residues involved in the contact of two subunits of a multimeric protein), are selected as targets for mutagenesis. In all this selections and structural data are good references. Each selected close amino acid(s) are members of group and each group can be considered as a library. The output of the screening effort will provide data as to which of the selected libraries provides an improvement in enzymatic activity.

2.5.3. Rational Design: Site-Directed Mutagenesis

Site-directed mutagenesis is the most widely used method of protein engineering. Site-directed mutagenesis was investigated by Michael Smith [26], who received the Nobel prize 1993 for this landmark method in molecular biology, together with Kary B. Mullis for his invention of the PCR reaction [27].

Site-directed mutagenesis provides an example of rational protein engineering, where an existing protein is modified by altering certain, pre-defined residues. These residues are chosen by an informed decision based on three-dimensional protein structure, homology models, or phylogenetic relationships, depending on available information, engineering purpose and desired outcome. Although technically simple, single-point mutations provide a very powerful tool in protein science.

The methods in use for site-directed mutagenesis generally involve mutagenic oligonucleotides, encoding the desired mutation and wild-type regions. Such mutagenic primers can contain single base pair mutations, or multiple substitutions, insertions or deletions. They are annealed to the DNA of interest, and the subsequently synthesized DNA will incorporate the desired mutations. Different PCR and non-PCR methods exist to amplify the desired mutant gene, and to prepare recombinant vectors for expression. In the site-directed mutagenesis, mutations are introduced with synthetic oligonucleotide mismatch primers. Both forward and reverse primers contain the desired mutation. Following a standard thermal cycling protocol, plasmid template dsDNA is denatured, the mutagenic primers anneal to the ssDNA template, and are extended by a proof-reading DNA polymerase during the elongation phase. After thermal cycling, the PCR product is treated with *DpnI* endonuclease to digest methylated parental DNA template, and the mutated plasmid is transformed into competent *E. coli* cells for nick repair, amplification and preservation.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Laboratory Equipments

Micropipette (Isolab-Rainin), autoclave (N-Biotek), vortex (N-Biotek), magnetic stirrer / heater (Wisd), electrophoresis power supply (Biorad), SDS-PAGE electrophoresis system (Bio-Rad), mini centrifuge (Sigma), centrifuge (Beckman Coulter), PCR-thermal cycler (Techne-5PRIMEG / 02), -80 deep freezer (Arctiko), refrigerator (Vestel), pure water device (Merck Milipore), shaking incubator (N-Biotek) pH meter (Hanna), oven (Nuve), water bath (Stuart), sonicator (Bandelin) microplate reader (Tecan), microplate shaker incubator (Bio-Rad), HisTrap Column (GE), Syringe Filters (Sartorius) Concentrators (Amicon Millipore)

3.1.2. Enzymes

Phusion High Fidelity DNA Polymerase (BioLabs), DpnI (Thermo Sci.), Lysozyme (Serva).

3.1.3. Chemicals and Kits

Yeast extract (LabM), Acrylamide (Fisher BioReagents), Bis acrylamide (Fisher BioReagents), Ammonium persulfate (Fisher BioReagents), Sodium dodecyl sulphate (Fisher BioReagents), Trypton (LabM), Glucose (LabM), Lactose (Fisher BioReagents), 2-mercaptoethanol (Alfa Aesar), Agar (Fluka), Agarose (Biomax), Imidazole (AppliChem), LB Broth (LabM), LB Agar, Ethidium bromide, Sodium chloride (Merck), Sodium Chloride, Sodium Bicarbonate, Sodium Phosphate (Fisher Scientific), Tris-Base (Formedium), Ampicillin (AppliChem), Hydrochloric acid (Sigma-Aldrich), Glycerol (Fisher BioReagents), Sodium Formate (Sigma), NAD⁺, NADH (Sigma), Citrate (Riedel-de Haën), Glycine (Sigma), SDS-PAGE Gel Stain (Generon), SDS-PAGE Gels, SDS-PAGE Running Buffer (CBS Scientific) dNTP

(Thermo Sci.), Plasmid isolation kit (Invitrogen), Invitrogen PureLink (TM), Quick Plasmid Miniprep Kit (Cat. No. K210010), BCA Assay Kit (Serva).

3.1.4. Plasmids and Cells

The *ctfdh* gene containing hexa-histidine sequence in pET-45b⁺ plasmid that previously constructed by BERC lab group was used in the experiments (Aslan, A. S., (2017), PEDS, 30(1), 47-55). Commercial *E. coli* BL21 (DE3) and NEB[®] 5 α (NEB) cells were used for transformation. The map of the pET45b⁺ vector is given in Appendix D

3.1.5. Mediums

In the thesis study, mediums described in the following were used.

3.1.5.1 Luria-Bertani (LB)

12 g LB were dissolved with 500 mL ddH₂O and this solution was sterilized at 121 ° C for 15 minutes by autoclave.

3.1.5.2. Luria-Bertani (LB) Agar Feeder

20 g of LB Agar was weighed and dissolved in 500 mL of ddH₂O and then autoclaved at 121 ° C for 15 min. After cooling to 55 ° C, 1 μ L / 1 mL ampicillin was added and distributed in petri dishes in a sterile cabinet. The covers of the petri dishes were closed, wrapped in parafilm, and stored at +4 °C for later use.

3.1.5.3. Autoinduction Studier Medium

5 g of yeast, 20 g of tryptone, 5 g of NaCl, 6 g of Na₂HPO₄, 3 g of KH₂PO₄, 0.5 g of glucose, 6.9 mL of 87% glycerol and 2.1 g of lactose were dissolved in distilled water and pH was adjusted 7 then the solution was completed 1 liter. The

autoinduction Studier medium [39] was sterilized in an autoclave at 121 ° C for 15 minutes.

3.1.6. Buffers and Solutions

3.1.6.1. Protein Purification Buffers

Proteins purification from bacterial lysate was done by using HisTrap protein purification columns. Purification buffers are given below:

- Buffer 1: 1.38 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (20 mM), 14.61 g NaCl (500 mM) and 1.02 g Imidazole (30 mM) were dissolved in 500 mL dH_2O and the pH was fixed to 7.4 using NaOH.
- Buffer 2: 1.38 g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (20 mM), 14.61 g NaCl (500 mM) and 17.02 g Imidazole (500 mM) were dissolved in 500 mL dH_2O and the pH was fixed to 7.4 using NaOH.
- Buffer 1 with 100 mM Imidazole (50 mL): 42.50 mL Buffer 1 and 7.50 mL Buffer 2 were mixed.
- Buffer 1 with 200 mM Imidazole (50 mL): 32 mL Buffer 1 and 18 mL Buffer 2 were mixed.
- Buffer 1 with 400 mM Imidazole (50 mL): 10.50 mL Buffer 1 and 39.50 mL Buffer 2 were mixed.

3.1.6.2. Ampicillin Solution

1 g ampicillin was dissolved in 10 mL ddH₂O and passed through a 0.45 mm sterile filter. Then the ependorfs were divided into 1 mL and stored at -20 ° C

3.1.6.3. Enzyme Activity Solution

- 16 mM NAD⁺ Solution

54.8mg NAD⁺ was weighed and dissolved in 5 mL 100 mM pH 5 sodium acetate buffer.

- 1mM NADH Solution

13.2 mg NADH was weighed and dissolved in 5 mL 100 mM pH 8 sodium phosphate buffer.

3.1.6.4. Enzyme Activity Assay Buffers

- Sodium Phosphate Buffer Solution (100 mM)

14.19 g Na₂HPO₄ and 13.79 g NaH₂PO₄·H₂O (100 mM) were dissolved in dH₂O and the final volumes were completed to 1 L. pH adjustments for NaPi buffer were done by using Na₂HPO₄ as a base and NaH₂PO₄·H₂O as an acid.

- Tris-HCl Buffer Solution (100 mM)

12.11 g C₄H₁₁NO₃ were dissolved in dH₂O and the final volume was completed to 1 L. pH adjustments were done by using HCl (100 mM).

- Glycine-HCl Buffer Solution (100 mM)

7.50 g NH₂CH₂COOH were dissolved in dH₂O and the final volume was completed to 1 L. pH adjustments were done by using HCl (100 mM).

3.2. Methods

3.2.1. Bioinformatic Analysis

The crystal structures of *Cb*FDH (PDB: 2j6i) and *Ps*FDH (PDB: 2nad) were used to guide selection of amino acid residues interacting with the substrate. The three candidate residues; Asn120Cys, Asn256Asp and Asp283Asn that close to the active site were identified, and the primers were designed for introducing these mutations into *Ct*FDH. The comparative structure modelling has been made in SwissModel server.

3.2.2. Rational Design

3.2.2.1. Primer Design

The forward and reverse primers (shown in Table 3.1) are designed to convert; Asparagine to Cysteine at position 120, Asparagine to Aspartic acid at position 256 and Aspartic acid to Asparagine at position 283 in *ctfdh* gene. OligoArchitect™ Primer and Probe Design tool was used for primer design.

Table 3.1: List of primer pairs used in study.

Primer	Sequence (5'-3')
Asn120Cys-F	GCAGAAGTGACCGGTTCA TGTGTGGTTTCG
Asn120Cys-R	CGAAACCACAC ATGA ACCGGTC ACTTCTGC
Asn256Asp-F	GTTCTTTGGCTGGTT GATACGGCGCGTGG
Asn256Asp-R	CCACGCGCCGT ATCA ACCAGCCAAAGAAC
Asp283Asn-F	GCGGCTACGGCGGTA AATGTCTGGTTCCCG
Asp283Asn-R	CGGGAACCAGAC ATTACG CCGTAGCCGC

3.2.2.2. Site-directed mutagenesis

Site-directed mutagenesis was performed to construct Asn120Cys, Asn256Asp and Asp283Asn mutants according to the following PCR procedure using the recombinant pET45b⁺ plasmid, which containing *CtFDH* gene as a template. Samples were set up in the amounts of reagents shown in Table 3.2.

Table 3.2: Reaction contents and quantities used for mutation.

Reactant	Amount
HF Reaction Buffer	10 μ L
The plasmid (~70 ng) containing the <i>ctfdh</i> gene	1 μ L
Forward Primers (~100 ng)	1 μ L
Reverse Primers (~100 ng)	1 μ L
10 mM dNTP mix	1 μ L
ddH ₂ O	35 μ L
Phusion High Fidelity DNA Polimerase	1 μ L
Total volume	50 μ L

PCR reactions are set up according to the procedure shown in Table 3.3.

Table 3.3: Cycling parameters for the Site-directed mutagenesis.

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 second
		60°C	50 second
		68°C	90 second
3	1	68°C	7 minutes

3.2.2.2.1. *Dpn* I Digestion of the PCR Products

After PCR, 10 μ L of PCR products were taken for agarose gel control. 1 μ L of *Dpn* I restriction enzyme was added into the remaining 40 μ L PCR product and incubated at 37 ° C for 2 hours.

3.2.2.2.2 Agarose Gel Electrophoresis

0.50 g agarose was dissolved in 50 mL 1X TBE buffer and boiled. After the cooling 5 μ L Serva DNA Stain Clear-G was added to the agarose gel. Than agarose gel tank was filled with this solution. After the gel freezing, the tank was filled with 1X TBE buffer and samples were loaded. The first 10 minutes were carried out at 80V and then continued at 100V. The bands were displayed under UV light.

3.2.2.2.3. Transformation of *CtFDH* to *E. coli*

Competent cell tubes were thawed on ice for 20 min. 1 μ l of the *Dpn* I-treated DNA from each sample reaction were separated aliquots of the chemically competent NEB[®] 5 α competent *E. coli* cells and swirled 4-5 times to mix DNA and cells. Samples was placed on ice for 30 min then heat shocked at 42 °C for 30 sec. Mixture were placed on ice for 5 min. 250 μ L of preheated SOC medium was added to the mixture and incubated at 37 °C at 250 rpm for 1 hour. Cells were mixed by flicking the tube then the mixture was separated by spreader to the selection plates and incubated overnight at at 37 °C. Individual colonies were picked and inoculated into overnight cultures for optimization of the expression conditions.

3.2.2.2.4. Plasmids Purification

Individual colonies were picked from LB-agar plates supplemented with 100 μ g/mL ampicillin, inoculated into 5 mL of LB medium containing ampicillin (100 μ g/mL) and incubated at 37°C at 225 rpm for overnight. After incubation, 5 mL of cell culture was precipitated to obtain the pellet. Plasmid purification was performed as follows according to the Invitrogen PureLink [™] Quick Plasmid Miniprep Kit protocol.

- Resuspension: 250 μ L of resuspension buffer (R3) containing RNase was added. The precipitate was homogeneously resuspended in the solvent and transferred to into a 2 mL tube.
- Lysis: 250 μ L, 37 ° C incubated lysis buffer (L7) was added. The tube was slowly mixed 4-5 times until the mixture became homogenous. It was incubated for 5 minutes at room temperature.
- Precipitation: 350 μ L of precipitation buffer (N4) was added and the mixture was stirred until it became homogeneous. Centrifuged at > 12000 xg for 10 minutes.
- Binding: After centrifugation, the supernatant was loaded into a column placed in a 2 mL tube. It was centrifuged at 12000 xg for 1 minute. passing liquid from the column was discarded.
- Wash: 700 μ L of wash buffer (W9) containing ethanol was loaded onto the colum. It was centrifuged at 12000 xg for 1 minute. passing liquid from the column was discarded. The same process was repeated once more. The column was centrifuged for an additional 1 minute, and the bottom liquid was again discarded.
- Collection: The colon was placed in a clean tube. 50 μ L, EDTA-free elution buffer was added to the colon and incubated at room temperature for 5 minutes, it was centrifuged at 12000 xg. The eluted plasmids were stored at -20 ° C.

3.2.2.2.5. DNA Sequence Analysis

20 μ L of the isolated plasmid sample was send to GATCBiotech for sequence analysis. The T7 promoter and terminator (5 'TAATACGACTCACTATAGGG 3' and 5 'GCTAGTTATTGCTCAGCGG 3') nucleotide sequence was used in the polymerase chain reaction for sequence analysis.

3.2.3. Expression of FDHs

Plasmid DNA of CtFDH variants were isolated from the obtained colonies and sequenced to ensure the correct DNA sequence of the generated constructs. The confirmed mutant plasmids were then transformed into competent *E. coli* BL21 (DE3) host cellst to express wild type and mutants CtFDH. Individual colonies were picked from LB-agar plates supplemented with 100 μ g/mL ampicillin, inoculated into 5 mL of LB medium containing ampicillin (100 μ g/mL) and incubated at 37 °C at 225 rpm

for overnight. The overnight cultures were added to the large autoinduction medium (500 mL) which were incubated at 30 °C at 225 rpm for 16 hours. After cultivation, the cells were harvested by centrifugation at 4000 rpm for 15 minutes and purification process was started.

3.2.4. Purification of *Ct*FDH Wild type and Mutants

The obtained pellets were resuspended in Buffer 1 (20 mM NaPi, 500 mM NaCl, 30 mM imidazole pH 7.4, 1.0 mg/mL lysozyme) and incubated on ice for 30 min. The resuspended cells were disrupted by sonication, and clarified by centrifugation (16000 rpm, 20 min). The lysate was filtered through a 0.45 µm filter then applied to a HisTrap column which was pre-equilibrated with the Buffer 1. The column was washed with 5 mL of Buffer 1 and the *Ct*FDH was eluted with Buffer 1 containing different imidazole concentrations (100-200 and 400 mM). Collected fractions were analysed by SDS-PAGE to identify fractions containing pure protein, which were then combined and diluted to 50 mL by addition of 100 mM of NaPi pH 7.4. Proteins were concentrated with Amicon Millipore centrifugal concentrators. The concentration of the purified *Ct*FDHs was determined by BCA Assay Kit. The expression efficiency was monitored by SDS-PAGE.

3.2.4.1. SDS-PAGE Analysis

Samples were prepared by mixing 15 µL sample and 5 µL 4X sample loading buffer. Samples were heated at 95 °C for 10 minutes then centrifuged at 13500 rpm for 10 minutes.

The SDS gel placed to tank than the tank was filled with running buffer. 5 µL protein standard was loaded into the first well and 5 µL of sample is loaded into the next wells. Then the running was started with 80 V for 10 minutes and it was continued 100 V for 1 hour.

3.2.5. Enzyme Activity Assay

The activity of wild type and mutants *CtFDH* were measured against 20 mM sodium formate and 20 mM sodium bicarbonate in 100 mM NaPi pH 7.4 containing 0.5 mM NAD⁺, NADH respectively. A unit was equal to the amount of pure enzyme required to consume 1 μ M NADH/NAD⁺ per minute by monitoring the change in absorbance at 340 nm. All experiments were performed in triplicates.

3.2.6. Effect of pH

The activity of wild type and mutants *CtFDH* were measured in different pHs to see the effect of them on *CtFDH* activity. The specific activity of *CtFDH* was measured in selected 100 mM buffers containing 20 mM sodium bicarbonate with 1 mM NADH. The selected 100 mM buffers were pH 4, 5, 6 Sodium citrate buffer, pH 5 Sodium asetate buffer, pH 6- 8 Sodium Phosphate buffer; pH 9 Tris-HCl buffer, pH 10 Glycine buffer.

3.2.7. Kinetic Analysis

The kinetic parameters of purified wild types and mutants *CtFDH* were determined through monitoring the change in absorbance at 340 nm due to NADH formation or consumption in optimum buffer. The reaction mixtures contained 1.0 mM NAD⁺ or 0.5 mM NADH and the concentration range used for measuring the kinetic parameters was 0-40 mM for formate and 0-25 mM for sodium bicarbonate. All reactions were performed at 25 °C with their control reactions in the absence of related substrates. Michaelis-Menten kinetic constants were determined from triplicate measurements of the initial velocities. The k_{cat} and K_M values were determined by using GraphPad Prism.

3.2.8. Molecular Modelling

Molecular dynamics simulations were used to further elucidate the interactions between the substrates and the two *CtFDH* variants (the wild type and the

Asn120Cys mutant). Initial models were constructed with Swiss-PDB Viewer, version 4.1. The PDB structure 2NAD was used as reference for the docking of the substrate pairs NAD⁺/formate and NADH/hydrogen carbonate into the active site. Formate and hydrogen carbonate were placed in the vicinity of the C4 position (C4N atom) of the nicotinamide ring of NAD⁺/NADH. In adherence to the simulation by Torres et al. [19], the hydride and its acceptor substrate were initially set within 3 Å from each other. The distances between the central carbon atoms of the substrates formate and hydrogen carbonate and the C4N were 2.86 Å and 2.50 Å, respectively.

Structure preparation for energy minimizations and molecular dynamics simulations were done with VMD, version 1.9.3 [28]. NAMD version 2.12 was used in the molecular dynamics calculations. The calculations were run with the CHARMM parameters from the CGenFF project [29] coupled with the default simulation settings of NAMD. At the temperature of 298 K, the structures underwent energy minimization for 10 picoseconds (10,000 timesteps in NAMD) followed by 90 picoseconds (90,000 timesteps) of molecular mechanics calculations. The results were visualized and analysed with VMD and Swiss-PDB Viewer.

4. RESULTS

4.1. Post-PCR Control

Mutations were performed on the pET45b⁺ vector containing the *ctfdh* gene using primers containing the mutant codon. The obtained PCR products were visualized by agarose gel electrophoresis. According to the obtained gel images, the PCR bands of the mutations expected to be theoretically about 4560 kb in size of the pET45b⁺ vector containing the *ctfdh* gene were obtained at these values as shown in Figure 4.1. and 4.2.

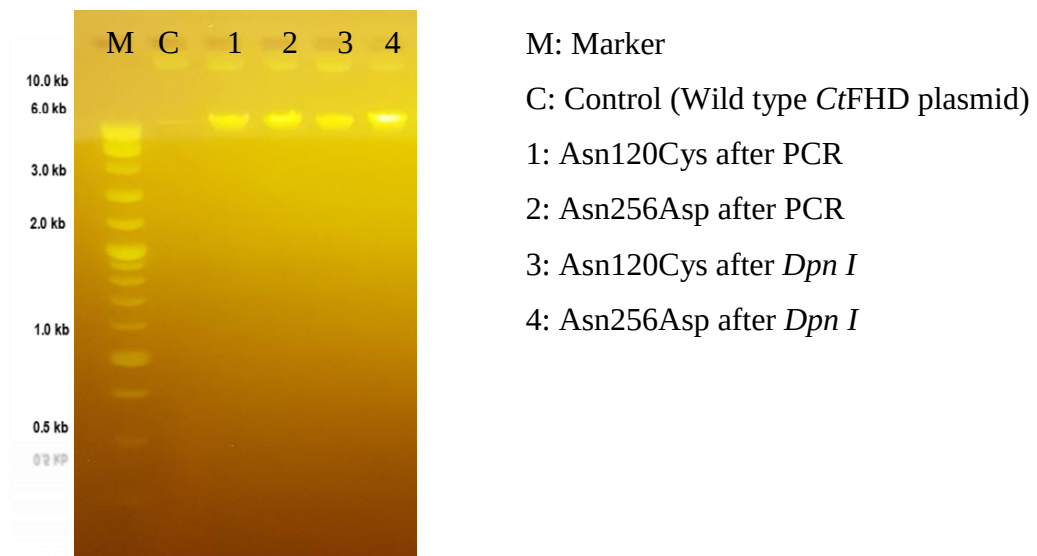


Figure 4.1: *CtFHDs* agarose gel image after PCR and after with *DpnI* incubation.

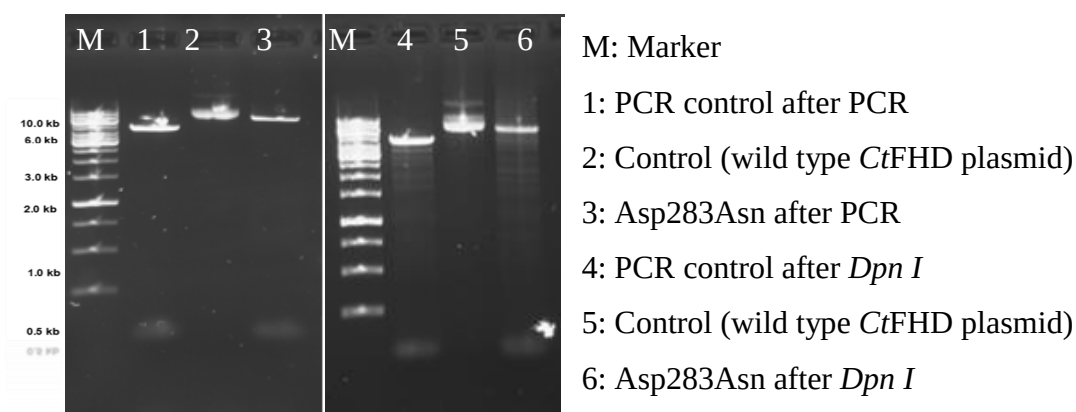


Figure 4.2: Asp283Asn *CtFHD* agarose gel image after PCR and *DpnI* incubation.

4.2. Expression of *CtFDHs*

Transformation of PCR product to and NEB[®] 5 α competent *E. coli* cells were successfully done. Bacterial colonies on the selection plates containing ampicillin (100 μ g/mL) can be seen in Figure 4.3.



Figure 4.3: Transformation plates of *CtFDHs*.

4.3. DNA Sequence Analysis Results

After PCR product transformation, plasmids containing the *ctfdh* genes were purified as described in section 3.2.9. DNAs were sent to sequence analysis. The analysis results were compared to the wild type *ctfdh* using the resulting BioEdit Sequence Alignment Editor program. Asn120Cys, Asn256Asp and Asp283Asn mutations have been identified. Sequence analysis results of the mutations for the relevant region are shown in Figure 4.4.

CtFDH wild type	GCAGAAAGTGACCGGGTTCAAACGTTTCG
Asn120Cys	GCAGAAAGTGACCGGGTTCAATGTGTTTCG
CtFDH wild type	GTTCTTTGGCTGGTTAATACGGCGCGTGG
Asn256Asp	GTTCTTTGGCTGGTTGATACGGCGCGTGG
CtFDH wild type	GCGGCTACGGCGGTGATGTCTGGTTCCCG
Asp283Asn	GCGGCTACGGCGGTAATGTCTGGTTCCCG

Figure 4.4: Sequence analysis results of wild-type and mutant CtFDHs.

4.4. Purification of CtFDHs

pET 45b+ vectors containing mutant CtFDH genes were transformed into *E. coli* BL21 (DE3) cells, protein expression and purification was performed as described in Sections 3.2.3 and 3.2.4 The SDS-PAGE gel image obtained by loading the collected fractions at the end of purification is shown in Figure 4.5. and 4.6. The gel image shows that CtFDH is ~ 45 kDa in size and pure in fractions were obtained for kinetic measurements.

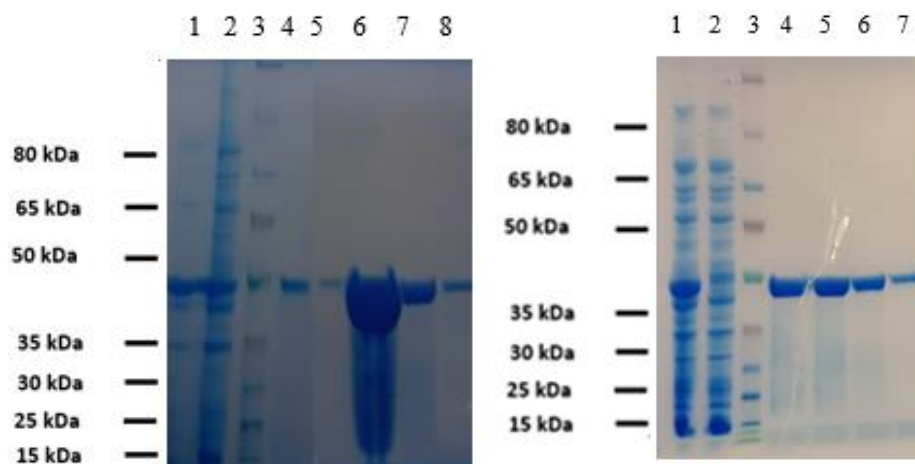


Figure 4.5: SDS-PAGE gel image for the CtFDH wild-type and Asp283Asn mutant.

In the Figure 4.6. the references of the numbers are as follows. 1. pellet 2. Post column washing 3. Protein standard 4.100 mM imidazole / third fraction, 5. 200 mM imidazole / fifth fraction, 6. 200 mM imidazole / sixth fraction, 7. 400 mM imidazole / ninth fraction, 8. 400 mM imidazole / tenth fraction.

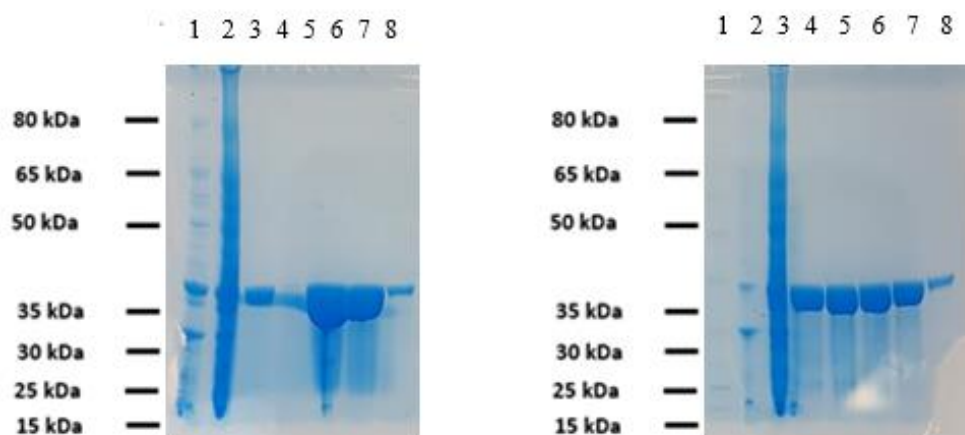


Figure 4.6: SDS-PAGE gel image for *CtFDH* Asn120Cys and Asn256Asp mutant.

In the Figure 4.6. the references of the numbers are as follows. 1. Protein standard 2. Post column washing 3. 100 mM imidazole / third fraction, 4. 200 mM imidazole / fifth fraction, 5. 200 mM imidazole / sixth fraction, 6. 400 mM imidazole / ninth fraction, 8. 400 mM imidazole / tenth fraction.

4.5. Effect of pH on *CtFDH* Activity

The effect of different pH on *CtFDH* activity for CO₂ reduction was measured (Figure 4.7.). The optimum pH is 8 for the wild type and Asn256Asp mutant, whereas for the Asn120Cys mutant the optimum pH is 7. When comparing the wild type FDH and mutants, Asn120Cys and Asn256Asp were showed lower activity than wild type *CtFDH* towards hydrogen carbonate at pH 8 However, the Asn120Cys showed slightly higher activity at pH 7 than the wild type *CtFDH*.

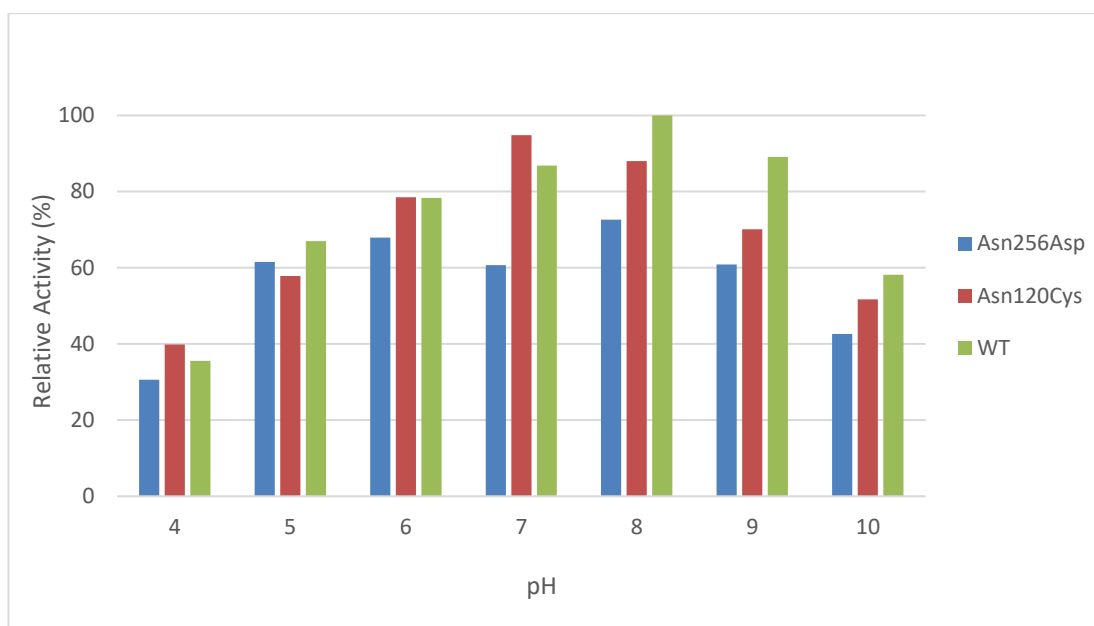


Figure 4.7: Effect of pH on relative activity of *CtFDHs*.

Sodium citrate buffers were used at pH 4. Sodium acetate buffers were used at pH 5. Sodium phosphate buffers were used at pH 6, 7 and 8. Tris-HCl buffer were used at pH 9. Glycine buffer was used pH 10 .

4.6. Kinetic Parameters Determined for the Wild Type and Mutant *CtFDHs*

Kinetic measurements were taken at room temperature as described in Section 3.2.16 and Michaelis-Menten kinetic values were determined. Asn120Cys and Asn256Asp *CtFDH* mutants did not show measurable activity against the formate also Asp283Asn lost activity both direction so kinetic studies were not performed. Michaelis-Menten graphs for formate and bicarbonate wild-type and mutants *CtFDH* are shown in Figures between 4.8. and 4.11. K_M and k_{cat} values for the wild-type and mutant *CtFDHs* are shown in Table 4.1.

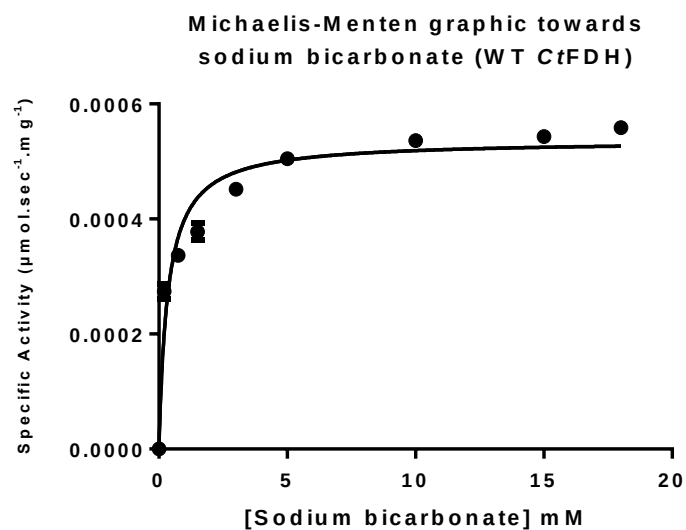


Figure 4.8: Michaelis-Menten chart versus sodium bicarbonate for the WT CtFDH.

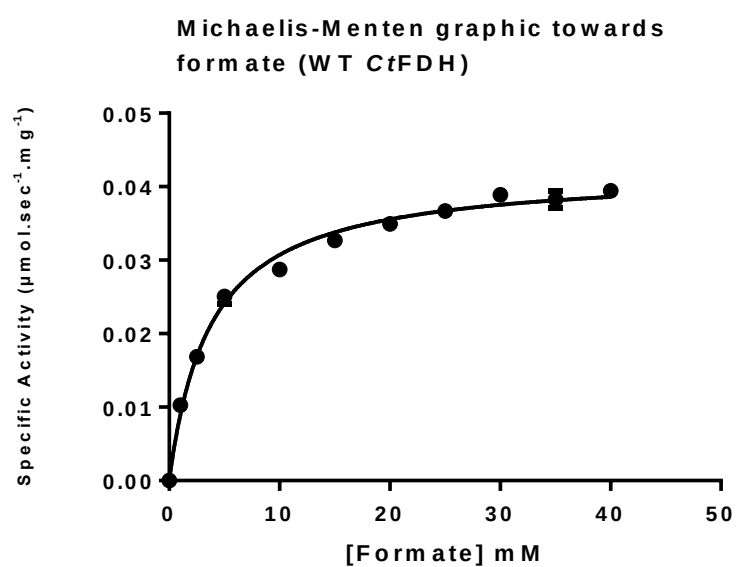


Figure 4.9: Michaelis-Menten chart versus formate for the WT CtFDH.

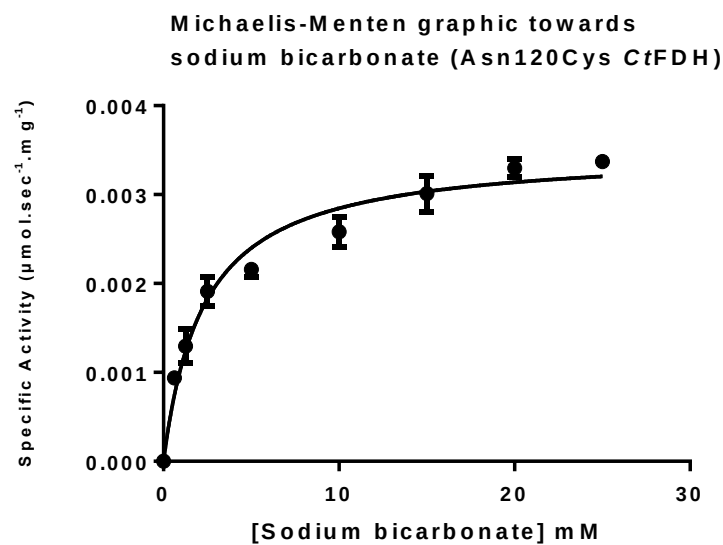


Figure 4.10: Michaelis-Menten chart versus sodium bicarbonate for the Asn120Cys.

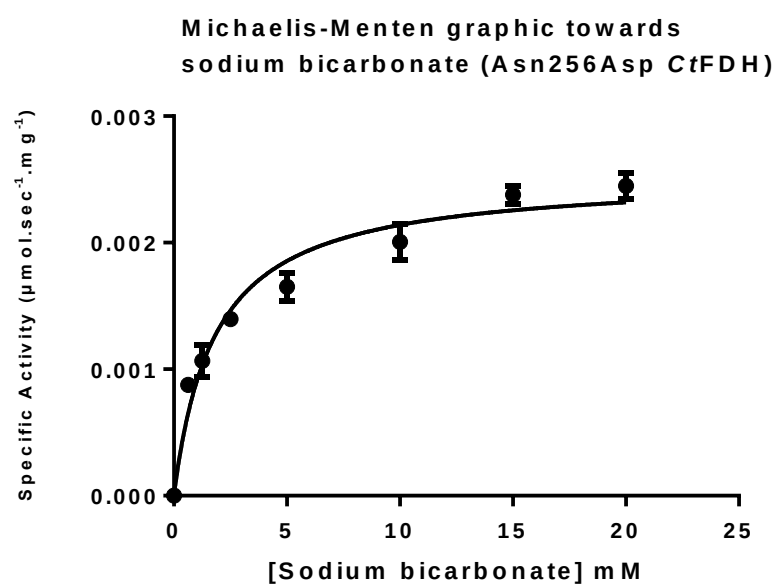


Figure 4.11: Michaelis-Menten chart versus sodium bicarbonate for the Asn256Asp.

Tablo 4.1: The kinetic constants of *Ct*FDH for hydrogen carbonate and formate.

		Substratlar					
<i>Ct</i> FDH	Specific Activity (U/mg)	Sodium Bicarbonate			Formate		
		K_M (mM)	k_{cat} (sec ⁻¹)	k_{cat}/K_M (sec ⁻¹ mM ⁻¹)	K_M (mM)	k_{cat} (sec ⁻¹)	k_{cat}/K_M (sec ⁻¹ mM ⁻¹)
Wild-Type	0.032 ± 0.03	0.35 ± 0.05	00.025 ± 0.003	0.071 ± 0007	3.70 ± 0.14	1.805 ± 0.004	0.490 ± 0.00
Asn120Cys	0.15±0.3	2.27 ± 0.33	0.162 ± 0.005	0.071± 0.015		No activity	
Asn256Asp	0.12±0.06	1.81 ± 0.30	0.115 ± 0.002	0.063± 0.006		No activity	
Asp283Asn			No activity			No activity	

4.7. Molecular Modelling

*Ct*FDH mutant Asn120Cys was further modelled to elucidate why it showed catalytic activity towards hydrogen carbonate reduction (the "reverse" FDH reaction) but was inactive on the "forward" formate oxidation. According to the molecular dynamic simulations, the structural differences were only 1.1 Å (RMS carbon alpha only) between the modelled wild type and Asn120Cys mutant with formate and hydrogen carbonate substrates. Moreover, the amino acid side chains of the active site mostly retained their positions. Six out of 11 common active site residues show hydrogen bond connections with substrates and NADH cofactors (Fig. 4.13). The binding of the wild type to hydrogen carbonate differs from formate so that the hydrogen carbonate has two hydrogen bonds more. The wild type FDH binds to the formate on three hydrogen bonds. One of the bonds is from guanidinium moiety (-NH₂) of Arg259 to the carbonyl of the formate and two other bonds from main chain (-NH) of Ile94 and side chain (-NH₂) of Asn120 are to the second carbonyl group. Whereas hydrogen carbonate binds to the wild type enzyme through five hydrogen bonds, three of which are from Arg259 to both carbonyl groups of the hydrogen carbonate and two are from hydroxide group of hydrogen carbonate to the main chain (-NH) of Ile94 and to the side chain (-NH₂) of Asn120.

Asn120Cys variant has only two hydrogen bonds with formate: one with main chain (-NH) of Ile94, and one with guanidinium amino (-NH₂) of Arg259. Our simulation indicates that when hydrogen carbonate is the substrate in the *Ct*FDH active site, there is room for two hydrogen carbonates: one of them can form hydrogen bonds with the side chains of Arg259 and His312, and the second hydrogen carbonate with main chain amino groups (-NH) of Ile94 and Cys120 as well as with carbonyl group (-C=O) of Gly315.

NADH as cofactor has a hydrogen bonding network that is similar for the wild type and the Asn120Cys FDH when hydrogen carbonate is the substrate. Both variants have two hydrogen bonds to the main chain groups of Thr257 (-NH) and Gly315 (-C=O) from the carboxamide group of NADH, and also two hydrogen bonds from the cofactor's phosphate hydroxyl groups to the main chain groups (-NH) of Ile176 and Gly315. When formate is the substrate there are three hydrogen bonds from carboxamide group of NAD(H). Main differences between these models are seen in

the mutant Asn120Cys with hydrogen carbonates. This mutant has space in the active site for two hydrogen carbonates, which can have hydrogen bonds to the residues Ile94, Cys120, Arg259, and His312 (Fig. 4.12 and Fig. 4.13).

Figure 4.12 shows the structure differences of the hydrogen bonding amino acid residues and the conformation of NAD⁺/NADH cofactor in the active site of *Ct*FDH models. In the upper part is the wild type with formate (left) and hydrogen carbonate (right). In the lower part is the mutant Asn120Cys with formate (left) and hydrogen carbonates (right). In the middle is shown the conformation of NAD⁺ in 2Nad.pdb structure. Formates are shown by one black star (*) and hydrogen carbonates by two black stars (**). Dotted green lines are hydrogen bonds. The images were created by Swiss-PdbViewer.

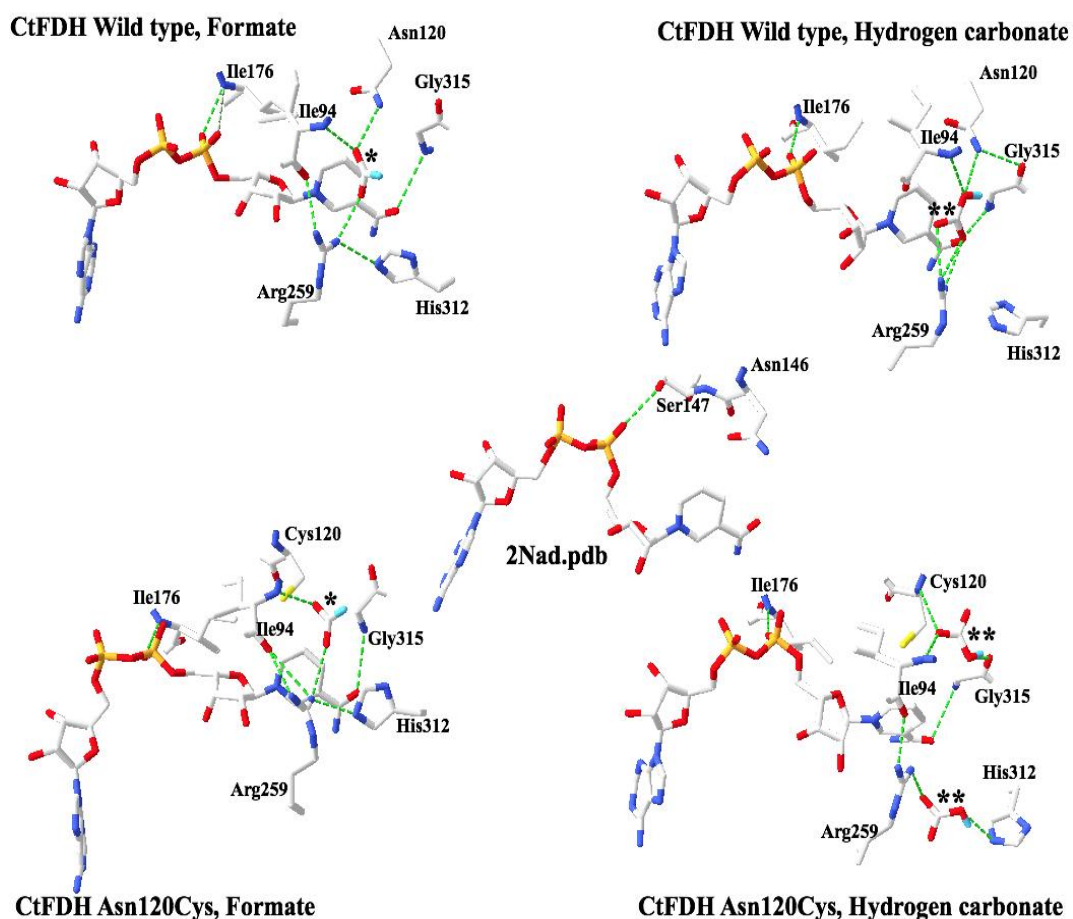


Figure 4.12: Structure of the hydrogen bonding amino acid residues of the *Ct*FDH.

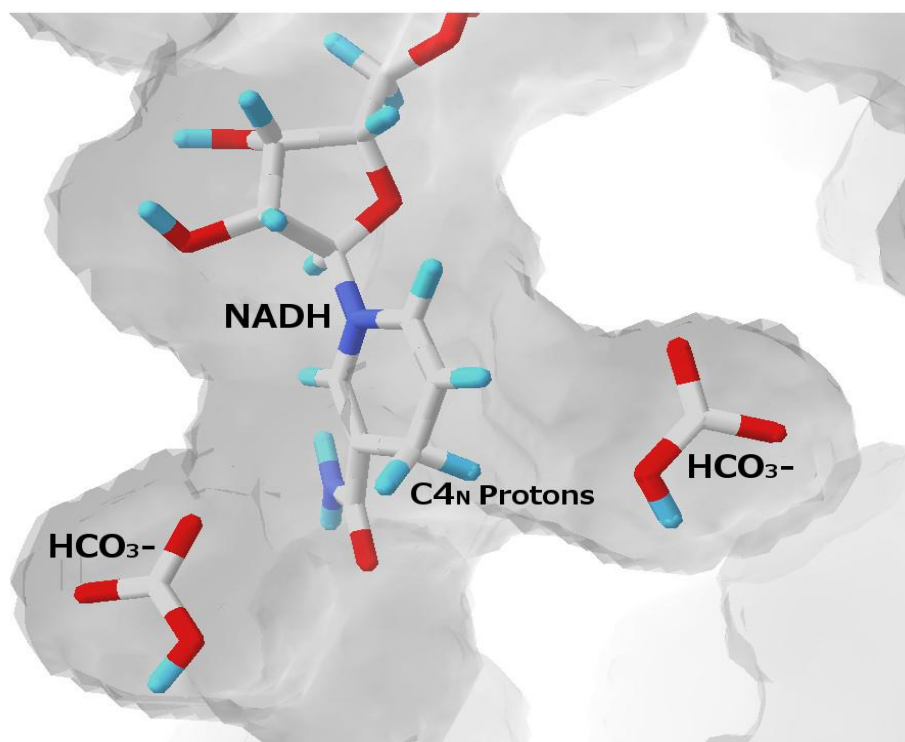


Figure 4.13: The hydrogen carbonate canal in the mutant Asn120Cys of *CtFDH*.

The Figure 4.13. shows the putative hydrogen carbonate canal in the mutant Asn120Cys of *CtFDH*. Two hydrogen carbonates are in the canal in close vicinity to C4_N protons. The images were created by Swiss-PdbViewer.

5. DISCUSSION

Designed mutations were made close to the catalytic centre of *CtFDH* to clarify structure-function relationships in the catalytic reaction, especially to find out how FDHs function with hydrogen carbonate as substrate. These mutations continue the earlier research with *CtFDH* (Aslan et al., 2017) [4]. Asn120 is one of the critical binding residues, its side chain is involved in coordinating the substrate into the catalytic centre. The side chain of Asn forms hydrogen bonds to the modelled formate and hydrogen carbonate, and also in the Asn120Cys mutant, cysteine has hydrogen bond between the main chain group (-NH) and substrate.

The modelling suggests that the mutation Asn120Cys loosens the tight control of substrate positioning by introducing more space and conformational flexibility to the active site, allowing the hydrogen carbonate substrates to move more easily. This was seen as a change in the position of the His312 side chain as well as fluctuation in the NADH conformation in the molecular dynamics simulation. In *CtFDH*, the conformational change of NADH is also due to the lack of the native hydrogen bonding between Val121 and NADH, while Ser or Thr in this position in many other FDHs can form hydrogen bonds with the NADH [3].

The modelling suggests an explanation for the differences between hydrogen carbonate and formate as substrates for the wild type and Asn120Cys mutant of *CtFDH*. Formate forms hydrogen bonds with side chains of Asn120 and Arg259, which may affect the formate resonance structure, therefore helping the hydride to leave towards the NAD^+ . In the reverse reaction the situation is different, hydrogen carbonate now being the acceptor of the hydride donated by NADH. To function as the FDH substrate, hydrogen carbonate must be in the carbonyl form, which might occur more easily in the Asn120Cys mutant active site than in the wild type (elevated k_{cat} in the mutant). The modelling indicates that in the wild type *CtFDH*, formate interacts through three non-symmetrical hydrogen bonds with the enzyme, whereas in the Asn120Cys mutant there are two hydrogen bonds with oxygen atoms of the formate. This study hypothesizes that this affects the reaction orientation and/or the

resonance structure of formate unfavourably, thus preventing the hydride transfer in the mutant *CtFDH*, but allowing an improved reaction with hydrogen carbonate.

Key features between the two substrates are that hydrogen carbonate (K_M 0.35 mM) binds much better than formate (3.7 mM) to the wild type *CtFDH* enzyme, but the catalysis rate is much faster with formate (1.805 sec⁻¹) than with hydrogen carbonate (0.025 sec⁻¹). Stronger binding of hydrogen carbonate could be one reason for the low catalysis rate by the wild type. Decrease of binding after the mutation Asn120Cys as shown by increased K_M (2.27 mM) increased the catalysis rate from 0.25 to 1.62 sec⁻¹. The 6.5-fold higher K_M indicates that the mutant Asn120Cys requires higher substrate concentration than the wild type *CtFDH* for the reaction to proceed, but despite of this, in the 0-25 mM substrate concentration the reaction rate was much higher in the mutant (Fig 4.10.).

Since the mutation increased the k_{cat} 6.5-fold, this suggests that the product release is a bottleneck in the enzyme reaction. As the modelling showed, the Asn120Cys mutation is likely to increase variation in the positioning of the substrate and in the nicotinamide ring conformation, which indicates that while the hydride transfer from NADH may have quite flexible limits for its occurrence, its efficiency is affected by the mutation. Albeit the difference between the substrates is only one additional hydroxyl group in hydrogen carbonate, the results show that the "forward" reaction of formate does not occur after the Asn120Cys mutation. This may also indicate that hydrogen carbonate must be in the mutant in a different position for the reverse reaction, relative to that of formate in the forward reaction [30]. Therefore, Cys120 can allow a favourable positioning for hydrogen carbonate but not for formate, as the modelling indicates. The mutation Asn120Cys may thus affect the FDH active site environment so that the cavity size increases, but only with hydrogen carbonate as the substrate and not with formate. Consequently, there is space for two hydrogen carbonates in the active site (Fig. 4.12. and 4.13.).

The mutation Asn256Asp in *CtFDH* was designed to change the electrostatic influences quite near to the NAD(H) cofactor. The distance to cofactor is 7.5 Å, and about 10 Å to the substrate. Streaming of additional electrons to NADH may not be beneficial for the substrate interaction, and K_M increased 5.7-fold for hydrogen carbonate. However, the k_{cat} also increased, probably due to the elevated K_M , in the same way as with Asn120Cys. Furthermore, this mutant enzyme was inactive with

formate indicating that the changed electrostatic balance nearby affects the active site quite severely.

The results of the present study show that the effect of a mutation can differ greatly between the *CtFDH* wild type and mutant enzymes and also between the substrates. It is likely that small structural changes cause these differences. The simulations revealed an intriguing possibility that the mutation Asn120Cys in *CtFDH* allows a structural change that provides more space to accommodate two hydrogen carbonates at the active site, thus allowing the "reverse" reaction to take place. Simulation with two hydrogen carbonates retained the conformation of the nicotinamide ring better than with one hydrogen carbonate and kept one hydrogen carbonate close to its C4_N protons.

Clarification of the role of active site residues with alternative substrates generates new understanding that is needed for engineering new more efficient enzymes for the conversion of CO₂ to formate.

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BIOGRAPHY

After completing Sevim Kurum High School in Van, Berin continued on to her undergrad degree in Molecular Biology and Genetics at Istanbul Arel University. Berin completed her internship at Imperial College of London Neurobiologic solution for Cancer Laboratory. After graduation from bachelor's degree Berin started her MSc. program at Gebze Technical University in Institute of Natural and Applied Sciences department of Molecular Biology and Genetics and also in this period she went to Biocatalysis Centre of Exeter University, Exeter, UK as a visiting academic under the supervision of Prof. Dr. Jennifer Littlechild to complete her MSc. studies.

APPENDICES

Appendix A: Publications related with the thesis

Yelmazer B., Valjakka J., Turunen O., Binay B., (2017), “ Engineering the activity of NAD⁺ dependent *Chaetomium thermophilum* Formate Dehydrogenase on carbon dioxide”, BioTrans 2017 13th International Symposium on Biocatalysis and Biotransformations, 270, Budapest, Hungary, 09-13 July.

Çakar M. M., Yelmazer B., Pala U., Çorbacıoğlu M., Aslan S. A., Binay B., (2017), “Problem of The Century: The Emission of Carbon Dioxide (CO₂): An Enzymatic Solution”, BioTrans 2017 13th International Symposium on Biocatalysis and Biotransformations, 270, Budapest, Hungary, 09-13 July.

Appendix B: Vector Map of Recombinant *ctfdh* Gene

The image was created with SnapGene software.

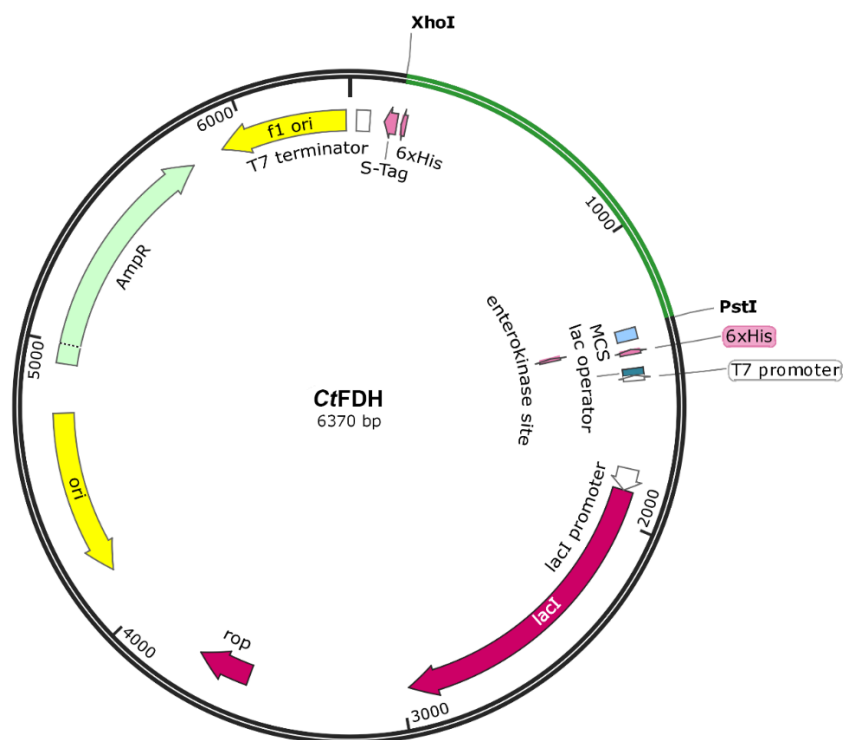


Figure B1.1: Cloned *ctfdh* gene into pET45b+ vector.