OMP Decarboxylase: Active site Labeling Using a Combination of Site-directed Mutagenesis and Chemical Modification

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Abstract

Orotidine 5'-monophosphate decarboxylase (ODCase) catalyzes the conversion of OMP to UMP. The enzyme functions without metals or cofactors. It has been proposed that an active site lysine donates a proton to the substrate OMP during decarboxylation. For a lysine to donate a proton, it must have an unusually low pK_a. To investigate the pK_a of the active site lysines in ODCase from yeast and *E. coli*, we attempted to specifically label the side chains of these lysines. The K93C and "C93 only" mutants of the yeast enzyme were generated and purified, but resulted in poorly soluble proteins after purification. The mutations C41S C161S C168S and K73C of *E. coli* ODCase were generated by site directed mutagenesis. The triple mutant, with no cysteine residues present, was insoluble regardless of efforts made to obtain soluble protein. Attempts to modify K73C with DTNB, in order to reversibly protect non-active site cysteines, were unsuccessful. Chemical modification of K73C with bromoethylamine restored partial decarboxylation activity to the inactive mutant protein. The results provide a basis for future efforts to generate mutated, chemically modified ODCase for NMR analysis.

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List of Symbols and Abbreviations

α	Alpha
β	Beta
Δ	Delta
γ	Gamma
ε	Epsilon
μ	Micro
μg	Microgram
μl	Microliter
μM	Micromolar
®	Registered
^{15}N	Isotopically labeled nitrogen
A	Adenosine
Abs	Absorbance
Amp	Ampicillin
Asp	Aspartate
ATP	Adenosine triphosphate
C	Cytosine
Cys	Cysteine
dH_2O	Deionized water
dNTP	Deoxynucleotide triphosphate
G	Guanidine
hr	Hour
Lys	Lysine
M	Molar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mM	millimolar
mol	moles
ng	nanogram
nm	nanometer
nmol	nanomoles
Ser	Serine
ssDNA	Single stranded DNA
T	Thymidine
UV	ultraviolet
V	volts
WT	Wild type

Chapter 1

Introduction

Orotidine 5'-monophosphate decarboxylase (ODCase) catalyzes the final step of pyrimidine nucleotide biosynthesis (Figure 1-1). In neutral solution, orotidine 5'-monophosphate (OMP) undergoes spontaneous decarboxylation to uridine 5'-monophosphate (UMP). This reaction proceeds with a half life of 78 million years. At the enzyme active site the same reaction has a half life of 18 msec.

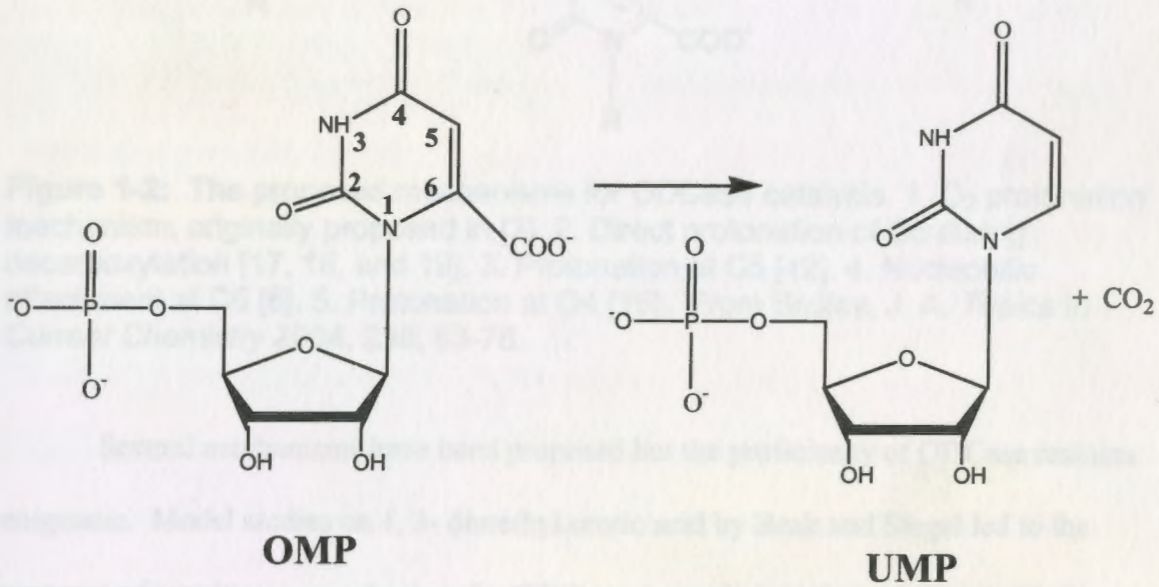


Figure 1-1: The ODCase reaction of OMP to UMP plus CO_2.

From the k_{cat} / K_m and the rate constant for uncatalyzed reaction, it is obvious that ODCase is a highly proficient enzyme with an astounding affinity for the altered substrate in the transition state. The enzyme appears to function without metals or other cofactors even though the substrate lacks a chemical moiety that could accommodate the negative charge that is generated at C6 when CO_2 is eliminated [1].

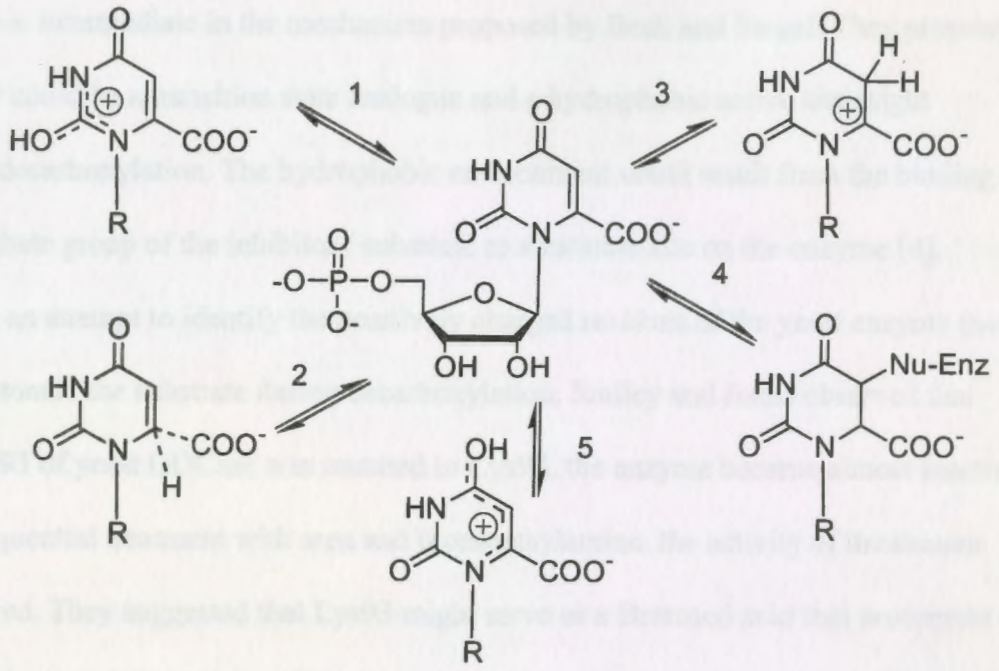


Figure 1-2: The proposed mechanisms for ODCase catalysis. 1. O_2 protonation mechanism, originally proposed in [3]. 2. Direct protonation of C6 during decarboxylation [17, 18, and 19]. 3. Protonation at C5 [42]. 4. Nucleohilic attachment at C5 [6]. 5. Protonation at O4 [15]. From Smiley, J. A. *Topics In Current Chemistry* **2004**, 238, 63-78.

Several mechanisms have been proposed but the proficiency of ODCase remains enigmatic. Model studies on 1, 3- dimethyl orotic acid by Beak and Siegel led to the proposal of a zwitterion mechanism for ODCase. According to their proposal, OMP decarboxylation proceeds with zwitterion or nitrogen ylide intermediate formation with protonation at O2 of the substrate. An acidic group on the enzyme might function to donate a proton to O2 of the substrate [2, 3].

Westheimer and associates reported that 1-(5'-phospho-β-D-ribofuranosyl) barbituric acid (BMP), an analogue of OMP, binds to ODCase about 100,000 times as strongly as does OMP. The K_i at pH 6 is 9×10^{-12} M. They observed that BMP was an extraordinarily powerful inhibitor of yeast ODCase with a structural resemblance to the

carbanionic intermediate in the mechanism proposed by Beak and Siegel. They proposed that BMP could be a transition state analogue and a hydrophobic active site might facilitate decarboxylation. The hydrophobic environment could result from the binding of the phosphate group of the inhibitor / substrate to a cationic site on the enzyme [4].

In an attempt to identify the positively charged residues of the yeast enzyme that might protonate the substrate during decarboxylation, Smiley and Jones observed that when Lys93 of yeast ODCase was mutated to Cys93, the enzyme became almost inactive and on sequential treatment with urea and bromoethylamine, the activity of the mutant was restored. They suggested that Lys93 might serve as a Brønsted acid that protonates OMP and is crucial for activity [5].

Silverman and Groziak proposed a covalent mechanism for ODCase. According to this proposal an enzyme nucleophile undergoes Michael addition at C5 of OMP and this complex dissociates via an acid-base catalyzed decarboxylative mechanism [6]. Experiments conducted by Wolfenden *et al.* with ^{13}C enriched BMP suggest that ODCase does not follow a covalent mechanism since no significant upfield shift of ^{13}C NMR C5 resonance was observed. Kinetic isotope effect experiments using OMP deuterated at C5 are negligible, also consistent with a non-covalent mechanism [7].

The observation of large ^{13}C isotope effects for ODCase by Smiley *et al.* provides further evidence against a covalent mechanism. They suggested that decarboxylation involves a protonation step [8].

Experiments with the alternate substrate 5-azaOMP suggest that the compound does not undergo nucleophilic attack as proposed in the Michael addition mechanism. When OMP was decarboxylated in ^{18}O water, the product UMP showed no signs of ^{18}O

incorporation, which indicates that there was no oxygen exchange between bulk solvent water and C2 of substrate, which is obligatory if the decarboxylation follows a Schiff base mechanism [9].

Ehrlich *et al.* reported that multiple isotope effects measured for *E. coli* ODCase are consistent with a stepwise mechanism in which a protonation step plays a crucial role in decarboxylation [10].

^{13}C kinetic isotope experiments on 1, 3- dimethyl orotic acid support the protonation of O4 rather than protonation of O2 and the formation of positive charge at N1 of the substrate during the non-enzymatic reaction [11, 12].

Rishavy and Cleland measured kinetic isotope effects of nitrogen at N1 with yeast ODCase and suggested that a carbanion intermediate formed during decarboxylation might be stabilized by electrostatic interaction with Lys93. They inferred that ^{15}N isotope effects ruled out the formation of a nitrogen ylide before or during C-C bond cleavage in the enzyme reaction [13]. However, this study was based on a model reaction that does not closely resemble the ODCase reaction, and the participation of N1 in the reaction mechanism cannot be ruled out [14].

Quantum mechanical calculations by Lee and Houk suggest that the ODCase mechanism involves formation of a carbene intermediate and that O4 is the site of protonation rather than O2. They also predicted that the ODCase active site must provide a low dielectric environment to facilitate proton transfer to O4 and the active site lysine should be properly oriented to affect this proton transfer [15].

Based on the crystal structures of ODCase from four different organisms, there appears to be no proton-donating group close enough to O4 of the inhibitors to suggest

protonation of the substrate during catalysis. Lee and Houk proposed a revised carbene mechanism in which an active site water molecule accepts a proton from the active site lysine and then transfers it to O4 of the substrate [16].

The X-ray crystallographic structures of four different ODCases - yeast ODCase – BMP complex [1], ODCase from *Bacillus subtilis* in complex with UMP [17], ODCase from *Methanobacterium thermoautotrohicum* in complex with 6-azaUMP [18], *E. coli* ODCase – BMP complex [19] - revealed that ODCase is a dimeric enzyme and each subunit folds as α / β barrels with eight central β sheets surrounded by nine α helices. The presence of metals or other cofactors that could participate in catalysis were not found. The binding of BMP initiates proton loop movements that envelope the ligand almost completely resulting in numerous favorable interactions with the phosphoryl group, ribofuranosyl group and the pyrimidine ring. The position of Lys93 of the yeast enzyme suggests that it could accommodate the negative charge that is developed at the C6 of the pyrimidine ring during decarboxylation. It might donate a proton that replaces the carboxylate group at C6 of the product. Hydrogen bonds from the active site to O2 and O4 also help to delocalize negative charge developed in the transition state. The phosphoryl group is proposed to contribute to catalysis by properly positioning the pyrimidine ring within the active site [1].

From the crystal structure of ODCase from *Bacillus subtilis*, Appleby *et al.* suggested that the anionic carboxylate of the substrate is positioned in a negatively charged region of protein close to Asp60 and C6, the carbon in the pyrimidine which subsequently becomes the carbanion, is placed near the positively charged ammonium group of Lys62. This arrangement leads to the destabilization of the ground state and

stabilization of the transition state. In this proposal, protonation of C6 by Lys62 results in the decarboxylation which follows an electrophilic substitution mechanism [17].

Larsen *et al.* proposed that, from the projected orientation of the orotate moiety of the substrate, the mechanism involving protonation of O2 or O4 seems unlikely. Based on the binding of BMP, OMP is projected to bind in a *syn* configuration and ODCase attacks directly on C6 and the carboxylate group of OMP. Based on the hydrogen bondings of the ODCase- BMP complex, they also proposed two different mechanisms for catalysis.

In the proposed charge repulsion mechanism, Lys44 is turned away from Asp71 to bind the ribose moiety. This makes the negatively charged carboxylate group face the negatively charged Asp71. This creates charge repulsion between the two moieties making decarboxylation favorable. Lys73 may transfer a proton to C6 and as UMP and CO_2 leave the active site, Lys73 is reprotonated by the solvent.

In a second proposed mechanism, binding of substrate to the active site might bring the carboxylate group of the substrate close to the carboxylate group of Asp71. A very short O - H - O hydrogen bond is formed between the carboxylate groups in the transition state. The proton on the O - H - O bond could come from Lys44 which makes a hydrogen bond to O3' of the ribose ring. As a result the pK_a of Asp71 increases. In a sequence of proton transfers, a proton is transferred to Asp71 and Lys73 donates a proton to C6 of OMP, which favors decarboxylation. In the final step Asp71 transfers a proton to Lys73. Lys44 might be reprotonated by solvent [19].

From the thio-substitution studies with OMP, Smiley *et al.* proposed that OMP binds to the active site in a manner distinct from binding of inhibitors. Possibly OMP

binding occurs with rotation of the pyrimidine ring by 180° about the glycosidic bond with respect to the binding of inhibitors. This in turn aligns O2 for protonation by Lys93 [20].

In all of the proposed mechanisms for ODCase, the active site lysine residue – Lys93 of the yeast enzyme and Lys73 of the *E. coli* enzyme – is making a critical contribution to catalysis. The experiments described in this thesis are designed to probe the nature of this active site lysine.

Concerted Site-directed Mutagenesis and Chemical Modification- Mechanistic Insights

Site-directed mutagenesis is a powerful technique for evaluating the contribution of individual amino acids to a catalytic mechanism, although this technique has its own limitations. The number of possible replacements is normally limited to the remaining 19 of the 20 naturally occurring amino acids.

Combining site-directed mutagenesis with chemical modification makes it possible to introduce unnatural amino acids into the proteins. This diverse technique has a possible disadvantage of introducing multiple modifications if the protein has the same amino acid residue of interest at a position different from that of the targeted one. This problem can be avoided either by sequential protection and modification that allows selective derivatization of the targeted amino acid or by modification of the interfering residues using site-directed mutagenesis prior to the modification of the target amino acid.

Chemical modification depends on the unique chemical reactivity of amino acid side chains particularly cysteine, lysine, histidine and tyrosine [21]. The usefulness of

introducing non natural amino acids by concerted site-directed mutagenesis and chemical modification is attributed to the fact that it allows greater variation in side-chain length and pK_a, making it easily detectable, than does the use of naturally occurring amino acids [22]. In this thesis, the focus will be on the examination of the role of lysine residues using concerted site-directed mutagenesis and chemical modification. The concept of this approach is shown in Figure 1-3.

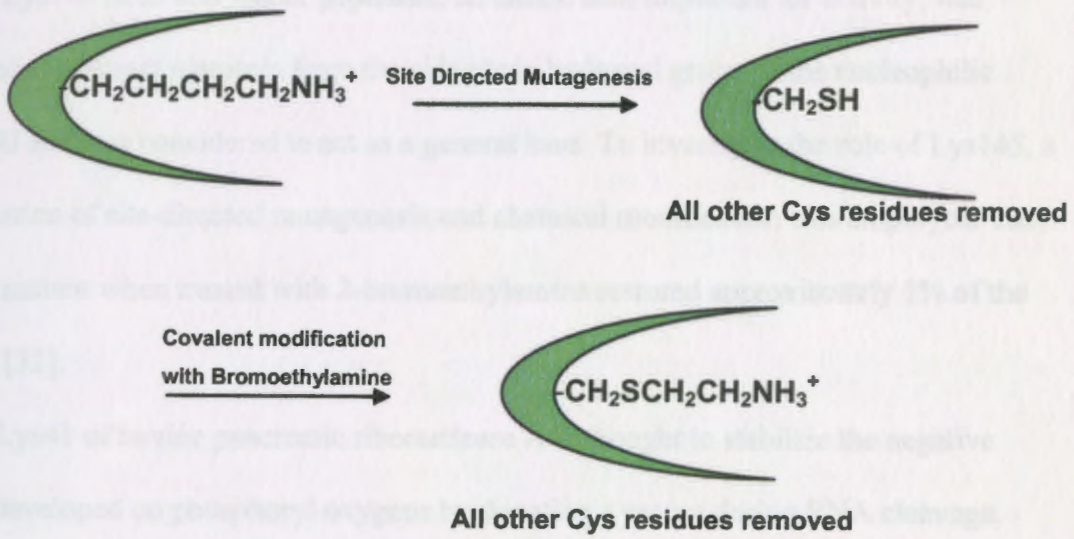


Figure 1-3: Concerted site-directed mutagenesis and chemical modification

Chemical modification using bromoethylamine also allows site-specific incorporation of isotope label in the aminoethyl side chain, which serves as a very good probe for the determination of pK_a of the chemically modified amino acid, by change in NMR signals with pH titration [23].

Previously, many inactive lysine to cysteine mutants were restored to partial activity by treatment with bromoethylamine [5, 21- 35]. The mutants K166C and K329C of ribulose bisphosphate carboxylase / oxygenase when treated with 2-bromoethylamine restored 20% and 60% activity respectively. This was likely due to the aminoethylation of the thiol group which creates a lysine-like side chain, aminoethylcysteine, at the active site [24].

Lys145 of *E. coli* leader peptidase, an amino acid important for activity, was proposed to abstract a protein from the side chain hydroxyl group of the nucleophilic serine 90 and was considered to act as a general base. To investigate the role of Lys145, a combination of site-directed mutagenesis and chemical modification was employed. The K145C mutant when treated with 2-bromoethylamine restored approximately 1% of the activity [32].

Lys41 of bovine pancreatic ribonuclease A is thought to stabilize the negative charge developed on phosphoryl oxygens by donating a proton during RNA cleavage. The Lys41 to Cys41 mutant was inactive. Rescue of K41C mutant with bromoethylamine resulted in restoration of 8% of wild type activity [22].

Lys239 of aspartate aminotransferase from *Bacillus* sp.YM-2 was proposed to mediate transaldimination and was therefore important for activity. It was thought that the ε-amino group of Lys239 binds to the pyridoxal phosphate of aspartate

aminotransferase and acts as a base in facilitating proton transfer between the substrate and C4' of pyridoxal phosphate. The activity of the K239C mutant of aspartate aminotransferase was restored by chemical modification with bromoethylamine. The modified mutant was $10^6 - 10^8$ times more active than unmodified K239C mutant [28].

Chemical modification of K93C mutant of yeast ODCase with bromoethylamine resulted in partial restoration of activity. Attempts were not made to specifically label the lysyl residue to enable the measurement of pK_a of active site lysine [5]. Lys93 of yeast ODCase (Lys73 in *E. coli*) was proposed to donate a proton to OMP during decarboxylation [5]. The lysine side chain has a pK_a of 10.5 in solution [36]. In order to donate a proton, it should probably have an unusually low pK_a.

To determine the pK_a of the active site lysine of ODCase, we plan to modify the Lys-to-Cys mutant with ^{15}N enriched modification reagent bromoethylamine, carry out NMR analysis of the modified mutant and try to find out whether the nitrogen of aminoethylcysteine is an ammonium ion ($-^{15}NH_3^+$) or a neutral amine ($-^{15}NH_2$). Our lab has both the yeast and *E. coli* genes available for this procedure. We plan to carry out a number of variations to this procedure in order to make the active site labeling as specific as possible. By adjusting the pH of the enzyme solution, we are able to observe the progression from $-NH_3^+$ to $-NH_2$ with increasing pH, and measure the pK_a of this active site functional group. This would be a unique spectrometric method for determination of the pK_a of an enzyme functional group.

Chapter 2

Attempted Expression and Purification of Wild Type and Mutant (K93C and "Cys93 Only") Yeast ODCase for Chemical Modification and Restoration of Activity

Introduction

Previously in our lab, the ODCase gene derived from the yeast *Saccharomyces cervisiae* was cloned into the vector pCal-n. This vector allows the purification of ODCase (or mutants) to be performed using the fusion protein encoded by pCal-n and the calmodulin affinity resin. Mutagenesis experiments were carried out to generate the mutants K93C and "C93 only" of yeast ODCase.

The purpose of carrying out site-directed mutagenesis and creating the mutant "C93 only" of yeast ODCase was to chemically modify Cys93 of the mutant with ^{15}N enriched bromoethylamine. Yeast ODCase has four naturally occurring cysteines (Cys33, Cys56, Cys155 and Cys263). The mutant K93C has the naturally occurring cysteines unmodified. These could also react with ^{15}N enriched bromoethylamine and create unwanted signals in NMR. We therefore generated the mutant "C93 only", which has all the naturally occurring cysteines mutated to serines and the only cysteine present in this protein is Cys93.

The sequence analysis of yeast ODCase from ExPASy proteomics server (Figure 2-1) and the crystal structure of ODCase from *Saccharomyces cerevisiae* revealed that the four naturally occurring cysteines are away from the important regions of the active site [1]. We thought that site-directed mutagenesis of these cysteine residues might not affect the activity of ODCase. For the chemical modification experiments relatively pure

ODCase was needed. Previously, initial attempts to purify mutant ODCases using Affi-gel Blue columns failed since the enzymes did not adhere to the columns. Hence we changed to calmodulin affinity column purification with the hope that it would result in better purification of mutant proteins.

```
        10         20         30         40
MSKATYKERA ATHPSPVAAK LFNIMHEKQT NLCASLDVRT

        50         60         70         80
TKELLELVEA LGPKICLLKT HVDILTDFSM EGTVKPLKAL

        90        100        110        120
SAKYNFLLFE DRKFADIGNT VKLQYSAGVY RIAEWADITN

       130        140        150        160
AHGVVGPGIV SGLKQAAEEV TKEPRGLLML AELSCKGSLA

       170        180        190        200
TGEYTKGTVD IAKSDKDFVI GFIAQRDMGG RDEGYDWLIM

       210        220        230        240
TPGVGLDDKG DALGQQYRTV DDVVSTGSDI IIVGRGLFAK

       250        260
GRDAKVEGER YRKAGWEAYL RRCGQQN
```

Figure 2-1: ODCase sequence from yeast. The active site residues are represented in red and cysteines are represented in green. The most critical portions of the sequence, as found in all sequences, are underlined.

Affinity® Protein Expression and Purification System

The affinity protein expression vector pCal-n allows fusion of the calmodulin-binding-peptide (CBP) affinity tag to the N terminus of the sequence encoding protein of interest. The CBP affinity tag is based on the fact that a 26-amino acid C-terminal fragment of muscle myosin light-chain kinase exhibits a high affinity for CaM at

physiological pH. The relatively small size of the CBP affinity tag makes it less likely to interfere with the function of the protein of interest. At low concentration of calcium and neutral pH, calmodulin binds to the CBP-tagged fusion protein. Calcium, calmodulin and CBP-tagged protein forms a ternary complex. The protein can be eluted by the addition of a neutral solution of 2 mM EGTA. Calcium now dissociates from the ternary complex and binds to the EGTA.

The pCal-n vector has a CBP-coding sequence inserted upstream of a multiple cloning site (MCS) that allows fusion of the CBP affinity tag at the N-terminus of the cloned protein-coding sequence.

This chapter describes the expression and purification of wild type, mutants K93C and "C93 only" of yeast ODCase in BL21 cells of *E. coli*.

Materials and Methods

LB medium contains 10 g of tryptone, 5 g of yeast extract (Amresco), and 5 g of NaCl per liter of dH_2O and is adjusted to pH 7.4 with NaOH. "LB-glycerol-amp medium" is LB medium containing 10% glycerol and ampicillin added to a final concentration of 50 μg/ml. IPTG was purchased from Amresco. Calcium chloride lysis buffer is composed of 2 mM $CaCl_2$, 50 mM Tris HCl, 0.15 M NaCl, 5 mM βME. Triton is added to a final concentration of 0.1%. The pH of the final solution is adjusted to 8.0. The composition of CBP elution buffer is 2 mM EGTA, 50 mM Tris. HCl, 5 mM DTT and 150 mM NaCl. The pH of the solution is adjusted to 8.0. Calmodulin resin was purchased from Sigma. pCal-n plasmid was purchased from Stratagene®. WT, K93C and

"C93 only" of yeast ODCase gene were already cloned into pCal-n and transformed into BL21 cells. BL21 cells were purchased from Stratagene®.

Optimizing IPTG concentration for ODCase over-production

Two 3 ml portions of overnight culture of BL21/pCal ODCase-f WT (hereafter wild type is represented as WT) were transferred to two 300 ml portions of secondary cultures of LB-glycerol-amp and incubated at 37°C with shaking for 3 hours. From one of the culture flasks, 50 ml and 1 ml portions were transferred into a 50 ml conical centrifuge tube and a 1.5 ml Eppendorf tube, respectively. The 50 ml culture was centrifuged at 3800 rpm for 10 min in an IEC Centra GP8 centrifuge and the 1 ml culture was centrifuged at 13,000 rpm for 2 min in accu Spin™ Micro centrifuge. The supernatants were discarded and the pellets were stored at - 20°C.

2.5 ml of 84 mM Isopropyl thio galactoside (IPTG) (final concentration 840 μM) was added to the flask from which 50 ml and 1 ml portions of culture were removed and the flask was labeled "High IPTG". 0.75 ml of IPTG (final concentration 210 μM) was added to a second flask and labeled as "Low IPTG". The two culture flasks were again incubated at 37°C with shaking. At 2, 4, 6 and 8 hr, 50 ml and 1 ml portions from the two flasks were removed and placed into a 50 ml conical centrifuge tube and a 1.5 ml Eppendorf tube respectively. The pellets were collected by centrifugation as before and stored at -20°C. The cell pellets from 50 ml samples were resuspended separately in 1 ml of calcium chloride lysis buffer. The resuspended samples were lysed using 0.1 mm glass beads in a Mini bead beater™ (BioSpec Products) for 3 min at 1 min intervals on ice. The glass beads were allowed to settle down and the supernatants were centrifuged at 13,000 rpm for 10 min in a microcentrifuge to remove the cell debris. The supernatants were

transferred to 1.5 ml Eppendorf tubes and protein concentrations were estimated by standard Bradford assay [37] using a UV spectrophotometer at 595 nm.

The SDS-PAGE gels samples were prepared and analyzed according to the following "Standard Procedure for SDS-PAGE gel electrophoresis". Hereafter this procedure was followed for preparation and analysis of SDS-PAGE gel samples.

Approximately 10 µg of protein from each sample was mixed with an equal amount of 2x SDS-PAGE loading buffer. Cell pellets from 1 ml samples were resuspended in 50 µl of 2x SDS-PAGE loading buffer. All the samples were denatured by heating at 95°C for 5 min followed by centrifugation at 13,000 rpm for 1 min. The gel samples were run on SDS-PAGE (12% acrylamide) using BioRad power Pak at 120V. The gels were soaked in Coomassie staining solution for 30 min and destained overnight using a solution containing methyl alcohol (5%) and glacial acetic acid (7%).

Attempted Expression of ODCase with 210 µM IPTG induction: Incubation time points up to 22 hours

6 ml of overnight culture of BL21/pCal ODCase-f WT was transferred to a secondary culture of 600 ml of LB-glycerol-amp and incubated at 37°C with shaking for 3 hours. 50 ml and 1 ml portions of culture were transferred into a 50 ml conical centrifuge tube and a 1.5 ml Eppendorf tube respectively. Each sample was centrifuged as described above. The supernatants were discarded and the pellets were stored at -20°C.

1.5 ml of IPTG (final concentration 210 µM) was added to the secondary culture and again incubated at 37°C with shaking. Samples were collected every 2 hr up to 22 hr of induction. Each sample was centrifuged as described above. The supernatants were

discarded and pellets were stored at -20°C. The cell pellets from 50 ml samples were resuspended separately in 1 ml of calcium chloride lysis buffer. The resuspended samples were lysed using a Mini bead beater™, and the samples were prepared as before. The supernatants were transferred to 1.5 ml Eppendorf tubes and protein concentrations were estimated by standard Bradford assay [37] using UV spectrophotometer at 595 nm.

The gel samples were prepared as mentioned above and run on an SDS-PAGE using Bio rad power Pak at 120V. The gels were soaked in Coomassie staining solution for 30 min and destained overnight.

Expression and Purification of WT, K93C and "C93 only" yeast ODCase Fusion Proteins

6 ml of overnight cultures of BL21/pCal ODCase WT, K93C, and "C93 only" were transferred to three different secondary cultures of 600 ml each of LB-glycerol-amp and incubated at 37°C with shaking for 3 hours. 1.5 ml of IPTG (final concentration 210 μM) was added to each of the secondary cultures and again incubated at 37°C with shaking. After 18 hr of induction, cell pellets were collected by centrifugation at 7000 rpm for 10 min in Sorvall® RC 5C plus. For every 1 ml of calcium chloride lysis buffer, 5 μl of each of protease inhibitors Leupeptin (0.1 mg/ml), Pepstatin (0.2 mg/ml) and Phenylmethylsulfonyl fluoride (PMSF) (0.2 M) were added. The supernatants were discarded and pellets were resuspended separately in calcium chloride lysis buffer containing protease inhibitors. Approximately 1 ml of buffer was used for every 2.5 g of pellet. The resuspended samples were divided into small portions and lysed using a Mini bead beater™, and the samples were prepared as before. The supernatants were

transferred to 15 ml conical centrifuge tubes and protein concentrations were estimated by standard Bradford assay [37] using a UV spectrophotometer at 595 nm.

Approximately 2 ml of Calmodulin resin was added to each of 3 affinity columns and allowed to form a resin bed. The alcohol solution in which the resin was stored was allowed to flow through the column. The resin was washed 3 times with 2 ml portions of calcium chloride lysis buffer to remove any alcohol adhering to the resin.

20 μg portions of protein from WT, K73C and "C93 only" were added to the 3 pre-equilibrated resin columns. The columns were kept shaking overnight at 4°C. The columns were allowed to stand to form a resin bed and the protein solutions were allowed to flow through the resin. The columns were washed with 2 ml portions of calcium chloride lysis buffer to remove any unadhered protein. The fractions were analyzed for protein by mixing 25 μl of sample with 500 μl of Bradford reagent. Washings were continued until no more protein came from the column. The fusion proteins were eluted by 2 ml portions of CBP elution buffer (hereafter mentioned as elution buffer). The fractions were again analyzed for protein using Bradford reagent. Elutions were continued until no detectable protein came off the column. The resin was regenerated by washing with wash buffer 1 (0.1 M $NaHCO_3$, 2 mM EGTA), wash buffer 2 (1 M NaCl, 2 mM $CaCl_2$) and wash buffer 3 (0.1 M acetate, 2 mM $CaCl_2$). The resin was stored in 20% ethanol. Protein concentrations of flow through, fractions, wash 1, wash 2 and elution fractions were estimated by standard Bradford assay [37] using UV spectrophotometer at 595 nm.

The gel samples were prepared according to the standard procedure mentioned above and run on an SDS-PAGE using Bio rad power Pak at 120V. The gels were soaked in Coomassie staining solution for 30 min and destained overnight.

Spectrophotometric Determination of ODCase Activity

The following procedure was adapted as a "Standard Procedure for ODCase assays" with variations made and mentioned wherever necessary.

The spectrophotometric assay of ODCase depends on the decrease in absorbance at 286 nm as OMP is converted to UMP. The UV spectrophotometer was set to a wavelength range of 220-320 nm. Readings were taken at 286 nm. 840 µl of dH_2O, 20 µl of 1 M Tris (pH 7.4) and 100 µl of eluent 2 of yeast ODCase WT were mixed and the spectrophotometer was blanked with this solution. 40 µl of 1.2 mM OMP was added and the absorbance readings were taken every 10 sec for a period of 90 sec. Specific activity was calculated as a measure of change in absorbance with time.

Elution fractions of yeast ODCase WT, K93C and "C93 only" were pooled and concentrated using an Amicon stirred-cell protein concentrator.

Results and Discussion

At 8 hr of induction, 840 µM IPTG and 210 µM IPTG produced ODCase bands of same width (data not shown). 210 µM IPTG concentration was considered optimum for ODCase production. ODCase production was attempted with 210 µM IPTG concentration to determine the optimum induction time.

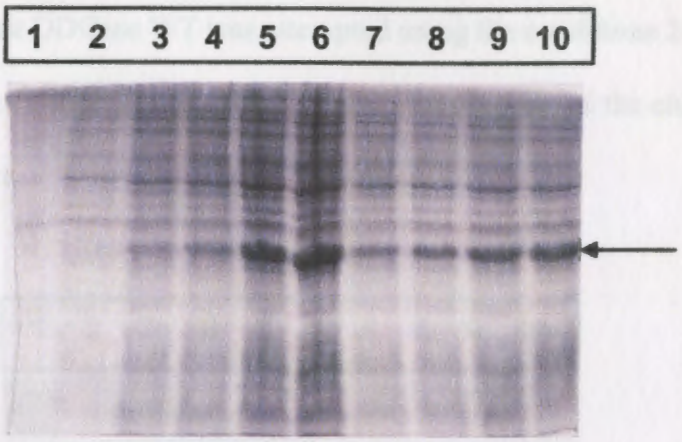


Figure 2-2: SDS-PAGE gel for time course analysis for the induction using whole cell samples of yeast ODCase WT. Arrow indicates the expected molecular weight for ODCase with the fusion protein, as produced from the pCal-n vector. Lane 1: molecular weight markers (not clearly seen). Lanes 2-10: protein from whole cell samples of ODCase for 0, 2, 4, 6, 8, 16, 18, 20 and 22 hr respectively.

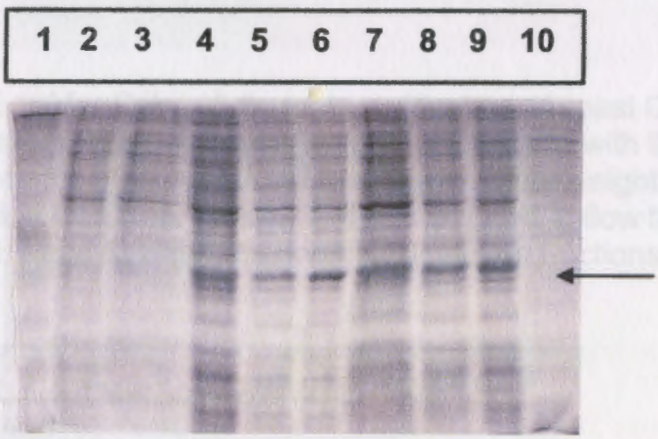


Figure 2-3: SDS-PAGE gel for lysate samples, time point induction of yeast ODCase WT. Arrow indicates the expected molecular weight for ODCase with the fusion protein, as produced from the pCal-n vector. Lane 1: molecular weight markers. Lanes 2-10: protein from lysate samples of ODCase for 0, 2, 4, 6, 8, 16, 18, 20 and 22 hr respectively.

The gels (Figures 2-2, 2-3) revealed that ODCase production increased with induction time and became almost constant after 18 hr induction. Hence we decided that 18 hr induction time is sufficient for maximum ODCase production.

Purification of yeast ODCase WT was attempted using the conditions 210 μM IPTG and induction time of 18 hr. The SDS-PAGE gels revealed that all the elution fractions were equally pure.

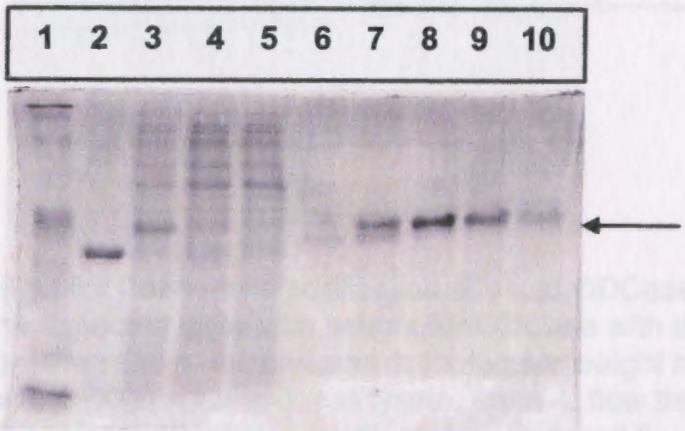


Figure 2-4: SDS-PAGE gel for Calmodulin resin purification of yeast ODCase WT. Arrow indicates the expected molecular weight for ODCase with the fusion protein, as produced from the pCal-n vector. Lane 1: molecular weight markers. Lane 2: purified non fusion ODCase. Lane 3: cell lysate. Lane 4: flow through fraction. Lane 5: wash 1. Lane 6: wash 2. Lanes 7-10: elution fractions 1, 2, 3 and 4 respectively.

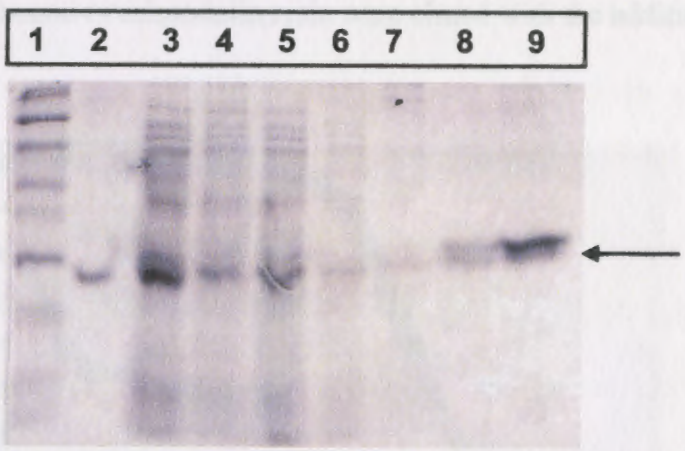


Figure 2-5: SDS-PAGE gel for Calmodulin resin purification of yeast ODCase K93C. Arrow indicates the expected molecular weight for ODCase with the fusion protein, as produced from the pCal-n vector. Lane 1: molecular weight markers. Lane 2: purified non fusion ODCase. Lane 3: cell lysate. Lane 4: flow through fraction. Lane 5: wash 1. Lane 6: wash 2. Lanes 7-9: elution fractions 1, 2 and 3 respectively.

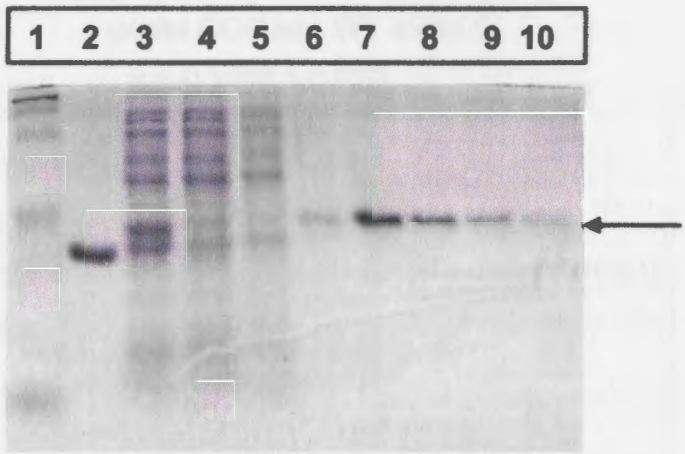


Figure 2-6: SDS-PAGE gel for Calmodulin purification of yeast ODCase "C93 only". Arrow indicates the expected molecular weight for ODCase with the fusion protein, as produced from the pCal-n vector. Lane 1: molecular weight markers. Lane 2: purified non fusion ODCase. Lane 3: cell lysate. Lane 4: flow through fraction. Lane 5: wash 1. Lanes 6-10: elution fractions 1, 2, 3, 4 and 5 respectively.

Almost all of the unwanted proteins came off as flow through. The remaining traces of interfering proteins came out in wash fractions. The pure ODCases (WT, K93C, "C93 only") which were bound to calmodulin resin were eluted with the addition of EGTA.

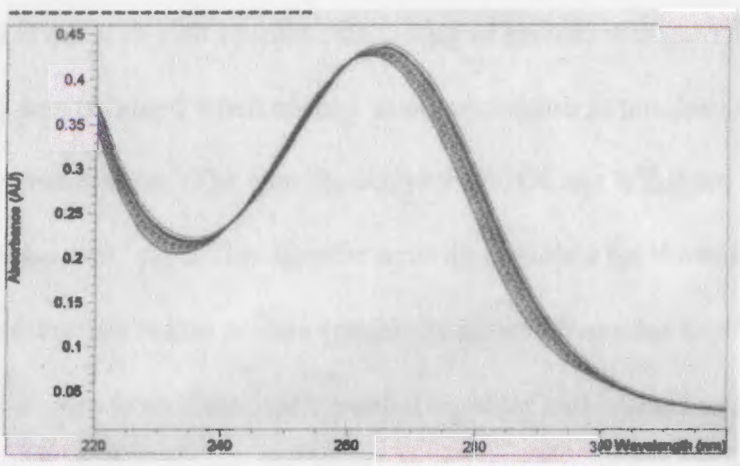


Figure 2-7: Spectrophotometric assay data for calmodulin resin purified ODCase WT.

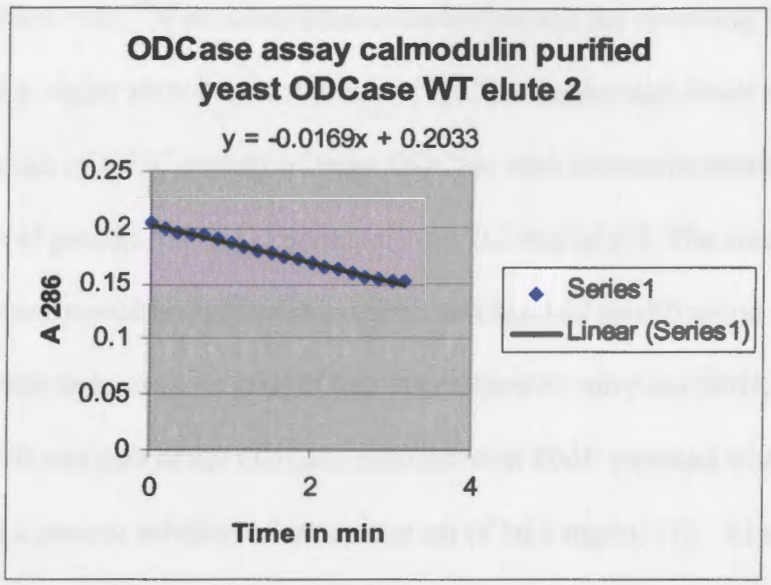


Figure 2-8: Graph of Spectrophotometric determination of ODCase WT activity. X-axis: Time in min. Y-axis: Absorbance at 286 nm.

WT ODCase with the CBP fusion was found to have catalytic activity (Figure 2-7). A graph of change in absorbance at 286 nm versus time was plotted (Figure 2-8). The slope of the graph which corresponds to change in absorbance was found to be -0.017 min^{-1}. The OMP to UMP reaction has a $\Delta\varepsilon_{286}$ of -0.00225 μM^{-1}. The change in absorbance (-0.017) when divided by -0.00225 μM^{-1} gave a change in concentration of 7.55 $\mu M\ min^{-1}$. This is equal to 7.55 nmoles min^{-1}. 9 µg of protein was used for assay. The specific activity was obtained when change in concentration in nmoles min^{-1} was divided by protein concentration. The specific activity of ODCase WT from elute 2 was found to be 0.84 nmoles $min^{-1}\ \mu g^{-1}$. This specific activity is quite a bit lower than that for the wild type enzyme with no fusion protein (generally about 40 nmoles $min^{-1}\ \mu g^{-1}$.)

The protein samples from elutes were pooled together and labeled as calmodulin purified WT ODCase, K93C and "C93 only" respectively and concentrated. The protein solutions seemed to precipitate above a concentration of 0.2 mg/ml. In order to carry out

chemical modification with ^{15}N enriched bromoethylamine and for obtaining NMR signals, we needed a higher protein concentration [7, 23]. Smiley and Jones carried out chemical modification of K93C mutant of yeast ODCase with bromoethylamine. The final concentration of protein in their experiments was 0.2 mg/ml [5]. The concentration of protein we obtained would be sufficient to carry out chemical modification with bromoethylamine and restoration of activity but insufficient to carry out NMR analysis [7, 23]. In the NMR analysis of the ODCase reaction with BMP enriched with ^{13}C at C5, Acheson *et al* used a protein solution of concentration of 16.1 mg/ml [7]. Li and Gershon used 0.15 mM solution of C272S K175C R209K mutant of Vaccinia virus protein VP39 in an attempt to measure the pK_a of Lys175 by NMR spectroscopy [23]. A concentration of our ODCase fusion protein of 0.15 mM would be about 4 mg/mL.

In conclusion, the Calmodulin resin purification of yeast ODCase fusion protein presented problems in terms of solubility and necessitated the change of strategy to obtain more soluble protein.

Chapter 3

Generation of *E. coli* ODCase Mutants ("C41S", "C41S C161S C168S" and "K73C") by Site-directed Mutagenesis and Chemical Modification of K73C mutant ODCase-f

Introduction

Yeast ODCase fusion protein resulted in solubility problems at concentrations higher than 0.2 mg/ml. We hoped that *E. coli* ODCase protein when expressed in *E. coli* would possibly produce more soluble protein. A literature search for the comparision of *E. coli* ODCase gene with the ODCase gene from *Saccharomyces cerevisiae* was performed. Sequence alignment of 53 ODCase enzymes revealed that there are 5 amino acid residues that are conserved in all the organisms [18]. The conserved amino acids corresponding to *E. coli* sequence are Lys44, Asp71, Lys73, Asp76 and Ile77. The degree of identity and homology between *E. coli* and the yeast enzyme are 24% and 45% respectively. Lys93 of yeast ODCase corresponds to Lys73 in the *E. coli* enzyme. From the molecular modeling using Swiss protein modeling, we identified that there are only 3 cysteines in the *E. coli* enzyme and that they are far from the active site. We propose that they might not be necessary for ODCase activity and their modification should not affect the ODCase activity.

The new experimental approach was to clone *E. coli* ODCase gene into pCal-n expression system. To ensure that the calmodulin resin purification system would not affect the protein structure and activity, we carried out purification of WT *E. coli* ODCase-f (wild type ODCase with the fusion protein attached). Site directed mutagenesis experiments were designed to mutate Cys41, Cys161, and Cys168 of *E. coli* ODCase to serines so that naturally occurring cysteines do not interfere with the chemical

modification experiments which we intend to carry out with bromoethylamine. If the naturally occurring cysteines were not important for substrate binding or ODCase activity, mutagenesis should result in a protein which is as active as wild type enzyme. For this we attempted to express the triple mutant (C41S C161S C168S) in BL21 cells. If the triple mutant posed the same solubility problems as the yeast enzyme, the strategy could be changed to utilizing the single K73C mutation. The 3 other cysteines in the K73C mutant of *E. coli* ODCase-f apart from Cys73, which would react with bromoethylamine, could be protected by reversible modification with DTNB; then modification of Cys73 with ^{15}N enriched bromoethylamine could be carried out followed by deprotection of the naturally occurring cysteines [23]. Control experiments of DTNB reaction with egg albumin and WT *E. coli* ODCase (non-fusion protein) were performed. Another control experiment tested whether modification of K73C *E. coli* ODCase-f with bromoethylamine restores activity as was the case with the yeast enzyme [5].

pCal-n Expression Vector

The pCal-n vector was used to clone *E. coli* gene of ODCase with fusion protein attached and carry out site directed mutagenesis and generate mutants "C41S, "C41S C161S C168S" and "K73C" of *E. coli* ODCase. This expression system with the fusion protein enables purification of ODCase WT and mutants with Calmodulin affinity resin.

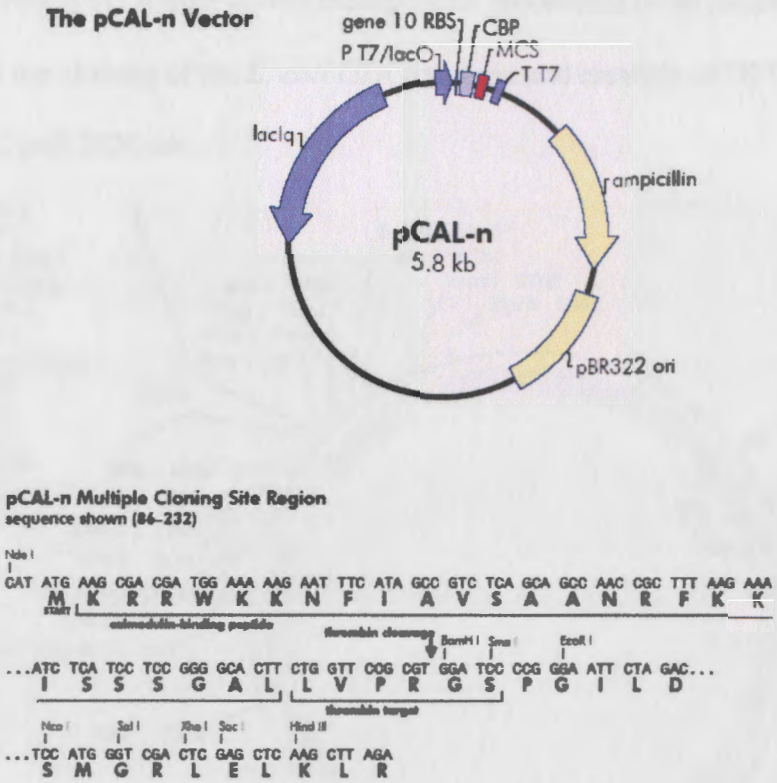


Figure 3-1: pCal-n vector (Affinity® Protein Expression and Purification System and Affinity® Protein Expression Vectors (Stratagene manual).

The pCal-n affinity protein expression vector is derived from pET-11 vector series. It has a T7 gene 10 promoter and leader sequence, which allows high selectivity of the promoter by T7 RNA polymerase. This selection leads to the repression of protein expression in the uninduced state and a high level expression in the induced state. The tight repression of protein expression is facilitated by the T7 *lac* promoter configuration and *lacI*^{q}. The pCal-n has a T7 promoter with *lac* operator, a T7 gene 10 ribosome binding site, a calmodulin binding peptide, a thrombin target, multiple cloning site, a T7 terminator and an ampicillin resistance site. It also has pBR322 origin of replication and *lacI*^{q} repressor open reading frame (ORF).

Vector M13mp19

The vector M13mp19 allows mutagenesis procedures to be carried out easily and was used for the cloning of the *E. coli* ODCase gene and creation of "K73C" and "K44C" mutants of *E. coli* ODCase.

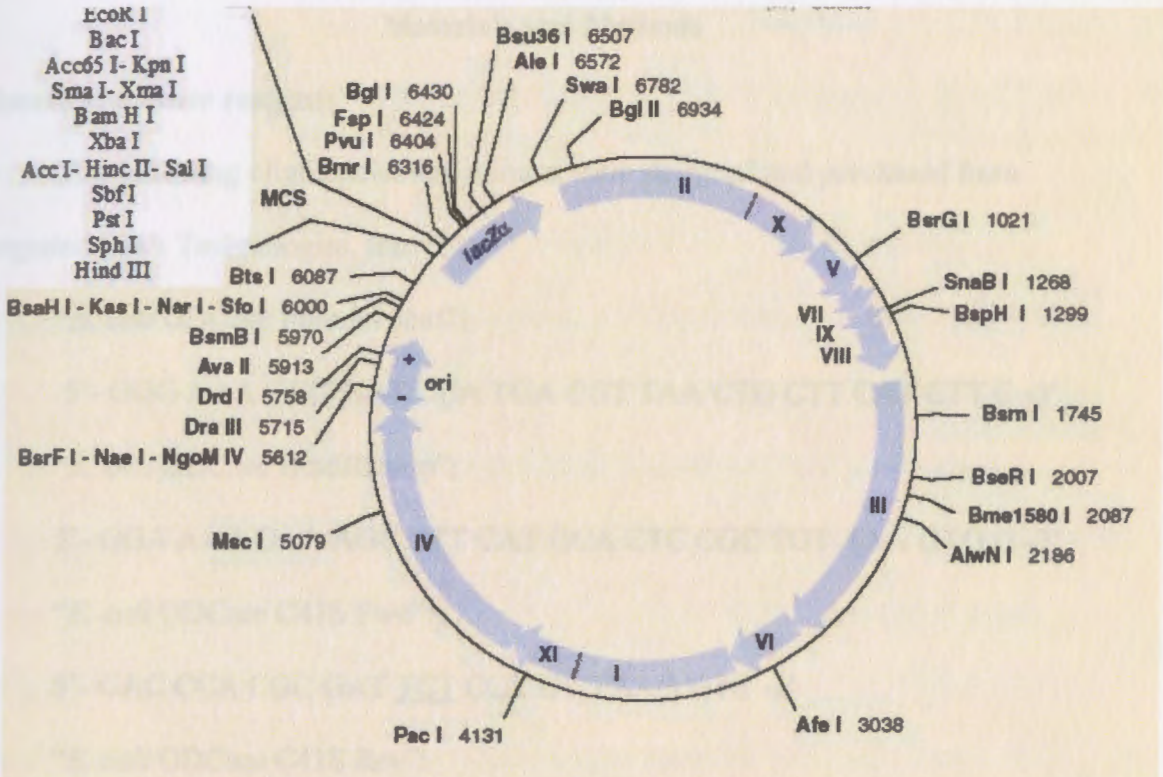


Figure 3-2: M13 mp19 vector (References: 1. Stewart, F.J. (2002) unpublished observations. 2. Messing, J. et al. (1977) *Proc. Natl. Acad. Sci.* USA 74, 3652-3646. 3. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) *Gene* 33, 103-119. Figure taken from New England bio labs manual)

The M13mp19 vector is derived from filamentous phage. This vector system has the unique advantage of recovering the inserted DNA as either single stranded or double stranded DNA. In M13mp19 vector, the foreign DNA can be inserted into a polylinker region that contains multiple restriction sites. The polylinker region is located near the alpha fragment of *lac* Z gene. This region is a nonessential region of the phage genome. When the *lac* Z gene is expressed in cells in the presence of X-gal (5-bromo-4-chloro-3-

indolyl-β-D-galacto pyranoside) and IPTG, a blue pigment is formed. Insertion of DNA into the poly linker region inactivates the alpha fragment of *lac* Z gene. When the insert-containing phages are plated under appropriate conditions, they form colorless plaques. The phages which do not contain insert forms blue plaques.

Materials and Methods

Primers and other reagents

The following oligo nucleotide primers were designed and purchased from Integrated DNA Technologies, Inc.

"*E. coli* ODCase Bam HI start":

5'- GGG AAA GGG GAT CCA TGA CGT TAA CTG CTT CAT CTT C -3'

"*E. coli* ODCase HindIII stop":

5'- GGA AAG GAA AGC TTT CAT GCA CTC CGC TGT AAA GAG G -3'

"*E. coli* ODCase C41S Fwd":

5'- GAC CCA CGC GAT TCT CGT CTG AAG GTC -3'

"*E. coli* ODCase C41S Rev":

5'- GAC CTT CAG ACG AGA ATC GCG TGG GTC -3'

"*E. coli* ODCase C161S C 168S Fwd":

5'- CGC AAA AAT CTG GCC TTG ATG GTG TGG TGT CTT CTG CTC -3'

"*E. coli* ODCase C161S C168S Rev":

5'- GAG CAG AAG ACA CCA CAC CAT CAA GGC CAG ATT TTT GCG -3'

"*E. coli* ODCase K73C Fwd":

5'- CTT TCT TGA CCT GTG TTT CCA CGA TAT CC -3'

"*E. coli* ODCase K73C Rev":

5'- GGA TAT CGT GGA AAC ACA GGT CAA GAA AG -3'

"*E. coli* ODCase K44C Fwd":

5'-CGT CTG TGT GTC GGC AAA GAG ATG -3'

(Mutation codons in the forward primers are shown underlined)

M13mp19 vector, Thermopol buffer, dNTPs, Vent DNA polymerase, restriction enzymes Bam HI and HindIII were purchased from New England Biolabs. X-gal was obtained from Sigma. XLI-Blue and BL21 cells are from Stratagene®. XLI-Blue competent cells were prepared according to the standard protocols. CJ236 cells were purchased from the *E. coli* genetic stock center. 10X T4 poly nucleotide kinase buffer and T4 poly nucleotide kinase were obtained from New England Biolabs. 5x PEG-NaCl solution consisted of 30% PEG 8000 (polyethylene glycol) in 1.6 M NaCl. The composition of 20x SSC solutions is 3 M NaCl, 0.3 M citric acid and the pH of the solution being 7.0. Qiagen® supplied kits for PCR product purification and plasmid purification. The compositions of calcium chloride lysis buffer and CBP elution buffers were the same as described in chapter 2. Calmodulin resin was purchased from Sigma.

Cloning and insertion of E. coli ODCase gene into pCal-n

Genomic DNA was prepared from *E. coli* BL 21 cells according to the standard procedure [38]. Oligonucleotides "*E. coli* ODCase Bam HI start" and "*E. coli* ODCase HindIII stop" were designed to amplify the ODCase gene and introduce restriction sites onto the ends. The lyophilized oligonucleotides were redissolved in 100 µl of sterile dH_2O and further dilutions were made to a final concentration of 5 µM.

The PCR reaction mixture of 100 µl, consisting of sterile dH_2O, 10X Thermopol reaction buffer (10 µl), 4dNTP mix 2 mM each (10 µl), oligonucleotides 5 µM (10 µl

each), genomic DNA (1 ng) and Vent DNA polymerase (1 µl) was assembled and the PCR was run in a thermal cycler according to the following program:

- Initial denaturation at 94°C for 5 min.
- 35 cycles of the following:
  - 94°C for 30 sec
  - 58°C for 60 sec
  - 72°C for 90 sec
- Single step at 72°C for 5 min

After this stage, the thermal cycler is programmed to lower the temperature to 4°C and hold this temperature indefinitely. Hereafter, this protocol is followed for PCR. Some modifications of the annealing temperatures were made when necessary and are described when used in the subsequent experiments.

10 µl of the reaction product was run on a 1.2% agarose gel at 120V using Biorad agarose gel electrophoresis tank. The gel electrophoresis was terminated when the blue tracking dye was about 3/4 to the end of the gel. The gel was stained in ethidium bromide for about 15 min and visualized under UV light. The remaining PCR product was spin column purified according to the Qiagen® PCR purification kit. 5 µl of the purified product was analyzed on 1.2% agarose gel to check successful purification.

The spin column purified PCR product and the pCal-n were then digested using restriction enzymes BamHI and HindIII. The reaction mixture contained PCR product or pCal-n, restriction enzyme buffer NEB 2, restriction enzymes BamHI and HindIII. The reaction was carried out for 2 hr at 37°C. The restriction digested products were again spin column purified using the Qiagen® kit. The products of purification were verified on

a 1.2% agarose gel. The concentration of DNA was estimated by comparing the brightness of bands of restriction products with the DNA markers on the gel. Approximately a 1:2 ratio (ng) of insert (PCR product) to vector (pCal-n) was used for ligation reaction. The ligation reaction mixture was comprised of restriction digested PCR product and pCal-n, 10X ligase buffer and T4 DNA ligase. The reaction was allowed to proceed for 1 hr at room temperature. A negative reaction was also carried out with the insert absent.

Transformation and screening of colonies for ODCase insert

Transformations of positive ligation mixture, negative ligation mixture, pCal-n, and no DNA, were carried out with XLI-Blue cells according to standard procedures. The cells were rescued by the addition of 1 ml of LB-glucose medium and were grown at 37°C with shaking for 1 hr. 200 µl of each sample was plated on LB-agar-amp plates and incubated overnight at 37°C. 20 different colonies were picked from the positive ligation plate and minipreps were prepared according to the standard protocol [39]. The DNA from the minipreps was analyzed on a 0.8% agarose gel.

The cells from 3 positive minipreps were inoculated separately into 3 different flasks containing 50 ml of LB-amp medium each. The cultures were incubated overnight at 37°C for Qiagen® purification. The plasmid constructions were verified by restriction digestion of 20 µl samples from the Qiagen® purification. The restriction digested products were run on a 1.2% agarose gel. The plasmid with the right construction was introduced into BL21 cells.

Expression and Specific activity Determination of *E. coli* ODCase fusion protein

The following growth procedure was followed as "Standard Trial Growth

Procedure" for all the time course induction experiments, with minor variations made and mentioned wherever necessary.

3 ml of overnight culture of BL21/pCal ODCase-f (*E. coli*) WT was transferred to a secondary culture of 300 ml of LB-amp and incubated at 37°C with shaking for 3 hours. 50 ml and 1 ml portions of culture were transferred into a 50 ml conical centrifuge tube and a 1.5 ml Eppendorf tube respectively. The 50 ml culture was centrifuged at 3800 rpm for 10 min and the 1 ml culture was centrifuged at 13,000 rpm for 2 min as before. The supernatants were discarded and the pellets were stored at - 20°C.

150 µl of 84 mM IPTG solution (final concentration is 50 µM) was added to the secondary culture and again incubated at 37°C with shaking. Samples were collected every 2 hr up to 6 hr of induction. The 50 ml and 1 ml samples were centrifuged as above. The supernatants were discarded and pellets were stored at -20°C. The cell pellets from 50 ml samples were resuspended separately in 1 ml of Calcium chloride lysis buffer.

The following procedure was used as "Standard Cell Lysis Procedure" for the preparation of cell lysates.

The resuspended cell pellet samples were transferred to the bead beater tubes containing 0.1 mm glass beads. The samples were then lysed by bead beating. The tubes were shaken in a Mini bead beater™ (BioSpec Products) for 1 min and kept on ice for 1 min. This bead beating procedure was carried out 3 times with 1 min intervals on ice. The glass beads were allowed to settle down and the supernatants were centrifuged at 13,000 rpm for 10 min in a microcentifuge to remove the cell debris. The supernatants were

transferred to 1.5 ml Eppendorf tubes and protein concentrations were estimated by standard Bradford assay.

Gel samples were prepared as before and run on an SDS-PAGE gel (12% acrylamide) using constant voltage at 120V. The gels were soaked in Coomassie staining solution for 30 min and destained overnight.

Spectrophotometric Determination of ODCase Activity in the WT fusion protein

The ODCase assays were performed as described previously. Four individual sets of spectra were developed using samples from 0 hr, 2 hr, 4 hr and 6 hr induction. 5 µl of each sample was used for assay. For 2 hr induction sample a 1:10 dilution, for 4 hr and 6 hr induction a 1:20 dilution was employed. Specific activity was calculated as a measure of change in absorbance with time, using protein concentrations and the value for $\Delta\varepsilon_{286}$ for the ODCase reaction of -2250 M^{-1} cm^{-1}.

Expression and Purification of WT E. coli ODCase fusion protein

The following procedure is followed as "Standard Growth Procedure for Purification" for the preparation of protein samples for Calmodulin purification.

5 ml of overnight culture of BL21/pCal ODCase (*E. coli*) WT, was transferred to a secondary culture of 500 ml each of LB-amp and incubated at 37°C with shaking for 3 hours. 300 µl of 84 mM IPTG solution (final concentration = 50 µM) was added to the secondary culture and again incubated at 37°C with shaking. After 6 hr of induction, cell pellet was collected by centrifugation at 7000 rpm for 10 min in Sorvall RC 5C plus. For every 1 ml of calcium chloride lysis buffer, 5 µl of each of protease inhibitors leupeptin (0.1 mg/ml), pepstatin (0.2 mg/ml) and PMSF (0.2 M) were added. The supernatant was

discarded and the pellet was resuspended in calcium chloride lysis buffer containing protease inhibitors. Approximately 1 ml of buffer was used for every 2.5 g of pellet.

The resuspended sample was subjected to lysis using 0.1 mm glass beads in a Mini bead beater™ as described previously. The protein concentration was estimated by standard Bradford assay [37] using UV spectrophotometer at 595 nm.

The following is used as the "Standard procedure for the purification of ODCase fusion proteins using Calmodulin affinity resin"

Approximately 2 ml of Calmodulin resin was added to an affinity column and allowed to form a resin bed. The alcohol solution in which the resin was stored was allowed to flow through the column. The resin was washed 3 times with 2 ml portions of calcium chloride lysis buffer to remove any alcohol adhering to the resin.

5 ml of protein was added to the pre-equilibrated resin column. The column was shaken overnight at 4°C. The column was allowed to stand to form a resin bed and the protein solution was allowed to flow through the resin. The column was washed with 2 ml portions of calcium chloride lysis buffer to remove any unadhered protein. The fractions were analyzed for protein by mixing 25 µl of sample with 500 µl of Bradford reagent. Washings were continued until no more protein came from the column. The fusion protein was eluted with 2 ml portions of CBP elution buffer. The fractions were again analyzed for protein using Bradford reagent. Elutions were continued until no detectable protein came off the column. The resin was regenerated by washing with wash buffer 1, wash buffer 2 and wash buffer 3 (same as mentioned in chapter 2) respectively. The resin was stored in 20% ethanol. Protein concentrations of flow through, wash 1, wash 2 and elutes were estimated by standard Bradford assay.

Gel samples were prepared as before and run on an SDS-PAGE gel (12% acrylamide) using constant voltage at 120V. The gels were soaked in Coomassie staining solution for 30 min and destained overnight.

Spectrophotometric Determination of ODCase Activity

The Elution fraction 2 was analyzed for ODCase activity as described before. 80 µl of elute 2 was used for assay. Specific activity was calculated as a measure of change in absorbance with time, using protein concentrations and the value for $\Delta\varepsilon_{286}$ for the ODCase reaction of -2250 M^{-1} cm^{-1}.

Mutagenesis and Attempted Expression of C41SC161SC168S *E. coli* ODCase-f

For the cysteine-to-serine mutagenesis experiments, the ODCase gene was amplified in two separate PCRs, each containing one piece of ODCase gene with a mutation. After amplification both pieces were joined by overlap extension. Oligonucleotides were designed to introduce mutations at codons 41, 161 and 168 of the *E. coli* ODCase gene. Codons 161 and 168 were mutated with a single overlap extension procedure. The lyophilized oligonucleotides were redissolved in 100 µl of sterile dH_2O and further dilutions were made to a final concentration of 5 µM.

Conversion of Cys41 to Ser41

E. coli ODCase-f WT plasmid was used as template. The two PCR reaction mixtures, consisting of sterile dH_2O, 10X Thermopol reaction buffer, 4dNTP mix, oligonucleotides ("*E. coli* ODCase Bam HI start" and "C41S Rev" in one PCR mix generating a small fragment, and "C41S Fwd" and "*E. coli* ODCase HindIII stop" in the other generating a large fragment), *E. coli* ODCase-f WT plasmid DNA and Vent DNA

polymerase were assembled. The PCR samples were run in a thermal cycler according to the protocol.

10 µl of each sample were run on a 1.2% agarose gel at 120V using a Biorad agarose gel electrophoresis tank. The remaining PCR products were gel slice purified according to Qiagen® and analyzed by gel electrophoresis.

The amount of DNA to be used in the overlap extension was calculated according to the size of the two pieces generated by PCR. A ratio of 300 ng of the small fragment to 1300 ng of the large fragment was used. The overlap extension reaction mixture of a total volume of 100 µl, consisting of sterile dH_2O, 10X Thermopol reaction buffer (10 µl), 4dNTP mix 2 mM each (10 µl), the two PCR products and Vent DNA polymerase (1 µl) was assembled and the reaction was run in a thermal cycler. The protocol for overlap extension reaction is given below.

35 cycles of the following:

- 94°C for 1 min
- 58°C for 1 min
- 72°C for 75 sec
- 4°C indefinitely.

This protocol is followed for overlap extension hereafter.

The overlap extension product was gel slice purified using Qiagen® kit. 5 µl of overlap extension product was run on a 1.2% agarose gel, and quantified as described previously.

Generation of pCal-ODCase-f C41S C161S C168S ("pCal triple mutant")

The overlap extension product from the C41S mutation was used as a template for

the creation of "pCal triple mutant". The two PCR reaction mixtures, consisting of sterile dH_2O, 10X Thermopol reaction buffer, 4dNTP mix, oligonucleotides ("*E. coli* ODCase Bam HI start" and "C161S C168S Rev" in one PCR mix generating the large fragment, and "C161S C168S Fwd" and "*E. coli* ODCase HindIII stop" in the other, generating the small fragment), overlap extension product from "C41S" mutation and Vent DNA polymerase were assembled and the PCR samples were run in a thermal cycler according to the protocol. 10 µl of each sample were run on a 1.2% agarose gel, and quantified as described previously. The remaining PCR products were gel slice purified according to Qiagen®. 5 µl of each of the purified products were analyzed on 1.2% agarose gel to check successful purification and quantification of DNA.

560 ng of the small piece and 1050 ng of the large piece were used for the overlap extension reaction. The overlap extension reaction mixture consisting of sterile dH_2O, 10X Thermopol reaction buffer, 4dNTP mix, the two gel slice purified PCR products and Vent DNA polymerase was assembled and the reaction was run in a thermal cycler according to the protocol. The overlap extension product was gel slice purified using Qiagen® kit. 5 µl of overlap extension product was run on a 1.2% agarose gel, and quantified as described previously.

The gel slice purified overlap extension product and the pCal-n were then restriction digested using restriction enzymes BamHI and HindIII. The reaction was carried out for 2 hr at 37°C. The restriction digested products were again spin column purified using Qiagen® kit. The products of purification were verified on a 1.2% agarose gel. The concentration of DNA was estimated by comparing the brightness of bands of restriction products with the DNA markers on the gel. Approximately a 1:2 ratio of insert

(PCR product) to vector (pCal-n) was used for ligation reaction. The ligation reaction mixture contained restriction digested PCR product (150 ng) and pCal-n (300 ng), 10X ligase buffer(1 µl) and T4 DNA ligase (0.5 µl) The reaction was allowed to proceed for 1 hr at room temperature. A negative reaction was also carried out with the insert absent.

Transformation and screening of colonies for "pCal triple mutant" ODCase insert

Transformations of positive ligation mixture, negative ligation mixture, pCal-n, and no DNA, were carried out with XLI-Blue cells according to standard procedures. The cells were rescued by the addition of 1 ml of LB-glucose medium and were grown at 37°C with shaking for 1 hr. 200 µl of each sample was plated on LB-agar-amp plates and incubated overnight at 37°C. 20 different colonies were picked from the positive ligation plate and minipreps were prepared according to the standard protocol [39]. The DNA from the minipreps was analyzed on a 0.8% agarose gel.

The cells from 3 positive minipreps were inoculated separately into 3 different flasks containing 50 ml of LB-amp medium each. The cultures were incubated overnight at 37°C for Qiagen® purification. The plasmid constructions were verified by restriction digestion of 20 µl samples from the Qiagen® purification. The restriction digested products were again run on a 0.8% agarose gel. The plasmids with the right construction were introduced into BL21 cells.

DNA sequencing of pCal-ODCase-f C41S C161S C168S ("pCal triple mutant")

The DNA from the 3 Qiagen® purified plasmids was further purified by phenol/chloroform extraction and ethanol precipitation, and analyzed by DNA sequencing using the CEQ 2000 dye terminator system (Beckman Coulter, Inc.).

Attempted Expression of C41S C161S C168S ODCase Fusion Protein

BL21 cells containing pCal ODCase-f C41S C161S C168S were grown according to the "Standard Trial Growth Procedure" for time course induction of cells. Cell lysates were prepared as described previously. The protein concentrations were estimated by standard Bradford assay [37] using a UV spectrophotometer at 595 nm.

Gel samples were prepared as before and run on an SDS-PAGE gel (12% acrylamide) using constant voltage at 120V. The gels were soaked in Coomassie staining solution for 30 min and destained overnight.

The following different conditions of protein expression were tried to obtain soluble protein.

1. LB medium, 50 μM IPTG induction at 37°C. $CaCl_2$ lysis buffer with 0.1% Triton pH 8.0 was used for lysis.

2. LB medium, 5 μM, 10 μM, 20 μM, 35 μM, 50 μM, 75 μM and 100 μM IPTG induction at 37°C. $CaCl_2$ lysis buffer pH 8.0 was used for lysis.

3. LB medium, 50 μM IPTG induction at 37°C. $CaCl_2$ lysis buffer pH 8.6 for lysis.

4. LB medium, 50 μM IPTG induction at 37°C. Cell lysates were prepared with $CaCl_2$–Tris lysis buffer pH 8.0 (Triton was absent in the lysis buffer).

5. Cells were grown in LB medium at 37°C, induced with 50 μM IPTG and lysed with $CaCl_2$–Hepes lysis buffer pH 7.4. (Hepes replaces Tris in the composition of lysis buffer.)

6. LB medium, 50 μM IPTG induction at 37°C; lysates were prepared with $CaCl_2$–Hepes lysis buffer pH 6.8.

7. LB medium with different glycerol concentrations (3%, 5% and 10%). Induction with 50 μM IPTG at 37°C. Cells were lysed with $CaCl_2$–Tris lysis buffer pH 8.0.

8. LB medium, 50 μM IPTG induction. Cells were grown at different temperatures 37°C, 30°C, 25°C and 17°C and lysed with $CaCl_2$–Tris lysis buffer pH 8.0

9. Cells were grown in enriched medium. The composition of enriched medium is as follows. 16 g of tryptone, 10 g of yeast extract and 5 g of NaCl were dissolved in 1 liter of dH_2O. The pH of the solution was adjusted to 7.4. The incubation temperature was 37°C. The cells were induced with 50 μM IPTG. Lysates were prepared with $CaCl_2$–Tris lysis buffer pH 8.0.

10. LB medium, 50 μM IPTG induction at 37°C. Cells were lysed with $CaCl_2$–Tris lysis buffer pH 8.0 containing different urea concentrations (1.3 M, 1.6 M, 1.9 M, 2 M and 2.3 M).

K73C mutant of *E. coli* ODCase

We attempted to generate the K73C mutant by PCR and overlap extension reaction. The pCal triple mutant plasmid DNA was used as a template to generate the two DNA fragments using primers "*E. coli* ODCase Bam HI start" and "K73C Rev" in one reaction and "K73C Fwd" and "*E. coli* ODCase HindIII stop" in a second reaction. The two DNA fragments thus generated failed to overlap. Different annealing temperatures were tried but failed to generate an overlap extension product. Hence we decided to generate the K73C mutant using M13 vector and *in vitro* replication.

Cloning of *E. coli* ODCase gene into M13mp19 vector

2 μg of *E. coli* pCal ODCase-f and 1 μg of M13mp19 were digested with restriction enzymes BamHI (from Roche) and HindIII (from NEB). Restriction enzyme

buffer NEB2 was used. The reaction was allowed to proceed for 2 hr at 37°C. The ODCase fragment of the restriction digested plasmid was gel slice purified using the Qiagen® kit. The digested M13mp19 was spin column purified according to Qiagen®. The purified products were run on a 1.2% agarose gel. 100 ng of restriction digested, gel slice purified ODCase fragment and 200 ng of restriction digested, spin column purified M13mp19 were used in a ligation reaction. 2 µl of T4 DNA ligase was used for the ligation reaction. Negative ligation reaction was also carried out with no insert. The reactions were allowed to proceed for 1 hr at room temperature.

Transformation of M13mp19 *E. coli* ODCase-f into XLI Blue competent cells

5 µl of the ligation mixture was used for transformation. The $CaCl_2$ treated XLI-Blue cells were removed from the -80°C freezer and thawed. Four 100 µl portions of cells were then transferred into 4 prechilled falcon tubes and were kept on ice. To the 4 tubes, 5 µl of positive ligation mixture, 5 µl of negative ligation mixture, 5 µl of M13mp 19 and no DNA were added respectively. The tubes were kept on ice for 10 min and then heat shocked at 42°C for 1 min. To each of the tubes, a mixture of 10 µl of IPTG (20 mg/ml), 40 µl of X-gal (20 mg/ml) and 3 ml of sterile top agar (LB containing 0.75% agar) maintained at 45°C was added and were plated on LB-agar plates. The top agar was allowed to solidify and the plates were incubated at 37°C overnight.

K73C Mutagenesis using M13mp19/ODCase

All the procedures followed hereafter are adapted from Current Protocols in Molecular Biology [40, 41]. Three colorless plaques were picked from the M13mp19 ODCase transformation plate with XLI-Blue cells, and phage supernatants were made according the standard procedure [40].

Preparation of replicative form DNA (RF DNA)

Three 2 ml portions of an overnight culture of XLI-Blue cells were added to three 200 ml portions of LB medium containing 1 ml of 20% glucose. The cultures were incubated at 37°C for 3 hr with shaking. The three phage supernatants previously prepared were now used to infect the three cultures. 200 µl of the phage supernatant was added to each culture and labeled as XLI-Blue cells infected by phage supernatant 1, 2 and 3 respectively and incubated at 37°C for 15 min with shaking. 0.3 ml of chloramphenicol (10 mg/ml) in ethanol was added to each culture and incubation was carried out at 37°C for 2 hr with shaking. 1.5 ml from each culture was taken and the minipreps were prepared and analyzed on a 0.8% agarose gel.

Amplification of the DNA from positive phage supernatants

Phage supernatant from a blue plaque was prepared. 500 µl of each of the three phage supernatants previously prepared from 3 positive (colorless) plaques and 500 µl of the phage supernatant from negative plaque (blue plaque) were purified by phenol chloroform extraction. The purified DNA pellets were redissolved in 50 µl of TAE buffer. 1 µl of each sample was amplified by PCR using "*E. coli* ODCase Bam HI start" and "*E. coli* ODCase HindIII stop" primers. A positive control reaction and a negative control reaction were also carried out with pCalODCase-f and no DNA respectively. 5 µl of each reaction product was analyzed on 1.2% agarose gel.

Preparation of single stranded DNA using CJ236 cells

Uracil-containing DNA is produced in *E. coli* CJ236 cells. Cultures of CJ236 cells infected with M13 phage allow the production of uracil-containing DNA. More frequent selection of mutants is thus possible with this strain [41].

5 ml of LB medium was inoculated with 50 µl of overnight culture of CJ236 cells and incubated at 37°C with shaking. 100 µl of phage supernatant (which was verified to have the ODCase insert) was added to 100 ml of LB medium containing 0.25 µg/ ml of chloramphenicol. 5 ml of a secondary CJ236 culture that had been grown for 3 hr (mid-log culture) was added and incubated at 37°C for 18 hr. The cells were centrifuged at 5000 x g for 30 min using Sorvall RC 5C plus. The pellet was discarded and the phage was precipitated by the addition of 1 volume of 5x PEG / NaCl solution (composition described in materials section) to 4 volumes of supernatant followed by incubation at 0°C for 1 hr. The phage pellet was collected by centrifugation at 6500 rpm for 15 min in the Sorvall RC 5C Plus centrifuge. The supernatant was discarded and the pellet was re-suspended in 5 ml of TE buffer and kept on ice for 1 hr. The solution was centrifuged at 6500 rpm for 15 min in the Sorvall RC 5C Plus centrifuge. The supernatant was transferred into an Eppendorf tube and the pellet was discarded. The supernatant which contains M13 phage with single stranded DNA was further extracted with phenol-chloroform. The pellet was re-dissolved in 1 ml of TE buffer. The DNA was quantitated spectrophotometrically at 260 nm.

Generation of mutations K73C and K44C in M13mp19/ODCase

The lyophilized oligonucleotides, "K73C Fwd" and "K44C Fwd" were re-dissolved in 100 µl of sterile dH_2O and further dilutions were made to a final concentration of 5 µM. Two kinase reactions, each containing 2 µl of 10X T4 polynucleotide kinase buffer, 2 µl of 10 mM ATP, 2 µl of 5 µM oligo (K73C Fwd in one reaction and K44C Fwd in another) and 2U of T4 polynucleotide kinase in a total volume of 20 µl were assembled and incubated at 37°C for 1 hr. The reactions were terminated

by the addition of 3 µl of 100 mM EDTA followed by denaturation of the enzyme at 70°C for 15 min. The reaction mixtures were then allowed to cool to room temperature.

The phosphorylated oligos K73C Fwd and K44C Fwd were used in the annealing reaction. Two reactions each containing 5 µl of phosphorylated oligo (K73C Fwd in one reaction and K44C Fwd in other), 13 µl of sterile dH_2O, 3 µl of SS DNA (1:10 dilution) and 1.25 µl of 20X SSC were assembled and spun in a microcentifuge at 13,000 rpm for 5 sec. The reaction tubes were placed in water at 70°C and allowed to cool to room temperature. The tubes were spun again in a microcentifuge at 13,000 rpm for 5 sec and kept on ice. A negative annealing reaction was also carried out in a similar way without the addition of phosphorylated oligo. To the annealing reaction mixtures, hybridization mixture containing 20 µl of 5X polymerase mix (prepared according to standard protocol), 2.5U T4 DNA polymerase, 2U T4 DNA ligase in a total volume of 100 µl was added and incubated at 0°C for 5 min, room temperature for 5 min and at 37°C for 2 hr.

XLI Blue competent cells were removed from the -80°C freezer, thawed and kept on ice. 100 µl of cells were transferred into a pre-chilled falcon tube. 2 µl of the K73C reaction was added to the cells and kept on ice for 10 min. The cells were then heat shocked at 42°C for 1 min. 3 ml of top agar was added and the mixture plated on LB-agar plate. The top agar was allowed to solidify and the plate was incubated at 37°C overnight. A similar procedure was followed with 10 µl of K73C reaction, 2 µl and 10 µl of K44C reaction, 10 µl of negative annealing reaction, 3 µl of SS DNA (positive control) and no DNA.

Preparation and sequencing of single stranded DNA from K73C and K44C Mutagenesis Reactions

8 tubes of LB medium (2 ml each) were inoculated with 20 µl of overnight culture of XLI-Blue cells. With sterile toothpicks single plaques were picked from mutagenesis reaction plates of K73C and K44C (four plaques from each). Secondary cultures were infected with individual plaques from K73C and K44C. The cultures were then incubated at 37°C for 8 hr. Volumes of 1.5 ml from each culture were transferred into sterile Eppendorf tubes and centrifuged at 13,000 rpm for 5 min in a microcentifuge. 1.25 ml of supernatant from each sample was transferred into fresh Eppendorf tubes. 250 µl of PEG/NaCl solution (composition described in materials section) was added to each tube and incubated at room temperature for 15 min. The tubes were again centrifuged at 13,000 rpm for 5 min in the microcentrifuge. Supernatants were discarded and the pellets were again centrifuged to remove traces of PEG solution. The pellets were then re-suspended in 200 µl of TE buffer and extracted with phenol and chloroform. The DNA was precipitated by addition of sodium acetate solution and ethanol. Finally the pellets were re-suspended in 25 µl of TE buffer. The concentration of DNA was estimated spectrophotometrically at 260 nm. The samples were then analyzed by DNA sequencing using the CEQ 2000 dye terminator system (Beckman Coulter, Inc.).

Introduction of K73C and K44C mutant ODCase genes into pCal-n

The SS DNA of K73C and K44C, from the samples which have the desired mutations were used as templates for PCR amplification using "*E. coli* ODCase BamHI start" and "*E. coli* ODCase HindIII stop" primers.

10 µl of each amplified product were run on a 1.2% agarose gel to determine the presence of amplified product. The remaining PCR products were spin column purified according to Qiagen®. 5 µl of each purified product were analyzed on 1.2% agarose gel to check for successful purification. Essentially the same procedures as for construction of other pCal-ODCase plasmids were followed.

The spin column purified pCal-n and the PCR products were then restriction digested using restriction enzymes BamHI and HindIII. The restriction digested and spin column purified pCal-n and the PCR products were then used in a ligation reaction. Transformations of ligation mixtures into XLI Blue cells were carried out as before and the colonies were screened for insert using miniprep DNA analysis.

The plasmids of the positive minipreps from K73C and K44C were Qiagen® purified and the constructions of plasmids were verified by restriction as described previously. The plasmids with right construction were transformed into BL21 cells. The Qiagen® purified plasmids were further purified by phenol chloroform extraction and analyzed by DNA sequencing using the CEQ 2000 dye terminator system (Beckman Coulter, Inc.).

Protein expression of K73C and K44C *E. coli* ODCase-f

BL21 cells containing pCal ODCase-f K73C and K44C were grown according to the "Standard Trial Growth Procedure" for time course induction of cells. Cell lysates were prepared as described previously. The protein concentrations were estimated by standard Bradford assay [37] using spectrophotometer at 595 nm.

Gel samples were prepared as before and run on an SDS-PAGE gel (12% acrylamide) using constant voltage at 120V. The gels were soaked in Coomassie staining solution for 30 min and de stained overnight.

Expression and Purification of K73C *E. coli* ODCase-f

BL21 cells containing pCal ODCase-f K73C were grown according to the "Standard Growth Procedure for Purification" for preparation of protein samples for Calmodulin resin purification. The resuspended cells were subjected to lysis using 0.1 mm glass beads in a Mini bead beater as described in the standard lysis procedure. The protein concentration was estimated by standard Bradford assay [37] using UV spectrophotometer at 595 nm.

Approximately 5 ml of protein was purified using Calmodulin affinity column as described previously. Protein concentrations of flow through, wash 1, wash 2 and elutes were estimated by standard Bradford assay [37].

Gel samples were prepared as described before and run on an SDS-PAGE (12% acrylamide) using constant voltage at 120V. The gels were soaked in Coomassie staining solution for 30 min and destained overnight.

Chemical Modification of K73C mutant ODCase with DTNB

10 ml of 0.1 M sodium phosphate buffer containing 1 mM EDTA (pH 8.0) was degassed using water aspirator for about 15 min. 0.02 g of DTNB was dissolved in the degassed buffer and again degassed for 15 min. Care was taken not to expose DTNB to excess light by covering the filter flask containing DTNB solution with aluminum foil.

The UV spectrophotometer was set for an absorbance range of 350-450 nm and the absorbance readings were taken at 412 nm. A positive reaction of DTNB with

sulfhydral groups results in an increase in absorbance at 412 nm. 100 µl of DTNB solution (5 mM) and 900 µl of calmodulin purified K73C ODCase (0.34 mg/ml) were mixed and the spectrophotometer was blanked. Absorbance readings were taken immediately after blanking the spectrophotometer then every 10 min for 2 hr.

Chemical Modification of Egg Albumin with DTNB

10 ml of 5 mM DTNB solution in 0.1 M sodium phosphate buffer containing 1 mM EDTA (pH 8.0) was prepared as before.

The UV spectrophotometer was set as before. 100 µl of DTNB solution (5 mM), 200 µl of Egg Albumin (5 mg/ml) and 700 µl of dH_2O were mixed and the spectrophotometer was blanked. Absorbance readings were taken immediately after blanking the spectrophotometer then every 10 min for 2 hr.

Chemical Modification of WT ODCase non fusion protein with DTNB in presence and absence of Urea

10 ml of 5 mM DTNB solution in 0.1 M sodium phosphate buffer containing 1 mM EDTA (pH 8.0) was prepared as before.The UV spectrophotometers were set as described before. Four different reactions were set up; the conditions are compiled in Table 3-1.

	DTNB	ODCase (7 mg/ml)	Urea (6 M)	dH_2O
Reaction without urea	100 µl	45 µl	0 µl	855 µl
Reaction with 1 M urea	100 µl	45 µl	150 µl	705 µl
Reaction with 4 M urea	100 µl	45 µl	600 µl	255 µl
Reaction with 5 M urea	100 µl	45 µl	750 µl	105 µl

Table 3-1: Chemical modification of ODCase non fusion protein with DTNB at different urea concentrations.

The reaction mixtures were then used to blank the spectrophotometers. Absorbance readings were taken immediately after blanking the spectrophotometers then every 10 min for 2 hr.

Chemical modification of K73C ODCase with Bromoethylamine

0.9 g of urea was dissolved in 2 ml of calmodulin purified K73C ODCase (0.3 mg/ml). Urea was dissolved slowly over a period of 10 min with gentle mixing. To 710 µl of the denatured protein 50 µl of 0.76 M bromoethylamine solution was added. The final concentration of urea in the solution was 7 M. The reaction mixture was incubated at room temperature (23°C) for 1 hr. A control reaction was also performed with 710 µl of denatured protein without adding bromoethylamine solution. Both the reactions were quenched by adding 5 µl of 1 M DTT to give a final concentration of 5 mM. The reaction mixtures were dialyzed using 0.1 M phosphate buffer pH 8.0 for 3 hr. The solutions were stored on ice for the determination of activity.

Spectrophotometric Determination of ODCase Activity

Bromoethylamine-treated K73C ODCase-f was assayed for ODCase activity as described previously. 20 µl of a 1:10 dilution of bromoethylamine treated K73C ODCase was used for assay. Specific activity was calculated as described before. The OMP to UMP reaction has a $\Delta\varepsilon_{286}$ of -0.00225 μM^{-1}. Assays for the control reaction mixture and untreated K73C ODCase were also performed.

Results and Discussion

Cloning and insertion of *E. coli* ODCase gene into pCal-n

The agarose gels from PCR products and spin column purification showed bands of the right size and confirms efficient PCR and purification. The agarose gels from

restriction digestion revealed two bands for pCal-n and one band for the PCR product, thus confirming successful restriction digestion.

The transformation of positive ligation mixture gave more colonies than negative ligation. There were no colonies on negative control plate. Miniprep purification revealed that some plasmids were slightly larger than pCal-n, indicating successful ligation and presence of insert. The restriction digestion of Qiagen® purified plasmids

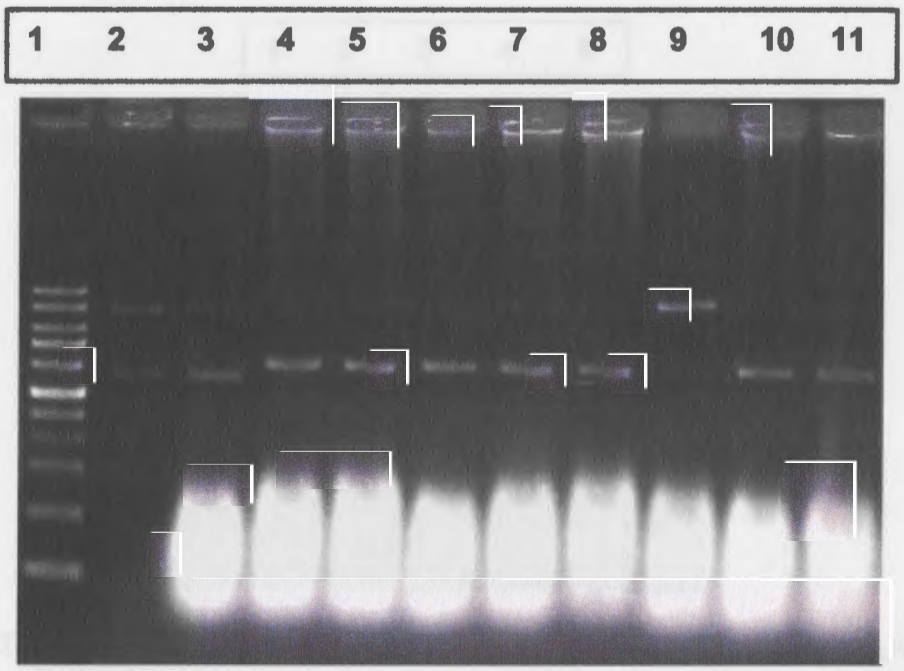


Figure 3-3: Miniprep analysis of colonies for ODCase insert. Lane 1: 1 Kb ladder molecular weight markers (New England Biolabs). Lane 2: purified pCal-n. Lanes 3-11: plasmids from mini-preps of screened colonies. Plasmids with desired insert are slightly larger than pCal-n.

verified that the construction of two plasmids were correct indicated by two bands, one of which was the same size as pCal-n and the other is of the same size as PCR product. For the other plasmid, the smaller band was not visible, indicating the incorrect construction of plasmid (data not shown).

The transformation of the recombinant plasmids with BL21 cells gave a good number of colonies indicating successful transformation.

Expression and Specific activity Determination of *E. coli* ODCase

The gel (Figure 3-4) revealed that ODCase production increased with induction time and became almost constant after 4 hr induction. We decided that 6 hr induction time is sufficient for maximum ODCase production.

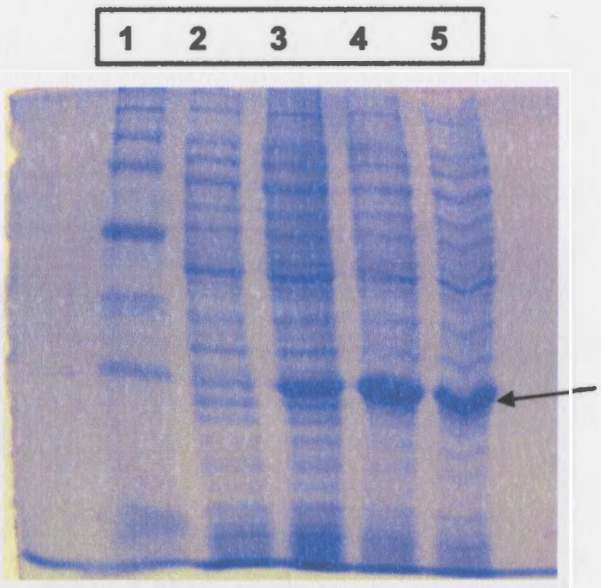


Figure 3-4: Time point induction of *E. coli* ODCase. Arrow indicates molecular weight for *E. coli* ODCase fusion protein. Lane 1: Molecular weight markers. Lanes 2, 3, 4 and 5: Cell lysates from 0, 2, 4 and 6 hr induction respectively.

ODCase activity was not measurable by spectrophotometric assay in the 0 hr induction sample. The specific activity for 2 hr induction sample was 2.23 nmoles min^{-1} μg^{-1} protein. For 4 hr and 6 hr, the specific activity was found to be 11.73 nmoles min^{-1} μg^{-1} protein and 9.34 nmoles min^{-1} μg^{-1} protein respectively (data shown in Appendix. Figures A-13, A-14 and A-15).

Expression and Purification of WT *E. coli* ODCase

Almost all of the unwanted proteins came off as flow through. The remaining traces of interfering proteins came out in wash fractions. Pure ODCase was eluted from the column with the addition of EGTA. The protein samples from the elute 2 were labeled as calmodulin-purified WT ODCase, and stored on ice. The specific activity was found to be 1.85 nmoles min^{-1} μg^{-1} protein. The fusion protein seems to retain activity even after calmodulin purification.

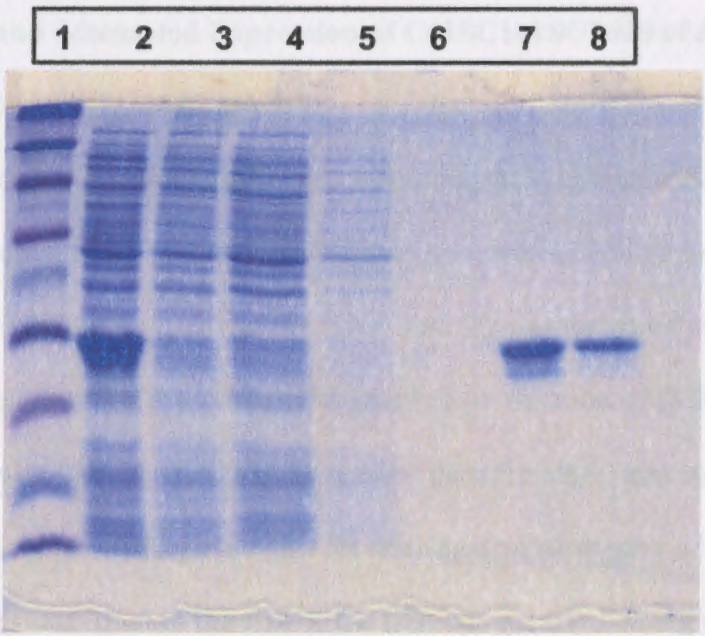


Figure 3-5: Calmodulin purification of *E. coli* ODCase-f. Lane 1: Molecular weight markers. Lane 2: cell lysate, lane 3: flow through. Lanes 4, 5: washes 1 and 2 respectively. Lanes 6, 7 and 8: Elutes 1, 2 and 3 respectively.

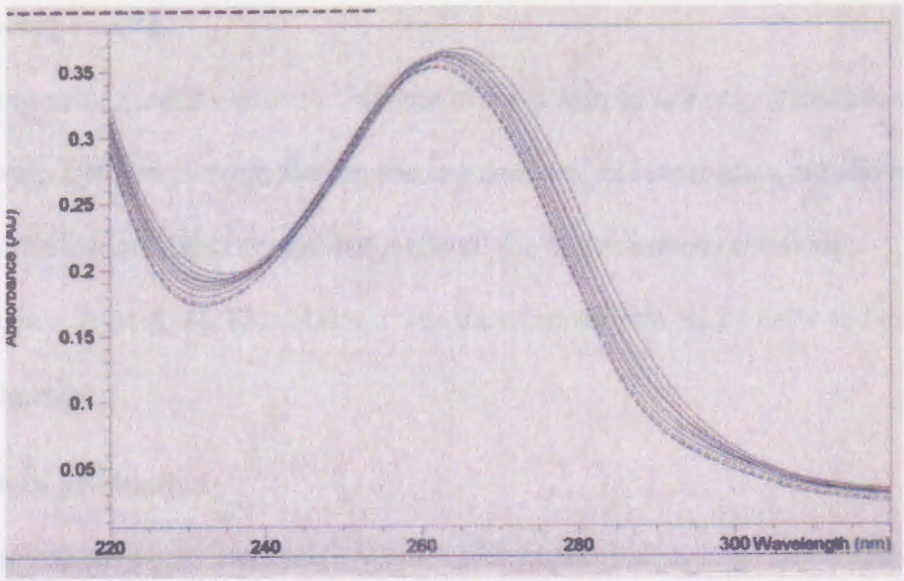


Figure 3-6: Spectrophotometric assay data of ODCase activity in Calmodulin-purified WT ODCase-f (*E. coli*)

Mutagenesis and Attempted Expression of C41SC161SC168S of *E. coli* ODCase-f

C41S and C41SC161SC168S mutation

In these site-directed mutagenesis reactions, the oligonucleotide primers were designed to contain mutations and the ODCase gene was amplified in two separate pieces. The two PCR amplification products were then annealed by overlap extension. The agarose gels from PCR products and gel slice purification of PCR products showed bands of right size (one band is slightly smaller than the other) and confirms efficient PCR and purification. The agarose gels for overlap extension gave a band that was larger than both pieces and equal to the size of the ODCase gene indicating that the overlap extension reaction worked. When the mutant plasmid for the triple mutant was constructed, the agarose gels from restriction digestion revealed two bands for pCal-n and one band for PCR product, thus indicating successful construction.

DNA sequencing results

The sequencing results indicate that one of the plasmids has only 2 mutations (data not shown). For one plasmid the sequencing data was not clear (data not shown). The data for the other plasmid reveals that it has all the three desired mutations (Appendix Figures A-1, A-2). This plasmid was transformed into BL21 cells and used for further experiments.

Mutant protein production

In experiments where incubation times were varied, there was no triple mutant ODCase production in 0 hr sample. There was increased production of ODCase in 2 hr, 4 hr and 6 hr whole cell samples. However, the lysates did not show the desired protein band, indicating the ODCase mutant protein was insoluble in the lysis buffer.

We therefore decided to try different conditions of protein expression to obtain soluble protein. The various experiments were conducted on the ODCase triple mutant and the results obtained are shown below.

Even though the protein was soluble using 2.3 M urea in lysis buffer, the protein precipitated when urea was removed during dialysis. Therefore, we switched our strategy to cloning K73C mutant ODCase.

Growth Medium and Induction	Lysis buffer	Growth Conditions	Result
LB medium, 50 μM IPTG	$CaCl_2$ –Tris lysis buffer with 0.1% Triton pH 8.0	37°C	Insoluble protein
LB medium, different IPTG concentrations (5 μM, 10 μM, 20 μM, 35 μM, 50 μM, 75 μM and 100 μM	$CaCl_2$–Tris lysis buffer pH 8.0	37°C	Insoluble protein
LB medium, 50μM IPTG	$CaCl_2$–Tris lysis buffer pH 8.6	37°C	Insoluble protein
LB medium, 50 μM IPTG	$CaCl_2$–Tris lysis buffer pH 8.0	37°C	Insoluble protein
LB medium, 50 μM IPTG	$CaCl_2$–Hepes lysis buffer pH 7.4	37°C	Insoluble

			protein
LB medium, 50 μM IPTG	$CaCl_2$–Hepes lysis buffer pH 6.8	37°C	Insoluble protein
LB medium with different glycerol concentrations (3%,5% and 10%), 50 μM IPTG	$CaCl_2$–Tris lysis buffer pH 8.0	37°C	Insoluble protein
LB medium, 50 μM IPTG	$CaCl_2$–Tris lysis buffer pH 8.0	37°C, 30°C,25°C, and 17°C	Insoluble protein
Enriched medium, 50 μM IPTG	$CaCl_2$–Tris lysis buffer pH 8.0	37°C	Insoluble protein
LB medium, 50 μM IPTG	$CaCl_2$–Tris lysis buffer pH 8.0, different urea concentrations (1.3 M, 1.6 M, 1.9 M, 2 M and 2.3 M,	37°C	Protein was soluble in 2.3 M urea

Table 3-2: Different growth conditions attempted for C41S C161S C168S ODCase-f

Cloning of *E. coli* ODCase gene into M13mp19 vector

The BamHI-HindIII ODCase gene fragment was gel sliced and purified using Qiagen® kit and used in the ligation reaction with BamHI and HindIII digested M13mp19. The blue plaques were formed by the phage containing no insert (the presence of IPTG and X-gal makes the plaques containing no insert blue) and colorless plaques were formed by phage containing ODCase insert. Some colorless plaques were picked and used for the preparation of replicative form DNA. Positive PCR results were obtained when amplifying the suspected M13-ODCase construction.

Mutagenesis using M13mp19 ODCase-f

The probable ODCase insert containing colorless plaques were picked and used in the preparation of phage supernatants. The preparation of RF DNA from phage supernatants did not produce the desired results. The DNA from the phage supernatants was amplified with *E. coli* ODCase BamHI start and *E. coli* ODCase HindIII stop primers. The agarose gels from the PCR amplifications revealed that the DNA from some

phage supernatants contain ODCase insert, which was confirmed by the presence of a band with the size of ODCase gene. The phage supernatants that contain ODCase insert were used in the preparation of single stranded DNA (ss DNA). The ss DNA was isolated from CJ236 cells. The concentration was found to be 300 ng/µl.

Generation of mutations K73C and K44C in M13mp19/ODCase

The oligonucleotide primers "*E. coli* ODCase K73C Fwd" and "*E. coli* ODCase K44C Fwd" were phosphorylated in a kinase reaction. The phosphorylated oligo nucleotides were used in a primer extension reaction. The reaction mixtures were used in transformation using XLI-Blue cells. No plaques were seen on the negative control plate. Negative reaction plate revealed fewer plaques than positive reactions. The plates with 2 µl reactions of K73C and K44C resulted in fewer plaques which can be easily isolated.

Sequencing of single stranded DNA from K73C and K44C M13mp19 ODCase-f

Three samples of ss DNA from K73C and two samples from K44C were used in DNA sequencing. The sequencing data revealed that the samples have the desired mutations (data shown in Appendix Figures A-3, A-4).

Introduction of K73C and K44C mutant ODCase genes into pCal-n

The agarose gels from PCR products and spin column purification showed bands of right size and confirms efficient PCR and purification. The agarose gels from restriction digestion revealed two bands for pCal-n and one band for each PCR product, thus confirming successful restriction digestion.

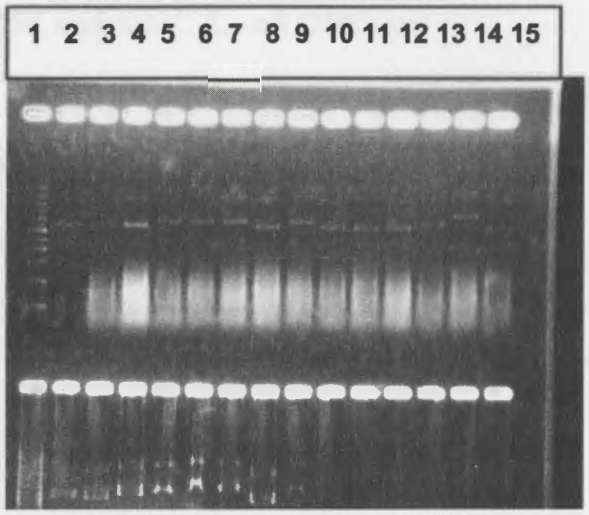


Figure 3-7: Screening of plasmids for K73C ODCase insert. Lane 1: 1 Kb ladder Molecular weight markers (New England Biolabs). Lane 2: purified pCal-n. Lanes 3-15: miniprep DNA from screened plasmids. Plasmids with insert are slightly larger than pCal-n.

The transformation of positive ligation mixtures gave more colonies than negative ligation. There were no colonies on negative control plate. Miniprep purification revealed that some plasmids were slightly larger than pCal-n , indicating the presence of insert in pCal-n plasmids. The restriction digestion of Qiagen® purified plasmids verified that the construction of plasmids for K73C and K44C were correct, indicated by two bands, one of which was the same size as pCal-n and the other is of the same size as PCR product. The DNA sequencing results indicated that the samples have the desired mutations (data shown in appendix Figures A-5, A-6).

Protein expression of K73C and K44C *E. coli* ODCase-f

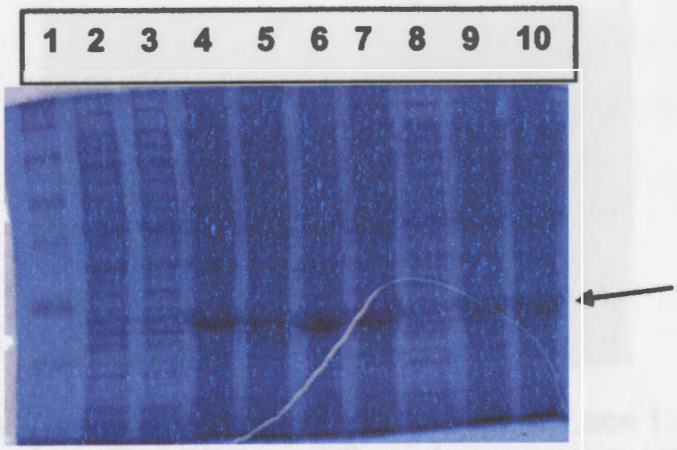


Figure 3-8: E. coli ODCase-f K73C, time point induction. Lane 1: MWM. Lane 2: whole cell sample of 0 hr induction. Lane 3: 0 hr lysate with triton in lysis buffer. Lane 4: whole cell sample for 3 hr induction. Lane 5: 3 hr lysate sample with triton. Lane 6: 6 hr whole cell sample. Lane 7: 6 hr lysate with triton. Lanes 8-10: 0, 3 and 6 hr lysates without triton respectively.

The gel (Figure 3-8) revealed that K73C ODCase production increased with induction time. 6 hr induction time was considered sufficient for maximum K73C production. Similar data was obtained with K44C (data not shown).

Expression and Purification of K73C *E. coli* ODCase-f

Almost all of the unwanted proteins came off as flow through and in wash fractions. The pure ODCase which was bound to calmodulin resin was eluted with the addition of EGTA. The K73C was about 90% pure and the presence of traces of unwanted proteins might not interfere with chemical modification which we wanted to carry out. The protein samples from the elution fractions were pooled together and labeled as calmodulin purified K73C ODCase, and stored on ice for future experiments.

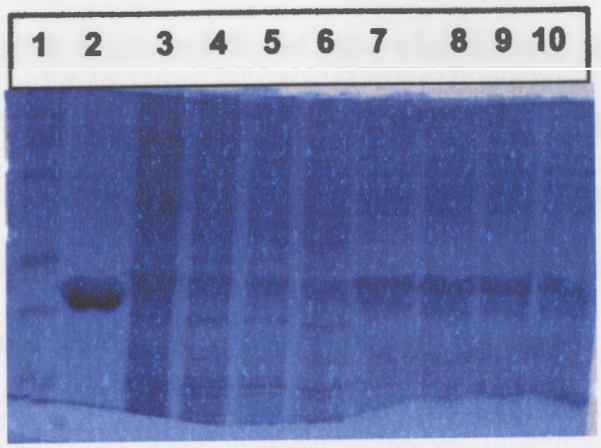


Figure 3-9: Calmodulin purification *E. coli* ODCase-f K73C. Lane 1: Molecular weight markers. Lane 2: purified ODCase WT non fusion protein. Lane 3: Lysate sample of K73C. Lane 4: flow through. Lanes 5, 6: wash fractions 1 and 2 respectively. Lanes 7-10: elution fractions 1, 2, 3 and 4 respectively.

Chemical Modification of K73C mutant ODCase with DTNB

An increase in absorbance at 412 nm was expected with the reaction of sulfhydryl groups of cysteines with DTNB, but no such increase was observed (data not shown). Probably the Tris - and especially the sulfhydryl groups of βME and DTT- present in the lysis and elution buffers are interfering with the reaction. $CaCl_2$ – MOPS lysis buffer and MOPS – EGTA elution buffer which do not contain DTT and βME were used for cell lysis and elution of protein from Calmodulin column. Even these changes did not help. Some control reactions were performed to test whether the DTNB reaction works for the above reaction conditions used. The egg albumin reacted with DTNB and an increase in absorbance was observed (Appendix Figure A-18). A graph was plotted with absorbance versus time (data not shown). The WT ODCase non fusion protein reacted well with DTNB in presence of 5 M urea. An increase in absorbance was observed (appendix figure A-19). A graph was plotted with absorbance versus time. Similar reactions were set up for K73C ODCase. The K73C did not react with DTNB as expected (data not

shown). Probably the mutant fusion protein has some what different properties which obscure the reaction with DTNB. The experimental strategy was changed to modify K73C ODCase with bromoethylamine without protecting naturally occurring cysteines and verify whether the modification restores activity to the inactive mutant.

Chemical modification of K73C ODCase with Bromoethylamine

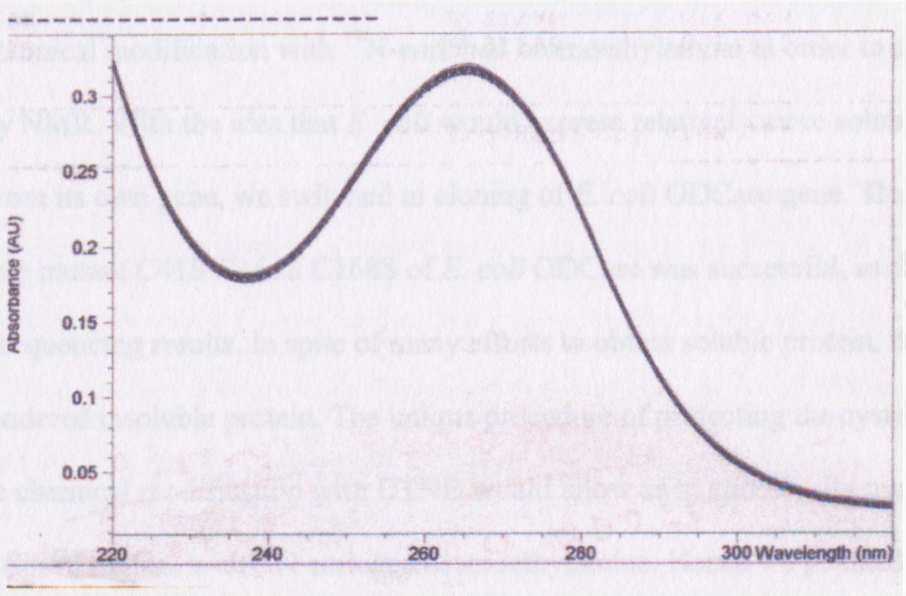


Figure 3-10: Spectrophotometric determination of ODCase activity, Bromoethylamine modified K73C (*E. coli*)

The bromoethylamine modified K73C ODCase-f was assayed for ODCase activity. The specific activity of the bromoethylamine modified K73C ODCase was found to be 12.04 nmoles min^{-1} μg^{-1} protein.). The unmodified K73C ODCase and control reactions did not show any activity. From the above results it can be stated that the restoration of activity to K73C ODCase is solely due to chemical modification by bromoethylamine.

Chapter 4

Conclusions

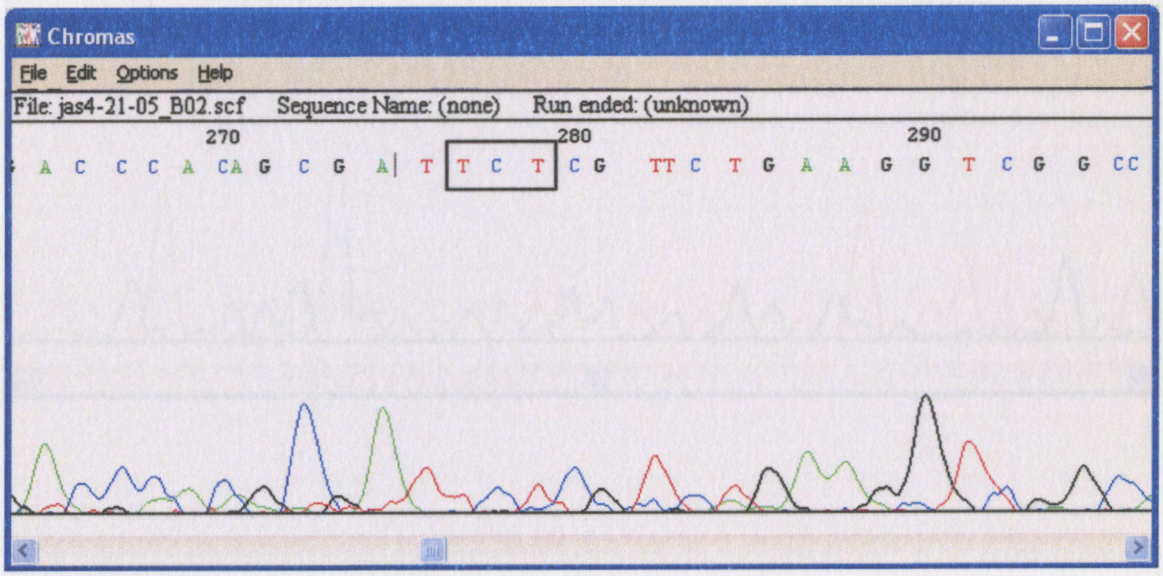
The WT yeast ODCase and mutants K93C and "C93 only," were purified by a calmodulin affinity system, however, their poor solubility made them unsuitable for further studies. Highly concentrated solutions of enzyme are necessary to carry out the desired chemical modification with ^{15}N-enriched bromoethylamine in order to analyze the ^{15}N by NMR. With the idea that *E. coli* would express relatively more soluble protein derived from its own gene, we switched to cloning of *E. coli* ODCase gene. The cloning of the triple mutant C41S C161S C168S of *E. coli* ODCase was successful, as determined by DNA sequencing results. In spite of many efforts to obtain soluble protein, the triple mutant rendered insoluble protein. The unique procedure of protecting the cysteines by reversible chemical modification with DTNB would allow us to specifically modify the Lys73 to Cys73 mutant with ^{15}N enriched bromoethylamine. Hence we planned to produce the K73C mutant. We encountered problems with the overlap extension reaction for this mutation of the *E. coli* ODCase gene. We switched to insertion of the ODCase gene into M13mp19 vector and carried out site directed mutagenesis, then transferred the mutant gene back into pCal-n. The DNA sequencing results verified our mutagenesis was successful. Calmodulin resin purification resulted in 80% pure protein. The K73C ODCase of *E. coli* did not react with DTNB in the same way as WT non- fusion ODCase of *E. coli*. From the observation of fusion proteins from yeast and *E. coli*, we think that the fusion tag is responsible for the poor solubility and reluctance to react with DTNB. The chemical modification of K73C ODCase with bromoethylamine resulted in partial restoration of activity. For future experiments it would be worthwhile to try the removal

of the fusion tag by proteolysis. This might make the protein more soluble and reactive towards DTNB.

An alternative strategy would be to attempt modification of one of the mutant proteins with ^{13}C-enriched bromoethylamine. The carbon atom adjacent to the amino group undergoes a small but measurable chemical shift upon protonation/deprotonation of the amino group. In the HSQC NMR analysis of K175C mutant of VP39 (the mRNA cap-specific 2'-O-methyltransferase from vaccinia virus) modified with ^{13}C-enriched aziridine, P. D. Gershon *et al.* observed an increase in chemical shift of 5 ppm for three different signals as $-NH_3^+$ is changed to $-NH_2$ with increase in pH from 7 to 12 [23]. The advantage with this strategy would be that low concentrated protein solutions are sufficient to carry out NMR analysis. The disadvantage, however, is that ^{13}C enriched bromoethylamine is not commercially available and is expensive to synthesize.

Appendix

Sequencing Data of pCal ODCase-f "C41S C161S C168S"



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CTG GTT CCG CGT GGA TCC TGA CGT CAN CTG CTT CAT CTT CTT CCC GCG
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ANC CCN GGN ANT GAA AGA AAT TAN GCN NNN ANT GNA NNA NNN NGT
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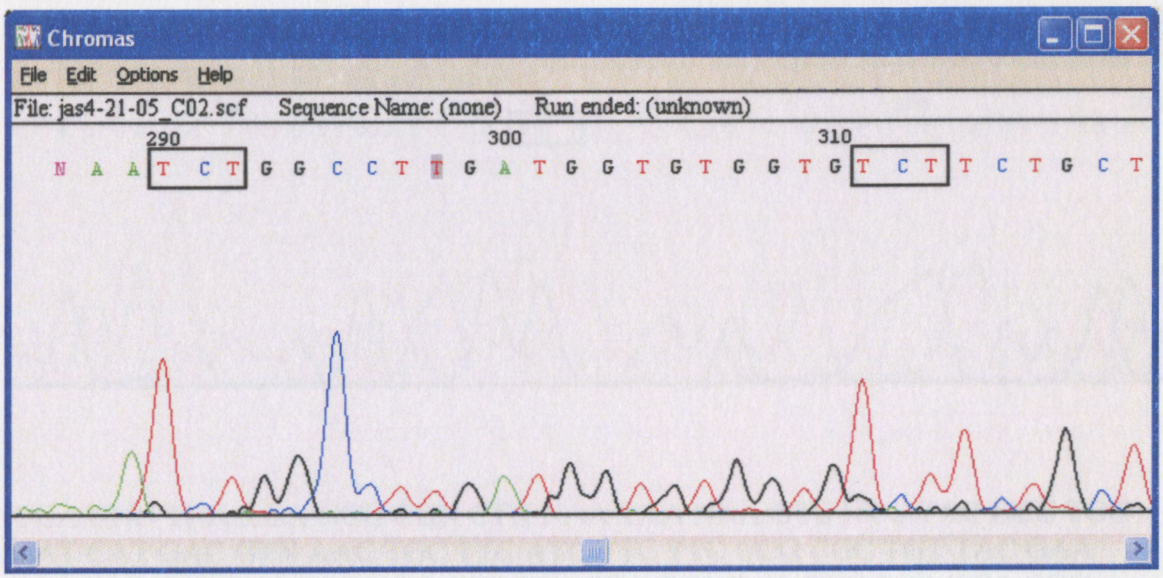
Figure A-1: C41S mutation indicated in bold

Sequencing Data of pCal ODCase-f "C41S C161S C168S"



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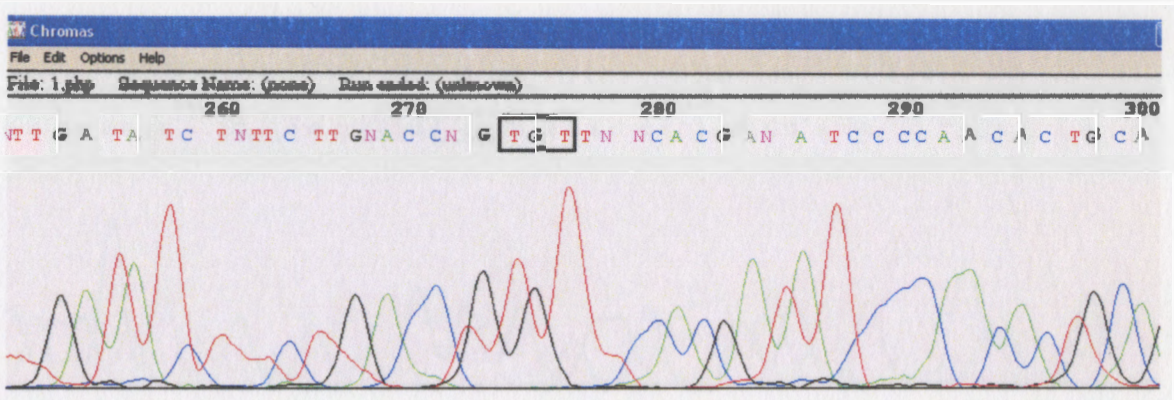
GCG CGT GAG GCA NTT GGT TCC GTT TGG CNN GAT GCA CCG CTT TTG
ATT GCT GTG ACA GTG TTG ACC AGC AAT AGG AAG CCA GCG ACC TGG
TCG ATC TTG GCA TGA CAC TGT CAC CTG CAG ATT ATG CAG AAC GTC
TGG CGG CAC TGA CGC ANA A**TC TGG** C CT T GA T GG T GT G GT G **TC T**
TC T GC TCA GGA AGC TGT GCG CTT TAA ACA GGT ATT CGG TCA GGA GTT
CAA ACT GGT TAC GCC GGG CAT TCG TCC GCA GGG GGG TGA AGC TGG
TGA CCA GCG CCG CAT TAN GNN CCA GAA CGG CGT GTC GGC NGG NTG
NNA TTA TAG TGG TGN NTG ATN NNA CCN GGG TAA NCN AAT GGN GTA
ATC GAG CCG AGA CGN TTG AGC GGA NTC GNA CGN NTT TTT CNG CNN
ANN NCN GNG NTG CGN TTC GNC GGG NCN TNN GAA NNT TNN NGC CGG

Figure A-2: C161S C168S mutation indicated in bold

Sequencing Data of M13mp19 ODCase-f "K73C" (ss DNA)



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GGG TCG GCA AAG AGA TGT TNA CNN NNG TNT GGG CCA CAG NTN TGN
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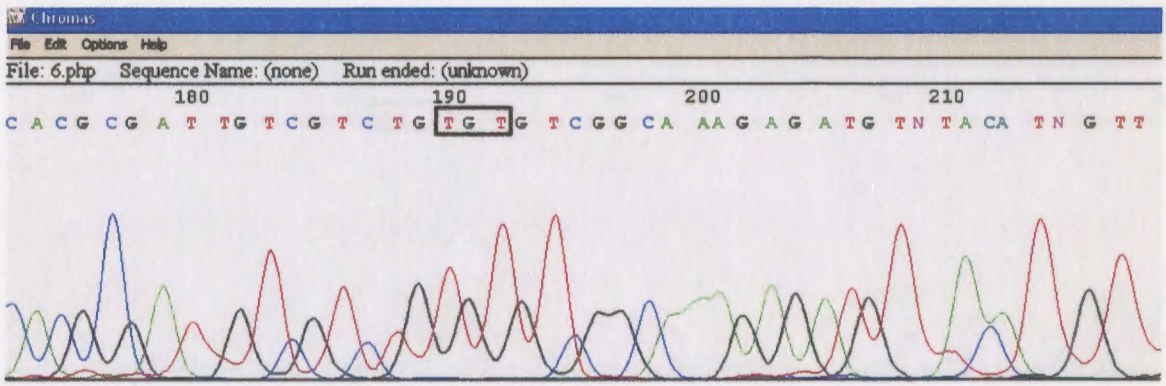
GNN NGG CGG ANN GAC AAA AAT GGG GNT GAT GNN NNT GTT GNG NTG
GTT CCC TGC CCT CAA GGG GAA AAG CNC NNG

Figure A-3: K73C mutation shown in bold

Sequencing Data of M13mp19 ODCase-f "K44C" (ss DNA)



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GAC GCG CTG GCC TTT GTC GAC AAG ATC GAC CCA CGC GAT TGT CGT
CTG **TGT** GTC GGC AAA GAG ATG TNT ACA TNG TTT GGG CCA CAG TTT
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Figure A-4: K44C mutation shown in bold

Sequencing Data of pCal ODCase-f "K73C"

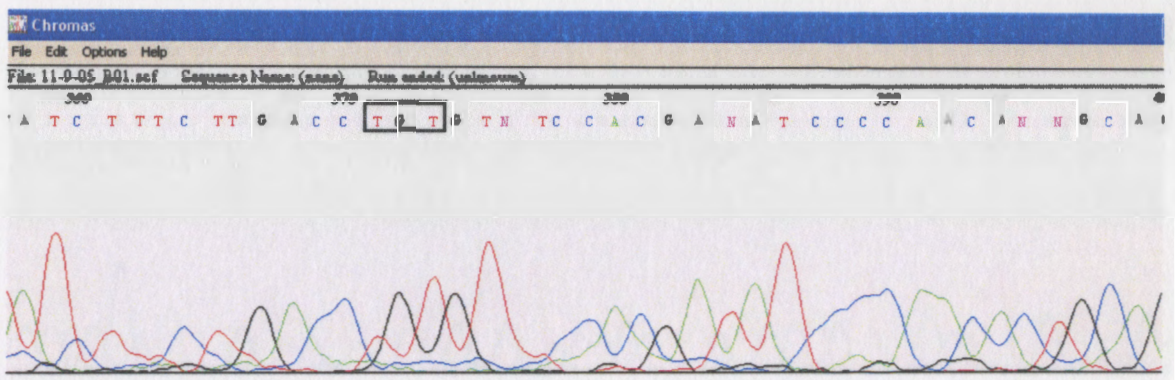

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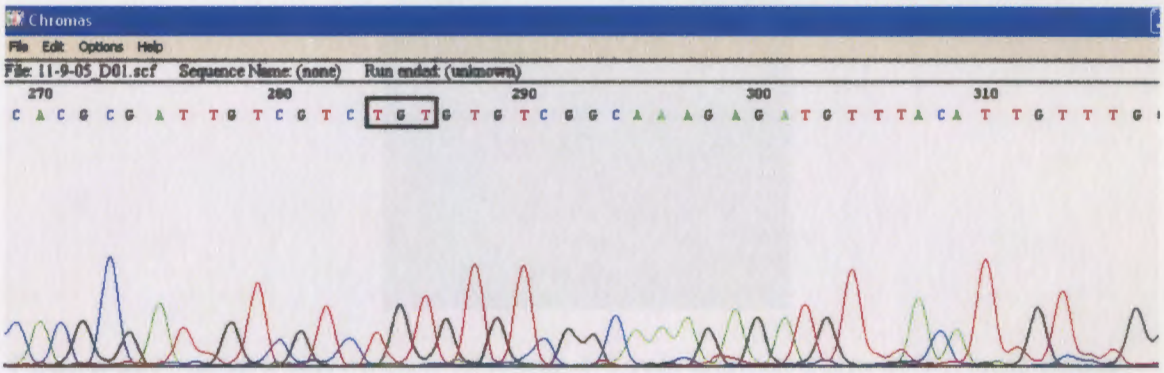
Figure A-5: K73C mutation shown in bold

Sequencing Data of pCal ODCase-f "K44C"



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CTT CTG GTT CCG CGT GGA TCC ATG ACG TTA ACT GCT TCA TCT TCT
TCC CGC GCT GTT ACG AAT TCT CCT GTG GTT GTT GCC CTT GAT TAT CAT
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AGT GTN GAC CAG CAT GCA AGC CAG CGA CCT GNT CGA TCT TGG CAT
GAC ACT GTC ACC TGC AGA TTA TGC AGN AAC GTC TGG CGG CAC TGN
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TTT AAA CAG GTA TNC GGT CAG GAG TTC AAA CTG GGT NAC GCC GGG
GCA TTC CGT CCG CAG GTG AAG TGN ANG CTG GGT GAC CAG CGC CGC
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Figure A-6: K44C mutation shown in bold

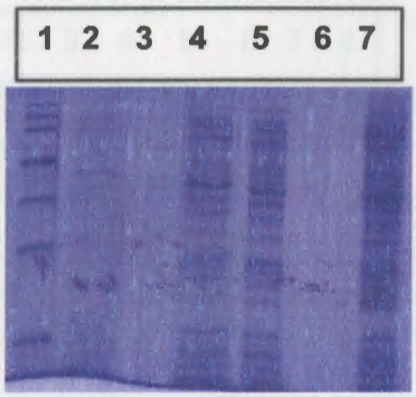

Figure A-7: pCal triple mutant ODCase-f. Lysis without triton in the lysis buffer. Lane1: MWM, Lanes 2, 4, 5 and 7: Cell lysates 0, 2, 4 and 6 hr induction.

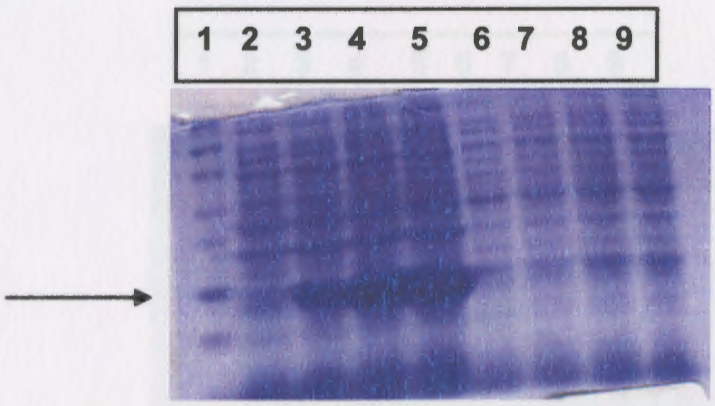

Figure A-8: pCal triple mutant ODCase-f. Lysis with triton in the lysis buffer. Molecular weight of pCal ODCase fusion protein indicated by arrow. Lane1: MWM, Lanes 2-5: Whole cell samples for 0, 2, 4 and 6 hr induction respectively. Lanes 6-9: Lysates for 0, 2, 4 and 6 hr induction respectively.

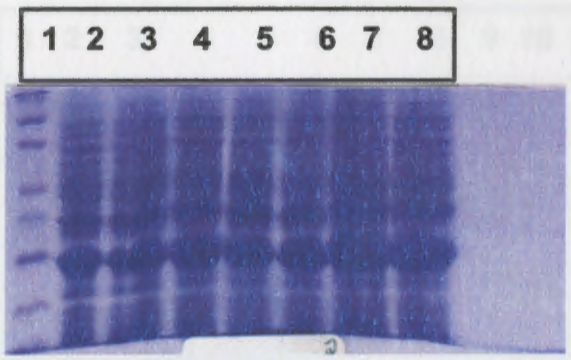

Figure A-9: pCal triple mutant ODCase-f. Different IPTG concentrations. Lane1: MWM, Lanes 2-8: Whole cell samples for 6 hr induction with 5 μM, 10 μM, 20 μM, 35 μM, 50 μM, 75 μM and 100 μM respectively.

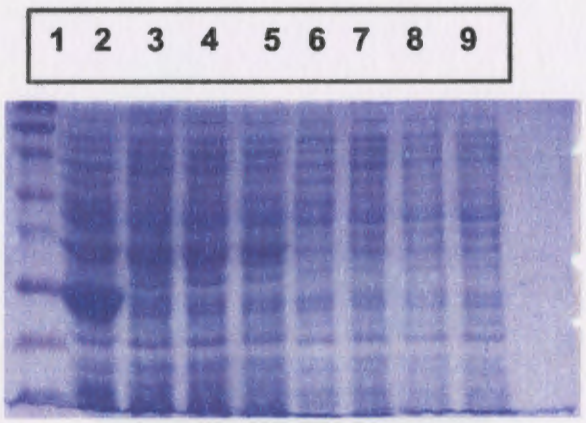

Figure A-10: pCal triple mutant ODCase-f. Different Glycerol concentrations. Lane1: MWM, Lanes 2-5: Whole Cell samples for 6 hr induction with 0, 3, 5 and 10% glycerol concentrations respectively. Lanes 6-9: Cell lysate samples for 6 hr induction with 0, 3, 5 and 10% glycerol concentrations respectively.

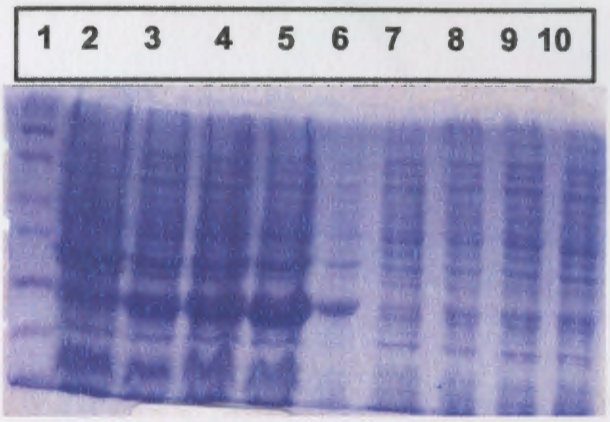

Figure A-11: pCal triple mutant ODCase-f. Enriched medium. Lane1: MWM, Lanes 2-5: Whole Cell samples for 0, 2, 4 and 6 hr induction respectively. Lanes 7-10: Cell lysate samples for 0, 2, 4 and 6 hr induction respectively.

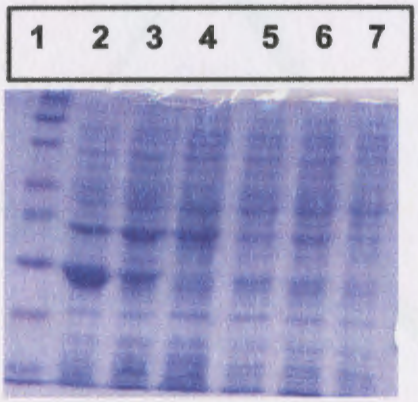

Figure A-12: pCal triple mutant ODCase-f. Different temperatures. Lane1: MWM, Lanes 2-5: Whole Cell samples for 6 hr induction at 37, 30, 25 and 17°C respectively. Lanes 6, 7: Cell lysate samples for 6 hr induction at 37, 30°C respectively.

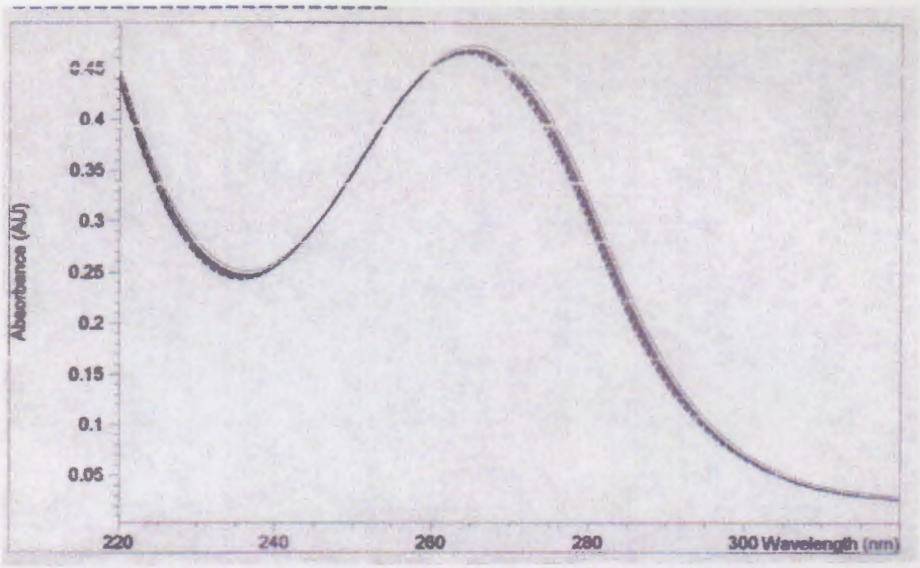

Figure A-13: WT pCal ODCase-f. *E. coli*. Spectrophotometric determination of activity. 2 hr induction.

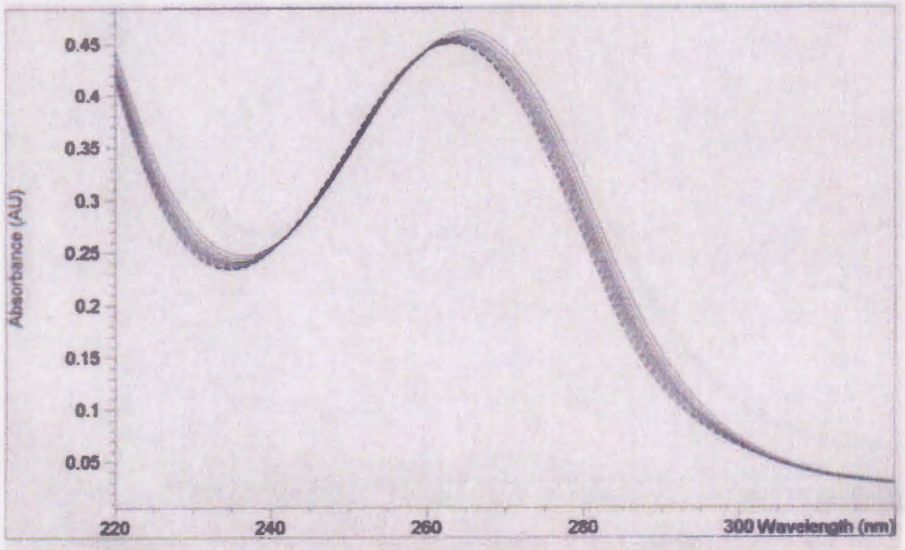

Figure A-14: WT pCal ODCase-f. *E. coli*. Spectrophotometric determination of activity. 4 hr induction.

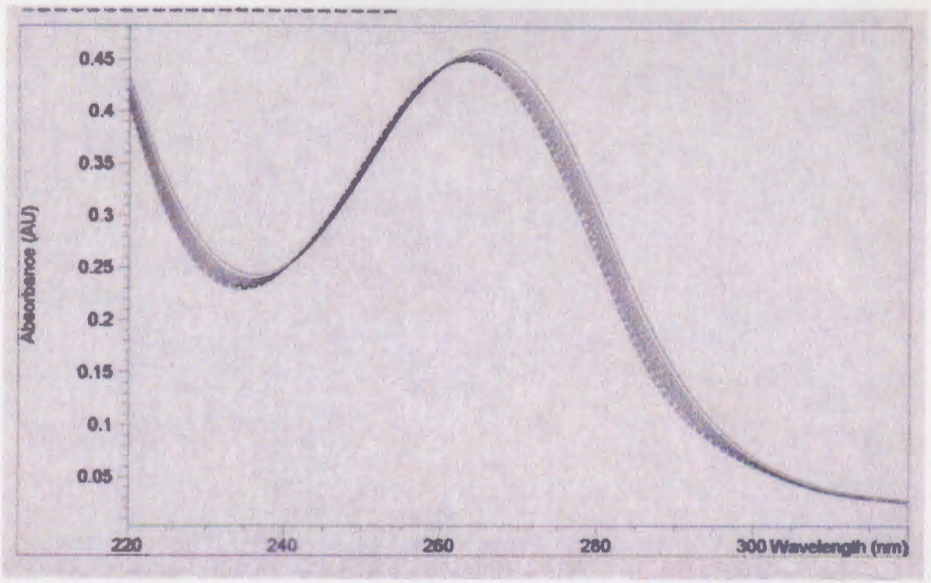

Figure A-15: WT pCal ODCase-f. *E. coli*. Spectrophotometric determination of activity. 6 hr induction.

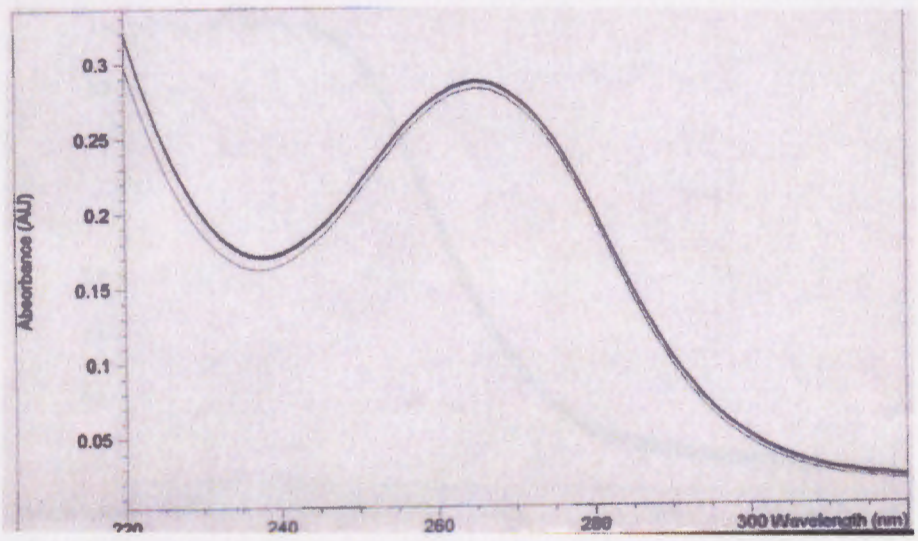

Figure A-16: K73C pCal ODCase-f. *E. coli*. Spectrophotometric determination of activity after Calmodulin purification.

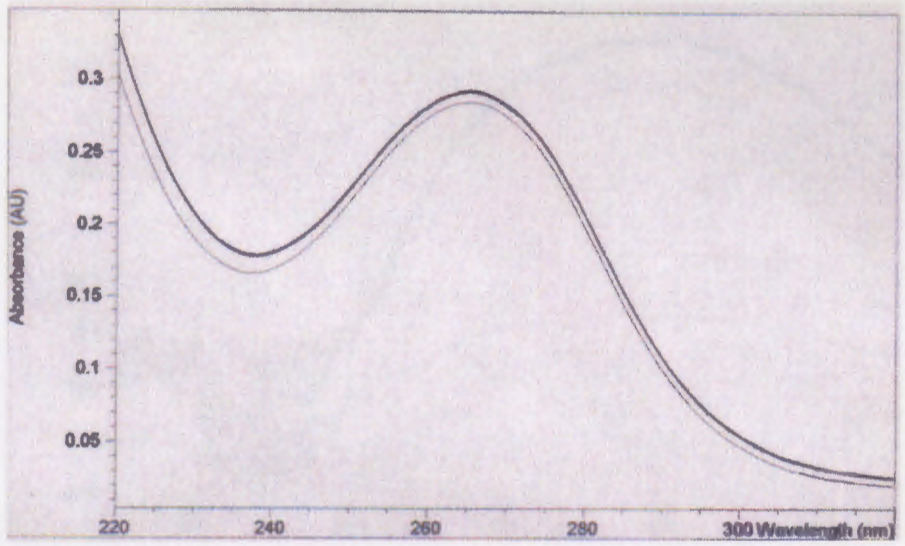

Figure A-17: Spectrophotometric determination of activity of Calmodulin purified K44C pCal ODCase-f (*E. coli*).

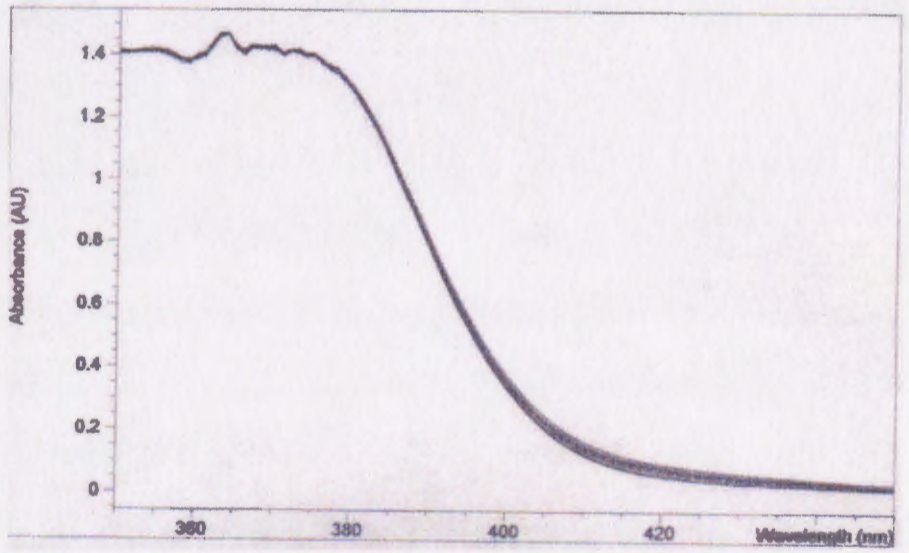

Figure A-18: DTNB reaction of Egg albumin

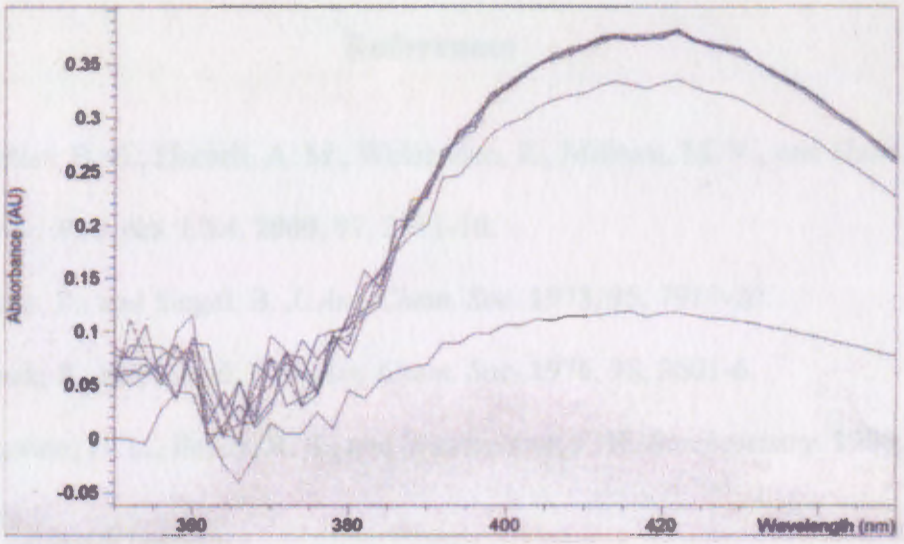

Figure A-19: DTNB reaction of WT *E. coli* ODCase non fusion protein, 5 M urea.

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