ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

EFFECTS OF CYSTEIN/CYSTINE ENGINEERING ON THERMAL STABILITY OF FORMATE DEHYDROGENASE FROM CANDIDA METHYLICA

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SİSTEİN / SİSTİN MÜHENDİSLİĞİNİN *CANDİDA METHYLICA* FORMAT DEHYDROGENAZININ

YÜKSEK LİSANS TEZİ

Osman DOLUCA

Tezin Enstitüye Verildiği Tarih : 20 Mayıs 2008

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HAZİRAN 2008

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TABLE OF CONTENTS

ABBREVIATIONS

ADH	:Alcohol Dehydrogenases
Cm	:*Candida methylica*
Cb	:*Candida boidinii*
FDH	:Formate deydrogenase
His	:Histidine
IPTG	:Isopropyl β-D-1-thiogalactopyranoside
K_m	:Michaels Menten Constant
LDH	:Lactate Dehydrogenase
NAD	:Nicotinamide adenine dinucleotide
NADH	:Nicotinamide adenine dinucleotide (reduced form)
Ni-NTA	:Nickel nitriloacetic acid
NMR	:Nuclear Magnetic Resonance
Ps	:*Pseudomonas* sp. 101

LIST OF TABLES

Page No.

LIST OF FIGURES

EFFECTS OF CYSTEINE/CYSTINE ENGINEERING ON THERMAL STABILITY OF FORMATE DEHYDROGENASE FROM CANDIDA METHYLICA

SUMMARY

Formate dehydrogenases (FDH) have been widely studied as a candidate for industrial NAD(P)H regeneration. Despite of its distinctive advantages, the limitation in the thermostability of the enzyme stays as an important problem. There are different approches to increase the thermostabilty of the enzymes, here we apply protein engineering based approach. The wild type FDH from *candida methylica* does not contain disulphide bridges, but does possess two cysteine residues buried in seperate hydrophobic pockets. Cysteine residues are particulary susceptible to atmospheric oxidation at elevated temperatures. One approach to increase thermostability is to replace both cysteine resides by valine, C23V and C262V. The second approach will be to stabilize via engineering disulphide bridges into the protein: I239C (to partner C262) and A153C (to partner C23 in the other subunit). If a disulphide bridge can be engineered into the native fold without introducing an enthalpic cost (strain), such a bridge would stabilise the native state by decreasing the entropy of the protein's unfolded state. Homology modelling has been used to detect possible mutations by modelling of FDH from *C. methylica*, using the Insight II molecular modeling program based on the X-ray crystal structure of the FDHs from *Pseudomonas sp*. 101 which has a about 50 % similarity and *Candida boidinii* which has > 90 % similarity in their amino acid sequences.

In this study, mutations have been introduced into *cm*FDH gene in 6xHis-tag pQE-2 expression vector by using Invitrogen Gene Tailor™ site-directed mutagenesis system. 6xHis-tag provides an eased purification with simple affinity chromatography step including Ni-NTA as resin.

Six mutant FDH strains were produced with C23V, C262V, C23V+C262V, A153C, I239C, A153C+I239C mutations. Kinetic and thermostability studies were performed for both wild type and mutant FDHs. Unable to show activity in disulfide mutants, tempscan experiments were performed with CD spectroscopy.

Experiments has shown that alteration of cysteines were unable to increase thermostability while activity was dramatically decreased in C262V introduced mutants. Disulfide mutants have lost their activity, showing formed disulfide bridges have disturbed conformation of active site inhibiting activity. A153C mutant, wşch forms disulfide bridges between subunits, was compared to wt FDH with CD specroscopy. Despite of lowered melting temperature, increased reversibility was detected, indicating that disulfide bridges can be used to enhance reversibility of proteins. Half life detection of proteins were suggested for further studies.

SİSTEİN / SİSTİN MÜHENDİSLİĞİNİN CANDIDA METHYLICA FORMAT DEHİDROGENAZININ TERMOSTABİLİTESİNE ETKİSİ

ÖZET

NAD(P)H rejenerasyonunda sıkça kullanılan enzimlerden biri de Format dehidrogenaz'dır (FDH). Ayırıcı avantajlarının yanı sıra termostabilitesinin sınırlı olması bir sorun olarak karşımıza çıkar. Termostabiliteyi arttırmak için uygulanacak değişik yaklaşımlar mevcuttur. Bu çalışmada protein mühendisliği tekniklerini kullanarak FDH'in termostabilitesini arttırmayı hedeflenmiştir. *Candida methylica*'da bulunan doğal FDH'in bünyesinde sulfur köprüleri tespit edilmemiş olup, bunun yerine hidrofoboik ceplere gömülü iki sistein tespit edilmiştir. Yüksek sıcaklıklarda atmosferik oksidasyona açık olduğu belirlenmiş sisteinlerin C23V ve C262V mutasyonlarıyla valinler ile değiştirilmesi, ayrıca (C23 ile eşleşecek) A153C ve (C262 ile eşleşecek) I239C mutasyonları yoluyla karşılıklı sulfur köprülerinin oluşturulması hedeflenmiştir. Entalpi açısından herhangi bir bedel ödemeksizin gerçekleştirilecek sulfur köprüleri, katlanmamış halin enropisini düşürerek, katlanmış yapıyı sabitleştirebilir. Bu amaçla *Pseudomonas sp*. 101 FDH'inin tespit edilmiş X-ray kristal yapısı kullanılarak *C. methylica* FDH'e ait homoloji modellemesi Insight II programı yardımıyla gerçekleştirilmiş olup mümkün olan mutasyonlar tespit edilmiştir.

Bu çalışmada, 6xHis.tag pQE-2 ekspresyon vektörüne yerleştirilmiş olan doğal *cm*FDH genini üzerinde, Invitrogen Gene Tailor^{TM} kullanılarak mutasyonlar gerçekleştirimiştir. 6xHis-tag Ni-NTA kulanılan affinite kromatografisi ile kolaylaştırılmış saflaştırma gerçekleştirilmesinde yardımcı olmaktadır.

C23V, C262V, C23V+C262V, A153C, I239C, A153C+I239C mutasyonlarına sahip altı mutant FDH üretildi. Hem mutant hem de doğal FDH için kinetik ve termostabilite çalışmaları yapılıp, aktivite görülmeyen sulfur köprülü mutantlar CD spektroskopi ile sıcaklık taramasından geçirildi.

Sisteinlerin değiştirilmesi ile termostabilitede özelikle de C262V mutantlarında düşüş gözlenmiş olup sulfur köprülerinin oluşturulması hedeflenen mutantlarda tamamen aktivite kaybı tespit edilmiştir. Bu durum sulfur köprülerinin oluşumu sırasında aktif bölgenin yapısının etkilenmiş olduğunu göstermektedir. Subunitler arası köprü oluşturan A153C mutantı doğal FDH ile CD spektroskopi ile karşılaştırılmış ve doğal FDH'den daha yüksek oranda geri katlanma yeteneği tespit edilmiştir. Bu göstermektedir ki sulfur köprüleri geri dönüşümün arttırılmasında etkili olmuştur. Bulgular ileriki yarı ömür çalışmalarıyla desteklenebilir.

1. INTRODUCTION

1.1. The Yeast *Candida methylica*

1.1.1. General information about *Candida methylica*

Candida methylica is a common type of methylotrophic yeast. Its class is Saccharomycetes and belongs to family of Saccharomyetaceae. Sharing the same genus with *Candida boidinii*, most of their DNA sequences are alike.

1.1.2. *Candida methylica* Methanol utilization pathway

Candida methylica has a metabolic pathway that enables them to use methanol as a sole carbon source like other methylotrophic yeast organisms (*Hansenula polymorpha* for maximum yield). Induction of methanol triggers production of a series of enzymes as shown in Fig 1.1.

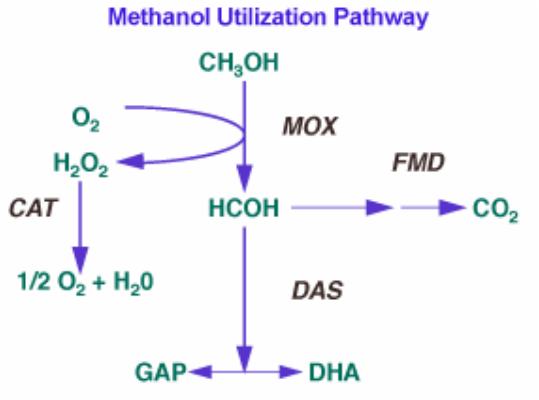

Figure 1.1: Methanol utilization pathway. Enzymes of pathway are shown in italics, are catalase (CAT), methanol oxidase (MOX), formate dehydrogenase (FMD), and dihydroxyacetone synthase (DAS). ("*Hansenula polymorpha* for Maximum Yield")

The last step of this pathway is reaction that consumes formate molecule (CH_2O_2 or methanoate) leaving CO_2. This last step is required for regeneration of NADH and performed by enzyme FDH (FMD, formate dehydrogenase or EC. 1.2.1.2).

1.2. NAD^+-dependent formate dehydrogenase (FDH)

Formate oxidation has been detected to be catalyzed with three FDHs found in living organisms. These are NAD.dependent FHD (E1.2.1.2.), NADP dependent FDH (1.2.1.43.) and cytochrome depended FDH (E.1.2.2.1.). Their coenzymes effect their selection for industrial usage.

NAD depended FDH is found in all methylotropic microorganisms. Since FDH is required for energy generation, it is abundant (up to %15 of total soluble protein) in cell. FDH gene is found in bacteria, yeast and plants but it is not yet detected in higher eukaryotes but a few trace like sequences.

1.2.1. Catalytic property of FDH

Most of FDH types catalyses an ordered Bi-Bi reaction, starting with substrate NAD^+. This is shown that Michaelis-Menten kinetic fits majority of them best. In recent studies, in activity tests applied at 30 ^oC pH 7.0-7.5, it is shown that FDHs of variety of organisms has similar activities ranging from 11.6 to 4.0 unit/mg. Their Km for formate also stays between 3 and 10 mM. For NAD^+ it is between 35 and 90 µM. These FDH activities changes a little in pH range between 5.5 and 11 (Tishkov V. I. and Popov V. O., 2004).

Although a FDH can catalyze in different pH values, its vulnerable against thermal changes above 50 ^oC. Most of FDHs lost half of their activity after 20 min. incubation between 50 and 60 ^oC. For example; *cb*FDH manages to keep most of its activity up to 45 ^oC. Thermal stability of FDH in low temperatures is so high that inactivation of FDH is almost 0 at 30 ^oC. As a result, increasing thermal stability of FDH is widely studied in order to apply FDH under harsh conditions so it can be more useful in industrial applications. In these studies, produced recombinant FDHs had slightly different activities then of native FDHs (Slusarcyzk H. et al., 2000).

As CO2 is a stable molecule it can be removed from system easily without engendering inhibition but other product, NADH has an inhibition effect on FDH. Also fast removal of CO_2 results irreversibility of the reaction. To overcome the problem, recombinant *cb*FDHs are produced that has slightly

higher K_i for NADH (*cb*FDH Ki = 17 ± 1.2, rec *cb*FDH Ki = 21 ± 1.8) (Slusarcyzk H et al., 2000).

Another inhibitor is thioformate which is a competitive inhibitor for formate. It was reported that Ki (for thioformate) is 80 µl for *C. boidinii* .Other inhibitors of FDH was reported as N_3^-, CN^-, Hg^{2+}, DTNP, PCMB, NO_3^-, Cu^{2+}, Ag^+. (Tishkov V. I. and Popov V. O., 2004) FDH is detected to be catalytically active in wide range of pH values (Mesentsev et al., 1997).

1.2.2. Structural properties of FDH

All known FDHs are homodimers and each subunit is formed of two domains. Two domains are identified as N terminus and C terminus domains. Although longer N terminus has a significant role in bacterial FDH's activity, no extra aminoacid sequence was reported for yeast and fungi FDHs. N terminus of bacterial enzymes are longer and increases thermostability with proline residues included. In all FDHs most of residues are related with stability of enzyme while only ten of them are catalytically important; Pro77, Phe78, Ile102, Asn118, Ala/Gly171, Gly173, Gly176, Arg267, Gln287 and His310 (for *cm*FDH and *cb*FDH). Altering these residues changing ligand from NAD^+ to $NADH^+$ was also succeeded (Tishkov V. I. and Popov V. O., 2004).

FDH is mentioned as very conventional enzyme. Its absolute homology is higher than 80-85 % between members of same group and 50-55 % between members of different groups. At the moment; total sequence of 36 enzymes and partial sequence of 25 enzymes are known (Tishkov V. I. and Popov V. O., 2004). 60 amino acid residues among known sequences are found to be conserved. For *candida* originated FDHs, approximately around 46000 Da, (*cm*FDH, *cb*FDH) is formed by 364 amino acids. When compared *cb*FDH and *cm*FDH has only 9 amino acids difference. But *ps*FDH has extra 47 amino acids in addition to many amino acid differences.

X.Ray studies have been performed for FDH from *Pseudomonas sp. 101* and *Mycobacterium vaccae* (Lamzin et al., 1994). As a stable and model protein *ps*FDH has been used for X-ray studies in different states and different ligands (Tishkov V. I. and Popov V. O., 2004). Unfortunately low stability of FDH from various organism failed crystallization attempts. Protein engineering

studies have been performed in order to obtain stable editions of FDHs from *Clostridium botulinum* for following crystallization studies (Schirwitz et al., 2005).

Homology modeling studies have been successfully achieved for FDHs from various organisms such as *C. boiidinii* (Slusarczyk et al., 2000), *Candida methylica* (Karaguler et al., 2004) and *Saccharomyces cerevisiae* (Serov et al., 2002).

Directed evolution studies have been successfully applied to *cb*FDH to improve thermal stability (Slusarczyk et al., 2003), although random mutagenesis has been successful to improve FDH, its noted that this approach takes time to obtain results (V.I. Tishkov, V.O. Popov, 2006).

1.2.3. Industrial usage of FDH

According to prescriptions of the Food and Drug Administration of the United States of America, optical purity of all chiral compounds used in drugs has to be higher then 99%. As requirement of producing these compounds in pharmaceutical, agrochemical industry, using enzymatic reactions are preferred because enantiosensitivity of non-enzymatic reactions can not be controlled. Using enzymes in industry enabled the option of producing pure chiral compounds.

One of these enzyme group is alcohol dehydrogenases. But they need energy provided by cofactor NADH or NADPH. They leave enzyme as NAD^+ and $NADP^+$. So they have to be provided in reduced form but this is expensive. Solution was achieved by regeneration of these cofactors using enzymes at the same time industrial reaction occurs. These enzymes should be using substrates that are cheap and easily found, should be active between a wide temperature and pH range, products and substrates shouldn't inhibit other reactions.

Several dehydrogenases have been characterized to be available as NADH regenerator such as Phosphite Dehydrogenase (Vrtis J.M. et al., 2002), Glutamate Dehydrogenase (Yamamoto H. et al., 2004) but by far mostly used enzyme as simultaneous regeneration of NADH in multi enzyme systems is Formate Dehydrogenase. With formate as cheap substrate, CO_2 as easily

removed product and irreversibility of the reaction FDH has clear advantage against other dehydrogenases (Donk W. A., and Zhaoz H., 2003).

The reaction initiated with alcohol dehydrogenase is kept continuous consuming cheap formate supplement while FDH provides NADH regeneration and producing CO_2 while reducing NAD^+ (Fig 2.2). As CO_2 product is gaseous, it is easily removed from system without causing any inhibition within system (Wichmann R. and Wandrey C., 1981).

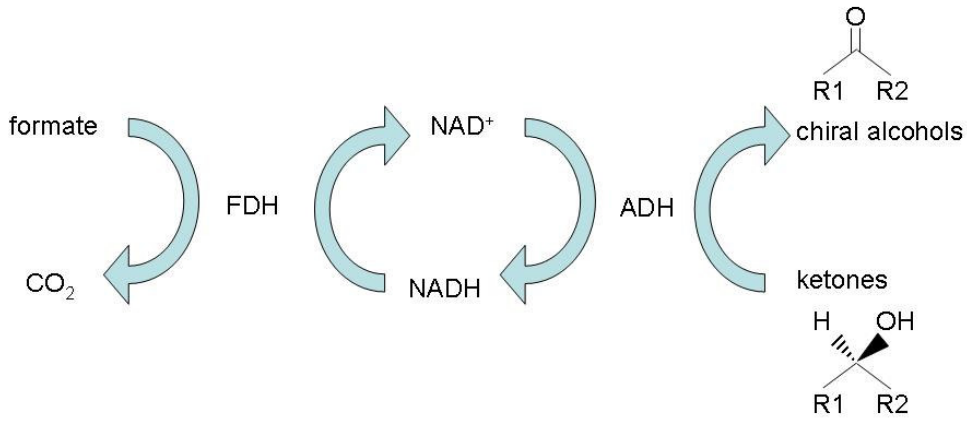

Figure 1.2: Continuous enzymatic synthesis and regeneration of NADH. Enzymes involved are alcohol dehydrogenase (ADH) and formate dehydrogenase (FDH).

1.2.4. Improving Industrial Formate Dehydrogenase

Such industrial proteins, like FDH are still required to be developed in different aspects to be more suitable for their applications. With wide range of improvements have been achieved or waiting to be achieved in properties such as purification, stabilization etc.

1.2.4.1. Protein purification

Proteins are most significant product of biotechnology and purity of them is very important in industry. As proteins mostly used in food and drug industry a contaminant can result production of harmful product and it should be removed immediately. As a result minimum purity was set by the Food and Drug Administration by % 99 (Rosen T.C. and Dauβmann T., 2003).

While protein was being purified its biological activity must be maintained, but most purification methods decrease its biological activity because of long purification steps.

Conventional protein purification methods can be categorized according to their separation methods. Ultra filtration, Size Exclusion chromatography and such methods uses the difference of proteins. Charge difference of proteins can also be used for purification of proteins. Such methods using charge differences are ion exchange chromatography or solvent precipitation. Hydrophobicity is also being used for purification principle in such as salting in / salting out method.

Common disadvantage of these methods is that different proteins may show similar characteristics in size, charge or hydrophobicity. On the other hand a new method has been developed. Using a unique ligand of certain protein has been proposed as chromatography method. Affinity chromatography method relies on using an immobilized ligand of protein. This ligand should be specifically recognized by interested protein so only desired product will be immobilized binding to ligand. While desired protein stays immobilized proteins and other molecules that doesn't recognize ligand is discarded. Usually this ligation is carried out in a column. After discarding undesired molecules ligand can be mobilized and moved out of column with desired protein. By combination of this method with recombinant DNA technology, a very efficient technique has been developed.

His tag is one of methods designed for easy purification of polypeptides, as long as desired protein has 6 residues of histidine tag at one terminus. This system, or so called 6xHis tag protein purification system**,** relies on selectivity and affinity of nickel-nitrilotriacetic acid (Ni-NTA). 6 histidine residues expressed at the N terminus of polypeptide is selectively recognized and bound by Ni-NTA at imidazole deficient condition. After ligation of polypeptide and immobilization of Ni-NTA, undesired molecules are removed. One of features of 6xHis tag protein purification system is ligation can occur easily because small molecule size of Ni-NTA ignores conformation problems so protein doesn't need to be denaturized. Also Factor Xa Protease can easily remove his tag after purification (Qiagen, 2003).

1.2.4.2. Protein Engineering

Protein engineering is a multidisciplinary field that uses biological, computational, chemical and physical techniques such as recombinant DNA technology or X-ray chromatography, to achieve alteration or addition new functions to proteins.

There are two methods in protein engineering. In rational design, desired modifications are applied to amino acid sequences at known positions of the protein. Site directed mutagenesis is a technique to change the amino-acid sequences of proteins by making mutations on the gene of the protein. Usually computational modeling is used to define the correct mutation. First of all, plasmid DNA or chromosomal DNA containing the gene of the desired protein is extracted from the micro-organism and multiplied by polymerase chain reaction. After the isolation of the true gene is conformed by electrophoresis and sequencing, another polymerase chain reaction is carried out with primers containing desired mutation. The primers are 30-40 bases long which are usually only 3 bases (1 codon) different than its original target gene. Thus, the primer hybridizes usually without any problem and taq polymerase multiplies the target gene with the mutation. The mutation is confirmed by electrophoresis and sequencing. Then, the mutated gene is transferred to a vector. To do that, the edges of mutated gene and the vector are cleaved by restriction enzymes to form sticky ends. Then, the gene is ligated to the vector by ligases. Finally, the vector carrying mutated gene is transformed to the target microorganism to produce engineered protein (Shaw W.V., 1987).

In order to make site directed mutagenesis, gene sequence of the protein must be known. If there is little information about the sequence or function of the desired protein, directed evolution, the second method of protein engineering, can be applied to modify the properties of the protein. This method mimics natural selection through making random mutations and growing the mutated microorganisms in selective media. The selection criteria are defined according to wanted properties. For example, if the aim is to increase the heat resistance of formate dehydrogenase which is required for microorganisms to obtain energy from formate molecule, the microorganism is cultured in a

media with low glycose concentration or in a glycose deficient media at slightly higher temperature than normal conditions. In order to achieve directed evolution, the desired property for a protein is obtained step by step. In the other words, It is almost impossible to bring 3^{o}C higher heat resistance in a single step because the microorganism must survive and must have time in order to adapt the new environment and to produce the mutated protein (Sen S., Dasu V.V. and Mandal B., 2007).

According to the known data and purposes of the study, one of the approaches can be applied to engineer proteins.

1.2.4.3. Homology Modeling

In enzyme engineering with rational design, it is crucial to know the 3D alignment of the atoms in the enzymes folded structure. Two common methods to obtain 3D data are X-ray Crystallography or NMR; however, it is not always possible to get 3D info by these basic methods.

Nuclear magnetic resonance (NMR) technique based on analyses of protons found in different functional groups. The phenomenon of magnetic resonance results from the interaction of the magnetic moment of an atomic nucleus with an external magnetic field. The cause of this magnetic moment is the quantum mechanical angular momentum of all nuclei that has even number of protons and neutrons. NMR has the advantage of application of proteins in solutions so their structural data belonging to natural conditions are obtained. This technique has proven its usability until 35000 Da proteins, however, for any larger protein, like FDH, this method fails.

X-ray chromatography is based on reflection of a beam of X-rays from a crystal with evenly spaced planes, producing unique spotted patterns for every molecule. The essential step in X-ray crystallography is the diffraction of X-rays from a crystalline material. A crystal is a solid in which a particular arrangement of atoms (its unit cell) is repeated indefinitely along three principal directions known as the basis (or lattice) vectors. X-ray diffraction method gives valuable information about protein's 3D structure but it needs highly purified protein crystals in solid state but not in solvent (Shafranovskii I.I. and Belov N.V., 1962). Crystallization of proteins is essential and these

crystals must be identical, free of inclusions and no larger then 0.5 mm. As building such crystals requires structurally stable molecules, crystallization is mostly problematic step. As for FDH, X-ray crystallization has been failed for *cb*FDH.

In order to overcome this issue, alternative approaches to predict the 3D structure like ab inito protein modeling or homology modeling (comparative modeling) can be used.

This study build on the data derived from homology modeling of *cm*FDH using the crystal structure of *ps*FDH. In this method it is assumed that similar amino acid sequences, form similar 3D structures in different proteins. Hence the enzymes with the same functions in different organism are generally produced by diverging and converging evolution, it is common to use data of the same enzyme from an evolutionarily close organisms (Zhang Y and Skolnick J, 2005).

1.2.4.4. Cysteine Cystine Engineering

Obtaining 3D structure itself is not enough for determining correct amino acids to alter to obtain thermostatically stable proteins. As random selection is a part of directed evolution, in rational design, selection of mutations requires certain approaches.

Comparative analyses of thermophilic proteins with their mezophilic and moderately thermophilic homologues are performed. Mutations are selected in order to make mezophilic proteins to get more similar to thermophilic homologues in 3D structure (Szilagyí A., Zavo´dszky P., 2000). This method requires detailed structural information of the protein and its homologues as more homologue data leads to more effective selection of mutations, although, FDH homologues' structural data are mostly undetermined.

Also using hydrophobization of α-helices of protein has been applied for thermostability enhancement. The principle of hydrophobization of α-helices is to stabilize the protein by enhancing electrostatic interactions between parallel and anti-parallel α-helices and increase rigidity of these motifs. Previous studies has been carried out for FDH based on this approach and resulted with positive stabilization effects (Rojkova A.M. et al., 1999).

Formation of hydrogen bonds and salt bridges are also a common method for enhancing the stability of proteins due to increased enthalpy stored within interactions. Two atoms would form a salt bridge on a protein if they have opposite partial charges within a distance of 3.5 Å. Where Asp or Glu side-chain carbonyl oxygen atoms is found to be within range from the nitrogen atoms in Arg, Lys and His side chains, salt bridges are detected to be formed and used in thermostability enhancement applications (Kumar S., Tsai C.J. and Nussinov R., 2000).

Decreasing surface functional groups which are exposed into environment is also another method for increasing stability. Proteins also start degradation through deformation on surface motifs. Amino acids with functional groups open to reactions on surface of proteins lead to disruption of the structure in time and stimulate of unfolding. Removal of functional surface groups has also been used as a mutation selection method for enhancing thermostability (Tsai C.J. and Nussinov R., 1997).

Cysteins are noted to show tendency to be oxidized or reduced when exposed and in time leads to instability of the enzyme. For example free SH groups of Human resistin has been detected to precipitate by forming S-S bonds between individual proteins (Aruna B et al., 2003). In order to overcome this problem Cysteins can either be removed or its functional SH groups can be occupied with disulfide bridges.

The disulfide bridges are the S-S bonds formed by the oxidation SH groups of two cysteine residues spatially located close approximation on the folded state of the protein (1.1).

$$2\ \text{-}CH_2SH + \tfrac{1}{2}O_2 \leftrightarrow \text{-}CH2\text{-}S\text{-}S\text{-}CH_2\text{-} + H_2O \qquad (1.1)$$

The sulfide bonds are mostly found in extracellular enzymes, which are exposed to various stresses rather then the physiological conditions, as they need to be more stable then their intracellular encounters. These strong bonds increase the overall stability of the enzyme compared to weak interactions that form the 3-D structure. Additional energy gained by formation of a disulfide bridge is generally equal or higher to the free energy of the folded state.

1..3. Enzyme Kinetics

Several Enzyme methods have been proposed up to date, however only a few of them has been preferred for their simplicity and accuracy.

Michaelis-Menten kinetics is a common method to describe the kinetics of many enzymes. In order to determine the maximum rate of an enzyme catalyzed reaction, a series of experiments or in other terms, activity assay, is carried out where the substrate concentration, [S], is increased until a constant initial rate of product formation is achieved. This is the maximum velocity (V_{max}) of the enzyme under the conditions of the experiment. In this state, enzyme active sites are saturated with substrate.

The reaction rate (V) is the number of reactions per second catalyzed per mole of the enzyme. The reaction rate increases with increasing substrate concentration [S], asymptotically approaching the maximum rate V_{max}. A more appropriate measure to characterize an enzyme is the substrate concentration at which the reaction rate reaches half of its maximum value ($V_{max}/2$). This concentration can be shown to be equal to the Michaelis constant (K_m) (Michaelis L. and Menten M. L., 1913). This K_m value gives the information about affinity of substrate for the enzyme. Higher K_m defines lower affinity of substrate for enzyme which means lower tendency of the substrate for undergoing the reaction. V_{max}/K_m values are used as a comparison data for efficiency of enzymes (Ryu K. and Dordick J.S., 1992).

2. EXPERIMENTAL PART

2.1. Materials

NAD^+ Free acid	: Roche
Formate	: Roche
IPTG	: EuroClone S.p.A.
Ampicillin	: Sigma-Aldric
Primers	: Operon
Bradford	: Sigma
Site directed mutagenesis kit	: Invitrogen GeneTailor
Expression and purification kit	: Qiagen QIAexpressionist
Crystalline bovine serum albumin	: Sigma-Aldric
Lysosyme	: Roche

2.2. Instruments

Balances	: Precisa XB 220°
	: Precisa BJ 610C

Centrifuges	: Beckman Coulter: Microfuge 18
	: Beckman Coulter, Allegro 25R
	: Beckman Coulter, Avanti J-30 I
UV Fluorescence Spectroscopy	: Shimadzu RF-530
CD Spectrophotometer	: Jasco J-810 CD Spectrophotometer
pH Meter	: inoLab pH720
Thermomixer	: Eppendorf Thermomixer comfort 1,5 ml
Magnetic stirrer	: VELP scientifica
Microplate Reader	: Biorad 3550-UV

2.3. Homology modeling

All mutations were selected based on homology modeling from pseudomonas sp. 101 Formate dehydrogenase (*ps*FDH) at Bristol University (Fig 2.1). During homology modeling studies Insight II software was used in previous studies.

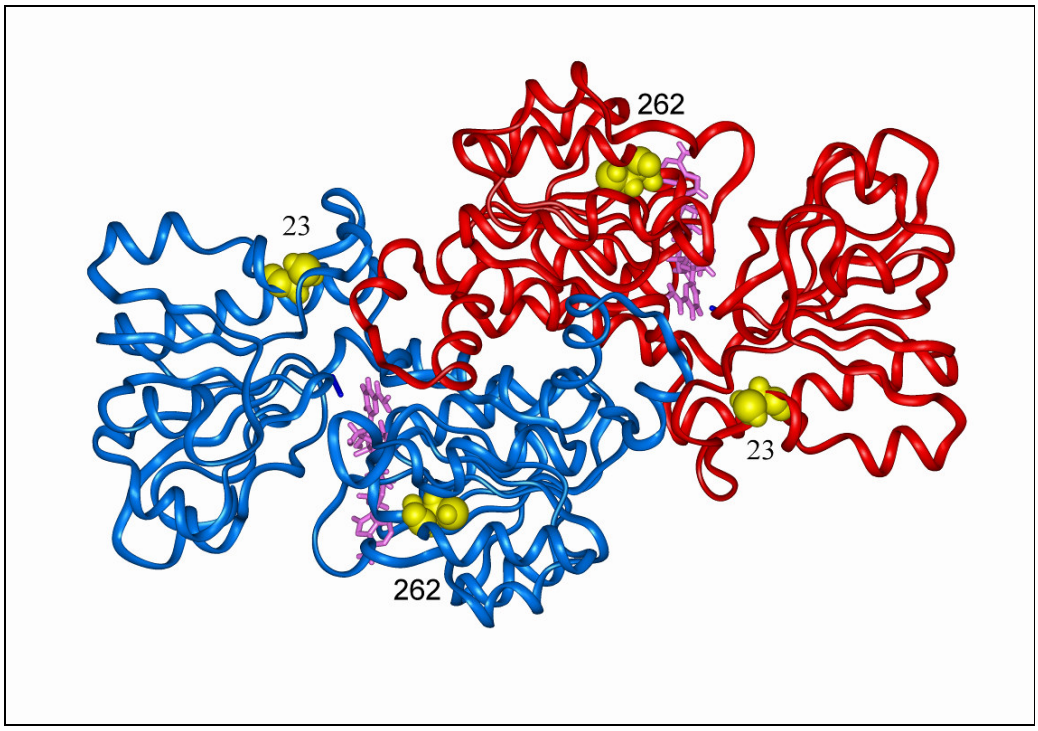

Figure 2.1.: 3 dimensional modeling of cmFDH with NAD+. Yellow spacefillings represent sidechains of cysteine residues of FDH at 23rd and 262nd positions.

Using data from homology modeling and molecular dynamics two possible approaches were suggested in order to increase thermostability of cmFDH.

2.4. Site Directed Mutagenesis

Detected mutants are created using Invitrogen Gene Tailor™ site directed mutagenesis kit using WT-FHD gene previously inserted into PQE2 vector. For mutagenesis reactions primers designed as shown in Table 2.1.

Table 2.1.: Primers used in mutagenesis reactions for each mutation.

A153C Forward
5- ATTAACCAGGATTGGGAGGTTTGTGCTATCGCTAA-3
A153C Reverse
5- AACCTCCCAATCGTGGTTAATAATTTTGTTCATGTGCTGG-3
C23V Forward
5-GCAGCTGCTGATGAAGAAAAATTATATGGTGTTACTG-3
C23V Reverse
5-ACCATATAATTTTTCTTCATCAGCAGCGTGCTTACCAGCATC-3
C262V Forward
5-TACCGCAAGAGGTGCTATTGTTGTTGCTGA-3
C262V Reverse
5-AATAGCACCTCTTGCGGTATTGACTAACC-3
I239C Forward
5-ACGCAGGTACAAAAGGTTTATGTAATAAGG-3
I239C Reverse
5-TAAACCTTTGTACCTGCGTGTAATGGAG-3

WT-FDH including PQE2 vector was first methylated with DNA methylase provided with Invitrogen Gene Tailor^{TM}. Methylated DNA was introduced and incubated for overnight before mutagenesis reaction performed in PCR with designed primers for each mutation. PCR products are run on agarose gel for initial control. Approved PCR samples are used to initiate transformation with competent DH5α *E. coli* cells. Heatshock transformation was performed by introducing 2 µl plasmid per 200 µl cell, incubated on ice for half hour and 60 seconds at 42 C, on ice for five minutes. 200 µl SOC medium added and incubated at 37 ^{o}C for 45 minutes and spread on plate for colony selection after overnight growth. Selected colonies are grown and plasmids isolated and

run on PCR with sequencing primers designed for PQE2 vector. After sequencing, cell lines of confirmed cell lines are selected and grown for expression.

2.5. Protein Expression

Innoculum development was carried on in two steps. Selected cell lines were grown in 10 ml LB with 1 mM amphacillin overnight and transferred into 100 ml LB with same concentration of amphacillin overnight at 37 C. Then 50 µl of culture is transferred and growth in 500 µl LB with amphacillin. As OD 600 was reached, cells were introduced IPTG so final concentration was 1 mM. After 5 hours of incubation cells are harvested by centrifugation.

2.6. Protein Purification

Purification was performed according to Qiagen QIAexpressionist protocol. Harvested cells are resuspended in 100 µl lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole) per gram cell harvest, lysozyme added and incubated on ice for 45 min. After gently vortexing cells are centrifuged at 15000 rpm for 15 min. Supernatant was collected and 100 µl of it was labeled as sample (CL). 10 µl of Ni-NTA resin per 50 µg protein to be isolated was added onto collected supernatant and mixed gently on ice for 30 min. Then sample was centrifuged at 13000 rpm for 5 min. Supernatant was collected and 100 µl was seperated as sample (FT). Resin as pellet was washed with 1 ml wash buffer (50 mM NaH2PO4, 300 mM NaCl) and centrifuged at 13000 rpm for 5 min. supernatant was removed and labelled as W1. Additional washing step was repeated and supernatant was labelled as W2. Pellet was added 500 µl elution buffer (50 mM NaH2PO4, 300 mM NaCl, 500 mM imidazole) and centrifuged at 13000 for 1 min. Supernatant was labeled as E1. Elution step was repeated two more times and supernatants were labeled as E2 and E3. Sample concentrations were measured with Bradford assay. Purity of samples was confirmed through SDS Page electrophoresis (Fig 3.4).

2.7. Kinetic Studies

Kinetic measurements of wild type and mutant FDHs were carried out at 25 °C in a reaction mixture containing 20 mM Tris Buffer at pH 8, 1 mM NAD^+, 0–40 mM formate and 0.4 μM enzyme. Initial rates were determined at 340 nm by measuring the NAD consumption and fitted using the Grafit 5 Kinetics Software.

2.8. Melting Temperature Studies

The proteins (wild type and mutant FDHs) were aliquot and incubated at different temperature (20- 60°C, 2°C intervals) for 20 min and as triplicate. Activity measurements were performed at 25 °C in a reaction mixture containing 20 mM Tris Buffer at pH 8, 1 mM NAD^+, 2 mM formate and 0.4 μM enzyme.

2.9. Tempscan Studies

Three disulfide mutants (A153C, I239C, A153C + I239C) and wild type *cm*FDH were applied to CD spectroscopy experiments in Umea University, Biochemistry department. Protein buffers were changed from Tris buffer to Wash buffer by dialysis overnight in order to enhance the signal in CD spectrophotometer. CD measurements were performed repeatedly with an increase in temperature (6 °C-88 °C, 6 °C intervals) and excisions were measured from 260 nm down to 210 nm wavelengths as triplicate for every temperature.

2.10. Post-Tempscan Measurements

After every tempscan for each sample during tempscan studies, additional triplicate measurements were performed after samples were cooled down to 20 °C and incubated for 20 minutes. Collected data were transferred to PC for plotting.

3. RESULTS AND DISCUSSION

3.1. Homology Modeling

Four possible mutations were detected to enhance thermostability of *cm*FDH using cysteine/cystine engineering; C23V, C262V, A153C and I239C. Single mutants with each of these mutations and double mutants of C23V+C262V and A153C+I239C are designed. Each double mutant was modeled in 3 dimensions for their structure (Fig 3.1 and 3.2).

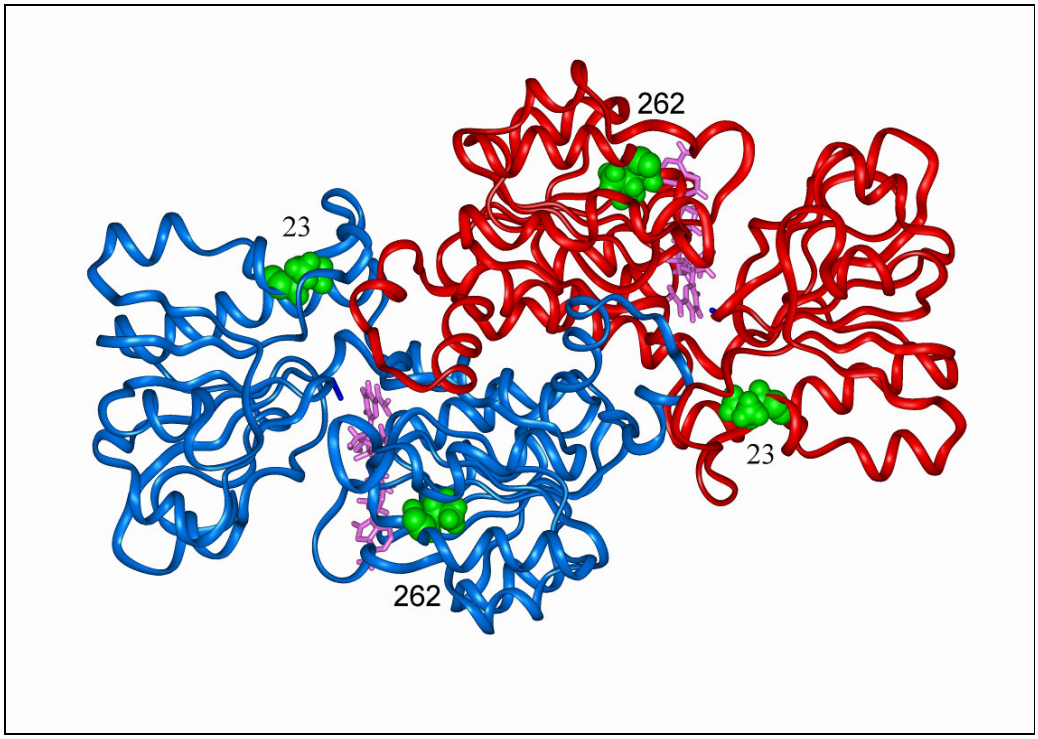

Figure 3.1.: 3 dimentional modeling of mutant *cm*FDH with NAD+. Green spaces represent modified mutations C23V and C262V in both subunits and their spacefill modelings of sidechains.

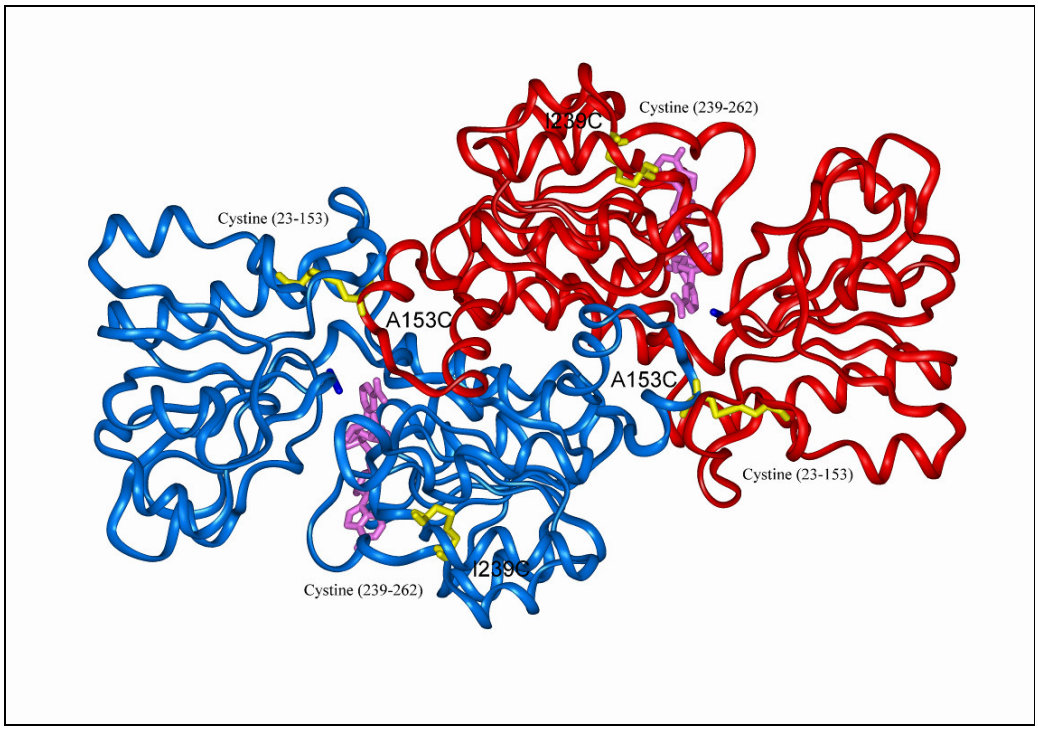

Figure 3.2.: 3 dimentional modeling of mutant *cm*FDH with NAD+. Yellow rods represent modified mutations A153C and I239C in both subunits and their disulfide bridges with C23 (pairing C153) and C262 (pairing C239)

C23 was detected to be located on N terminus of each subunit. Due to its conformation, cysteine side chain faces N terminus domain of partner subunit (Fig 3.1).

3.2. Site Directed Mutagenesis

Six mutant *FDH* genes were successfully built and confirmed through sequencing and screened with agarose electrophoresis (Figure 3.3). Double mutants were built by introducing C262V mutation to C23V mutant gene and I239C mutation to A153C mutant gene.

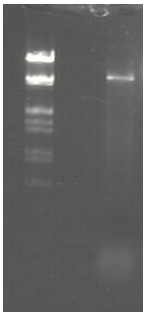

Figure 3.3. Mutant FDH gene inserted PET vector.

3.3. Protein Purification

Samples were stored in various steps of purification. These samples were run on SDS Page gel (Fig 3.4). FDH was observed at marker level around 40000 kDa. Lanes 2, 3 and 4 were loaded with elutions E1, E2 and E3. As seen on these lanes protein samples have been purified successfully. Additional elution steps can be repeated in order to obtain more protein unless protein concentration is not important. Lane 5 belongs to washing step W1. As seen various sizes of proteins are separated from resin including low amount of FDH. A second washing step shows no other washing step is required in lane 6 since no more protein can be detected on gel. Lane 7 belongs to cell lysate, CL, and shows induced FDH production with IPTG.

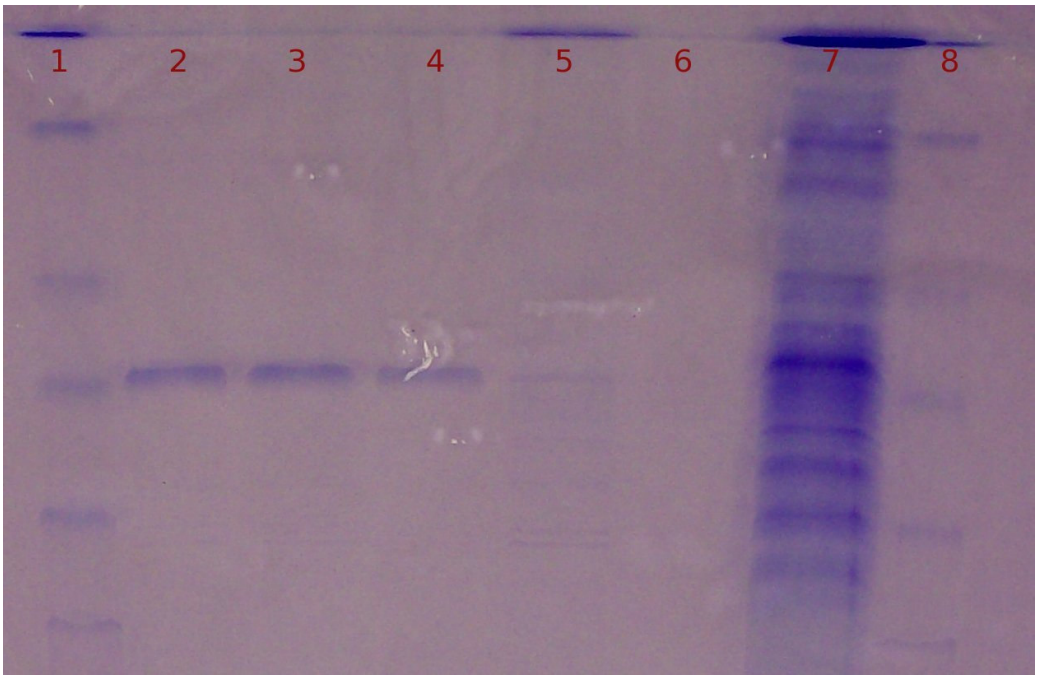

Figure 3.4.: SDS PAGE Gel electrophoresis of samples collected through protein purification. 1. Marker, 2. first elution sample E1, 3. Second elution sample E2, 4. third elution sample E3, 5. first washing sample, 6. Second washing sample, 7. Cellular protein content CL, 8. Marker

3.4. Kinetic Studies

After purification and concentration detection proteins were initially used for activity assays, detecting change in absorbance given by NAD+ consumption through reaction per minute (ΔA/min) (Table 3.1.). Previous data obtained from WT FDH was also included in table in order to compare. Data has been used to compute a curve fit using EZFit software for calculation of micheals menten variables.

Table 3.1.: Initial velocities of wt and mutant FDHs versus changing formate concentration. No K_m or V_{max} was available to calculate for A153C, I239C and A153C+I239C mutants due to no activity.

	Velocity (ΔA/min)						
Formate (mM)	**Native FDH**	**C23V**	**C262V**	**C23V+C 262V**	**A153C**	**I239C**	**A153C+ I239C**
2	0,0543	0,0378	0,024	0,0239			
4	0,0806	0,0601	0,031	0,03			
6	0,0945	0,0692	0,0342	0,0330			
8	0,1008	0,0855	0,038	0,037			
10	0, 1079	0,0914	0,04	0,0434			
15	0,1249	0,0912	0,0461	0,0476			
20	0,1351	0,1037	0,0489	0,0494			
25	0,1408	0,1149	0,0525	0,0540			
30	0,1432	0,1191	0,0571	0,0593			
35	0,1533	0,1217	0,0617	0,0603			
40	0,1512	0,1297	0,0652	0,0628			
Km	4,75	5,88	5,72	5,61	No activity	No activity	No activity
Vmax	0,167	0,14	0,068	0,068			

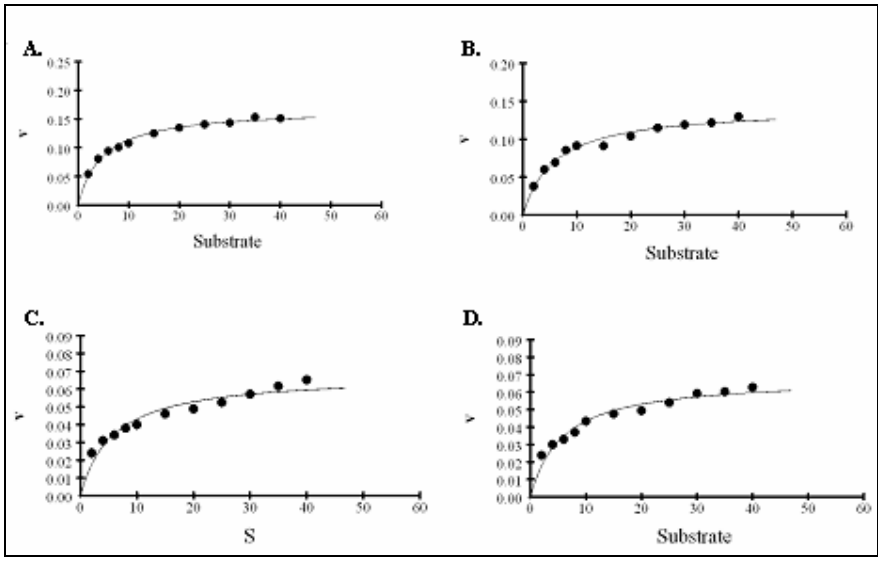

Figure 3.5.: Activities of FDH versus substrate concentration. A. Wild type *cm*FDH. B. C23V mutant. C. C262V mutant. D. C23V+C262V mutant.

It has been detected that the mutation C23V has slightly lowered the activity of the protein and increase in Km represents the slight decrease in affinity for formate (Fig 3.5). This decreased in V_{max} could be tolerable if thermostability was achieved. On the other hand, mutants introduced C262V mutation, show relatively low, even less then half of wildtype *cm*FDH. This shows how C262 configuration is essential for activity and conformation of active site. Also this shows C262 is positioned more close to active site.

Inactivity has been detected for disulfide mutants. As we suggested that C262 has relatively higher effect on conformation of active site, decrease in activity of mutants, introduced I239C mutation, was expected due to conformational disruption at C262 position and spontaneously active site by formation of disulfide bridge. On the other hand A153C mutant was expected to give some activity, however, no activity was observed. Most obvious effect of A153C mutation is decrease in flexibility different then C23V mutation, as disulfide bridge was intended to be formed. This shows importance of flexibility between subunits for activity. Although mutations were selected from positions that would have interfered with active site least, still indirect effect

of mutations had negative effect on activity possibly due to conformational disruption.

3.5. Melting Temperature Studies

In order to measure melting temperature of active proteins, proteins were incubated in different temperatures and measured for activity. (Table 3.2.) The data collected was used to plot and fit curves were used to detect temperatures where proteins has half of initial activity.

Table 3.2.: Activity of C23V, C262V, C23V+C262V mutants in 20 ^{o}C after incubation in various temperatures for 20 min.

tempreature	***C23V***	**C23V+C262V**	**C262V**
20	0,0398	0,0191	0.0160
30	0,0517	0,0207	0.0170
32	0,0421	0,0221	0.0165
34	0,0420	0,0200	0.0164
36	0,0435	0,0174	0.0150
38	0,0423	0,0175	0.0152
40	0,0430	0,0175	0.0143
42	0,0435	0,0117	0.0137
44	0,0447	0,0097	0.0108
46	0,0404	0,0021	0.0071
48	0,0376	0,0036	0.0037
50	0,0262	0,0020	0.0014
52	0,0237	0,0020	0.0011
54	0,0059	0,0011	0.0003
56	0,0035	0,0014	-0.0004
58	0,0021	0,0007	0.0006
60	0,0007	0,0003	-0.0007

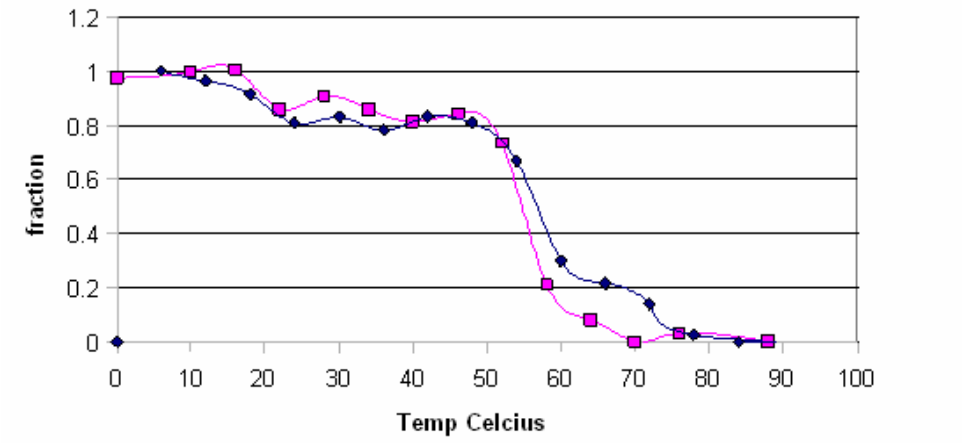

Figure 3.6.: Unfolding tempscan of FDHs using CD spectroscopy . Blue points represent WT FDH and purple points represent A153C mutant fraction with increasing temperature.

Data collected through tempscan was plotted (Figure 3.6.).

A slight low melting temperature was detected for A153C mutation comparing to wild type FDH. It has been shown that even a bridge formation may not increase thermostability. Decreased flexibility might have disrupted the favorable concats between subunits.

Unable to provide data for other two disulfide mutants, results couldn't be compared. For further investigation of S-S bridge on stability, experiments may be required to repeated with other secondary structure characterization methods like FTIR and etc.

3.6. Post-Tempscan Measurements

As temperature reached up to 80 ^{o}C during temperature scan, it was assumed that unfolding ratio of proteins in sample solution was hundred percent. Temperature was decreased down to 20 ^{o}C and ratio of refolded protein was measured. It has been detected that A153C mutant has higher refolding ratio then wild type FDH (Fig 3.7.).

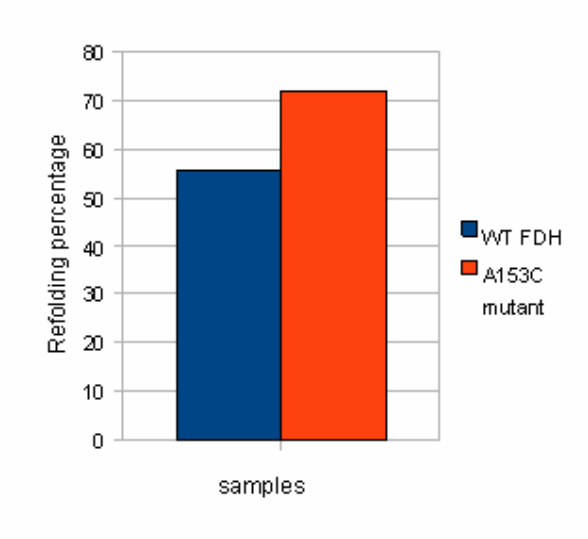

Figure 3.7. Percent of refolded protein during 20 min incubation at 20 ^{o}C following tempscan with CD spectroscopy.

Despite of low melting temperature of A153C mutant, high reversibility of A153C mutation points out that although disulfide bridge didn't increase thermostability due to decrease in local favorable contacts between subunits, overall reversibility of the FDH has been increased. The contact points of the subunits are not only around mutation and FDH stability is also favored by contacts through neighboring sites. Disulfide bridge between subunits may disrupt favorable contact formation around mutation area but favoring dimerization of subunits must have been induced by two disulfide bridges. These bridges quicken attachments of subunits and induce refolding favoring formation of overall intersubunitary interactions.

3.7 Discussion

We have shown the modifications of cysteine residues effect protein thermostability depending on the position and favored conformation. As C23V mutation has affected activity slightly, C262 position shows more dramatic effect in both thermostability and activity. Exchange of amino acids with their electrochemical equivalents may not be enough to enhance thermostability, especially for amino acids, positioned inside hydrophobic core.

A153C mutation has showed enhanced refolding ability, however, protein was inhibited. Although active and more reversible protein was failed to achieve it was shown that disulfide bridges between subunits can be used to induce refolding.

The effects of removal of SH groups might be observed in form of increase in reversibility or half life instead of melting temperature. Further studies should be carried on to detect possible effects on these.

4. CONCLUSION

Confirmed by literature, our study showed that as well as an increase in thermal stability, the contrary should be expected. Moreover, our results reveal possible side effects of cystein/cystine engineering experiments. Due to lack of information on 3 dimensional structure, homology modeling studies do not give adequate knowledge to base a rational design to increase stability. More conformational data is required for more precise results.

We have also seen that increasing reversibility may show decrease in thermostability, which indicates that inversely proportional relation between stability and reversibility might be observed in cystein/cysyine engineering studies.

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