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Enantioselective epoxidation catalyzed by manganese-substituted human carbonic anhydrase

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TABLE OF CONTENTS

ABBREVIATIONS

hCAII[Zn]	Human Carbonic Anhydrase II
hCAII[Mn]	Manganese-Substituted Human Carbonic Anhydrase
PCR	Polymerase Chain Reaction
DNA	Deoxyriobonucleic Acid
RNA	Ribonucleic Acid
Ala	Alanine
Thr	Threonine
Leu	Leucine
Ser	Serine
Pro	Proline
His	Histidine
Lys	Lysine
Asn	Asparagine
dNTP	Deoxynucleoside Triphosphate
DMSO	Dimethyl Sulfoxide
SOB	Super Optimal Broth
SOC	Super Optimal Broth with Catabolite Repression
BES	*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid
LB	Luria-Bertani
dd	Distilled, Deionized
OD	Optical Density

IPTG	Isopropyl β-D-1-thiogalactopyranoside
PMSF	Phenylmethylsulfonyl Fluoride
TAME	N_{α}-*p*-Tosyl-L-Arginine Methyl Ester Hydrochloride
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
PDCA	2,6-Pyridinedicarboxylate
HPLC	High Pressure Liquid Chromatography
ee	Enantiomeric Excess
6XM9	Minimal Medium Salt

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ABSTRACT

Artificial metalloenzymes are an important class of hybrid catalysts for enantioselective, regioselective and chemoselective organic transformations. Despite several limitations associated with this type of hybrid catalyst system and their limited development relative to small molecule catalysts, significant advances have been achieved over the past few decades. This thesis describes the background, applicability of such hybrid catalysts, anchoring strategies and methods involved in the improvement of catalytic activities of artificial metalloenzymes. The generation of a thermostable human carbonic anhydrase mutant, activity determination, generation of an artificial metalloenzyme (manganese-substituted human carbonic anhydrase) and its use as a catalyst for enantioselective epoxidation of olefins is also described.

CHAPTER I

INTRODUCTION

General introduction

Enzymes are a class of macromolecules with three-dimensional structures composed of folded linear amino acid chains. Enzymes are one of the most important types of catalysts used for biological transformations in nature. Some enzymes require an additional non-protein component called a cofactor in order to achieve proper activity. These cofactors can either be metal ions, metal clusters or organic components. The enzyme with its cofactor is called the haloenzyme and the enzyme without the cofactor is called the apo-enzyme. Enzymes that have metal cofactors, are called metalloenzymes and the metal cofactor is bound to the amino acid residues inside the active site of the enzyme. Nearly one third of structurally characterized proteins are metalloenzymes[1] and many of these metalloenzymes are capable of catalyzing otherwise challenging reactions in biological systems.[2, 3]

The synthesis of enantiopure compounds is challenging, but is becoming increasingly important in the pharmaceutical industry,[4] in the food industry and in agricultural fields. Recently, the metalloenzymes and the design of artificial metalloenzymes has gained the attention of the synthetic chemistry community due to the ability of metalloenzymes to catalyze enantioselective, regioselective and chemoselective organic transformations. Thus, the use of metalloenzymes and the development of new artificial metalloenzymes with improved activities and selectivities have increased in recent years.[5, 6, 7, 8, 9] Small-molecule, homogeneous catalysts typically use chiral ligands to achieve high levels of enantioselectivity, but by combining enzymatic catalysis with transition metal catalysis the

potential exists to eliminate the use of chiral, small-molecule ligands, which are expensive and/or difficult to make.[10] The combination of transition metal catalysis and enzymatic catalysis leads to the generation of hybrid catalyst systems capable of selective organic transformations with high functional group tolerance and high turnover numbers. Also, the ability to fine-tune the ligand environment by modifying the secondary coordination sphere of the enzyme in these systems may lead to further improvements.

The essential components for a hybrid catalyst system are a biomolecular host and a catalytically active metal moiety. In the case of metalloenzymes, the biomolecular host provides a chiral pocket for enantioselective transformations. The "lock-and-key" hypothesis explains that the selectivity of these transformations results from the shape and the size of the active site, and these parameters matter greatly when it comes to enzymatic catalysis.[11] Changing the active metal complex that is incorporated in the active site of the enzyme can increase the activity and broaden the scope of transformations that can be catalyzed by metalloenzymes. Akaburi, Sakurai, Izumi and Fujii reported the first use of biomolecular host and metal moiety incorporation (a protein-palladium complex prepared by adsorption of palladium chloride on silk fibroin fiber) for an asymmetric hydrogenation to synthesize optically active amines and amino acids.[5] One of the earliest reports of artificial metalloenzyme catalysis is the oxidation of ascorbic acid by carboxypeptidase[Cu] by Yamamura and Kaiser.[6] The use of artificial metalloenzyme catalysis for synthetic transformations has undergone dramatic development in the decades following these initial reports.

The structure of an artificial metalloenzyme consists of a biomolecular host, an active catalytic metal moiety and an optional anchoring group or a spacer group. The classification

of an artificial metalloenzyme is based on these primary components. Biomolecular hosts can either be a metalloenzyme (carbonic anhydrase, bovine serum albumin, etc.), a protein (adipocyte lipid-binding protein) or a polynucleotide (DNA, RNA).[12] The metal moiety can be a metal cation (Cu^{2+}, Mn^{2+}, Rh^{1+}, etc.) that is directly bound to the active site of the enzyme or incorporated into the enzyme through a spacer group or a linker. The linker can consist of an organic or inorganic moiety (Cu-phthalocyanin,[13] Rh-diphosphine,[14] etc.). The precise location of the metal catalyst and the strategy used to connect the biomolecular host and the metal catalyst is important in terms of classifying metalloenzymes. The three major anchoring strategies used to attach the metal moiety to the artificial metalloenzyme are dative anchoring, supramolecular anchoring and covalent anchoring.[12]

Dative anchoring is a common method used in artificial metalloenzyme generation in order to incorporate a metal cation to the biomolecular host. The metal cation found in the natural metalloenzyme is removed by introducing a chelating agent using dialysis. Then, a new metal cation is introduced into the same site of the apo-enzyme through dialysis, thereby generating the artificial metalloenzyme (Figure 1). The normal function of the metalloenzyme will be lost as a result of the metal cation exchange in the active site. However, novel catalytic activity can be introduced by this method. Yamamura and Kaiser reported the first example of using dative anchoring to generate carboxypeptidase[Cu].[6] Carboxypeptidase[Zn] shows peptidase and esterase activity but the replacement of Zn^{2+} with Cu^{2+} in the active site generates carboxypeptidase[Cu], which catalyzes the oxidation of ascorbic acid to dehydroascorbic acid.[6] Recently the Kazlauskas group[7] and the Soumillion group[15] have reported on the same strategy to generate carbonic anhydrase[Mn]. Carbonic anhydrase[Zn] catalyzes the hydration of carbon dioxide and facilitates the removal of carbon

dioxide from mammalian bodies. However the artificial metalloenzyme generated by the replacement of Zn^{2+} with Mn^{2+} in the active site showed a novel catalytic activity by catalyzing the epoxidation of alkenes. The Kazlauskas group also used this same strategy to generate carbonic anhydrase[Rh] from carbonic anhydrase[Zn] for the catalytic hydrogenation of *cis*-stilbene.[16]

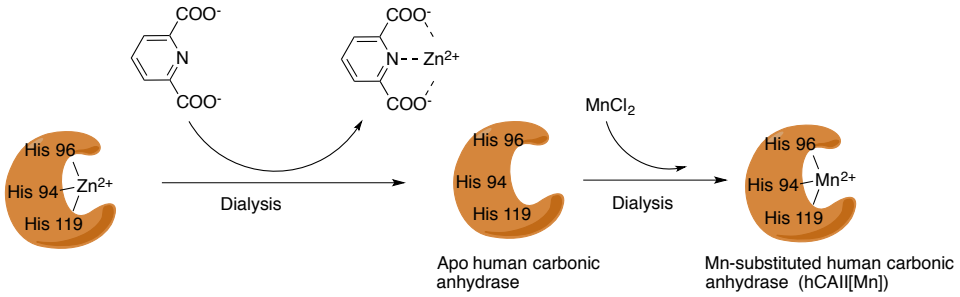


Figure 1: Dative anchoring strategy for the generation of an artificial metalloenzyme

Supramolecular anchoring is the second strategy used to anchor the catalytically active metal moiety to the biomolecular host. In this method the metal catalyst is anchored to the biomolecular host such as a protein,[17, 18] DNA,[8] or antibody[14] through organic ligands and/or spacer groups. C.C. Lin, C.W. Lin and S.C. Chan reported this anchoring strategy to generate an artificial metalloenzyme in which a rhodium(I)-biotin complex was bound to streptavidin and used as a catalyst for hydrogenation of itaconic acid with moderate enantioselectivity.[17] Roelfes and Feringa have reported the use of this method to generate an artificial metalloenzyme (an artificial DNAzyme) using a DNA double helix as the biomolecular host and a Cu^{2+} metal ion (Figure 3).[8] They use small aromatic molecules

(nitrogen containing heterocycles) to link the Cu^{2+} catalyst to the DNA double helix and the resulting complex catalyzes an asymmetric Diels-Alder reaction.

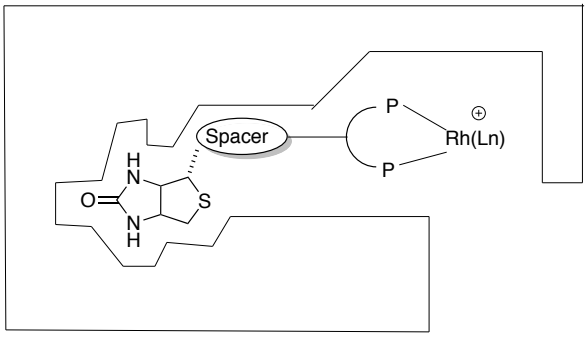


Figure 2: Supramolecular anchoring strategy for the generation of an artificial metalloenzyme [$Rh(L_n)$(biotin-ligand)-streptavidin.[18]

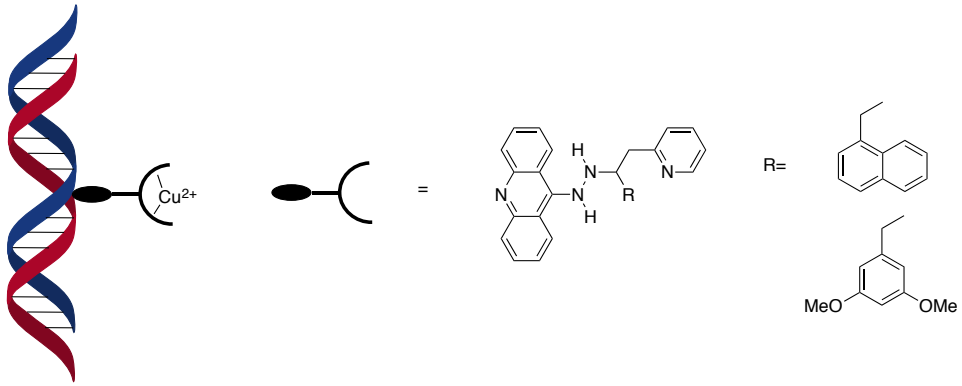


Figure 3: Supromolecular anchoring strategy for the generation of DNAzyme[8]

Covalent anchoring is the third strategy known for the generation of artificial metalloenzymes. This method involves a modification of the biomolecular host (which can be an enzyme,[19] DNA,[20] or RNA[20]) and the active metal catalyst is attached to the host with or without a linker. The first semi-synthetic enzyme was generated by Kaiser by the modification of cysteine-25 in the active site of papain to be used as an oxidoreductase.[19]

More recent examples on the use covalent anchoring are reported by de Vries by the modification of cysteine-25 in the active site of papain to complex $[Rh(COD)_2]BF_4$ (Figure 4, equation 1).[21] A phosphorus ligand was directly attached to the nucleophilic thiol group of cysteine-25 and the rhodium complex was introduced to the active site of papain through this linker. This novel artificial metalloenzyme was used for the hydrogenation of methyl-2-acetomidoacrylate.[21, 22] Salmain has used the same strategy to generate an artificial metalloenzyme from papain for a catalytic Diels-Alder reaction (Figure 4, equation 2).[23] In this report, the cystein-25 inside the active site was modified to include a nitrogen containing aromatic ligand which complexes the ruthenium(II) catalyst inside active site of the enzyme. Jaschke[20] reported the use of DNA-diene-iridium(I) hybrid catalyst for allylic amination in an aqueous medium. This novel catalyst contains a oligodeoxynucleotide carrying a diene ligand (a bicyclo[2.2.2]octadiene) and an iridium[I] complex that is combined with a complementary DNA strand.

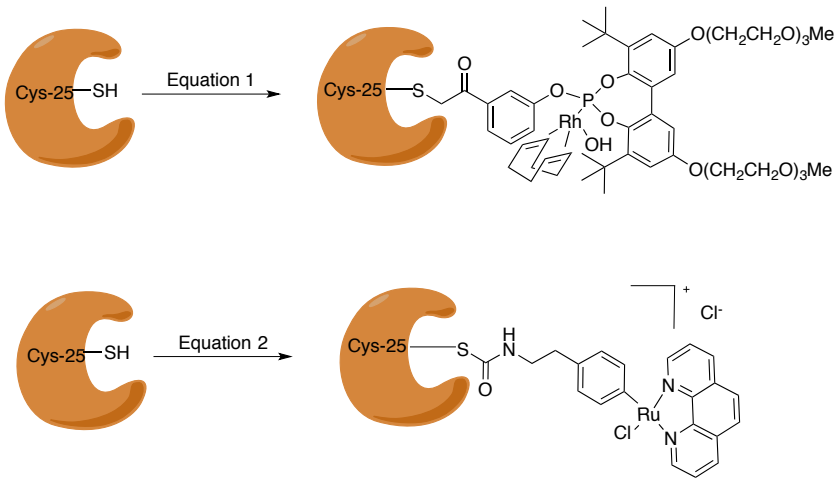
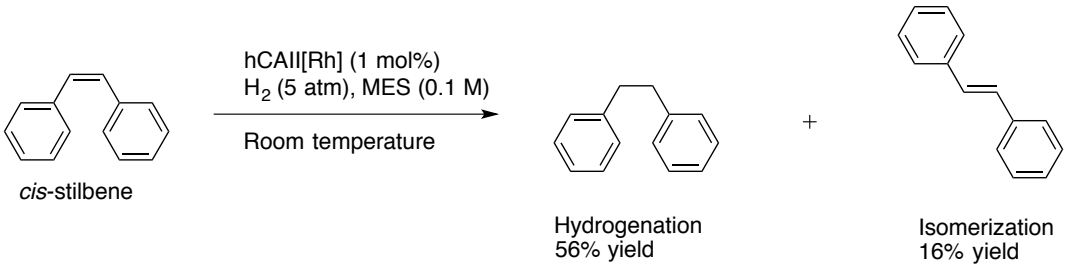


Figure 4: Covalent anchoring of papain at cysteine-25 inside the active site: equation 1,[21, 22] equation 2[23]

The novel activities obtained through the generation of artificial metalloenzymes have been exploited extensively towards the identification of hybrid catalyst systems for enantioselective, regioselective and chemoselective organic transformations. Promising developments have been made in reactions such as the hydrogenation of olefins (Figure 5A),[14, 16, 17, 21, 22] the oxidation of alcohols (Figure 5B),[6, 24] sulfoxidation (Figure 5C),[25] the epoxidation of olefins (Figure 5D),[7, 15] the hydroformylation of olefins,[9, 26, 27] and Diels-Alder reactions (Figure 5E).[8, 20, 23, 28]

A)



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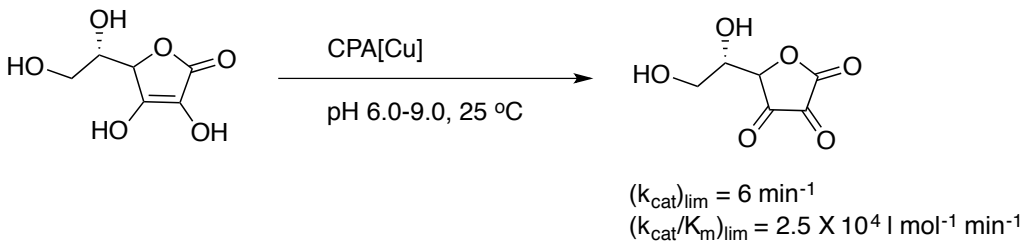

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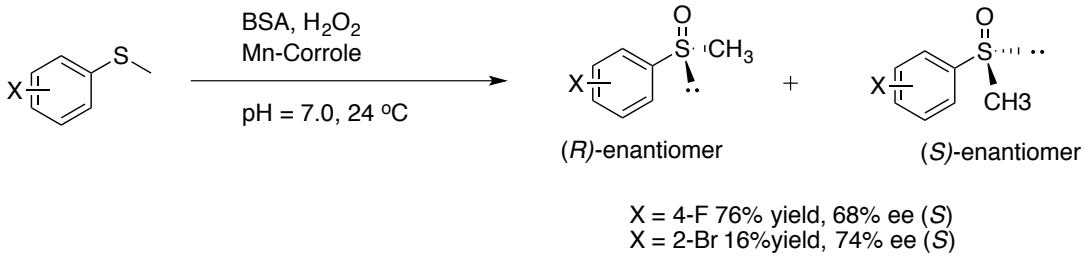

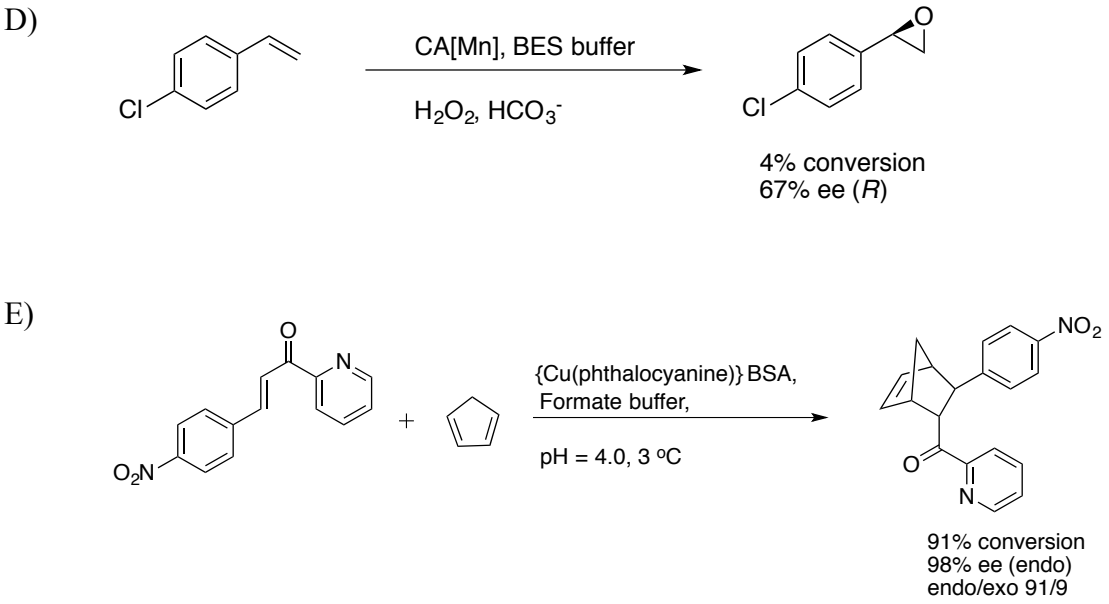

Figure 5: Reactions catalyzed by artificial metalloenzymes; A) hydrogenation of olefins,[16] B) oxidation of alcohols,[6] C) enantioselective sulfoxidation,[25] D) epoxidation of olefin,[7] E) enantioselective Diels-Alder reaction[28]

Enzymes play a major role in the design of artificial metalloenzymes, but the numbers of potential enzyme candidates are limited as the active site of the enzyme must be capable of accommodating the metal catalyst moiety as well as substrates and linkers or spacer groups if any in order to achieve selective organic transformations. Some of the most frequently used enzymes for the generation artificial metalloenzymes are carbonic anhydrase,[7, 15, 16] papain,[19, 23] albumin,[27, 28] myoglobin,[29, 30] avidin[17, 24, 31] and streptavidin.[13, 18, 24, 31] Among those enzymes carbonic anhydrase is most extensively used metalloenzyme to generate artificial metalloenzymes by the dative anchoring strategy. Carbonic anhydrase (CA) catalyzes the

hydration of carbon dioxide and the dehydration of bicarbonate. The natural metalloenzyme contains a Zn^{2+} in the active site, which is bound through the imidazole groups of three histidine residues (His-94, His-96 and His-114) and one hydroxide ion (or a water molecule).[32, 33] Carbonic anhydrases are categorized into five classes: α-CA in animals (all mammalians), β-CA in plants and prokaryotes, γ-CA in bacteria, δ-CA in marine diatoms and ζ-CA in bacteria.[32] Among these α-CA is the most widely studied class and it has 16 known isoforms (CA-I, CA-II and so on). Carbonic anhydrase is a water-soluble, single-chain, monomeric enzyme and the molecular weight is ~30 kDa (CA-II 29 kDa, CA-IV 35 kDa).[34] There are many reasons why carbonic anhydrase is an excellent candidate for the generation of artificial metalloenzymes: 1) it is relatively easy to express and purify as compared to other commonly used metalloenzymes, 2) it has a very good stability under standard laboratory conditions and as a result, it is easy to handle, 3) it is easy to prepare the apo-enzyme and Zn^{2+} can be replaced with other divalent cations without a loss of the enzyme stability, 4) the structure of the enzyme and the active site are well established and the amino acid sequences are known for most of the isoforms.

This thesis details the generation of manganese-substituted human carbonic anhydrase (hCAII[Mn]) using a dative anchoring strategy and the use of this artificial metalloenzyme for the catalytic, enantioselective epoxidation of olefins. Furthermore, this thesis describes the development of a thermostable artificial metalloenzyme derived from the wild-type of human carbonic anhydrase.

Thesis Organization

This thesis consists of four chapters. Chapter 1 is a general introduction to the background and work described in chapter 2 and chapter 3. The research described in chapter 2 and 3 has not yet been submitted to any journal for publication. Chapter 4 is the conclusion on the thesis.

Chapter 2 describes the background, methods involved in the generation of a thermostable variant of human carbonic anhydrase isoenzyme II from the wild-type, and the experiments carried out to confirm the thermostability.

Chapter 3 describes the generation of manganese-substituted human carbonic anhydrase from wild-type and thermostable mutants, and the use of those catalysts for the enantioselective epoxidation of olefins. The problem associated with this system and possible solutions to overcome these problems are also discussed.

Chapter 4 is a general conclusion for the thesis.

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CHAPTER 2

GENERATION OF A THERMOSTABLE HUMAN CARBONIC ANHYDRASE

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Abstract

The thermostability of the wild-type of human carbonic anhydrase[Zn] was improved with the use of site-directed mutagenesis to introduce six mutations in defined positions of the wild-type enzyme. The improved thermostability of the mutant was confirmed by an esterase activity assay and comparison to the wild-type. The importance of the generation of metalloenzymes with improved thermostability is to use them to generate artificial metalloenzymes with improved catalytic activities. This thermostable mutant will be used to generate a manganese-substituted human carbonic anhydrase for the catalytic enantioselective epoxidation of olefins.

Introduction

Artificial metalloenzymes are a promising class of hybrid catalysts for enantioselective, regioselective and chemoselective organic transformations. However, limitations such as poor stability at elevated temperatures, in organic solvents and at varied pH must be overcome before metalloenzymes can be utilized as catalysts for additional reaction types. Currently, reactions catalyzed by artificial metalloenzymes have low turnover

numbers, limited catalyst lifetime and a limited scope of substrates. Therefore, the need and the search for new approaches for further improvement of the activity and stability of artificial metalloenzymes have become increasingly important over the last couple of decades. One successful method for the generation of artificial metalloenzymes with improved activity and/ or selectivity is directed evolution. This concept is based on the repeated cycles of random gene mutagenesis, expression, screening and isolation of evolved genes. This sequence is repeated until the desired activity and/or selectivity is achieved. Reetz published the first report on the directed evolution of lipase (from *Pseudomonas aeruginosa*) and used the resulting mutant in the catalytic, enantioselective hydrolysis of racemic *p*-nitrophenyl-2-methyldeconate.[1]

A number of different methods are available for gene mutagenesis, of which site-saturation mutagenesis,[2, 3] site-directed mutagenesis,[4, 5] combinatorial mutagenesis [6, 7, 8] and insertional mutagenesis[9] methods are most common. Site-saturation mutagenesis is the process of introducing all possible random mutations into a specific position of the amino acid sequence of the enzyme in order to generate a library of mutant genes (Figure 1). This process is followed by a protein expression, purification of the enzyme and finally screening the activity and/ or selectivity for a specific reaction. If any improvement is observed, the evolved gene is isolated and exposed to further rounds of site-saturation mutagenesis for additional improvement.[10]

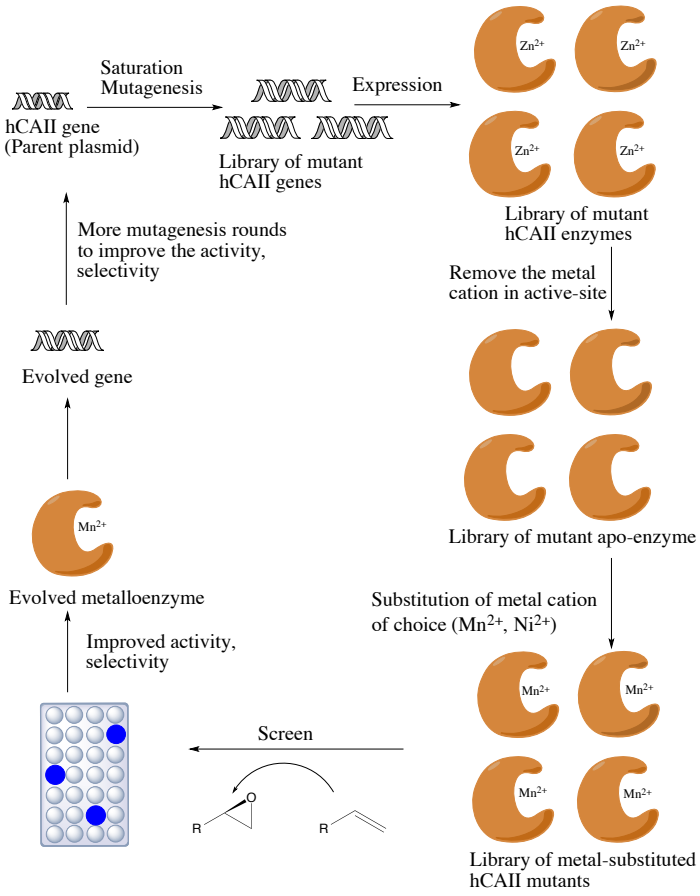


Figure 1: A schematic representation of directed evolution on human carbonic anhydrase

Site-directed mutagenesis is the introduction of a specific mutation into a defined position of the amino acid sequence of the enzyme. Polymerase chain reaction (PCR) is an important biotechnological tool used to create these precise mutations and to amplify these DNA sequences.[11] The short oligodeoxyribonucleotide fragments (primers) carry the specific mutation designed and these are complementary to the template DNA. Denaturation of the DNA template, annealing of mutagenic primers containing the desired mutation and the extension of the primers are the three basic steps of site-directed mutagenesis by PCR (Figure

2). Repeated cycles of these three steps rapidly amplify the new DNA sequence with the desired mutation at a pre-defined position.

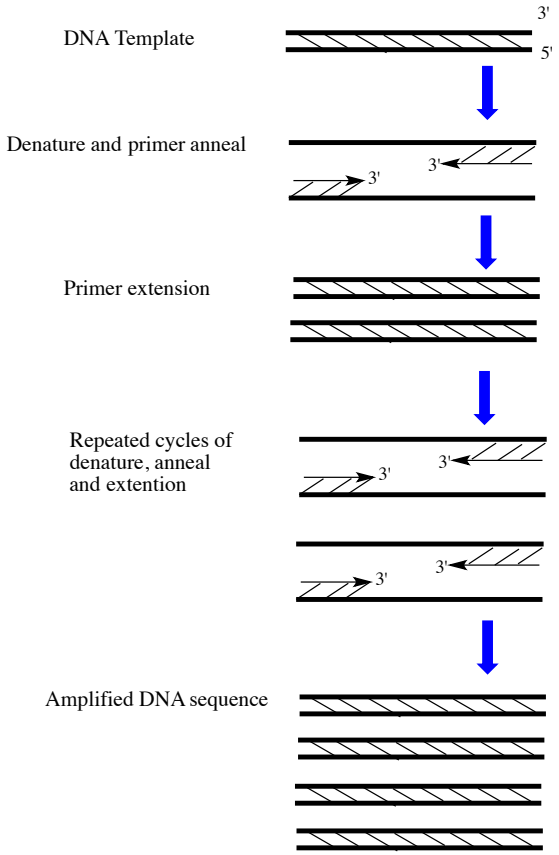


Figure 2: Schematic representation of site-directed mutagenesis by PCR

This chapter describes the generation of thermostable variant of human carbonic anhydrase (hCAII) to be used as an artificial metalloenzyme catalyst for the epoxidation of 4-chlorostyrene. The current literature precedents on this topic show that manganese-substituted carbonic anhydrase works as a hybrid catalyst to achieve good enantioselectivity for the epoxidation reaction, but yields of the epoxide product are poor.[12, 13] Therefore, site-directed mutagenesis was used to introduce thermostability to the wild-type of human

carbonic anhydrase in order to improve the catalytic activity of the artificial metalloenzyme generated. The thermostable variant was generated by the sequential introduction of six mutations into the amino acid sequence of the wild-type of human carbonic anhydrase.[14] PCR was used to amplify each mutant DNA sequence followed by expression and the isolation of mutant plasmids. Hydratase activity assays[14, 15] and esterase activity assays[15, 16] are the common methods used to determine the activity of the mutants of human carbonic anhydrase. In this study, esterase activity assay was used to study the thermostability.

Results and Discussion

1) Generation of a thermostable variant of human carbonic anhydrase

The generation of a thermostable variant of human carbonic anhydrase begins with the sequential introduction of six single-point mutations to the amino acid sequence of the wild-type enzyme.[14] QuikChange II XL site-directed mutagenesis kit, a double stranded circular DNA (parent plasmid) as the template DNA and a two stage polymerase reaction protocol (Wang protocol)[17] are used for the site-directed mutagenesis method. The primers that carry these specific mutations were designed and ordered from Integrated DNA Technologies (IDT) and a list of primers used is shown in the Table 1.

Table 1: List of primes used for the site-directed mutagenesis of human carbonic anhydrase

Primer	Type	Sequence
Ala65 Thr	Forward	5'(ATCCTCAACAATGGTCATACATTCAACGTGGAGTTTGAT)3'
	Reverse	3'(TAGGAGTTGTTACCAGTATGTAAGTTGCACCTCAAACTA)5'
Leu223 Ser	Forward	5'(GTCAGCAGCGAGCAGGTGAGCAAATTCCGTAAACTTAAC)3'
	Reverse	3'(CAGTCGTCGCTCGTCCACTCGTTTAAGGCATTTGAATTG)5'
Leu239 Pro	Forward	5'(GAGGGTGAACCCGAAGAACCAATGGTGGACAACTGGCGC)3'
	Reverse	3'(CTCCCACTTGGGCTTCTTGGTTACCACCTGTTGACCGCG)5'
Ala247 Thr	Forward	5'(GTGGACAACTGGCGCCCAACACAGCCACTGAAGAACAGG)3'
	Reverse	3'(CACCTGTTGACCGCGGGTTGTGTCGGTGACTTCTTGTCC)5'
Leu100 His	Forward	5'(CACTTTCACTGGGGTTCACATGATGGACAAGGTTCAGAG)3'
	Reverse	3'(GTGAAAGTGACCCCAAGTGTACTACCTGTTCCAAGTCTC)5'
Lys153 Asn	Forward	5'(TTGAAGGTTGGCAGCGCTAACCCGGGCCTTCAGAAAGTT)3'
	Reverse	3'(TTGAAGGTTGGCAGCGCTAACCCGGGCCTTCAGAAAGTT)5'

The site-directed mutagenesis method is used for the sequential introduction of mutations into defined positions of the wild-type enzyme. After the PCR amplification, template DNA (parent plasmid) is digested and the plasmid containing the resulting mutation is transformed into BL-21 cells, grown overnight on agar plates, and the plasmids are isolated and sequenced. The sequenced plasmid carrying the mutation is used as the template DNA for the second round of site-directed mutagenesis and the same procedure is done to introduce the second mutation. All six mutations mentioned in Table 1 are introduced by this

process and were introduced in the following order: Alanine 65 to Threonine, Leucine 239 to Proline, Leucine 223 to Serine, Alanine 247 to Threonine, Lysine 153 to Asparagine and Leucine 100 to Histidine. The DNA sequences of the six mutants generated are shown in Figures 3-8.

NNNNNNNNNNNTNNNNNNNANTATTTTNTTNANNTTAAGAAGGAGATATACCATGGC
CCATCACTGGGGGTACGGCAAACACAACGGACCTGAGCACTGGCATAAGGACTTCCC
CATTGCCAAGGGAGAGCGCCAGTCCCCTGTTGACATCGACACTCATACAGCCAAGTA
TGACCCTTCCCTGAAGCCCCTGTCTGTTTCCTATGATCAAGCAACTTCCCTGAGGAT
CCTCAACAATGGTCAT**ACA**TTCAACGTGGAGTTTGATGACTCTCAGGACAAAGCAGT
GCTCAAGGGAGGACCCCTGGATGGCACTTACAGATTGATTCAGTTTCACTTTCACTG
GGGTTCACTTGATGGACAAGGTTCAGAGCATACTGTGGATAAAAAGAAATATGCTGC
AGAACTTCACTTGGTTCACTGGAACACCAAATATGGGGATTTTGGGAAAGCTGTGCA
GCAACCTGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTGGCAGCGCTAAACC
GGGCCTTCAGAAAGTTGTTGATGTGCTGGATTCCATTAAAACAAAGGGCAAGAGTGC
TGACTTCACTAACTTCGATCCTCGTGGCCTCCTTCCTGAATCCCTGGATTACTGGAC
CTACCCAGGCTCACTGACCACCCCTCCTCTTCTGGAATGTGTGACCTGGATTGTGCT
CAAGGAACCCATCAGCGTCAGCAGCGAGCAGGTGTTGAAATTCCGTAAACTTAACTT
CAATGGGGAGGGTGAACCCGAAGAACTGATGGTGGACAACTGGCGCCCAGCTCAGCC
ACTGAAGAACAGGCAAATCAAAGCTTCCTTCAAATAAGATGGTCCCATAGTCTGTAT
CCAAATAATGAATCTTCGGGTGTTTCCCTTTAGCTAAGCACAGATCCGGCTGCTAAC
AAAGCCCGAAAGGAAGCTGAGTT

Figure 3: DNA sequence of the Ala65Thr mutant

NNNMNNNNANNNCNCTARATATTTTGTTTACTTTAAGAAGGAGATATACCATGGCCC
ATCACTGGGGGTACGGCAAACACAACGGACCTGAGCACTGGCATAAGGACTTCCCCA
TTGCCAAGGGAGAGCGCCAGTCCCCTGTTGACATCGACACTCATACAGCCAAGTATG
ACCCTTCCCTGAAGCCCCTGTCTGTTTCCTATGATCAAGCAACTTCCCTGAGGATCC
TCAACAATGGTCAT**ACA**TTCAACGTGGAGTTTGATGACTCTCAGGACAAAGCAGTGC
TCAAGGGAGGACCCCTGGATGGCACTTACAGATTGATTCAGTTTCACTTTCACTGGG
GTTCACTTGATGGACAAGGTTCAGAGCATACTGTGGATAAAAAGAAATATGCTGCAG
AACTTCACTTGGTTCACTGGAACACCAAATATGGGGATTTTGGGAAAGCTGTGCAGC
AACCTGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTGGCAGCGCTAAACCGG
GCCTTCAGAAAGTTGTTGATGTGCTGGATTCCATTAAAACAAAGGGCAAGAGTGCTG
ACTTCACTAACTTCGATCCTCGTGGCCTCCTTCCTGAATCCCTGGATTACTGGACCT
ACCCAGGCTCACTGACCACCCCTCCTCTTCTGGAATGTGTGACCTGGATTGTGCTCA
AGGAACCCATCAGCGTCAGCAGCGAGCAGGTGTTGAAATTCCGTAAACTTAACTTCA

ATGGGGAGGGTGAACCCGAAGAA**CCA**ATGGTGGACAACTGGCGCCCAGCTCAGCCAC
TGAAGAACAGGCAAATCAAAGCTTCCTTCAAATAAGATGGTCCCATAGTCTGTATCC
AAATAATGAATCTTCGGGTGTTTCCCTTTAGCTAAGCACAGATCCGGCTGCTAACAA
AGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC
CCTTGGGGGCCTCTAAACGGGGTCTTGAGGGGGTTTTTTGCTGAA

Figure 4: DNA sequence of the (Ala65Thr + Leu239Pro) mutant

NNNMNNNANTCNTCTANANATTTTGTTTACTTTAAGAAGGAGATATACCATGGCCCA
TCACTGGGGGTACGGCAAACACAACGGACCTGAGCACTGGCATAAGGACTTCCCCAT
TGCCAAGGGAGAGCGCCAGTCCCCTGTTGACATCGACACTCATACAGCCAAGTATGA
CCCTTCCCTGAAGCCCCTGTCTGTTTCCTATGATCAAGCAACTTCCCTGAGGATCCT
CAACAATGGTCAT**ACA**TTCAACGTGGAGTTTGATGACTCTCAGGACAAAGCAGTGCT
CAAGGGAGGACCCCTGGATGGCACTTACAGATTGATTCAGTTTCACTTTCACTGGGG
TTCACTTGATGGACAAGGTTCAGAGCATACTGTGGATAAAAAGAAATATGCTGCAGA
ACTTCACTTGGTTCACTGGAACACCAAATATGGGGATTTTGGGAAAGCTGTGCAGCA
ACCTGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTGGCAGCGCTAAACCGGG
CCTTCAGAAAGTTGTTGATGTGCTGGATTCCATTAAAACAAAGGGCAAGAGTGCTGA
CTTCACTAACTTCGATCCTCGTGGCCTCCTTCCTGAATCCCTGGATTACTGGACCTA
CCCAGGCTCACTGACCACCCCTCCTCTTCTGGAATGTGTGACCTGGATTGTGCTCAA
GGAACCCATCAGCGTCAGCAGCGAGCAGGTG**TCC**AAATTCCGTAAACTTAACTTCAA
TGGGGAGGGTGAACCCGAAGAA**CCA**ATGGTGGACAACTGGCGCCCAGCTCAGCCACT
GAAGAACAGGCAAATCAAAGCTTCCTTCAAATAAGATGGTCCCATAGTCTGTATCCA
AATAATGAATCTTCGGGTGTTTCCCTTTAGCTAAGCACAGATCCGGCTGCTAACAAA
GCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCC
CCTTGGGGGCCTCTAAACGGG

Figure 5: DNA sequence of the (Ala65Thr + Leu239Pro + Leu223Ser) mutant

KTTNTCCARAANTCTCTGATATTTTGTTTACTTTAAGAAGGAGATATACCATGGCCC
ATCACTGGGGGTACGGCAAACACAACGGACCTGAGCACTGGCATAAGGACTTCCCCA
TTGCCAAGGGAGAGCGCCAGTCCCCTGTTGACATCGACACTCATACAGCCAAGTATG
ACCCTTCCCTGAAGCCCCTGTCTGTTTCCTATGATCAAGCAACTTCCCTGAGGATCC
TCAACAATGGTCAT**ACA**TTCAACGTGGAGTTTGATGACTCTCAGGACAAAGCAGTGC
TCAAGGGAGGACCCCTGGATGGCACTTACAGATTGATTCAGTTTCACTTTCACTGGG
GTTCACTTGATGGACAAGGTTCAGAGCATACTGTGGATAAAAAGAAATATGCTGCAG
AACTTCACTTGGTTCACTGGAACACCAAATATGGGGATTTTGGGAAAGCTGTGCAGC
AACCTGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTGGCAGCGCTAAACCGG
GCCTTCAGAAAGTTGTTGATGTGCTGGATTCCATTAAAACAAAGGGCAAGAGTGCTG
ACTTCACTAACTTCGATCCTCGTGGCCTCCTTCCTGAATCCCTGGATTACTGGACCT
ACCCAGGCTCACTGACCACCCCTCCTCTTCTGGAATGTGTGACCTGGATTGTGCTCA

AGGAACCCATCAGCGTCAGCAGCGAGCAGGTG**TCC**AAATTCCGTAAACTTAACTTCA
ATGGGGAGGGTGAACCCGAAGAA**CCA**ATGGTGGACAACTGGCGCCCA**ACA**CAGCCAC
TGAAGAACAGGCAAATCAAAGCTTCCTTCAAATAAGATGGTCCCATAGTCTGTATCC
AAATAATGAATCTTCGGGTGTTTCCCTTTAGCTAAGCACAGATCCGGCTGCTAACAA
AGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACC
CCCTTGGGGCCTCTAAACGGGGTCTTGAAGGGGTTTTTTGCTGAAANGGAGGAACCT
ATATCCGGAAGGGGAATTCCCSGCGCCGCGATGCCCCTTTCGGTCTTCGAATAAAAT
ACCTKGTGACGGAAGRATCMMYTTCGCAGAAATAAATAAAATCCTGGGGGTCCCTGG
TTGAWWACCGGGGAARCCCCTGGGGCCAACTTTTTGGGCGAAAAATGGAAAMCGTTT
GATTCGGN

Figure 6: DNA sequence of the (Ala65Thr + Leu239Pro + Leu223Ser + Ala247Thr) mutant

NNNMNNANNTCTCTARATATTTTGTTTACTTTAAGAAGGAGATATACCATGGCCCAT
CACTGGGGGTACGGCAAACACAACGGACCTGAGCACTGGCATAAGGACTTCCCCATT
GCCAAGGGAGAGCGCCAGTCCCCTGTTGACATCGACACTCATACAGCCAAGTATGAC
CCTTCCCTGAAGCCCCTGTCTGTTTCCTATGATCAAGCAACTTCCCTGAGGATCCTC
AACAATGGTCAT**ACA**TTCAACGTGGAGTTTGATGACTCTCAGGACAAAGCAGTGCTC
AAGGGAGGACCCCTGGATGGCACTTACAGATTGATTCAGTTTCACTTTCACTGGGGT
TCACTTGATGGACAAGGTTCAGAGCATACTGTGGATAAAAAGAAATATGCTGCAGAA
CTTCACTTGGTTCACTGGAACACCAAATATGGGGATTTTGGGAAAGCTGTGCAGCAA
CCTGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTGGCAGCGCT**AAC**CCGGGC
CTTCAGAAAGTTGTTGATGTGCTGGATTCCATTAAAACAAAGGGCAAGAGTGCTGAC
TTCACTAACTTCGATCCTCGTGGCCTCCTTCCTGAATCCCTGGATTACTGGACCTAC
CCAGGCTCACTGACCACCCCTCCTCTTCTGGAATGTGTGACCTGGATTGTGCTCAAG
GAACCCATCAGCGTCAGCAGCGAGCAGGTG**TCC**AAATTCCGTAAACTTAACTTCAAT
GGGGAGGGTGAACCCGAAGAA**CCA**ATGGTGGACAACTGGCGCCCA**ACA**CAGCCACTG
AAGAACAGGCAAATCAAAGCTTCCTTCAAATAAGATGGTCCCATAGTCTGTATCCAA
ATAATGAATCTTCGGGTGTTTCCCTTTAGCTAAGCACAGATCCGGCTGCTAACAAAG
CCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAACAATAACTAGCATAACCCC
TTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCCG
GATGGGAATTCCCCGCGCGCGATGCCCTTTCGTCTTCGAATAAATACCTGGTGACGG
AAGATCMCTTCGCAAAATAAATAAATCCTGGGTGTCCCKGTTGAAACCGGGGAAGCC
CTGGGGCCAACYTTTTGGSCRAAAATGAAAMMGTTTGATCGGSMMCGTAAAAAGGTT
CCAACTTTTCACCNATAATGRAAATAAGAWTCMCTAMCCGGGGSSGN

Figure 7: DNA sequence of the (Ala65Thr + Leu239Pro + Leu223Ser + Ala247Thr + Lys153Asn) mutant

NNNNGGANTNCNCTAGAATATTTTGTTTACTTTAAGAAGGAGATATACCATGGCCCA
TCACTGGGGGTACGGCAAACACAACGGACCTGAGCACTGGCATAAGGACTTCCCCAT
TGCCAAGGGAGAGCGCCAGTCCCCTGTTGACATCGACACTCATACAGCCAAGTATGA
CCCTTCCCTGAAGCCCCTGTCTGTTTCCTATGATCAAGCAACTTCCCTGAGGATCCT
CAACAATGGTCAT**ACA**TTCAACGTGGAGTTTGATGACTCTCAGGACAAAGCAGTGCT
CAAGGGAGGACCCCTGGATGGCACTTACAGATTGATTCAGTTTCACTTTCACTGGGG
TTCA**CAT**GATGGACAAGGTTCAGAGCATACTGTGGATAAAAAGAAATATGCTGCAGA
ACTTCACTTGGTTCACTGGAACACCAAATATGGGGATTTTGGGAAAGCTGTGCAGCA
ACCTGATGGACTGGCCGTTCTAGGTATTTTTTTGAAGGTTGGCAGCGCT**AAC**CCGGG
CCTTCAGAAAGTTGTTGATGTGCTGGATTCCATTAAAACAAAGGGCAAGAGTGCTGA
CTTCACTAACTTCGATCCTCGTGGCCTCCTTCCTGAATCCCTGGATTACTGGACCTA
CCCAGGCTCACTGACCACCCCTCCTCTTCTGGAATGTGTGACCTGGATTGTGCTCAA
GGAACCCATCAGCGTCAGCAGCGAGCAGGTG**TCC**AAATTCCGTAAACTTAACTTCAA
TGGGGAGGGTGAACCCGAAGAA**CCA**ATGGTGGACAACTGGCGCCCA**ACA**CAGCCACT
GAAGAACAGGCAAATCAAAGCTTCCTTCAAATAAGATGGTCCCATAGTCTGTATCCA
AATAATGAATCTTCGGGTGTTTCCCTTTAGCTAAGCACAGATCCGGCTGCTAACAAA
GCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCC
CTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGGAGGAACTATATCC
GGATGGGAATTCCCCGCGCGCGATGCCCTTTCGTCTTCGAATAAATACCTGTGACGG
AAGATCMCTTCGCARAAWWAAATAAATCCTGGGKGTCCCTGTTGAWACCGGGAAASC
CCTG

Figure 8: DNA sequence of the (Ala65Thr + Leu239Pro + Leu223Ser + Ala247Thr + Lys153Asn + Leu100His) the thermostable mutant

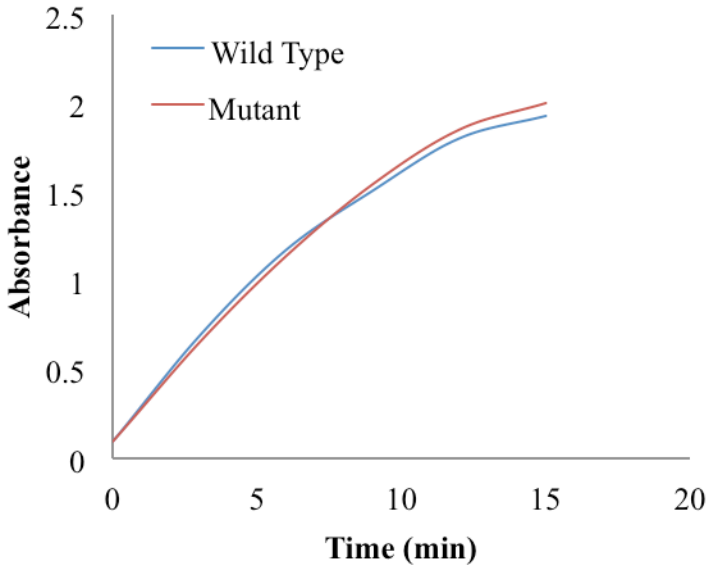
2) Determination of the thermostability of the human carbonic anhydrase mutant

The thermostability was studied using an esterase activity assay and the activity of the thermostable mutant was compared to the wild-type enzyme to determine the level of thermostability introduced to the mutant generated by site-directed mutagenesis.[17] The ester hydrolysis activity of human carbonic anhydrase was studied by monitoring the change in the UV-Vis absorbance (at 348 nm) of the hydrolysis of 4-nitrophenylacetate to 4-nitrophenylate ion in the presence of the human carbonic anhydrase. The purified wild-type and mutant hCAII[Zn] were each used for the esterase activity assay. A range of temperatures (50, 55, 60, 65, 70 ^{o}C) was selected and enzyme solutions used for the assay were incubated at each

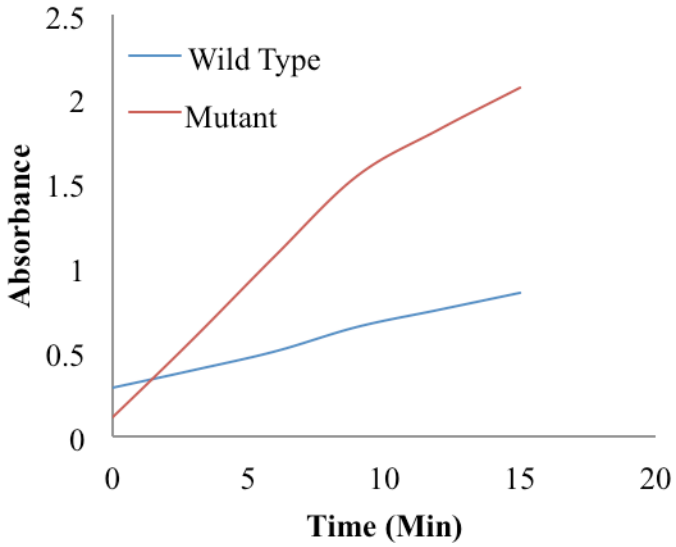
temperature for 2 hours prior to the assay. The percentage esterase activity is determined by the following equation.

$$\% \text{ Esterase activity} = \frac{\text{(Activity after treatment)}}{\text{(Activity before treatment)}} \times 100$$

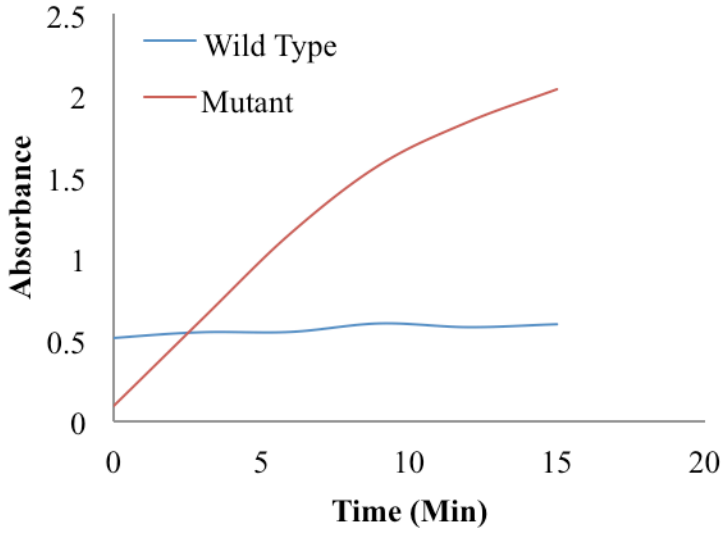
A) No incubation



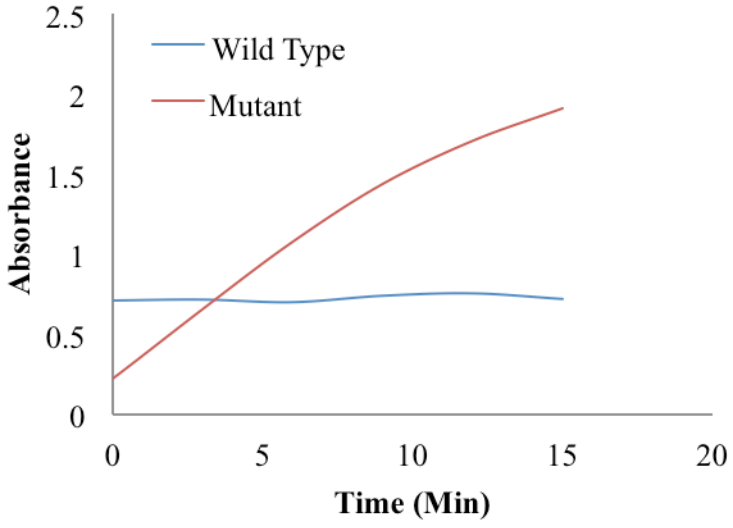
B) Incubation at 50 ^{o}C



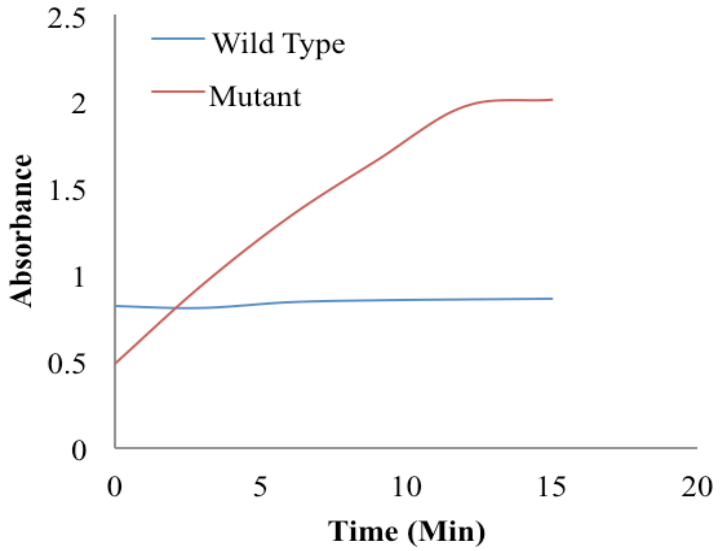
C) Incubation at 55 °C



D) Incubation at 60 °C



E) Incubation at 65 °C



F) Incubation at 70 °C

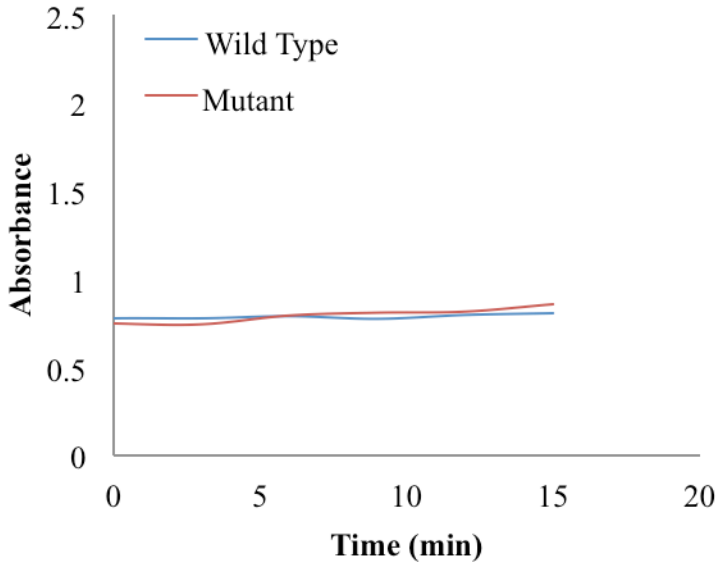


Figure 9: Esterase activity of wild type and thermostable mutant at elevated temperatures

Table 2: % Esterase activity of wild-type and mutant human carbonic anhydrase[14]

Temperature (°C)	% Esterase activity (Experimental)		% Esterase activity (Literature report)	
	Wild-Type	Mutant	Wild-Type	Mutant
50	31	100	Not reported	Not reported
55	5	100	12	100
60	1	94	0	100
65	2	79	0	88
70	1	6	0	0

3) Levels of protein expression of wild-type, mutants and thermostable mutant

Wild-type and mutated plasmid DNA were used for protein expressions to generate wild-type and mutant hCAII[Zn]. Separation and purification carried out obtain pure hCAII[Zn]. Table 3 summarizes the amounts of pure wild-type and mutant apo-hCAII obtained from each protein expression. Apo-enzyme was generated by the addition of a chelating agent 2,6-pyridinedicarbxylate followed by a dialysis with BES buffer until the chelating agent was completely removed.

Table 3: Protein expression levels of wild-type and mutant

Entry	Mutant hCAII[Mn]	Amount of apo-hCAII (mg/L of culture)
1	Wild-type	189.0[a]
2	Ala65Thr	184.0
3	Leu239Pro	195.0
4	Leu223Ser	205.0
5	Ala247Thr	71.0
6	Ala65Thr+Leu239Pro	66.0
7	Ala65Thr+Ala247Thr	81.0
8	Ala65Thr+Leu239Pro+Ala247Thr	160.0
9	Ala65Thr+Leu239Pro+Leu223Ser	136.0
10	Ala65Thr+Leu239Pro+Leu223Ser+Ala247Thr	77.0
11	Ala65Thr+Leu239Pro+Leu223Ser+Ala247Thr+ Leu100His	75.0
12	Ala65Thr+Leu239Pro+Leu223Ser+Ala247Thr+ Lys153Asn	108.0
13	Ala65Thr+Leu239Pro+Leu223Ser+Ala247Thr+ Leu100His+Lys153Asn (Thermostable mutant)	173.0[b]

a- average of 13 protein expressions
b- average of 6 protein expressions
all others single protein expression

Conclusion

A thermostable variant of human carbonic anhydrase is generated successfully using site-directed mutagenesis. This thermostable mutant is a combination of six single-point mutations in defined positions namely Alanine 65 to Threonine, Leucine 100 to Histidine, Lysine 153 to Asparagine, Leucine 223 to Serine, Leucine 239 to Proline and Alanine 247 to Threonine. Protein expressions are successfully carried out to obtain wild-type and mutant hCAII[Zn]. The wild-type and thermostable mutant show similar levels of protein expressions. The thermostability of the mutant is confirmed by conducting an esterase activity assay compared to the wild-type of human carbonic anhydrase. The mutant generated shows a remarkably high stability at elevated temperatures (65 ^{o}C) compared to the wild-type enzyme, which is not stable at temperatures above 55 ^{o}C. This thermostable variant is a good candidate for the generation of an artificial metalloenzymes since it has improved stability at elevated temperatures, which can lead to improved catalytic activity.

Experimental

Polymerase chain reaction for site-directed mutagenesis and transformation

The reaction buffer, dNTP, DMSO and *Pfu-Ultra* High Fidelity DNA polymerase were used from a QuikChange II XL site-directed mutagenesis kit for PCR. Polymerase chain reaction mixtures were made in two separate PCR tubes (with forward and reverse primers) and each contained; 10X reaction buffer (2.5 μL), template DNA (93 ng/ μL, 1.07 μL), primer (100 ng/ μl, 0.6 μL), dNTP (2 mM, 2.5 μL) dd H_2O (16.9 μL), DMSO (0.5 μL) and *Pfu-Ultra* High Fidelity DNA polymerase (0.5 μL). Table 3 shows two-stage thermal cycling used for the reaction.

Table 4: Two-stage thermal cycling

Stage	Step number	Temperature (^{o}C)	Time
1	1	95	2 min 30sec
	2	55	1 min
	3	68	10 min
2	1	95	30 sec
	2	55	1 min
	3	68	10 min

The thermal cycling began with stage 1 in the order of step 1, step 2, step 3 and then step 2 and 3 were repeated as a cycle (step 2 followed by step 3) for five times. Once stage 1 was completed forward and reverse reaction mixtures were combined and mixed properly in a new PCR tube and subjected to stage 2. Stage 2 was carried out as a cycle with step 1, step 2, step 3 and repeated for sixteen times. Then the reaction tubes were placed on ice for 2 minutes followed by the addition of *Dpn*-1 restriction enzyme (1 μL) to the reaction mixture to digest the template DNA. Then the reaction mixture was incubated at 37 ^{o}C for 1 hour. Next, a microcentrifuge tube containing a pre-frozen BL-21 cell aliquot (50 μL) was thawed on ice and transferred into a pre-chilled 14 mL BD Falcon polypropylene round bottom tube. Then 2 μL of β-mercaptoethanol mix was added to cells, mixed properly and incubated on ice for 10 minutes swirling the mixture gently every 2 minutes. The 5 μL of *Dpn*-1 treated PCR product was added to cell mixture, mixed thoroughly and incubated on ice for another

30 minutes. The mixture was heat shocked at 42 ^{o}C for 30 seconds in water bath and then incubated on ice for 2 minutes. The cell mixture was rescued with SOC (250 μL of SOB, 5 μL of 1M filter sterilized glucose, which was pre-heated at 42 ^{o}C for 2 minutes) and placed in a shaker for 1 hour (37 ^{o}C, 250 rpm). Finally the culture was plated on pre-heated (1 hour at 37 ^{o}C) LB-ampicillin agar plates (250 μL of culture on one agar plate, no dilution with dd H_2O) and incubated for 16 hours at 37 ^{o}C in an incubator. Sterile conditions were maintained throughout the procedure.

Isolation of plasmids for DNA sequencing

QIAprep Spin Miniprep kit (50) purchased from QIAGEN was used for the following procedure. A single colony from an agar plate (grown for 16 hours) was picked using an autoclaved toothpick and introduced to a mixture of LB broth (5 mL) and ampicillin (100 mg/mL, 5 μL) in a 14 mL BD Falcon polypropylene round bottom tube and placed in a shaker for incubation for 16 hours (37 ^{o}C, 250 rpm). Next, cells were pelleted by centrifuging at 5000 rpm for 3 minutes at room temperature. The cell pellet was re-suspended in buffer P1 (250 μL) and transferred to a microcentrifuge tube. Then buffer P2 (250 μL) was added and mixed thoroughly by inverting the tube 4-6 times until the solution become clear. Next, buffer N3 (350 μL) was added and mixed immediately by inverting the tube 4-6 times and centrifuged at 13000 rpm for 10 minutes. Then the supernatant was transferred to a QIAprep spin column, centrifuged at 13000 rpm for 1 minute and the flow through was discarded. The spin column was washed with buffer PB (500 μL), centrifuged at 13000 rpm for 1 minute and the flow through was discarded. Next the spin column was washed with buffer PE (750 μL), centrifuged at 13000 rpm for 1 minute and the flow through was discarded. The spin

column was centrifuged at 13000 rpm for another 1 minute to remove any residual washing buffers. Finally the QIAprep column was placed on a clean 1.5 mL microcentrifuge tube followed by the addition of buffer EB (30 μL) to the center of the spin column and it was left to stand for 2-3 minutes before centrifugation at 13000 rpm for 1 minute to elute the plasmid DNA. All of these DNA samples were sequenced by the DNA facility, Office of Biotechnology at Iowa State University.

Transformation of BL-21 chemically competent cells

First, a microcentrifuge tube containing a pre-frozen BL-21 cell aliquot (50 μL) was thawed on ice and transferred into a pre-chilled 14 mL BD Falcon polypropylene round-bottom tube. Then, wild-type or mutant plasmid containing the DNA encoding hCAII (300 ng) was added to the cells, mixed thoroughly and left on ice for 30 minutes. Next, a heat shock was given to the mixture at 42 ^{o}C for 30 seconds in a water bath and incubated on ice for 2 minutes. After that the cell mixture was rescued with SOC (250 μL of SOB, 5 μL of 1M filter sterilized glucose, which was pre-heated at 42 ^{o}C for 2 minutes) and placed in a shaker for 1 hour (37 ^{o}C, 250 rpm). Then, culture was plated on pre-heated (1 hour at 37 ^{o}C) LB-ampicillin agar plates (90:10 μL of dd H_2O: culture) and incubated for 16 hours at 37 ^{o}C in an incubator. Next, a single colony from the agar plate was picked up using an autoclaved toothpick and introduced to a mixture of LB broth (5 mL) and ampicillin (100 mg/ mL, 5 μL) in a 14 mL BD Falcon polypropylene round-bottom tube and placed in a shaker for incubation for 16 hours (37 ^{o}C, 250 rpm) overnight.

Protein expression

First, one overnight culture was added to each shaker flask with 490 mL of autoclaved induction media (10.0 g of bacto-tryptone, 5.0 g of yeast extract, 2.34 g of NaCl, 30 mL of 6XM9 salt, 0.333 mL of 0.3 M $ZnSO_4$ diluted to 490 mL), ampicillin (100 mg/ mL, 0.5 mL) and 20% filter-sterilized glucose (10.0 mL). Then shaker flasks were placed in the shaker (37 ^oC, 250 rpm) and allowed to grow until OD_{600}~ 1.0. Once the OD_{600} value reached that number protein expression was induced by adding $ZnSO_4$ (0.3 M, 0.75 mL) and IPTG (100 mM, 1.25 mL) and shaking was continued for another 6 hours. After that inhibitors PMSF (1 mg/ mL, 2.0 mL) and TAME (1 mg/ mL, 1 mL) were added and cells were pelleted by centrifuging at 12000 rpm for 45 minutes at 4 ^oC. Next the cell pellet from a 1 L culture was re-suspended in 200 mL of lysis buffer with dithiothreitol (50.0 mL of 1M tris-sulfate, 50.0 mL of 1 M NaCl, 40.0 mL of 0.25 M EDTA, 0.66 mL of 0.3 M $ZnSO_4$, 1.0 mL of 10 mg/ mL PMSF, 1.0 mL of 1 mg/ mL TAME and 0.1542 g of dithiothreitol diluted to 1 L, pH = 8.0), lysozyme was added (1 mg/ mL of buffer), and the mixture was placed inside the shaker for 1 hour (37 ^oC, 250 rpm). After that the cell-buffer mixture was centrifuged (5000 rpm, 75 minutes, 4 ^oC), the supernatant was collected, 10% streptomycin sulfate (1/10 volume of the supernatant) was added and the solution was stirred at 4 ^oC for 15 minutes. Next the supernatant was centrifuged at 5000 rpm for 60 minutes at 4 ^oC, and the clear supernatant containing the crude hCAII[Zn] was collected without any cell materials which contains the crude hCAII[Zn]. Then the supernatant was transferred to centrifugal dialysis tubes (MWCO 10 kDa) and centrifuged with tris-sulfate buffer (pH = 8.0, 10 mM) five times each for 50 minutes.

Protein purification

First, DEAE-sephacel gel (30.0 mL per 1 L culture) was equilibrated with 2 column volumes of 1 M tris-sulfate (pH = 8.0, 60 mL) followed by 5 column volumes of 10 mM tris-sulfate (pH = 8.0, 150 mL). Then the gel was poured in to an flask with supernatant collected at the end of the protein expression (crude hCAII[Zn]) and equilibrated at 4 ^{o}C for 30 minutes. Next the mixture was transferred into a fritted filter funnel and washed with 1 column volume of 10 mM tris-sulfate (pH = 8.0, 30 mL) and 1 column volume of 20 mM tris-sulfate (pH = 8.0, 30 mL) and fractions were collected into centrifuge tubes containing purified hCAII[Zn]. SDS-PAGE technique was to determine the purity of the hCAII[Zn] and a clear band was observed at ~29 kDa with no other major bands.

Esterase activity assay

The thermostability of human carbonic anhydrase[Zn] wild-type and thermostable mutant were determined by the esterase activity assay method. During the assay *p*-nitrophenylacetate was hydrolyzed to *p*-nitrophenylate ion and the change in the absorbance of the assay solution was measured at 348 nm using NanoDrop 2000/2000c spectrophotometer over a time period of 15 minutes. Enzymatic assay solution was prepared in a cuvette by adding tris-sulfate buffer (50 mM, 1.4 mL, pH = 8.0), *p*-nitrophenylacetate (3 mM in acetone, 1.0 mL), dd H_2O (0.5 mL) and hCAII[Zn] solution (0.1 mM, 0.1 mL). The solution was mixed properly immediately after the addition of the enzyme solution and an initial absorbance was measured. The absorbance was then monitored over a 15 minute time period at 3-minute time intervals. Two separate assay solutions were monitored for changes in absorbance, one with the wild-type enzyme and another one with the thermostable mutant.

A second set of assay solutions were made using the same reagents, the same volumes, but with hCAII[Zn] wild-type and thermostable mutant solutions incubated at 50 ^{o}C for 2 hours in a water bath prior to the assay solution preparation. The absorbance was monitored over a 15 minute time period at 3-minute time intervals. A control was made in a cuvette by adding tris-sulfate buffer (50 mM, 1.4 mL, pH = 8.0), *p*-nitrophenylacetate (3 mM in acetone, 1.0 mL) and dd H_2O (0.6 mL) to observe the non-catalyzed *p*-nitrophenylacetate hydrolysis in this system and to subtract this effect to report the hydrolysis driven by the enzyme. This experiment was done to cover a range of temperatures from 50 – 70 ^{o}C in 5 ^{o}C temperature increments.

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CHAPTER 3

ENANTIOSELECTIVE EPOXIDATION CATALYZED BY MANGANESE-SUBSTITUTED HUMAN CARBONIC ANHDRASE

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Abstract

A thermostable human carbonic anhydrase and wild-type of human carbonic anhydrase were used to generate manganese-substituted human carbonic anhydrase using the dative anchoring strategy. The manganese-substituted human carbonic anhydrase (hCAII[Mn]) was used as a catalyst for enantioselective epoxidation. The identification of reaction conditions, impact of the oxidant on the catalytic activity, enzyme stability, and selectivity of both hCAII[Mn] catalysts were studied. The hypothesis was that the thermostable mutant of hCAII[Mn] might overcome the problem of catalyst degradation under oxidative reaction conditions leading to improved yields and enantioselectivities compared to the wild-type hCAII[Mn].

Introduction

Artificial metalloenzymes are an important class of hybrid catalysts that has been developed and used for enantioselective, regioselective and chemoselective organic transformations over the past couple of decades. Artificial metalloenzymes are widely studied as catalyst systems for hydrogenation[1, 2, 3, 4] hydroformylation[5, 6, 7] and for Diels-Alder reaction[8, 9] and have been used as catalysts for sulfoxidation[10] epoxidation of olefins[11,

[12] and oxidation of alcohols[13, 14] although these reactions remain underdeveloped as compared to variants that use homogeneous catalyst systems.

The enantioselective epoxidation of alkenes is an important organic transformation in synthetic chemistry. Sharpless reported the first example of asymmetric epoxidation of allylic alcohols in the presence of a titanium catalyst (Figure 1).[15] Since the initial report, many examples of metal-catalyzed asymmetric epoxidation of alkenes have been developed.[16, 17, 18, 19] Enantioselective epoxidation can also be achieved using organocatalysts such as chiral sulfonium ylides,[20] chiral dioxaranes [21] or chiral hydroperoxides.[22]

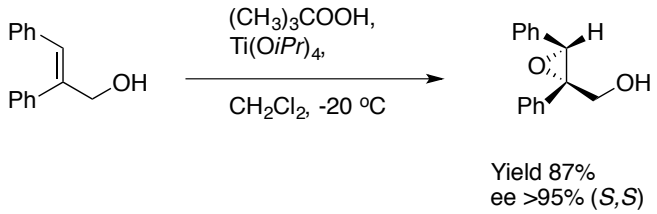

Figure 1: Metal-catalyzed asymmetric epoxidation (Sharpless epoxidation of allylic alcohols)[15]

Although these methods are effective, artificial metalloenzymes are a potential class of catalysts for enantioselective epoxidation that would eliminate the need for small-molecule, chiral ligands. The metal center can catalyze the desired epoxidation reaction and the active site of the enzyme can be utilized as the chiral pocket to impart enantioselectivity. Manganese-substituted carbonic anhydrase has been used as a catalyst for enantioselective epoxidation of alkenes and promising selectivities have being achieved, but the yields of these reactions are poor. There are only two literature reports of this reaction type; one published by Kazlauskas[11] in 2006 the other by and Soumillion[12] in 2006. Kazlauskas' report

shows that reactions using an artificial metalloenzyme for epoxidation inherently result in degradation of the catalyst over the course of the reaction.[11] Therefore the need for the generation of artificial metalloenzyme catalysts with improved catalytic activity and improved oxidative stability to prevent oxidative degradation is the largest challenge associated with this type of reactions. In this chapter, the use of wild-type and a thermostable manganese-substituted human carbonic anhydrase as catalysts for enantioselective epoxidation of 4-chlorostyrene is described (Figure 2). Also, the catalytic activity of the thermostable hCAII[Mn] mutant is evaluated under epoxidation reaction conditions to determine if the problem of oxidative degradation can be overcome.

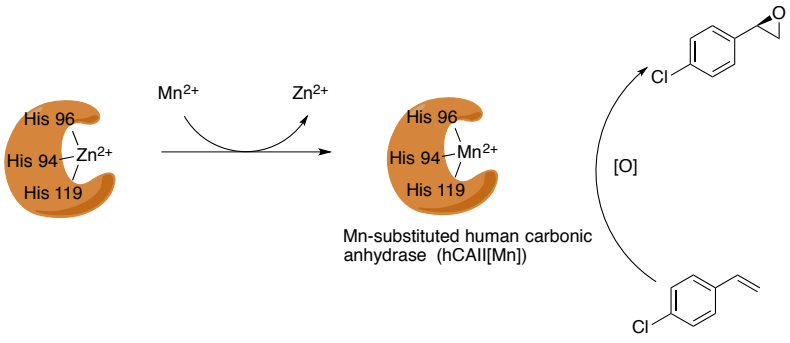

Figure 2: Enantioselective epoxidation of 4-chlorostyrene catalyzed by manganese-substituted human carbonic anhydrase

Results and Discussion

This section describes the generation of wild-type and thermostable variants of manganese-substituted human carbonic anhydrase (hCAII[Mn]) that functions as an enantioselective olefin epoxidation catalyst. The identification of reaction conditions and the

impact of the oxidant on the catalytic activity, stability, and selectivity of hCAII[Mn] will also be discussed.

Enantioselective epoxidation is carried out using 4-chlorostyrene as the olefin and hCAII[Mn]as the catalyst in the presence of BES buffer (*N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid), potassium bicarbonate and hydrogen peroxide. This reaction was optimized relative to the loading of hCAII[Mn] catalyst, potassium bicarbonate, hydrogen peroxide, type of the buffer and the pH. In an early evaluation of reaction conditions, the best conditions observed generated the epoxide product in 7% yield and 74% ee with the wild-type of hCAII[Mn] as the catalyst (Figure 3). The use of other buffers like phosphate buffer or a mixture of BES and phosphate buffer did not generate the product with good enantioselectivity.

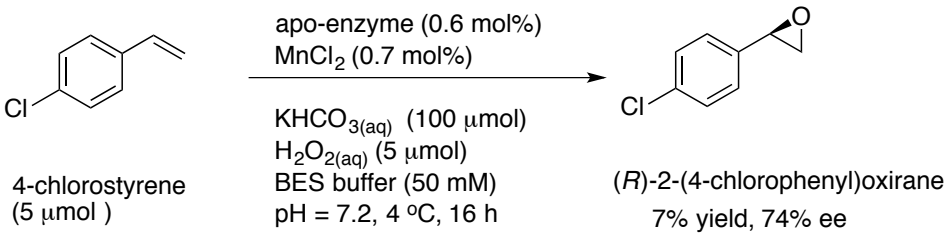

Figure 3: Enantioselective epoxidation of 4-chlorostyrene with wild-type hCAII[Mn]

All the mutants generated during the process of making the thermostable mutant are transformed for protein expressions and pure mutant carrying hCAII[Zn] are obtained followed by the generation of mutant hCAII[Mn]. Epoxidation of 4-chlorostyrene is carried

out using these mutant hCAII[Mn] catalysts under the optimized conditions shown in Figure 3 (Table 1).

Table 1: Enantioselective epoxidation of 4-chlorostyrene with mutant hCAII[Mn] catalyst

Entry	Mutant hCAII[Mn]	% Yield	% ee
1	Ala65Thr	3	59
2	Leu239Pro	7	72
3	Leu223Ser	6	73
4	Ala247Thr	3	76
5	Ala65Thr+Leu239Pro	5	65
6	Ala65Thr+Ala247Thr	2	71
7	Ala65Thr+Leu239Pro+Ala247Thr	3	72
8	Ala65Thr+Leu239Pro+Leu223Ser	1	72
9	Ala65Thr+Leu239Pro+Leu223Ser+Ala247Thr	2	71
10	Ala65Thr+Leu239Pro+Leu223Ser+Ala247Thr+ Leu100His	2	70
11	Ala65Thr+Leu239Pro+Leu223Ser+Ala247Thr+ Lys153Asn	5	73
12	Ala65Thr+Leu239Pro+Leu223Ser+Ala247Thr+ Leu100His+Lys153Asn (Thermostable mutant)	3	67
13	Wild-type	7	74

The yields and enantioselectivities reported for thermostable mutant are not improved compared to reaction conducted with the wild-type hCAII[Zn]. These mutant artificial metalloenzyme catalysts showed poorer catalytic activity upon the increasing the number of mutations compared to the wild-type catalyst (Table1). Leucine 239 to Proline and Leucine 223 to Serine mutant catalysts showed similar catalytic activity as wild-type (Table 1, entries 2 and 3) and these single-point mutants would be better candidates for site-saturation mutagenesis for further activity improvements.

Further optimization of the reaction conditions was required to improve the yield of the reaction. Reaction optimizations were conducted using the wild-type hCAII[Mn] and thermostable hCAII[Mn] side-by-side under the same conditions in order to compare the catalytic activity of the two enzymes. The following variables were evaluated under conditions shown in Figure 3 with the following changes; 1) reduced H_2O_2 amounts gave further reduced yields (4.0-1.0 μmol), 2) increased $KHCO_3$ amounts further reduced the yields (100-140 μmol), 3) increased catalyst loadings did not make any improvement in yields (2.0-10.0 mol%), 4) increased H_2O_2 amounts did not show an improvement in yields (6.0-8.0 μmol).

The epoxidation reactions were then carried out at higher catalyst loadings (10 mol% apo-enzyme) to ensure that small changes in yields and enantioselectivities could be properly observed, since varying reaction conditions did not initially appear to have an effect. An increased amount of H_2O_2 (8 μmol) at higher catalyst loading led to an improvement in yield with both the wild-type and mutant enzymes (Figure 4).

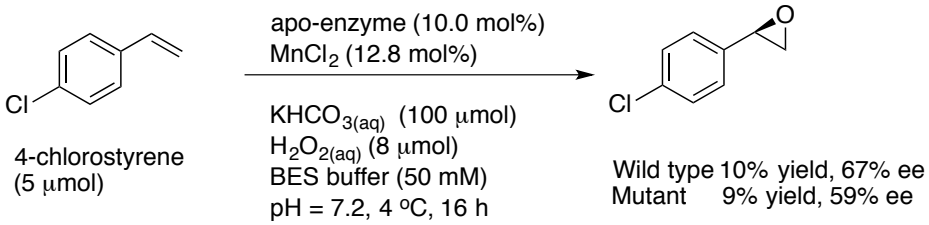

Figure 4: Enantioselective epoxidation with 10 mol% catalyst loading

This promising observation led us to study the effect of reduced $KHCO_3$ amounts under otherwise identical reaction conditions, since previous observations showed that the increased $KHCO_3$ led to reduced yields in this reaction (Figure 2, Table 2).

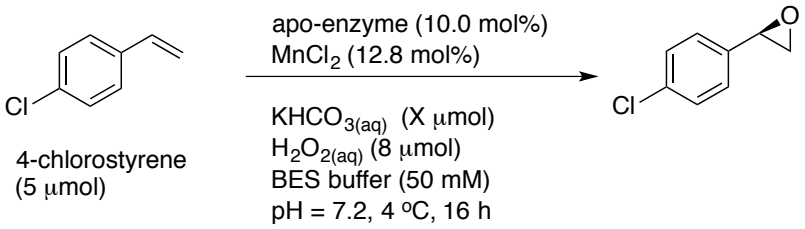

Figure 5: Effect of $KHCO_3$ on the enantioselective epoxidation of 4-chlorostyrene

Table 2: Effect of $KHCO_3$ on the enantioselective epoxidation of 4-chlorostyrene

Entry	**$KHCO_3$ (X μmol)**	**Wild Type**		**Thermostable Mutant**	
		%Yield	**%ee**	**%Yield**	**%ee**
1	60	11	70	9	62
2	80	11	68	10	62
3	100	10	67	9	59

Table 2 shows that reduced $KHCO_3$ amounts does not affect the yield but small improvement in enantioselectivity were observed. Therefore the amount of $KHCO_3$ was kept at 60 μmol for future experiments (Table 2, entry 1) and the effect of H_2O_2 on the yield and enantioselectivity of this reaction was evaluated instead.

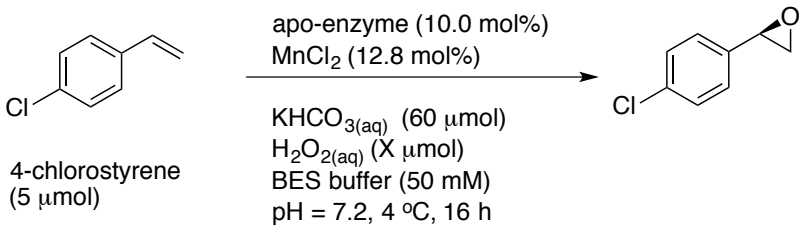

Figure 6: Effect of H_2O_2 on the enantioselective epoxidation of 4-chlorostyrene

Table 3: Effect of H_2O_2 on the enantioselective epoxidation of 4-chlorostyrene

Entry	H_2O_2 (X μmol)	Wild Type		Thermostable Mutant	
		%Yield	%ee	%Yield	%ee
1	8	11	70	9	62
2	10	14	68	11	61
3	20	22	66	19	55
4	40	29	61	26	49
5	60	34	57	31	45
6	80	34	55	33	43
7	100	39	53	38	40

A significant effect on the yield and enantioselectivity of the epoxidation of 4-chlorostyrene is observed as the amount of H_2O_2 is varied (Figure 6, Table 3). The role of H_2O_2 and $KHCO_3$ in this reaction is to generate a peroxycarbonate anion, which is necessary to drive the epoxidation. However, the peroxycarbonate anion also facilitates the degradation of hCAII[Mn] during the course of the reaction.[11] The enantioselectivity decreases due to the degradation of hCAII[Mn] as a result of a manganese(IV)-oxo species generated during the reaction. However, a certain level of selectivity is retained even upon the degradation, possibly due to an unfolded enzyme structure or due to the oxidation of some amino acid residues inside the active site. Therefore it is important to have a higher amount of H_2O_2 to improve the yield, but a low enough loading to prevent the deterioration of the enantioselectivity.

Until this point, the catalyst loading was maintained at a ratio of apo-enzyme: Mn^{2+} 10:12.8 mol% (a slight excess of Mn^{2+} to facilitate the complete dative anchoring of metal catalyst to the active site). However, it was found that a 1:1 ratio of apo-enzyme: Mn^{2+} leads to slightly improved yields in both the wild type and mutant enzyme catalyst systems (Figure 7).

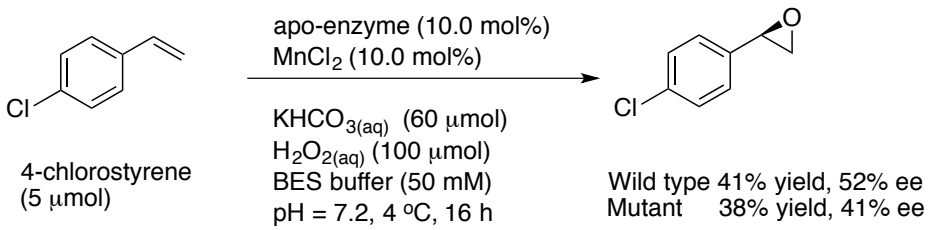

Figure 7: Modified apo-enzyme: Mn^{2+} ratio for the enantioselective epoxidation of 4-chlorostyrene

Next a set of control experiments were conducted to confirm that the hCAII[Mn] is the active catalyst in the epoxidation of 4-chlorostyrene (Figure 8, Table 4). The wild-type and thermostable hCAII[Zn] did not generate any epoxidation product (Figure 8 conditions, 10 mol% hCAII[Zn]). In the absence of apo-enzyme a racemic product is observed (Table 4, entry 1). In the absence of $MnCl_2$ or H_2O_2 this reaction does not proceed to give an epoxide product (Table 4, entries 2 and 4). In the absence of $KHCO_3$ an epoxide product is observed in a poor yield but with a good enanatioselectivity showing that H_2O_2 is also capable of driving this epoxidation reaction but as not as efficient oxidant relative to the peroxycarbonate anion (Table 4, entry 3). All of these observations support the claim that hCAII[Mn] is the active catalyst species for the enantioselective epoxidation of 4-chlorostyrene.

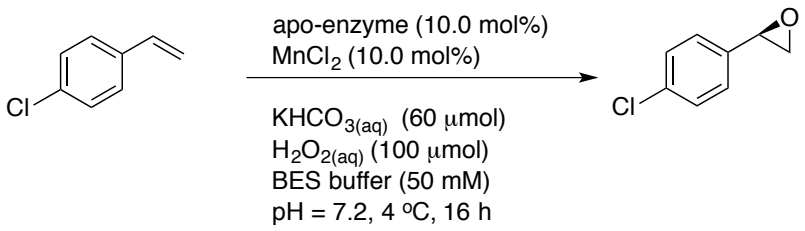

Figure 8: Control experiments

Table 4: Control experiments

Entry	Apo-E	$MnCl_2$	$KHCO_3$	H_2O_2	Wild Type		Thermostable Mutant	
					%Yield	%ee	%Yield	%ee
1	No	Yes	Yes	Yes	3	Racemic	3	Racemic
2	Yes	No	Yes	Yes	No reaction	-	No reaction	-
3	Yes	Yes	No	Yes	3	66	2	57
4	Yes	Yes	Yes	No	No reaction	-	No reaction	-

Next, the effect of reduced catalyst loading was evaluated. The catalyst loading was reduced from 10 mol% - 1 mol% (Figure 9, Table 5). The yield and the enantioselectivity slightly dropped upon the reduction of the catalyst loading from 10 mol% - 1 mol% in both systems. However improved turnover numbers were observed (13 turnovers at 2 mol% of wild-type apo-enzyme loading, Table 5, entry 3). In fact, reduction of the hCAII[Mn] loading to 1.0 mol% resulted in a significant decrease in enantioselectivity (Table 5, entry 4).

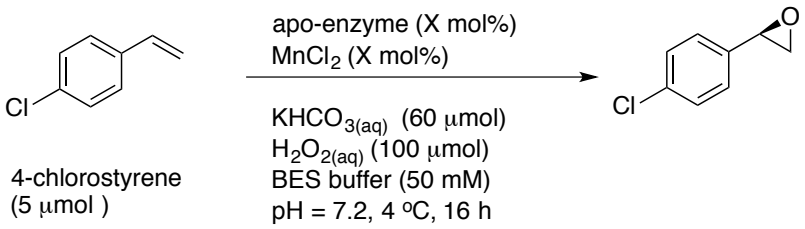

Figure 9: Effect of catalyst loading

Table 5: Effect of catalyst loading

Entry	X mol%	Wild Type		Thermostable Mutant	
		%Yield	%ee	%Yield	%ee
1	10.0	41	52	38	41
2	5.0	33	55	30	45
3	2.0	26	52	24	49
4	1.0	14	38	12	40

Finally, the epoxidation was conducted at elevated temperatures (up to 65 ^{o}C) to study the stability of the thermostable hCAII[Mn] catalyst compared to the wild-type (Figure 10, Table 6). Even though the thermostable hCAII[Zn] mutant showed an excellent thermostability at elevated temperatures (Chapter 2, Table 2), the artificial metalloenzyme generated from the mutant hCAII[Zn] did not prove to be stable at elevated temperatures under these oxidative reaction conditions (Table 6). Yields of the epoxidation product decreased dramatically above 37 ^{o}C, and no reaction was observed above 55 ^{o}C for either wild-type enzyme or the thermostable mutant catalysts.

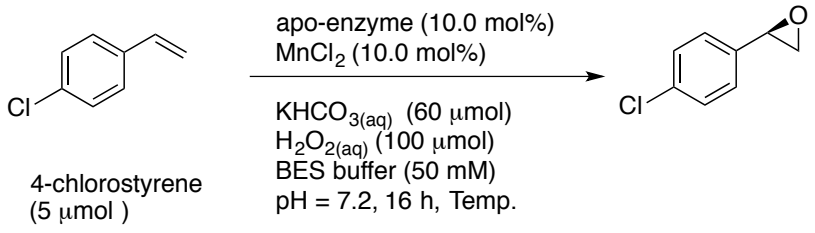

Figure 10: Effect of temperature on the enantioselective epoxidation of 4-chlorostyrene

Table 6: Effect of temperature on the enantioselective epoxidation of 4-chlorostyrene

Entry	Temperature (^{o}C)	Wild Type		Thermostable Mutant	
		%Yield	%ee	%Yield	%ee
1	4	41	52	38	41
2	RT	18	52	19	43
3	37	7	51	8	43
4	45	2	48	2	42
5	50	1	44	2	43
6	55	-	-	1	39
7	60	-	-	-	-
8	65	-	-	-	-

Conclusion

Through a dative anchoring strategy, wild-type and a thermostable manganese-substituted human carbonic anhydrase were successfully generated as catalysts for the epoxidation of olefins. Although the thermostability of hCAII[Zn] is improved by the introduction of mutations, these mutations do not significantly affect the activity of the artificial metalloenzyme hCAII[Mn] generated under oxidative reaction conditions. The best reaction conditions for the epoxidation of 4-chlostyrene were identified by the careful screening of a variety of reaction variables. Both wild-type and thermostable manganese-substituted human carbonic anhydrase show good catalytic activity at lower temperatures, but

not at elevated temperatures. The thermostability introduced to the wild-type did not translate to oxidative stability of the artificial metalloenzymes generated. Upon the reduction of the catalyst loading improved turnover numbers were observed. (13 turnovers at 2 mol% of wild-type apo-enzyme loading). The yields and turnover numbers of the epoxidation of 4-chlorostyrene are improved, despite some erosion of the enantioselectivity, which is an improvement upon the literature precedents.[11, 12]

Experimental

1) Transformation, protein expression, purification and preparation of apo-human carbonic anhydrase

Transformation of BL-21 chemically competent cells

First, a microcentrifuge tube containing a pre-frozen BL-21 cell aliquot (50 μL) was thawed on ice and transferred into a pre-chilled 14 mL BD Falcon polypropylene round-bottom tube. Then, wild-type or mutant plasmid containing the DNA encoding hCAII (300 ng) was added to the cells, mixed thoroughly and left on ice for 30 minutes. Next, a heat shock was given to the mixture at 42 ^{o}C for 30 seconds in a water bath and incubated on ice for 2 minutes. After that the cell mixture was rescued with SOC (250 μL of SOB, 5 μL of 1M filter sterilized glucose, which was pre-heated at 42 ^{o}C for 2 minutes) and placed in a shaker for 1 hour (37 ^{o}C, 250 rpm). Then, culture was plated on pre-heated (1 hour at 37 ^{o}C) LB-ampicillin agar plates (90:10 μL of dd H_2O: culture) and incubated for 16 hours at 37 ^{o}C in an incubator. After that a single colony from the agar plate was picked up using an autoclaved toothpick and introduced to a mixture of LB broth (5 mL) and ampicillin (100 mg/

ml, 5 μL) in a 14 mL BD Falcon polypropylene round-bottom tube and placed in a shaker for incubation for 16 hours (37 ^{o}C, 250 rpm) overnight.

Protein expression

First, one overnight culture was added to each shaker flask with 490 mL of autoclaved induction media (10.0 g of bacto-tryptone, 5.0 g of yeast extract, 2.34 g of NaCl, 30 mL of 6XM9 salt, 0.333 mL of 0.3 M $ZnSO_4$ diluted to 490 mL), ampicillin (100 mg/ mL, 0.5 mL) and 20% filter-sterilized glucose (10.0 mL). Then shaker flasks were placed in the shaker (37 ^{o}C, 250 rpm) and allowed to grow until OD_{600}~ 1.0. Once the OD_{600} value reached that number protein expression was induced by adding $ZnSO_4$ (0.3 M, 0.75 mL) and IPTG (100 mM, 1.25 mL) and shaking was continued for another 6 hours. After that inhibitors PMSF (1 mg/ mL, 2.0 mL) and TAME (1 mg/ mL, 1 mL) were added and cells were pelleted by centrifuging at 12000 rpm for 45 minutes at 4 ^{o}C. Next the cell pellet from a 1 L culture was re-suspended in 200 mL of lysis buffer with dithiothreitol (50.0 mL of 1M tris-sulfate, 50.0 mL of 1 M NaCl, 40.0 mL of 0.25 M EDTA, 0.66 mL of 0.3 M $ZnSO_4$, 1.0 mL of 10 mg/ mL PMSF, 1.0 mL of 1 mg/ mL TAME and 0.1542 g of dithiothreitol diluted to 1 L, pH = 8.0), lysozyme was added (1 mg/ mL of buffer), and the mixture was placed inside the shaker for 1 hour (37 ^{o}C, 250 rpm). After that the cell-buffer mixture was centrifuged (5000 rpm, 75 minutes, 4 ^{o}C), the supernatant was collected, 10% streptomycin sulfate (1/10 volume of the supernatant) was added and the solution was stirred at 4 ^{o}C for 15 minutes. Next the supernatant was centrifuged at 5000 rpm for 60 minutes at 4 ^{o}C, and the clear supernatant containing the crude

hCAII[Zn] was collected without any cell materials which contains the crude hCAII[Zn]. Then the supernatant was transferred to centrifugal dialysis tubes (MWCO 10 kDa) and centrifuged with tris-sulfate buffer (pH = 8.0, 10 mM) for five times each for 50 minutes.

Protein purification

First, DEAE-sephacel gel (30.0 mL per 1 L culture) was equilibrated with 2 column volumes of 1 M tris-sulfate (pH = 8.0, 60 mL) followed by 5 column volumes of 10 mM tris-sulfate (pH = 8.0, 150 mL). Then the gel was poured in to an flask with supernatant collected at the end of the protein expression (crude hCAII[Zn]) and equilibrated at 4 ^{o}C for 30 minutes. Next, mixture was transferred into a fritted filter funnel and washed with 1 column volume of 10 mM Tris-sulfate (pH = 8.0, 30 mL) and 1 column volume of 20 mM tris-sulfate (pH = 8.0, 30 mL) and fractions were collected into centrifuge tubes containing purified hCAII[Zn]. This procedure produces ~ 50-240 mg of hCAII[Zn]. SDS-PAGE technique was used to determine the purity of the hCAII[Zn] and a clear band was observed at ~29 kDa with no other major bands.

Preparation of apo-human carbonic anhydrase

A solution of purified hCAII[Zn] solution was transferred to dialysis tubes (MWCO 10 kDa) and centrifuged with BES buffer five times for 30 minutes (50 mM, pH = 7.2) and 2,6-pyridinedicarboxylate solution (250 mM in 50 mM phosphate buffer) was added and let stand for overnight. Then the enzyme solution with PDCA

was transferred to dialysis tubes again and centrifuged with BES to remove the entire chelating agent added (until a constant A_{280} value is given from the enzyme solution) which produce the human carbonic anhydrase apo-enzyme.

Preparation of chemically competent BL-21 cells for transformation

A frozen glycerol stock of BL-21 cells was streaked onto a LB plate without ampicillin and grown overnight at 37 ^{o}C in the incubator for 16 hours. Then, a single colony was selected and used to inoculate a 10 mL starter culture (LB broth, no ampicillin) that was grown for 16 hours in a shaker (37 ^{o}C, 250 rpm). And also LB broth (1 L), 100 mM $MgCl_2$ (500 mL), 100 mM $CaCl_2$ (500 mL), $CaCl_2$/glycerol solution (85/15 v/v, 100 mL), four nalgene bottles with caps, microcentrifuge tubes and two shaker flasks were autoclaved and all the solutions were chilled overnight at 4 ^{o}C. Next, LB-broth in shaker flasks were inoculated with 10 mL starter culture and grown in the shaker at 37 ^{o}C until OD_{600}~ 0.35-0.45. Once the OD_{600} reached that value, the shaker flasks were placed on ice immediately and chilled for 30 minutes swirling occasionally to provide even cooling and nalgene bottles were started to cool at the same time. The culture was split in between four nalgene bottles, centrifuged at 4000 rpm for 15 minutes at 4 ^{o}C in a pre-chilled rotor. After that the supernatant was decanted and each cell pellet was re-suspended in ice cold $MgCl_2$. (100 mM, 100 mL). Cell suspensions were combined into two nalgene tubes and centrifuged at 3000 rpm for 15 minutes at 4 ^{o}C. Next, supernatant was decanted and each cell pellet was re-suspended in ice cold $CaCl_2$. (100 mM, 100 mL). Cell suspensions were combined to one nalgene bottle, incubated on ice for 20 minutes and centrifuged at 3000 rpm for

15 minutes at 4 ^{o}C. Next the supernatant was decanted, the cell pellet was re-suspended in $CaCl_2$/ glycerol solution (85 mL), transferred to a 50 mL conical tube and centrifuged at 2100 rpm for 15 minutes at 4 ^{o}C. At the same time, autoclaved microcentrifuge tubes were placed on ice to chill. Next the supernatant was decanted and the cell pellet was re-suspended in $CaCl_2$/ glycerol solution (2 mL). Finally these cells were transferred in to microcentrifuge tubes in 50 μL aliquots, flash froze with liquid nitrogen and stored at – 80 ^{o}C until use.

Preparation of ampicillin-LB agar plates

First 37 g of LB agar was dissolved in 1 L of water (LB- agar formulation: 10 g of casein peptone, 5.0 g yeast extract, 10.0 g of NaCl and 12.0 g of agar per liter). Then the pH of the solution was adjusted to 8. Next the solution was autoclaved at 121 ^{o}C for 20 minutes, cooled and ampicillin (100 mg/ mL, 1 mL) was added. Finally the agar solution was poured into petri dishes, allowed to solidify.

2) Generation of manganese-substituted human carbonic anhydrase and epoxidation of 4-chlorostyrene

First, the manganese-substituted human carbonic anhydrase was generated by pre-complexing $Mn^{2+}_{(aq)}$ and apo-enzyme in a 4-dram closed glass vial prior to the reaction by adding $MnCl_{2(aq)}$ (2 mM) and apo-enzyme in BES buffer (50 mM, pH = 7.2). The mixture was stirred at room temperature for 15 minutes. Then, 4-chlorostyrene (200 mM in acetone, 25 μL), $KHCO_{3(aq)}$ (1M) and $H_2O_{2(aq)}$ (1M) were added in the mentioned order (total aqueous volume ~ 1.1 mL) and stirred for 16

hours. Next the reaction mixture was extracted with 1.5 mL of hexane (with 0.5 mM 4-chloroanisole as the internal standard), and the reaction was analyzed by HPLC (chiral OJH column, 99.5:0.5 Hexane: *i*-Propanol) for analysis. Retention times are 11 minutes (major enantiomer) and 12 minutes (minor enantiomer)

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CHAPTER 4

CONCLUSION

Artificial metalloenzymes are a promising class of hybrid catalysts for selective organic transformations. A new artificial metalloenzyme was generated to achieve thermostability and to improve the catalytic activity for the epoxidation of olefins. Site-directed mutagenesis and polymerase chain reaction were used to introduce six mutations sequentially to defined positions of the wild-type enzyme in order to generate the thermostable mutant. The mutant hCAII[Zn] showed an improved stability at elevated temperatures compared to the wild-type of hCAII[Zn] as determined by an esterase activity assay.

A dative anchoring strategy was used to generate manganese-substituted human carbonic anhydrase from the wild-type enzyme and the thermostable mutant, and both of these artificial metalloenzymes demonstrate catalytic activity for the epoxidation of 4-chlorostyrene. Both the wild-type and thermostable hCAII[Mn] showed catalytic activity for epoxidation of 4-chlorostyrene at low temperatures and conditions were finely optimized to improve the yields of this reaction. The thermostable mutant hCAII[Mn] did not have increased oxidative stability as expected and undergoes oxidative degradation similar to the wild-type hCAII[Mn], which limits further improvement of yield and enantioselectivity of this reaction.

One possible approach to improve the catalytic activity of hCAII[Mn] would be the use directed evolution to improve the activity by site-saturation mutagenesis of the wild-type enzyme. This approach would generate a library of mutants to screen for improved activity and/ or selectivity of the enantioselective epoxidation of olefins. Another approach would be

the evaluation of activity of these catalysts for the epoxidation of olefins in the presence of different oxidants.