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1. Introduction

Global warming and the resultant problems it could cause has made the scientific community and general public realize that there is a need to decrease dependency on oil for energy. It has also shown the need to increase the efficient use of energy in manufacturing and industrial processes. Green processes would be an alternative way of decreasing oil dependency and biotechnology is promoted as a way both to decrease oil dependency and as a source for renewable bio-based products. Biocatalysis (using enzymes or whole systems) can provide a valuable alternative to traditional chemical processes because it has many advantages such as; efficiency in enhancing the rate of chemical reactions and for their ability to discriminate between potential substrates. Therefore there is the possibility of using biological molecules to catalyse any reaction or modify any product of interest to industry. Biological catalysts from animal, plant and bacterial sources have evolved to perform most types of organic reactions, producing chirally pure and complex molecules with interesting biological properties. Biocatalysts have thus become important tools in medicine, the chemical industry, food processing and in agriculture. Industrial processes often require extreme conditions such as high pressure, temperature and extreme pH which require a large amount of energy to achieve and may produce unwanted toxic waste. Biological enzymes do not require such conditions and produce chirally pure products often without the disadvantages of unwanted toxic by-products. They offer a number of advantages over conventional chemical catalysts (Davies, 2003; Kirk et al., 2002; Perez, 2010; Tao, 2009; Wojtasiak, 2006): i) Most enzymes catalyze their reactions under mild conditions such as physiological temperature and pH (6-8) and so are therefore often compatible with one another. Compatible enzymes can be used together either in sequence or cooperatively to catalyze multistep reactions. ii) Enzymes are regioselective, and also stereospecific and this allows the production of exact chiral products from racemic mixtures. Enantiomerically pure compounds are specially demanded by the pharmaceutical, food and cosmetics industries. iii) They may be cheap and easy to use because many enzymes are commercially available. iv) They are regarded as environmentally friendly because catalysis is achieved without organic solvents or the heavy metal toxic waste.
Biotransformations can be carried out using pure enzymes in solution or inside intact organisms. It remains an unanswered question as to which method is better as each has advantages and disadvantages. Enzymatic transformation usually gives a single product so they can be specific for chosen reactions. However, they are expensive and many potentially useful enzymes need cofactors. These co-factors may also be expensive, therefore it would be useful to develop a co-factor recycling system in these cases. This has been achieved for oxidoreductases, but it is not always possible and with high development costs it may not be cost effective. Whole organisms do not have this disadvantage because enzyme co-factors and their recycling systems are already present in cells. However, growing and harvesting whole cells is very labor intensive and the end product is not always pure.

2. Protein engineering methods

Protein engineering, which is the design and construction of novel proteins, usually by manipulation of their genes, is a promising approach which can be used to create enzymes with the desired properties. Proteins are engineered with the goal of better understanding the molecular basis for their functions and also so they will be able to synthesize novel products in non-native environments. Success would greatly expand the possible applications of enzymes in industrial processes.

Protein engineering methods comprise three main strategies; rational design, directed evolution and a combination of both methods, semi rational design (site saturation mutagenesis) (Figure 1).

Fig. 1. Protein engineering methods
2.1 Rational design

Rational design in other words computational design of proteins requires the amino acid sequence, 3D structure and function knowledge of the protein of interest. This method provides controllable amino acid sequence changes (insertion, deletion or substitution). Controlled changes are important to determine the effect of individual residue changes on the protein structure, folding, stability or function.

Knowledge of three-dimensional structure is a key for understanding the biological function. Although understanding of 3D structure of proteins is crucial in terms of their function, only about 1 % of proteins (68,812 proteins with known structures have accumulated in the PDB database in the date of July 2011) for which the amino acid sequence is known, had their 3D structure determined because of the time consuming nature and difficulty of crystallographic experimental methods (Sanchez & Sail, 1997). As a result, the gap between the numbers of known sequences and structures continuously grows. In addition to enlarging databases, improvements in sequence comparison, fold recognition and protein modelling algorithms have supported the enhancement of protein structure prediction studies based on computer modelling methods to bridge this gap (Hillisch, 2004).

In the absence of 3D structure of the interested protein, homology modelling, which is used to predict the 3D structure of a target protein by using an experimentally (x-ray crystallography or NMR) determined protein structures as a template, is already the most promising and easiest technique among the computer based structure prediction methods (Sali & Blundel, 1993). Therefore the importance of homology or comparative modelling which can provide a useful 3D models for many proteins is steadily increasing (Suarez, 2009).

When the mutants obtained and characterized from both computational and random mutagenesis methods have been compared, it is often found that the best mutants obtained from both methods have the same residue changes (Binay et al., 2009). On the other hand strongly destabilized mutants obtained from the computational method can not be found by random mutagenesis. This explains the advantages of rational design in terms of either increasing stability or determination of individual residue effect on the protein stability, folding or function (Wunderlich et al., 2002).

The first step in rational design is the development of a molecular model by using an appropriate algorithm. This is followed by experimental construction and analysis of the properties of the designed protein. Besides the improvement of several enzyme properties like coenzyme and substrate specificity (Chul Lee et al., 2009), stability towards to oxidative stress (Slusarczyk et al., 2000), rational protein design has also been applied to improving the thermostability of several cases (Annaluru et al., 2006; Spadiut et al, 2009; Voutilainen et al., 2009; Wei et al., 2009).

Mechanisms for altering these properties include manipulation of the primary structure. Just a single point mutation may cause significant structural or functional changes in the protein. There are many rational strategies to change protein characteristics such as introducing disulfide bridges, optimization of electrostatic interactions, improved core packing, shorter and/or tighter surface loops etc. These changes are put in practice by site-directed mutagenesis. In this technique, mutations are created at computationally defined sites in the gene sequence via PCR using primers containing nucleic acids changes which correspond to the desired amino acid changes (Walker & Rapley, 2008).
2.2 Directed evolution

Unlike rational design, directed evolution (in vitro evolution or random mutagenesis) does not require any knowledge about sequence, structure or function of proteins. Directed evolution mimicks natural evolution in vitro by reducing the time scale of evolution from millions of years to months or weeks. This method has been used since 1980s to enhance or alter various enzyme functions. It has become a powerful technology through the work of Arnold and Stemmer in the 1990's which enhance the existing methods (Arnold, 2001; Stemmer, 1994a, 1994b). Today, directed evolution methods can be divided into two classes; (i) non-recombinative, random mutagenesis of genes (e.g. Sequence saturation mutagenesis (SeSaM), Error Prone PCR (epPCR)) and (ii) recombinative methods, recombination of gene fragments of homologous enzymes from different sources (e.g. DNA Shuffling, Family DNA Shuffling, Random Chimeragenesis on Transient Templates (RACHITT)) (Bornscheuer & Pohl, 2001; Williams et al., 2004).

Directed evolution requires two essential steps; one is the generation of random genetic libraries and the other one is screening and selection of variant enzymes that possess the desired characteristics, for example increased catalytic activity, enhanced selectivity or improved stability. Choice of the right strategy for both steps is very important to achieve the desired goal. In order to select a target protein from a large pool of mutant proteins, an efficient screening strategy, such as high-throughput solid phase digital imaging, phage display and other different screening techniques, is the most important requirement for the success of this method. The disadvantage of this method is the time-consuming process of screening and the selection of desired mutants and generally it requires robotic equipment to screen large libraries of enzyme variants (Turner, 2003). Screening of libraries on the order of $10^3-10^4$ variants seems sufficient for reliable selection (Tao & Cornish, 2002).

2.3 Semirational design-site saturation mutagenesis

Although either rational design or directed evolution gives effective results, to overcome the time consuming screening and selection process of directed evolution and the necessity of amino acid sequence and 3D information for rational design, a new approach would be useful. A combination of both strategies represented the new route to improve the properties and function of an enzyme (Bommarius, 2006).

Site saturation mutagenesis (SSM) method has some advantages when it is compared to other directed evolution methods. In directed evolution methods such as DNA shuffling (Stemmer, 1994) or error-prone PCR (Wong et al., 2004), random or targeted mutations in the whole sequence coding for the protein generates a large mutant library which is very time consuming to screen. With saturation mutagenesis, it is possible to create a library of mutants containing all possible combination of 22 different amino acids at one or more pre-determined target positions in a gene. Choice of the correct mutagenesis, positions that can be responsible for desired changes is determined by homology modelling which requires 3D information (Lehmann & Wyss, 2001).

Because of the rational approach of this method, it is possible to obtain more effective results by combining it with high throughut screening methods. Site saturation mutagenesis has been successfully used to improve several enzymatic properties as well as thermostability (Andreadeli et al., 2008; Reetz et al. 2006; Wu et al., 2009; Zheng, 2004).
3. *Candida methylica* NAD⁺-dependent formate dehydrogenase

A wide range of organisms use formate in a variety of metabolic pathways. From aerobic to anaerobic organisms formate dehydrogenase is the last enzyme in the metabolic pathway which catalyses the oxidation of formate to CO₂ and water. The use of formate and formate dehydrogenase (FDH) have been extensively studied and reviewed (Thiskov & Popov, 2004, 2006).

There are three families of FDH namely; complex non-Nicotinamide Adenine Dinucleotide (NAD⁺)-dependent FDH, complex, soluble NAD⁺-dependent type and simple, soluble NAD⁺-dependent FDH types. Two of them are complex and use heavy metals such as molybdenum, selenium, iron, etc. The third one which is the simplest and is called NAD⁺-dependent FDH because it only requires NAD⁺ as a coenzyme. It is also the slowest class of FDH with regard to catalytic rate. The third class is represented by proteins devoid of any prosthetic groups. The molecular properties of these FDHs from prokaryotes reveal that their FDHs belong to the same family as the NAD⁺-dependent FDHs from yeasts and higher plants. In other words, the molecular masses, affinities for formate and the substrate specificities of the enzymes from bacteria, yeast and plants all resemble one another. This has been confirmed in many cases, by comparison of gene-derived amino acid sequences (Popov & Lamzin, 1994).

In the last decades active sequencing of genomes resulted in the discovery of FDH genes in various organisms such as *Staphylococcus aureus*, *Mycobacterium avium* subsp. *paratuberculosis* str.k10, different strains of *Bordetella*, and *Legionella, Francisella tularensis subsp. tularensis* SCHU S4, *Histoplasma capsulatum*, *Cryptococcus neoformans* var. neoformans JEC21, *A. thaliana*, potato, rice, barley, cotton plant, English oak, *Mesembryanthemum crystallinum*, (S. cerevisiae, C. boidini, C. methylica, *Hansenula polymorpha*, and *Pichia pastoris*, *A. nidulans*, *N. crassa*, *G. zeae* PH-1, *M. grisea*, *M. graminicola*, *U. Maydis* (Figure 2). (The detailed information about organisms can be found in the web page of The National Center for Biotechnology Information). Among the large number of microorganisms that have formate dehydrogenase, attention has been mainly focused on the yeasts. In yeast, the ability to utilize methanol as the sole carbon source is limited to members of 4 genera, namely *Candida, Hansenula, Pichia* and *Torulopsis*. An investigation of FDHs led to the selection of *Candida* species NAD⁺-dependent FDH as the best candidate for the NADH regeneration system because it is stable and it has relatively good activity. However, while several FDH enzymes have been isolated, crystal structures of FDHs from the bacterium *Pseudomonas sp.*101 and yeast *Candida boidini* have been solved (Lamzin et al., 1992; Schirwitz et al., 2007).

NAD⁺-dependent FDH is a dimeric enzyme with two identical subunits each has an independent active site, containing no metal ions or prosthetic groups. They are unable to use one-electron carriers as oxidizers and are highly specific to both formate and NAD⁺. FDH catalyzes the oxidation of formate to carbon dioxide coupled with reduction of NAD⁺ to NADH (Thiskov & Popov, 2004, 2006):

\[
\text{HCOO}^- + \text{NAD}^+ \rightarrow \text{CO}_2 + \text{NADH}
\]
Fig. 2. Sequence alignment of *C. methylica* FDH with other FDHs from *hp* fdh (Hansenula Polymorpha), *nc* fdh (Neurospora Crassa), *st* fdh (Solanum Tuberosum), *an* fdh (Aspergillus Nidulans), and *ps* fdh (Pseudomonas sp. 101). Residues conserved in all FDHs, including *C. methylica* FDH, are in red and residues conserved in all FDHs but not in *C. methylica* FDH are in blue.
The FDH enzyme was first discovered in 1950 (Uversky, 2003) but it has attracted attention in recent years due to its practical application in the regeneration of NAD(P)H in the enzymatic processes of chiral synthesis. FDH is widely used for coenzyme regeneration with enzymes used for optically pure product synthesis in the pharmaceutical, food, cosmetic and agriculture industries (Patel, 2004; Jormakka et al., 2003). The FDH catalysed reaction is also a suitable model for investigating the general mechanism of hydride ion transfer because of direct transfer of hydride ion from the substrate onto the C4-atom of the nicotinamide moiety of NAD\(^+\) without stages of acid-base catalysis (Serov et al., 2002a).

NAD\(^+\)-dependent FDH from *Candida methylica* (\(cm\)FDH) was previously isolated by Allen & Hollbrook, 1995). Its N-terminal amino acid sequence was determined and it was cloned into pKK223-3 and overproduced in *Escherichia coli*. \(cm\)FDH in pKK223-3 vector has been used in several studies but purification of enzyme was a time consuming and costly process. Therefore, in order to eliminate difficulties in the purification of FDH and to produce quick and highly purified-homogeneous-recombinant protein, \(cm\)FDH was subcloned into pQE-2 expression vector and the amount of purified protein improved about 3 times. It was observed that the N-terminally His tagged FDH has similar activity to the FDH enzyme without the His-tag after digestion with exopeptidases (Ordu and Karagüler, 2007). Since then, the recombinant FDH from *Candida methylica* has been intensively studied to improve the properties for the NAD(P)H regeneration by using protein engineering techniques.

### 3.1 Kinetic and thermodynamic properties of the folding and assembly of \(cm\)FDH

Although there has been much empirical work on stabilizing FDHs versus increasing temperatures and other environmental factors such as oxidation (Thiskov & Popov, 2006), the thermodynamic and kinetic properties of its folding and unfolding pathway have not been dissected in detail. Whereas, in order to control the stability of proteins by genetic modification of sequence we need to understand the full mechanism of folding and unfolding of the active system. This, ideally, requires elucidation of the kinetic and equilibrium properties of each step and to target certain critical steps in the process, so that the sequence engineering would be more rationally directed.

While many of the proteins of interest in biotechnology are oligomeric, as are many of the structures in biological systems where we want to understand the dynamics of assembly and disassembly, our understanding of folding and assembly processes in multi-chain proteins is less comprehensive than kinetics and thermodynamics of folding in single-chain proteins. For these reasons it is useful to examine mechanisms such as formate dehydrogenase enzyme containing two identical subunit with a view to provide a framework for their analysis. The native form of \(cm\)FDH is a dimer and each subunit has 364 residues folded into two distinct domains, each comprising a parallel \(\alpha\)-sheet core surrounded by \(\alpha\)-helices arranged in a Rossmann-type fold. While one domain is functionally defined by co-enzyme binding, the other domain is defined for catalysis; coenzyme and the substrate are encapsulated in the inter-domain cleft during the catalytic reaction. The molecule dimerizes by 2-fold symmetrical interactions between the co-enzyme-binding domains while the catalytic domains are distal to the dimer interface (Schirwitz et al., 2007).

In this section, we define the rates of steps in the minimal model of folding and assembly reaction and deduce the equilibrium properties of the system with respect to its thermal and
denaturant sensitivities. These results act as a basis for understanding the effects of site-specific engineering or forced evolution on the stability of this molecule.

Equilibrium denaturation data of cmFDH yielded a dissociation constant of about $10^{-13}$ M. Findings showed that homodimeric cmFDH unfolds by two state single transition model without intermediates in equilibrium and in the equilibrium one dimer is equal to two unfolded monomers including both folding and dissociation processes.

Thermodynamics of the folding-unfolding transition showed that, at the reference temperature of 25°C the enthalpy change on folding ($\Delta H$) is unfavourable (approximately $+27 \pm 18$ kcal mol$^{-1}$), while the entropy change is favourable ($-T\Delta S = -46 \pm 18$ kcal mol$^{-1}$). The heat capacity change ($\Delta C_p$) is $-10.5 \pm 1.8$ kcal mol$^{-1}$·K$^{-1}$; a value that is dominated by the degree of desolvation of non-polar surface during the folding process (Ordu et al, 2009).

The refolding process of cmFDH enzyme is rate-limited by at least two steps occurring on the same time scale. Further, one of these steps must be multi-molecular since the half-time of refolding is clearly sensitive to the protein concentration. The intermediate state (or states) lie between the two rate-limiting steps is devoid of activity. The simplest model that accounts for the data is an essentially irreversible uni-bi reaction: $2U \rightarrow 2M \rightarrow D$, where U is the unfolded chain, M the folded monomer and D the active dimer.

According to this kinetic model, rate constant yields yielded as yielded values of $1.9 \pm 0.4 \times 10^{-3}$ s$^{-1}$ for the unimolecular folding step and $1.6 \pm 0.5 \times 10^{4}$ M$^{-1}$s$^{-1}$ for the bimolecular association of subunits (Ordu et al, 2009). The monomeric intermediate of cmFDH forms active dimers at a rate which is slower than expected for a process limited by subunit diffusion in solution. Considerations based on orientational constraints and Brownian diffusion for protein associations have suggested that the basal rate should lie in the range of $10^5$–$10^6$ M$^{-1}$s$^{-1}$ (Northrup & Erickson, 1992; Schlosshauer & Baker, 2004; Zhou et al., 1997).

The relatively slow bimolecular rate constant is combined with a slow rate of dissociation to yield a high stability for the native dimer ($\Delta G^0 = -14.6$ kcal mol$^{-1}$), in keeping with other dimeric proteins of high molecular weight. This level of stability ($K_d \sim 10^{-12}$ M) means that at a micromolar concentration, less than one millionth of the enzyme is in the inactive and unstable monomeric state. It is interesting to note that the rate constant for FDH dissociation is so slow that, once formed, the dimer has a dissociative half-life of one and a half years.

The heat inactivation of cmFDH, as for most proteins, is not reversible and follows a first-order decay. As a result of this, denaturation cannot be formally treated as an equilibrium system to which the orthodox analysis can be applied, rather it should be thought of as being defined by a temperature-sensitive rate constant for the irreversible step (Ordu et al, 2009).

### 3.2 Coenzyme regeneration

Production of optically pure compounds is important for product quality and customer safety in industry. In traditional industrial chemical synthesis of chiral compounds, the products are usually racemic mixtures which contain both forms of optically active compounds. In the pharmaceutical industry, when only one enantiomer has the appropriate physiological activity, problems from side effects of another enantiomer can arise like the case of thalidomide. While the R-enantiomer of thalidomide has an analgesic activity, the S-
enantiomer causes defects in the fetus (Muller, 1997). In food industry, chiral molecules are indicator of quality and purity of products. Natural food components are optically pure but extreme industrial process like high pH, temperature and irradiation cause racemic mixture in the food. Chirality is also important in taste and odour applications in food industry. For example, while the L-form of asparagine, tryptophan, tyrosine and isoleucine are characterized by bitter taste, they are characterized as sweet taste in the D-form (Wojtasiak, 2006).

Enzymatic reactions catalyzed by oxidoreductases (e.g. lactate dehydrogenase, hydroxyisocaproate dehydrogenase, xylitol dehydrogenase, mannitol dehydrogenase and limone monooxygenase) are highly stereospecific and very important for the production of chirally pure products. Lactate dehydrogenase and hydroxyisocaproate dehydrogenase can be used in the production of optically pure hydroxyacids which are used for the production of semi synthetic antibiotic (S - α-hydroxyisocaproic acid) and medical diagnosis (S - phenyl pyruvate in diagnosis of phenylketonuria and S - ketoisocaproic acid for some urine disease) (Nakamura, 1988; Van der Donk & Zhao, 2003). Xylitol dehydrogenase and mannitol dehydrogenase are used in D-xylitol and D-mannitol synthesis, respectively and limone monooxygenase used in aroma synthesis also provides stereospecific compounds in the food industry (Kaup et al, 2005; Mayer et al, 2002). The reduction of a carbonyl group particularly to generate a new chiral center, is one of the most widely used biotransformation in industry. Enzymatic synthesis of the final product may reach 100 %, while the processes based on chemical synthesis of racemic mixtures can only provide the theoretical yield of 50 %. Enzymatic reactions take place under mild conditions, this minimizes problems from side reactions and they have a regio and stereochemical specificity that can be difficult to achieve chemically. However use of these enzymes and similars is still limited because of the requirement for stoichiometric amounts of the very expensive NAD(P)H coenzyme. Enzymes remain unchanged after the reaction is completed, but coenzymes react with the substrate chemically and they are usually more expensive than desired product. It is possible to recover used coenzymes via recycling reactions, but existing methods for regenerating NAD(P)H are still a significant expense and are not cost-effective in the manufacturing process. Therefore, there is a need for a low-priced method of coenzyme regeneration (Liu and Wang, 2007; Patel, 2004; Vrtis, 2002).

Several approaches including chemical, electrochemical, photochemical, microbial and enzymatic synthesis have been investigated for coenzyme regeneration. Enzymatic regeneration is the most promising one in the industrial process due to its high selectivity, efficiency, aqueous solvent as operational medium and environmentally safe waste (Van der Donk and Zhao, 2003).

There are two different approaches for enzymatic regeneration. One of them is substrate coupled reaction systems in which one enzyme that reacts with both the reduced and oxidized forms of coenzyme to catalyze both the desired synthesis of the product from one substrate and the coenzyme regeneration with a second substrate. But in this system it is difficult to find thermodynamically favourite conditions for both reactions in the same medium. The other approach, enzyme coupled, is the usage of second enzyme to catalyze the coenzyme regeneration (Eckstain et al., 2004; Popov & Thiskov, 2003). In this way second substrate must be very cheap or can be regenerated easily.

Several enzymes have been studied for NAD(P)H regeneration based on enzyme coupled approach. Formate dehydrogenase (FDH) (Bolivar et al., 2007; Gül-Karagüler et al., 2001;
Seelbach et al., 1996), phosphite dehydrogenase (PTDH) (Johannes et al., 2007; Relyea et al., 2005; Woodyer et al., 2006), alcohol dehydrogenase (ADH), glucose-6 phosphate dehydrogenase, glucose dehydrogenase (GDH) (Endo & Koizumi, 2001; Xu et al., 2007) are currently available systems.

PTDH, catalyzes the oxidation of phosphite to phosphate by reducing the NAD\(^+\) to NADH, works in very narrow pH range (pH 7.0-7.6) and destabilize above 35 °C. Although the cost of phosphite is cheaper than formate, which is the substrate of FDH, 90 g of phosphite is necessary to regenerate 1 mol of NADH by PTDH while formate dehydrogenase needs only 45 g of formate to produce 1 mol of NADH. GDH which catalyzes the hydrolization of gluconolactone to gluconic acid has both coenzyme specificity, NAD\(^+\) and NADP\(^+\) (Nicotinamide Adenine Dinucleotide Phosphate). Although this enzyme has higher specific activity than FDH (FDH, 2.5-10U/mg; GDH, 20-100U/mg), 172 g of glucose is needed for reduction of 1 mol of NAD(P) to 1 mol of NAD(P)H. Moreover, gluconic acid should be removed from the reaction medium to produce pure chiral product.

When FDH is compared to other studied dehydrogenases it offers several advantages. The enzyme is commercially available at low cost and has a favourable thermodynamic equilibrium. It is used for the industrial scale regeneration of NADH in bioreactors with recycle numbers for the cofactor in the order of 130,000 during the production of 640 g l\(^{-1}\) d\(^{-1}\) of L-leucine (Wandrey, 1986) at present. Its reaction results in a 99–100 % yield of the final product. Since the reaction of FDH is essentially irreversible and the product (CO\(_2\)) can be easily removed from the reaction system and formate or CO\(_2\) does not inhibit the other reaction and does not interfere with the purification of the final product. FDH also has a wide range pH optima so that it can work with lots of different enzymes (Popov & Thiskov, 2003). Because of its advantages FDH is very suitable for NAD(P)H regeneration. Therefore use of a cheap reductant is possible. Wilks et. al. (1986) adapted the general scheme for cofactor recycling using formate dehydrogenase (Figure 3).

![Diagram](https://www.intechopen.com)

Fig. 3. General scheme for cofactor recycling using formate dehydrogenase. This diagram is the conventional Wandrey-Kuhla route for the bulk synthesis with reducing equivalents from formic acid (Wilks et. al., 1986).
In laboratory scale, FDH was used for NADH regeneration with several enzymes like lactate dehydrogenase, xylitol dehydrogenase and mannitol dehydrogenase in the production of hydroxyacids, xylitol and mannitol, respectively, (Kaup, 2005; Mayer et al., 2002; Van der Donk & Zhao, 2003). FDH from the yeast Candida boidinii was used in the first commercial scale process of chiral synthesis of tert-L-leucine with leucine dehydrogenase by German Degussa company (Popov & Thiskov, 2003). NADP+-dependent enzymes are less common than their NAD+-dependent counterparts. Engineered FDH is also a favourite enzyme to regenerate NADPH among other investigated enzymes such as ADH and glucose-6 phosphate dehydrogenase because of its cheap substrate. Engineered FDH that can accept NADP+ and it is preferred over glucose-6 phosphate dehydrogenase given the expense of its substrate (Andreadeli et al., 2008; Wu et al., 2009). The number of studies on FDH and its application for coenzyme regeneration in the processes of chiral synthesis with NAD(P)H-dependent enzymes is getting larger year by year (Holbrook et al., 2000; Tishkov & Zaitseva, 2008).

Unfortunately, native FDHs have some disadvantages. These are low $k_{cat}$, high $K_M$, its limited coenzyme specificity solvent tolerance and lack of thermostability. Hence it is important to improve the stability of FDH to cope with the harsh conditions like high temperature, pressure or pH required for the most of manufacturing processes in food or pharmaceutical industries. To make FDH a more suitable enzyme for cofactor regeneration these disadvantages should be removed. Protein engineering is a promising approach to improve the industrial parameters of FDH.

4. Protein engineering studies on cmFDH

During the evolution, organisms adapted to extremes of pH, salinity, pressure and temperature by means of mutations that affect stability, catalytic activity or substrate specificity of enzymes (Bloom et al., 2006). Proteins are the best candidates to evolve because they may change their biochemical function with just a few mutations (Razvi & Scholtz, 2006). These molecules have evolved by different strategies to protect their stability and function under unusual conditions.

4.1 Increasing the thermostability

Studies in the literature show that thermophilic proteins typically have increased number of van der Waals interactions, hydrogen bonds, salt bridges, dipole-dipole interactions, disulphide bridges, hydrophobic interactions, aromatic stacking interactions, improved core packing, shorter and/or tighter surface loops, enhanced secondary structure propensities, decreased conformational entropy of the unfolded state and oligomerization at the molecular level (Karshikoff & Ladenstain, 1998; Kumar et al., 2000; Robinson-Rechavi et al., 2006). Thermophilic enzymes are already used in industrial applications, e.g. the $\alpha$-amylase from Bacillus licheniformis is among the most thermostable natural enzymes used in biotechnological processes. However, the use of thermophilic enzymes depends on their availability. Identification and purification of new industrial enzymes suitable to harsh conditions is not always straightforward. Therefore, improving of wild type enzymes for a desired function or increased stability by using protein engineering is an important approach to overcome this restriction. Several efforts have been attempted to identify the most efficient method to enhance thermostability of a mesophilic protein by using protein
engineering methods (Annaluru et al., 2006; Rodriguez et al., 2000; Yokoigawa et al., 2003). Unfortunately, there is no general rule by which a mesophilic protein can be converted into a thermophilic one.

In the laboratory, protein stability studies have been performed against to several denaturating agents like extremes of pH, salinity, pressure and temperature. Among these extreme conditions, high temperature stability (thermostability) is particularly important in the industrial and biotechnological enzymatic production process where the enzymes are often inactivated due to high temperature. For example in the starch sector, which is one of the largest users of enzymes, conversion of starch include liquefaction and saccharification steps. The temperature has to be 105-110 ºC during these processes. Otherwise, below 105 ºC, gelatinisation of starch granules is not achieved successfully and causes filtration problems in the other steps (Synowiecki, 2006; Haki, 2003). This causes an economical challenge for the manufacturer. There are many advantages for industry by using thermostable enzymes in commercial applications. Higher temperatures can lead to i) Increased activity, reaction rates typically increase two to three fold for each 10 ºC increase of temperature, ii) increased yield of product because many substrates are more soluble at higher temperatures, and by a shift of the thermodynamic equilibrium for endothermic reactions, iii) increased storage and operational stability, iv) increased stability against other denaturing conditions, for example the possibility of sterilizing products in the course of the production. If such problems can be solved by high temperature, any FDH enzyme that would be used for NAD(P)H regeneration in this kind of high temperature process would need to be thermostable.

The lack of thermostability is the most important disadvantage of FDH. Studies in the literature with the aim of improving the thermostability of FDH have been focused on *Pseudomonas* sp 101 which has the best thermostability among the known FDHs and the FDH from *Candida boidini* by using rational design or directed evolution. The FDH enzymes from these organisms have the advantage of solved crystal structures.

In the case of FDH from *Candida methyllica*, the first attempt was made to produce a more thermostable enzyme by the DNA shuffling method because the 3D structure of FDH from *C. methyllica* is not yet solved. In DNA shuffling, the ability to select an improved protein from a large pool of protein variants depend on sensitive screening or selection methods. In this study host proteins could not be inactivated through a heat step screening method. In the light of this problem a homology model of cmFDH was generated to design rational mutations for optimizing the surface electrostatic interaction on the protein surface and introduction of disulphide bridges into protein structure.

After the determination of importance of hydrophobic interaction by Langmuir in 1938, hydrophobic interactions are one of the widely studied and observed driving forces behind the folding and stability of globular proteins (Dill, 1990; Folch, et al., 2008; Reiersen & Rees, 2000). Recent results show that two other strategies have attracted attention to increase protein thermostability. One of them is improving the electrostatic interactions on the protein surface to optimize the surface electrostatic interactions (Eijsink, 2004; Kumar & Nussinov, 2001; Roca et al., 2007; Takita et al., 2008). The second one is the introduction of disulphide bonds between Cys residues (Chu et al., 2007; Hamza & Engel, 2007; Yang et al., 2007). Although the contribution of surface electrostatics or disulphide bridges to protein stability is still not fully understood, it is clear that these interactions are important in protein thermostability.
In order to generate homology model of *Candida methylica*, crystal structures of FDH from *Pseudomonas sp.*101 (Lamzin et al., 1992) which has 49% amino acid sequence identity with *cm*FDH and *Candida boidinii* which has about 90% identity with *cm*FDH (Schirwitz et al., 2007) were used as the basis of model. Richard Sessions within the University of Bristol, helped with the computer simulations presented in this section. Amino acid changes were performed using InsightII 2005 (Accelrys). All the differences (insertions and/or deletions) between template (*Pseudomonas sp.*101 and *Candida boidinii*) and target (*Candida methylica*) sequences in the alignment appeared at the surface of the structure, the loop building facility in InsightII was used to model these differences in the protein backbone. Energy minimization of modelled native *cm*FDH and various mutant FDHs were carried out using the steepest descents and conjugate gradients methods to minimize the energy because of the large number of atoms in FDH protein from *Candida methylica*. All energy minimizations were calculated using Discover, Version 2.95 (Accelrys) on a Linux workstation, which can be used for minimization and molecular dynamic simulations.

Gratifyingly, we find that the two models are closely similar with the following Root Mean Squared Deviations of the Cα positions: complete dimer, 2.7 Å; NAD⁺-binding domain, 1.7 Å; catalytic domain, 1.8 Å. Likewise, this result validates the designs and interpretations presented in our previous mutagenesis (Karaguler et al., 2007).

The all mutants designed either to optimize the surface electrostatic interactions or to introduce a disulphide bridge were applied to *cm*FDH by site directed mutagenesis to generate a thermostable *cm*FDH.

**4.1.2 Optimization of electrostatic interactions on the surface of cmFDH**

Compared with positions buried in the core, stabilizing surface mutations are less likely to disrupt the tertiary structure, which may be considered as the evidence of evolutionary selection (Alsop et al., 2003; Robinson-Rechavi, 2006). Electrostatic interactions also affect the protein flexibility that is significant for movement of residues with respect to each other and their environment. The changes in electrostatic interaction of charged side chains of residues are critical for protein folding and stability thus biological activity of the protein.

In order to increase the thermostability of formate dehydrogenase enzyme, Fedorchuk et al. (2002) tried to optimize electrostatic interactions by engineering residues in positions 43 and 61 of *ps*FDH by comparison to the *Mycobacterium vaccae* N10 FDH that only differs in two amino acid residues but has a lower stability than *ps*FDH. They showed the thermostability effect of loop regions of bacterial formate dehydrogenases which are absent in analogous eukaryotic enzyme and the replacement of Asp43 and in the *ps*FDH molecule does not result in an increase in stability. Rojkova et al. (1999) applied the hydrophobization of alpha helix strategy. They selected the 5 serine residues occupying positions 131, 160, 163, 184 and 222 of *ps*FDH. Their results showed that a combination of mutations had an additive effect to FDH stabilization and obtained a four-point mutant FDH which has a thermal stability 1.5 times higher, compared to the wild type enzyme. Serov et al. (2005) tried to optimize the polypeptide chain conformation to increase thermostability of *ps*FDH.

In our study, according to model mentioned in the section 4.1.1, a set of surface-charge mutations, simply based on inspection of the *cm*FDH homology model and chemical
intuition, was designed. Comparison of the thermodynamic properties of mutants and native cmFDH shows that additional electrostatic interactions have different effects on the enthalpy and entropy changes at room temperature. Based on the thermodynamic results, it is suggested that three of nine catalitically active mutants resulted in increased protein stability by the measures of free energy of folding, Tm values and persistence of activity. Except relatively improved mutants, melting temperature increased between 2 and 6 °C while the folding and unfolding patterns of native cmFDH was not altered.

This result corresponds to a 33 % accuracy of designing mutant to increase thermostability. On the other hand, all the other mutation positions were significant such that they all had a measurable effect on the cmFDH folding and activity, and hence contributed to our understanding of the interactions. Another point of interest concerns the effect of double mutants on stability. It is known that, stabilization effect is generally additive allowing significant stabilization to be achieved with a number of single-point mutations (Serov et al., (2005). In contrast, a stabilizing effect of double mutants was not observed even though the corresponding single mutants had stabilizing effects.

4.1.3 Introduction of disulphide bridges into the structure of cmFDH

Disulphide bridges are also thought to stabilize the three-dimensional structure of proteins (Thornton, 1981). This hypothesis is based on observations that the disruption of disulphide bridges leads to a decrease in the thermodynamic stability of proteins (Anfinsen & Scheraga, 1975; Creighton, 1978). Although the mechanism by which disulphide bonds confer stability is not known yet in detail, one major aspect is that introducing disulphide bridges into proteins is the most obvious way to decrease the number of conformations available to the unfolded protein. Since part of the driving force to denature a protein is conformational entropy (the unfolded state has many more configurations than the folded state) reducing the number of conformations available to the unfolded state should stabilise the folded state.

FDH enzyme is one of several enzymes which have been subjected to disulphide bridge or Cys residue engineering to stabilize it against several factors or to overcome atmospheric oxidation of Cys residues. Odintseva et al. (2002) have engineered Cys residues in an attempt to increase the stability of PSDFH, obtaining mutants that showed the same kinetic parameters as the wild type enzyme, but its thermal stability dropped four-fold. Slusarczyk et al. (2000) have engineered the Cys residues of cbFDH and they showed that mutations affecting Cys23 are more effective than Cys262 on the stability. The first attemption to introduce a disulphide bridge into the FDH enzyme from Candida methylica to improve the thermostability was applied by Karagüler et al, in 2007b. Three pairs of cysteine residues were introduced into the cmFDH gene to construct three different disulphide bridged (T169/T226, V88/V112, M156/L159) mutants by site directed mutagenesis. The wild type cmFDH contain two cysteine residues buried in separate hydrophobic pockets but does not contain disulphide bridges, hence it is a good candidate for attempted stabilization by disulphide bridge approach.

No formate-dehydrogenase activity could be measured for the mutants V88C/ V112C (N-domain bridge) and M156C/ L159C (inter-subunit bridges) in either reducing or oxidising conditions. Presumably these mutations have distorted the structure of the FDH dimer to such an extent that the catalytic machinery is rendered ineffective. In contrast, the mutations T169C and T226C in the C-domain of cmFDH are tolerated. The catalytic efficiency (k_{cat}/K_{M})
of the mutant protein in both its oxidised and reduced form is about four fold lower than the wild type cmFDH. Interestingly, the individual $k_{cat}$ and $K_M$ data show that the introduction of the cysteine residues in the reduced mutant raises $K_M$ by about two fold and reducing $k_{cat}$ by about two fold, with respect to the wild type. Oxidising the mutant and forming a cystine bridge restores $K_M$ close to that of the wild type, consistent with forming a more native-like substrate binding site but reduces $k_{cat}$ a further two fold (Karagüler et al, 2007b).

Another attempt to introduce a disulphide bridge into cmFDH on the positions of A153 and I239 by mutating these residues to cysteine have been performed by the same group (unpublished data). The disulphide bridge mutants investigated in this work and these mutants did not increase the thermostability of cmFDH. Moreover, only one of them could be tolerated to give a functional enzyme. Hence it is clear that choosing sterically ideal sites for the insertion of disulphide bridges is crucial.

In all previous studies, to generate homology model of Candida methylica, crystal structures of FDH from Pseudomonas sp.101 (Lamzin et al., 1992) which has 49 % amino acid sequence identity with cmFDH was used as the basis of model. In our latest attempt, according to homology modelling of cmFDH based on Candida boidinii FDH crystal structure (Schirwitz et al., 2007) which has amino acid sequence similarity 97 % to cmFDH, two residues selected to change by Cys residue lie on the start point of two different $\beta$-strands. This is a good position to introduce a disulphide bridge in the N-terminal of catalytic domain of cmFDH and investigate the effect of Met to Cys replacement for stabilizing the structure.

Altough the mutant cmFDH which has disulphide bridge did not show the expected improvement in the stability, characterization studies of individual mutants showed that Met to Cys change is related to temperature stability. Catalytic efficiency ($k_{cat}/K_M$) of this single mutant was 63 % better than that of the native cmFDH and $T_m$ value was measured as 2 °C higher than that of the native cmFDH. Substitution of methionine to cysteine, which converts a hydrophobic residue into a more hydrophilic one, can markedly alter the properties of a protein (Daia et al., 2007). Methionine and cysteine are two sulfur-containing amino acids but side chain of Cys is shorter than Met. Therefore, probably, change of Met to Cys affects the catalytic efficiency of protein and increase the thermostability by providing a more sterically compact structure.

The Far –UV CD spectra of native cmFDH and Met to Cys mutant exhibits similar shape at 25 °C. This result shows that Met to Cys mutant and native cmFDH have a nearly identical secondary structure content and indicates that the presence of the disulphide bridge does not affect significantly the enzyme secondary structure. During the heating to different temperatures, the CD spectra of both enzymes suggest that the replacement of methionine to cysteine leads to an increase of the thermostability of the secondary structure of cmFDH. On the other hand, it is known that introduction of disulphide bonds is one of the strategies to increase stability of a protein, which arises from a decrease of the conformational entropy (Mårtensson et al., 2002; Ordu, 2011, in preparation).

4.2 Alteration of coenzyme specificity

In biological systems, the majority of redox enzymes involved in anabolic processes use the coenzyme NADPH whereas those of catabolic processes generally use the coenzyme NADH (Stryer, 1988). NADPH is distinguished from NAD$^+$ by the presence of phosphate group esterified to the 2' hydroxyl group of its adenosine moiety.
The majority of NAD+-dependent FDHs are highly specific towards NAD+ and do not utilise NADP+ as a coenzyme (Popov & Lamzin, 1994). Since many enzymatic synthesis of chemical compounds in industry require cofactors like NAD(H) or NADP(H) as discussed in section 3.2., it has been problem that the known FDHs only work with NAD+ as a cofactor but not NADP+. However, Pseudomonas sp. 101 FDH is exceptional in that it turns over NADP+ at 25 % of the rate of NAD+ under optimal reaction conditions (Popov & Lamzin, 1994). A number of positively charged amino acid side chains (Arg222, His223 and His379) are located close to the region which binds the 2'-O-PO₄²⁻ of NADP+ and may provide a suitable electrostatic stabilisation for the bulky negatively charged phosphate group of NADP+.

One challenge is to convert NAD+-specific formate dehydrogenase to an NADPH-specific enzyme. It would be an advantage of NAD+-dependent FDHs if they could be modified to work with NADP+ as well because many of enzymatic synthesis of chemical compounds in industry require NADPH. Therefore, the dual activity enzyme could be used to input cheap reducing equivalent in a commercial process, normally using an NADP+ enzyme, as seen in figure 3.

Many attempts using both rational design and site saturation mutagenesis approaches have been made to change the coenzyme specificity of FDH from different sources (Andreadeli et al., 2008; Karaguler et al., 2001; Serov et al., 2002b; Wu et al., 2009). In our previous experiments we achieved cmFDH to use NADP+ by the single mutation Asp195Ser (Karaguler et al., 2001). However, this mutant binds NADP+ weakly and we suggested that cmFDH possesses an aspartic acid residue (195) which binds the hydroxyl groups of the adenine ribose moiety of NAD+, in common with many NAD+-dependent dehydrogenases containing the Rossmann fold. D195S shows similar catalytic constants to wild type in the reaction with NAD+. In contrast with wild type, the reaction of NADP+ catalysed by the mutant is clearly discernible. However, the accessible concentrations of NADP+ are well below K_M, hence only (k_cat/K_M)_{NADP} can be determined. The ratio of the catalytic efficiencies for NAD+ versus NADP+ for the mutant protein is 40:1 in favour of NAD+. Likewise, the D195S enzyme is at least 8300-fold more efficient at turning over NADP+ than the wild type. These results demonstrate that D195S is a major determinant of cofactor specificity in cmFDH. Wu et al., (2009) revealed that site saturation mutagenesis application on residues Asp195, Tyr196 and Gln197 of Candida boidinii FDH produce more mutants with significant NADP+ specificity, which indicate the critical roles of these residues in determining the enzyme’s cofactor specificity. In the light of these foundations, we have also explored further mutations (Ozgun et al., submitted in 2011) in the coenzyme binding domain to improve the K_M of cmFDH for NADP+. The single mutations at D195, Y196 and Q197 in the coenzyme binding domain are introduced by using site saturation mutagenesis. In the library, two single mutants exhibit the highest catalytic efficiency (k_cat / K_M) in the presence of NADP+. Mutation on the residue 195, which has a proven role for the substrate specificity of FDH, is selected from the first generation mutant library as a template to construct the secondary generation mutant library. Second generation mutation is introduced into Y196 and Q197 residues. 2 double mutants are selected as promising NADP+ specific FDH mutants from the library. These mutants increased the overall catalytic efficiency of NADP+ to 56000 and 50000-fold, respectively. Results emphasize that; SSM is an efficient method for creating ‘smarter libraries’ for improving the properties of cmFDH.
5. Conclusion

In order to make FDH more suitable enzyme for its industrial applications, protein engineering is a promising approach. The work presented in this chapter demonstrate the difficulty of engineering hyper-stability into highly evolved enzyme structures using these methods. Each method has its own advantages and disadvantages. Although a combination of site-directed mutagenesis and model building is now being used to engineer novel proteins the rational design still can be inefficient because it is so time-consuming and only a limited number of amino acid variants can ever be evaluated for function. The results of DNA shuffling experiments showed that a very efficient screening technique is necessary to make this approach possible. Screening limits the search for beneficial mutations, because the basic rule of DNA shuffling is you get what you screen for, therefore it is vital to know exactly what the screen is actually selecting. The application of site saturation mutagenesis which is a combination of both strategies represent the new route to obtain the biocatalysts with the desired properties

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7. References


Protein Engineering Applications on Industrially Important Enzymes: Candida methylica FDH as a Case Study


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