OMP Decarboxylase: Preparation of Purified Protein and Inhibitors for X-ray Crystallography and NMR

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Abstract

OMP decarboxylase is the final enzyme of the *de novo* pyrimidine biosynthetic pathway and it displays one of the largest rate enhancements ever measured. The mechanism by which the enzyme carries out decarboxylation remains unknown. Several mechanisms have been proposed to explain the activity of ODCase. Our group tried to support the mechanism involving Lys93 protonation of O2 by crystallographic and NMR studies using the inhibitor, UMP-6-thiocarboxamide (UMP-$CSNH_2$), a substrate isostere. The *E. coli* ODCase gene was cloned for overproduction of protein, and the resulting ODCase was purified by applying to the Affi-Gel Blue column and eluting using different inhibitors. The inhibitor-free protein used for different protein/inhibitor samples was obtained by eluting protein from the column with UMP, and dialyzing the pooled fractions three times. Unenriched and ^{15}N-enriched UMP-$CSNH_2$ were synthesized and purified, for use in the preparation of ODCase-inhibitor samples for NMR and crystallography. The bond distances of hydrogens attached to ^{15}N and hydrogens attached to C5' were measured using molecular modeling, in order to determine whether or not NMR signals resulting from close proximity of these hydrogens is possible. An attempt was made to synthesize BMP, a potent ODCase inhibitor, by enzymatic catalysis using OPRTase. Different protein and protein inhibitor samples were prepared for crystallographic and NMR studies. The NMR spectra of ODCase with ^{15}N labeled UMP-$CSNH_2$ showed only faint signals. Crystallization of ODCase with UMP-$CSNH_2$, carried out at the University of Toledo, appears promising.

Acknowledgements

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List of Symbols and Abbreviations

ODCase	Orotidine 5'-monophosphate decarboxylase
OMP	Orotidine 5'-monophosphate
UMP	Uridine 5'-monophosphate
OPRTase	Orotate phosphoribosyltransferase
BMP	1-(5'-phospho-β-D-ribofuranosyl) barbituric acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
βME	β mercapto ethanol

Chapter 1

Introduction

Decarboxylation is the displacement of a carboxyl group by a proton with the formation of an anion intermediate whose stability determines the rate of the reaction.

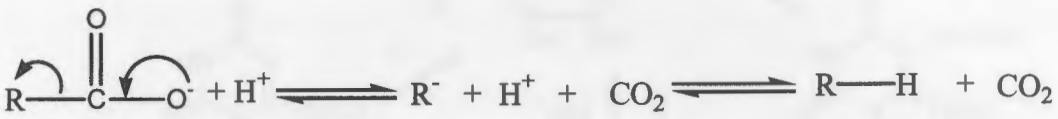


Figure 1-1: General decarboxylation reaction.

Orotidine 5'-monophosphate decarboxylase (ODCase) catalyzes the conversion of orotidine 5'-monophosphate (OMP) into uridine 5'-monophosphate (UMP) in the final step of the *de novo* biosynthesis of pyrimidine nucleotides. UMP is a necessary precursor for all pyrimidine nucleotides [1].

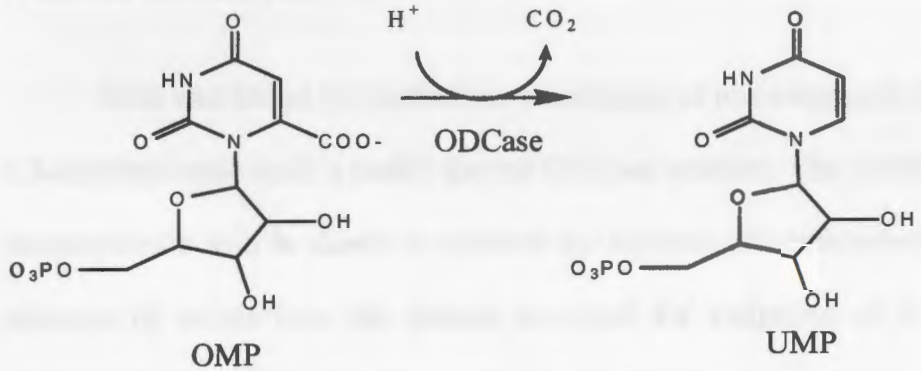


Figure 1-2: Reaction catalyzed by ODCase.

ODCase displays one of the largest rate enhancements ever measured. Although ODCase has been the subject of study of many enzymologists, its catalytic mechanism remains unclear. Various mechanistic hypotheses have been proposed to explain the highly proficient rate of decarboxylation. Based on different experimental evidence five mechanisms for the generation of a more reactive intermediate have been proposed [2].

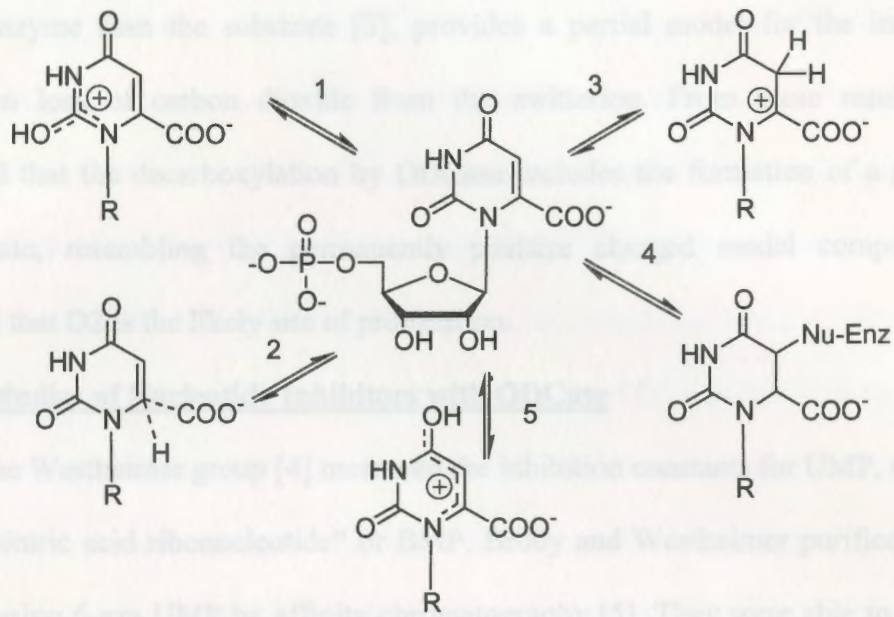


Figure 1-3: Five proposed enzyme-catalyzed reactions for the activation of OMP to a more reactive intermediate by ODCase [2]. **1**. O2 protonation mechanism. **2**. Direct protonation of C6 during decarboxylation. **3**. Protonation at C5. **4**. Nucleophilic attachment C5. **5**. Protonation at O4. Adopted from reference: Smiley JA (2004) Topics in Current Chemistry 238:63.

Beak and Siegel [3] studied the mechanism of non-enzymatic decarboxylation of 1,3-dimethylorotic acid, a model for the ODCase reaction. The decarboxylation of 1,3-dimethylorotic acid is shown to proceed by separate pH-determined pathways; in the presence of excess base the process involved the ionization of 1,3-dimethylorotate, whereas decarboxylation is initiated by zwitterion formation in the neutral solvent. By comparing the rates of decarboxylation of various analogs, the authors concluded that the analogs which are incapable of forming such a decarboxylated zwitterion had lower or unmeasurable rates of decarboxylation. Another analog, dimethoxypyrimidine carboxylate, with a permanent positive charge distributed around the heterocycle, showed a rate of decarboxylation 10^7 times faster than 1, 3-dimethylorotate. It was also observed

that the inhibitor 6-azauridine monophosphate (6-azaUMP), which has a greater affinity for the enzyme than the substrate [3], provides a partial model for the intermediate formed on loss of carbon dioxide from the zwitterion. From these results it was postulated that the decarboxylation by ODCase includes the formation of a protonated intermediate, resembling the permanently positive charged model compound, and suggested that O2 is the likely site of protonation.

Affinity studies of Nucleotide Inhibitors with ODCase

The Westheimer group [4] measured the inhibition constants for UMP, 6-azaUMP and "barbituric acid ribonucleotide" or BMP. Brody and Westheimer purified the yeast ODCase using 6-aza UMP by affinity chromatography [5]. They were able to purify the protein by 3200-fold in one purification step. From simple additional steps they achieved 6700-fold purification. With this purified enzyme they studied the binding of BMP to ODCase. Inhibition constants for UMP and 6-azaUMP were found to be 4.6×10^{-4} M and 5.1×10^{-7} M, respectively, at pH 6.0 and BMP binds 100,000 times as strong as does the substrate with the K_i of 9×10^{-12} M at pH 6.0. The authors hypothesized that the anionic form of the inhibitor was the predominant form binding to the enzyme. The analogues with anionic pyrimidine ring were proposed to be stronger inhibitors than UMP with a neutral uracil. Unlike UMP and 6-azaUMP, BMP possesses an amide moiety similar to that of substrate, which may be responsible to achieve maximum interaction with the enzyme's active site.

To probe the active site of ODCase, Smiley and Saleh [6] used the thio-substituted analogues of UMP as inhibitors of yeast ODCase. They measured the inhibition constants for UMP and thio-substituted analogues 4-thioUMP and 2-thioUMP

and calculated the inhibition constants for the respective anionic forms. The measured K_i value for 4-thioUMP anion is 0.4×10^{-7} M, which is significantly less than that for UMP anion, indicating a portion of the active site with a stronger attraction for S4 of 4-thioUMP than for O4 of UMP. The calculated K_i value for 2-thioUMP anion is 2.7×10^{-7} M, which is equal to that for UMP anion. From these observations, in combination with the measurement of the absence of ODCase catalysis toward 2-thioOMP and nearly full activity toward 4-thioOMP, it was proposed that ODCase catalysis may involve protonation of the substrate at O2 in the transition state of the reaction and interaction of Zn^{+2} with O4 of the substrate and product. The authors synthesized the alternate substrate, cytidine-6-carboxylate (CMP-6-carboxylate) to measure the ODCase catalyzed decarboxylation of this compound. But they observed lack of catalytic activity and binding of CMP-6-carboxylate to the ODCase, which emphasizes the importance of the substrate structure at position 4 of the pyrimidine.

Previously, Smiley and Jones [7] identified the proton donating critical amino acid side chain in ODCase by site-directed mutagenesis. They replaced yeast ODCase Lys93 with a cysteine which resulted in a mutant protein (K93C) with no measurable activity, 2×10^{-8} M times less than the activity of the wild enzyme. Treatment of this mutant protein with 2-bromoethylamine to yield S-(2-aminoethyl) cysteine, restored the activity by a factor of at least 5×10^{5} M over the untreated mutant protein. The binding affinity of the substrate OMP was not measurably changed by the mutation, indicating that Lys93 has an essential role in catalysis which is mechanistically distinguishable from substrate binding. In contrast with the wild type enzyme, the K93C protein has an affinity for the neutral ligand UMP which is greater than that for the anionic 6-azaUMP. These

observations support that Lys93 is apparently critical for catalysis of the substrate to product and for the binding of anionic inhibitors.

The enzymatic decarboxylation of OMP may proceed by an addition-elimination mechanism involving a covalently bound intermediate [9] or by elimination of CO_2 to generate a nitrogen ylide. To investigate this, Jones and Wolfenden [8] synthesized BMP with ^{13}C at the 5-position. If decarboxylation mechanism proceeds by addition-elimination mechanism, BMP might be expected to be bound as a covalent adduct by the attack of an enzymic nucleophile at C5 of the pyrimidine ring. If true, a significant up field shift of the ^{13}C NMR C5 resonance (25-50 ppm) would be expected due to the rehybridization of C5 from sp^2 to sp^3. For the nitrogen ylide mechanism, protonation of either carbonyl oxygen or N-1 to generate the zwitterion intermediate would be expected to result in little or no change in the C5 resonance. But the interaction of ^{13}C labeled BMP with ODCase resulted in only a small (0.6 ppm) downfield displacement of the C5 resonance, indicating no rehybridization that would necessarily accompany 5,6-addition of an enzyme nucleophile. When k_{cat} for the synthesized substrate OMP with deuterium at C5 position was calculated no significant change was observed, suggesting that C5 does not undergo significant changes in geometry before or during the step that determines the rate of the catalytic process. These results are consistent with a nitrogen ylide mechanism and offer no support for the intervention of covalently bound intermediates in the catalytic process.

Study of Isotope effects

The O'Leary and Jones groups [10] measured the carbon isotope effects of the reaction under a number of different conditions in an attempt to investigate the

mechanism of ODCase. They showed that ODCase displays large ^{13}C isotope effects that can be altered by changing the pH, temperature, or glycerol content of the reaction medium. A quantitative kinetic analysis using their results excludes the possibility of an enzymatic mechanism involving covalent attachment of an enzyme nucleophile to C5 of the pyrimidine ring. The observed carbon isotope effect did not rise to the level of the intrinsic isotope effect at the high pH levels, but reached a maximum of about 1.035. This data supports the protonation step in a catalytic mechanism similar to that proposed by Beak and Siegel.

Decarboxylation reactions result in the formation of an anionic transition state or intermediate as a result of the neutralization of the negative charge on the carboxyl group. Catalysis results from the stabilization of this anionic species. In the case of ODCase the substrate has no facile mechanism for delocalization of the negative charge. And there are no co-factors or metal ions [12] are found to be involved in the catalysis of ODCase. Then the highly accelerated rate achieved by the ODCase must be from interactions between enzymic functional groups and the substrate. To investigate this Rishavy and Cleland [11] studied the nitrogen isotope effects on the ODCase reaction with two model compounds.

The ODCase reaction shows a ^{15}N isotope effect of 1.0036 at N1. Picolinic acid, a model compound, shows an ^{15}N isotope effect of 0.9955 for decarboxylation in ethylene glycol at 190 °C, while *N*-methyl picolinic acid, second model compound shows a ^{15}N isotope effect of 1.0053 at 120 °C. The transition state for enzymatic decarboxylation of OMP may resemble the transition state for *N*-methyl picolinic acid, which includes no bond order changes at N1. This suggests a carbanion intermediate

stabilized by simple electrostatic interaction with Lys93 and ground-state destabilization resulting from charge repulsion between the carboxyl of the substrate and Asp91, which was part of a Asp-Lys-Asp-Lys chain of interacting side chains containing the catalytic lysine.

Affinity studies of Altered Substrates with ODCase

Shostak and Jones [13] synthesized a series of alternate substrates for yeast ODCase to study the catalytic mechanism. They made substitutions at 5-position of pyrimidine ring of the OMP. They observed that the 5-bromo- and 5-chloro analogs of OMP are potent inhibitors while the 5-fluoro derivative is a good substrate with a turnover number 30 times that observed with the substrate. If ODCase uses an electrophilic substitution mechanism, protonating the substrate somewhere along the 5, 6 double bond, an electrophilic substitution pre-decarboxylation step would be slower with 5-fluoroOMP. Studies with 5-aza OMP (substituted with nitrogen at 5-position) rules out the mechanism involving the nucleophilic addition of the enzyme at the 5-position because 5-aza group is not electrophilic. The enzyme showed the catalytic activity when the substitution is at 4-position, that is 4-ketogroup can be replaced with a thioketone. No catalysis is observed when the same substitution is made at the 2-position.

To evaluate the effects of individual binding determinants on transition state stabilization, Miller and Wolfenden [14] compared the binding properties of substrates and competitive inhibitors of the human ODCase with the fragments obtained by truncating these ligands at various positions. By comparing the enzymatic decarboxylation of orotidine and orotate, the k_{cat}/K_m value of orotidine is 10-folder larger than that of orotate. This indicates the contribution of ribofuranosyl group to transition

state stabilization. However, both substrates are decarboxylated much slower than the OMP. When the K_m values of these nonphosphorylated substrates are compared, the ribofuranosyl substituent appears to increase binding affinity 127-fold. In contrast, addition of a ribofuranosyl substituent to the product uracil, or to the inhibitors barbituric acid and 6-aza uracil, weakens binding affinity slightly. These results give the impression that direct interactions between the active site of ODCase and substituent ribose may be of moderate strength in the enzyme-substrate complex. These interactions diminish as the transition state is formed and collapses to form the enzyme-product complex. The phosphoryl group of OMP contributes approximately 10 kcal/mol of binding free energy to transition state stabilization, as indicated by comparison of the k_{cat}/K_m value of OMP with that of orotidine, and of the K_i value of the transition state analogue inhibitor BMP with that of the corresponding ribonucleoside. In addition to these enzyme-ligand contacts and active site residues, it was supposed that Thr100 and Asp37, contributed by both subunits of the dimeric enzyme are positioned to form hydrogen bonds with the 2'- and 3'-OH groups of the ligand's ribosyl moiety [15].

Structural study of ODCase

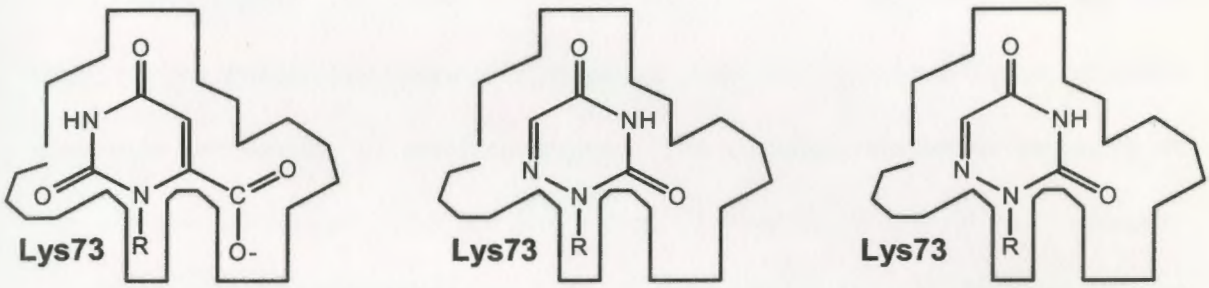
The crystal structures of ODCase from different microbial sources are published with different inhibitors bound to the active site. Begley and Appleby group [16] obtained the crystal structure of *Bacillus subtilis* ODCase with bound UMP. From the obtained crystal structure it was found that ODCase is a dimer of two identical subunits containing an active site that is located across one end of the barrel and near the dimer interface. The UMP is bound to the active site in such a way that the phosphate group is most exposed and the C5-C6 edge of the pyrimidine base is most buried. From these

results the authors proposed that the ground state of the substrate is destabilized by electrostatic repulsion between the carboxylate of the substrate and the carboxylate of Asp60. This repulsion is reduced in the transition state by shifting negative charge from the carboxylate to C6 of the pyrimidine, which is close to the protonated amine of Lys62.

The crystal structure of the recombinant *Saccharomyces cerevisiae* enzyme has been determined in the absence and presence of the proposed transition state analog BMP [17]. From these crystal structures, Lys93 appears to optimize electrostatic interactions with developing negative charge at C6 of the pyrimidine ring, and donates the proton that replaces the carboxylate group at C6 of the product. In addition, H-bonds from the active site to O2 and O4 help to delocalize negative charge in the transition state. These results are supported by the crystal structures of the ligand-free and the 6-aza UMP complexed *E. coli* ODCase [18]. The crystal structure of *E. coli* ODCase in complex with BMP [19] shows the similar results that Lys73 is in close proximity to O6 of the ligand BMP and the proximity of the OMP carboxylate group with Asp71 appears to be instrumental for the decarboxylation of OMP, either through charge repulsion or through the formation of a very short hydrogen bond between the two carboxylate groups. The structures of ODCase in complex with the above inhibitors show that O2 is away from the Lys93, which is against to the mechanism involving Lys93 protonation of O2.

Our group assumes that UMP and 6-azaUMP could fit into ODCase active site either with the ring atoms superimposed with that of the substrate during catalysis, or with the glycosidic bond rotated from the structure by about 180°. The above explanations from crystal structures are not sufficient to explain this. It has been assumed that these inhibitors are binding in an orientation that is the same that of the substrate

OMP. It is not possible to get the crystal structure of ODCase in complex with substrate OMP, as the enzyme is very active it immediately converted to product UMP. To know how the substrate OMP is binding in the ODCase active site, we propose to crystallize *E. coli* ODCase in complex with UMP-6-thiocarboxamide, a substrate isostere.



1 2 3

Figure 1-4: Any enzyme active site (depicted by the irregular geometric figure) capable of accommodating OMP (1) should be capable of binding 6-azaUMP in the same orientation (2) or in an orientation with the pyrimidine ring turned 180° (3).

To crystallize the ODCase in complex with UMP-6-$CSNH_2$, consistent amount of pure protein was required. With this intention we cloned the *E. coli* ODCase gene and purified the protein by applying to the Affi-Gel Blue column and eluting with different inhibitors like 6-azaUMP, UMP and UMP-6-$CSNH_2$. We planned to synthesize BMP, a potent ODCase inhibitor by enzymatic reaction catalyzed by OPRTase. We cloned the *E. coli* OPRTase gene and purified in the same way as ODCase using Affi-Gel Blue column and eluting with high salt buffer. To synthesize BMP by enzymatic reaction we used barbituric acid as substrate and checked the conversion of barbituric acid to BMP on HPLC. For crystallographic and NMR studies we synthesized the unlabeled and ^{15}N labeled UMP-6-$CSNH_2$ in our lab. To know the distances between hydrogens attached to the ^{15}N labeled nitrogen and hydrogens attached to the C5, when the UMP-6-$CSNH_2$ is in

syn & anti confirmations in the active site, we used the Spartan programme to model the UMP-6-$CSNH_2$ in the active site and measured the bond distances.

Chapter 2

Cloning of *E.coli* ODCase gene and Production and Purification of *E. coli* ODCase

Introduction

Investigating the decarboxylation mechanism of OMP decarboxylase and obtaining the crystal structures of ODCase in complex with the different inhibitors necessitate preparation of purified enzyme. The complete nucleotide sequence of ODCase was determined from the pyr-4 gene of *Neurospora crassa* which encodes orotidine-5' -phosphate decarboxylase [20], [21]. Cloning of yeast ODCase was done by Bell and Jones [22], using 15C yeast cells and they used the plasmid, pGU2, which carries the *URA3* gene that encodes for ODCase. This plasmid, pGU2, is under control of the promoter for *GAL1*, which allows galactose induction for overexpression of the protein.

Authors induced the yeast culture with galactose and lysed the cells and purified the ODCase by 60-90% ammonium sulfate fractionation and affinity chromatography on an Affi-Gel Blue column. The CM-52 cellulose column removes a small amount of contaminating proteins remained after elution from the Affi-Gel Blue column. They used 6-azaUMP to elute the ODCase from this column and the fractions containing this enzyme were pooled and concentrated. Concentration of purified ODCase was determined by Bradford assay and the purity was checked on SDS-polyacrylamide gel. Bound 6-azaUMP was removed from the enzyme by sequential dialysis using dialysis buffer containing sodium phosphate, 2-mercaptoethanol, glycerol and UMP in different

concentrations. They achieved 60 mg of ODCase with a specific activity of 39-43 units/mg from 4.8 liters of cell culture.

In the following experiments, we cloned the *E. coli* ODCase gene into plasmid pCal-n and overproduced the ODCase by IPTG induction. The protein was then purified by applying to Affi-Gel Blue column after ammonium sulfate fractionation.

Materials and Methods

Materials

Primers for the cloning of ODCase gene are obtained from Integrated DNA Technologies, Inc and have the following sequences (restriction sites underlined):

"*E. coli* ODCase NdeI start":

5'-GGGAAAGGCATATGACGTTAACTGCTTCATCTTC-3'

"*E. coli* ODCase HindIII stop":

5'-GGAAAGGAAAGCTTTCATGCACTCCGCTGTAAAGAGG-3'

Thermostable DNA polymerase and restriction enzymes (NdeI and HindIII) were from New England Biolabs. Bradford dye and Affi-gel blue column material are obtained from Bio-Rad Laboratories, Inc. UMP was purchased from Sigma. Bead Beater from BioSpec products, Hewlett Packard 8453 UV spectrophotometer and Waters 1525 HPLC are used. LB medium was prepared by using 10 g/L of tryptone, 5 g/L of yeast extract and 5 g/L of sodium chloride and pH was adjusted to 7.4 (1 ml of 0.5 mg/ml ampicillin per liter for LB-Amp medium).

Cloning of *E. coli* ODCase gene

Plasmid pCal-n (Figure 2-1) with ampicillin resistant gene was used for cloning of the *E. coli* ODCase gene. Plasmid pCal-n from *E. coli* carrying this plasmid was isolated

and purified using Qiagen QIAFilter Plasmid Midi kit. The ODCase gene was amplified from genomic *E. coli* DNA by Polymerase chain reaction (PCR) using DNA polymerase and primers listed above. The PCR reaction was set to cycle 35 times using the following temperatures: 95° C for 30 sec, 58° C for 60 sec and 72° C for 75 sec. The size of the DNA was checked by running on an agarose gel (1.2%) in each step. PCR product and the plasmid pCal-n were digested using restriction digested enzymes NdeI and HindIII. The restriction enzyme digested PCR product was ligated to the restriction enzyme digested plasmid pCal-n using T4 DNA ligase. The ligated product was inserted into $CaCl_2$-treated XLI-Blue *E. coli* cells and the transformed cells were grown on LB-Amp agar medium. The insertion of the ODCase gene into plasmid pCal-n was confirmed by running the agarose gel with minipreparations of the colonies taken from the LB-Amp agar plates (Figure 2-2). The Qiagen purified plasmid with cloned *E. coli* ODCase gene was inserted into BL21 *E. coli* cells.

Overexpression and Purification of *E. coli* ODCase

Over-production of ODCase from transformed *E. coli* cells was confirmed by time point induction with isopropyl-β-D-thiogalactopyranoside (IPTG). 5 ml of LB-Amp medium was inoculated with the cells having cloned plasmid for *E. coli* ODCase and incubated at 37° C overnight by constant shaking. 300 ml of LB-Amp medium was inoculated with 3 ml of the overnight culture. After 3 hrs of incubation at the same conditions as above, 50 µM of IPTG (62.5 µL of 20 mg/ml) was added. The cells were collected and lysed with lysate buffer containing 50 mM potassium phosphate (pH 7.0), glycerol 10%(v/v), 5 mM β-mercaptoethanol, 2 µM pepstatin, 0.6 µM leupeptin and

1 µM Phenyl methyl sulfonyl fluoride (PMSF). Samples of the culture were taken every 2 hrs for up to 8 hrs and the lysates were electrophoresed on an SDS-polyacrylamide gel.

Large scale production of *E. coli* ODCase was carried out with 2L culture of LB-Amp. The produced ODCase was used in attempted purification by chromatography with the Affi-Gel Blue column. The protein was eluted with 1 µM 6-aza UMP (or other nucleotide, in subsequent experiments) and the purified fractions are checked on the SDS-polyacrylamide gel.

In subsequent experiments, 40-70% ammonium sulfate fractionation of the lysate was done before the lysate was applied to column. The resuspended final pellet was dialyzed 2 times with dialysis buffer (50 mM Tris at pH 7.4, 5 mM β-mercaptoethanol and 10 % (v/v) glycerol), first dialysis for overnight and the second dialysis for 2 hrs. The solution was applied to Affi-Gel Blue column and the non-specific proteins were removed by washing the column with dialysis buffer. The ODCase was eluted with buffer containing 1 µM 6-azaUMP. Different fractions before and after elution with 6-azaUMP were checked on SDS-polyacrylamide gel. Fractions containing ODCase were pooled and the concentration of protein was estimated by Bradford assay.

Inhibitor-free Protein samples

In order to get protein free of 6-azaUMP, the protein was applied to DEAE Sephagel column and eluted without azaUMP by HEPES dialysis buffer but elution was not achieved even with 1 M NaCl-dialysis buffer. We were unsuccessful in removing 6-azaUMP by applying protein-inhibitor sample to this Sephagel column and eluting with dialysis buffer.

As an alternate approach to the previous Affi-Gel Blue chromatography method, the protein was eluted using 1 mM UMP from Affi-Gel Blue column and then the protein with UMP was dialyzed by changing dialysis buffer 3 times in 24 hrs in order to get inhibitor-free protein. The dialyzed samples collected at different time intervals were checked on HPLC in comparison with standard UMP concentrations, in order to determine the extent of removal of UMP. In addition we attempted to elute the protein with another inhibitor, 1 µM UMP- thiocarboxamide. This inhibitor is desired for enzyme-inhibitor samples, so replacement of the eluting nucleotide with UMP-thiocarboxamide would not be necessary.

Bradford Assay

Bradford assay is the method used commonly to determine the total protein concentration of a sample [24]. The dye used in this method is the Coomassie brilliant blue G-250 with A_{max} at 465 nm. The UV spectrophotometer was blanked at 595 nm using 900 µL of Bradford dye and 100 µL of 1.5M NaCl. The absorbances of the protein, BSA (Bovine serum albumin, 0.5mg/ml) at different concentrations (5, 10, 15, 20 µL) are then measured. Standard protein concentration versus absorbance at 595 nm curve with the protein BSA was plotted (Figure 2-5). Then the absorbance of protein sample, whose concentration has to be determined, was measured. From the standard curve the unknown amount of the protein sample was calculated.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to separate DNA fragments by size. The migration of the DNA molecule can be detected under UV light after the gel has been stained in ethidium bromide (EtBr). The DNA samples along with DNA marker loaded

with agarose gel loading buffer are run over the agarose gel at 110 V and stained in EtBr for 15 min and observed under the UV light.

SDS-Polyacrylamide Gel Electrophoresis

In SDS-PAGE proteins are separated on the basis of their molecular weights, where those with lower molecular weights migrate farther. SDS-PAGE gels are prepared [25] and after loading the protein samples and the protein molecular weight marker with added SDS buffer, gel was run at constant voltage (100 V). Bands are visible after staining and destaining of the gel.

ODCase Assay

Enzyme assay of ODCase was carried out by using substrate OMP synthesized in our lab. When the enzyme is active it catalyzes the reaction of substrate OMP to product UMP. The UV absorbances for these two compounds are slightly different and the spectra for these compounds have peaks at different wavelengths. When the enzyme is added, the spectrum of the substrate can be observed to change towards the product. This can be detected by observing the change in absorbance of the reaction mixture at 286 nm. This change in absorbance is in correspondance with the conversion of OMP to UMP. From the graph, change in absorbance versus time, we can calculate the enzyme activity and specific activity (Figure 2-7). The factor that relates the change in absorbance (286 nm) to change in concentration for the ODCase reaction is -2250 M^{-1}. Thus, the measured slope in the absorbance vs. time plot is divided by -2250 M^{-1} to obtain the change in concentration vs. time. This value is then divided by the amount of protein used in the assay to obtain nmol $min^{-1}mg^{-1}$ or nmol $min^{-1}\mu g^{-1}$.

Determining the amount of UMP remaining in the protein sample after dialysis by HPLC analysis

The protein samples containing UMP are collected before and after dialysis at different time intervals and analyzed on HPLC (Waters 1525 HPLC; Hamilton column, PRP-X100 with length 250mm and diameter of 4.6mm; solvent A: water; solvent B: 0.8M ammonium bicarbonate; gradient: 100% A to 100% B over 20 min). UMP can be separated on the HPLC at the retension time of 6 min. The amount of UMP can be determined based on the peak area. The graph of dialysis time versus peak area was plotted.

Results and Discussion

After successfully constructing the plasmid with ODCase gene by inserting *E. coli* ODCase gene into plasmid pCal-n using restriction enzymes NdeI and HindIII, the conditions that are favorable for the maximum yield of ODCase were determined. Maximum yield of ODCase can be obtained by growing the *E. coli* cells in LB-Amp medium and induction by 50 µM IPTG for 6 hrs at 37° C with continuous shaking (Figure 2-3). The pure ODCase with a single band on the SDS-polyacrylamide gel was obtained in a single purification step using Affi-Gel Blue column following ammonium sulfate fractionation (Figure 2-4). Elution of highly pure ODCase from Affi-Gel Blue column using different inhibitors UMP, 6-aza UMP and UMP-6-thiocarboxamide was shown to be possible (Table 2-1). 60-70 mg of protein was obtained from 4 L culture of *E. coli* with the specific activity of 54.8 nmol $min^{-1}\mu g^{-1}$. The pure protein free of inhibitors can be achieved by eluting the protein with UMP and dialyzing this protein inhibitor sample by changing the dialysis buffer 3 times (Figure 2-7).

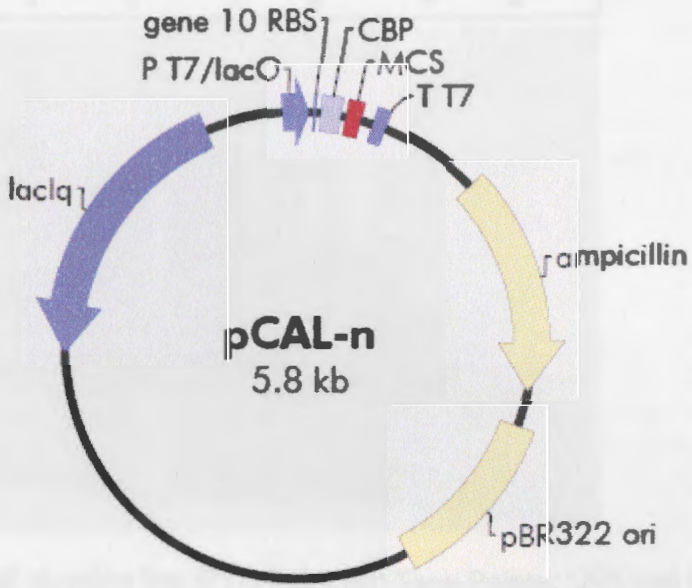


Figure 2-1: Plasmid pCal-n used for *E. coli* ODCase gene cloning with ampicillin resistant site. Adopted from www.stratagene.com.

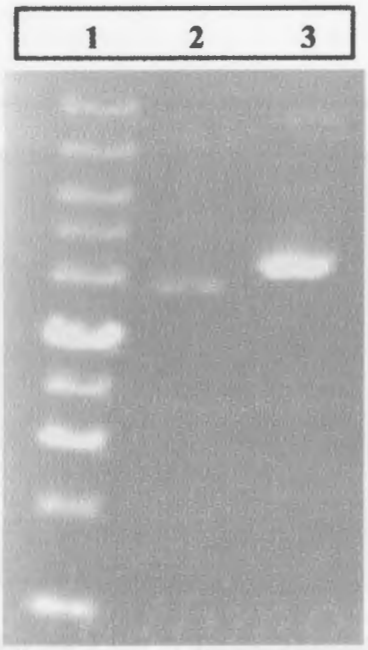


Figure 2-2: Agarose gel showing the plasmid with inserted ODCase gene.
Lane 1: DNA molecular weight markers.
Lane 2: Plasmid pCal-n.
Lane 3: Plasmid pCal-n with inserted gene.

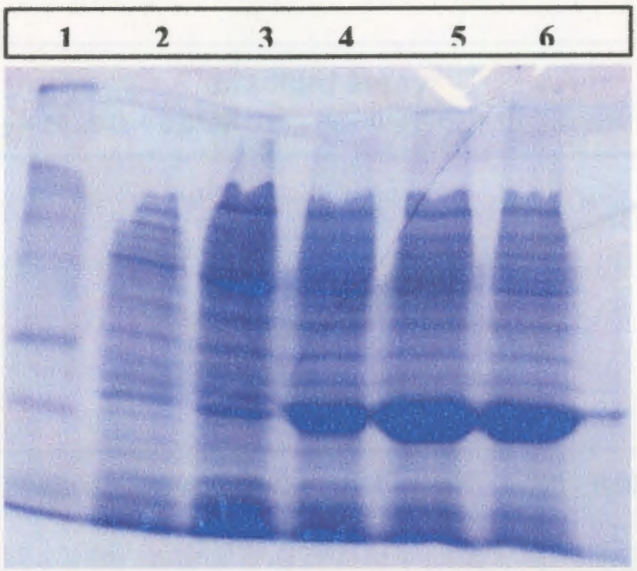


Figure 2-3: SDS-PAGE showing the IPTG Induction Time Points: ODCase is produced in large amounts after 6-8 hours.
Lane 1: protein molecular weight marker.
Lane 2: 0 hr induction.
Lane 3: 2 hr induction.
Lane 4: 4 hr induction.
Lane 5: 6 hr induction.
Lane 6: 8 hr induction.

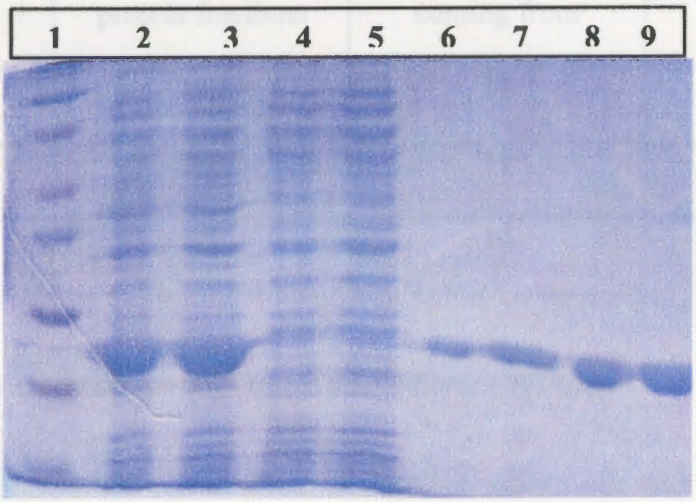


Figure 2-4: SDS-PAGE showing samples in the purification of ODCase.
Lane 1: protein molecular weight marker.
Lane 2: cell lysate.
Lane 3: resuspended protein from 40-70% ammonium sulfate fractination.
Lane 4, 5: Flow through from Affi-Gel Blue column.
Lanes 6, 7, 8, 9: purified ODCase fractions eluted from Affi-Gel Blue column.

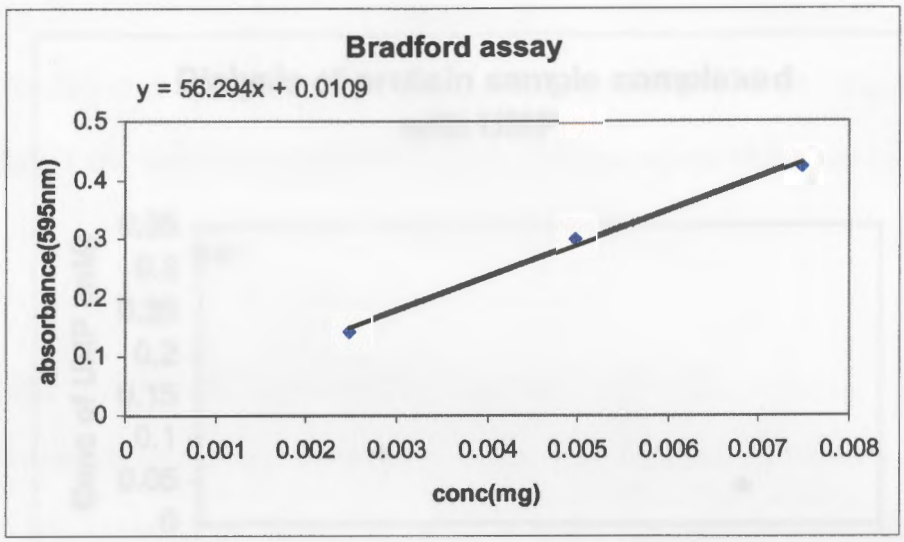


Figure 2-5: Standard Bradford assay curve.

Inhibitor	No. of non specific protein fractions	ODCase start coming from	No. of fractions of ODCase
6-azaUMP	102	12	20
UMP-$CSNH_2$	100	11	22
UMP	100	14	17

Table 2-1: Affinity chromatography results with different inhibitors

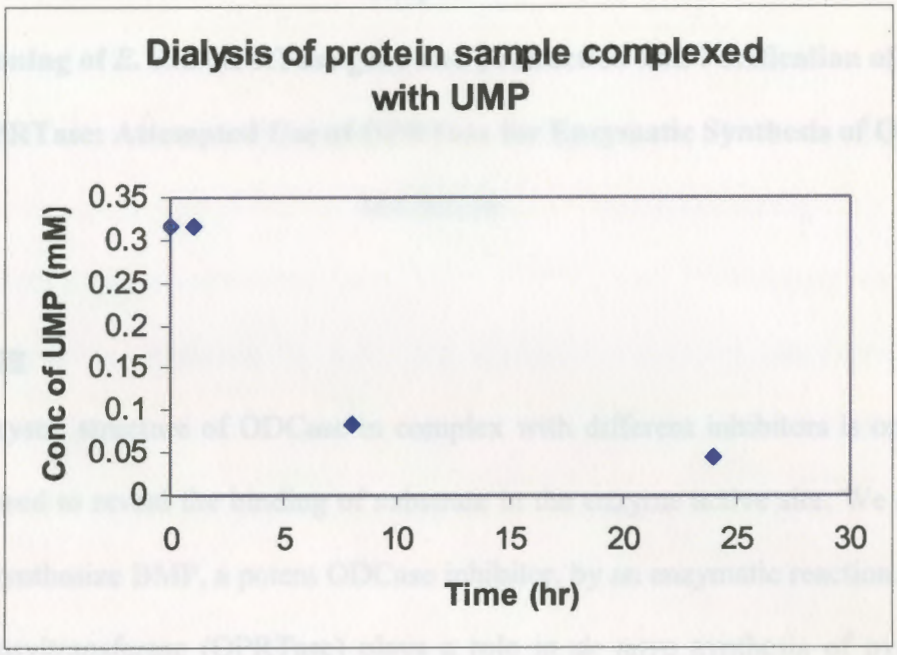


Figure 2-6: 0-24 hr Dialysis Curve showing the decrease in the concentration of UMP.

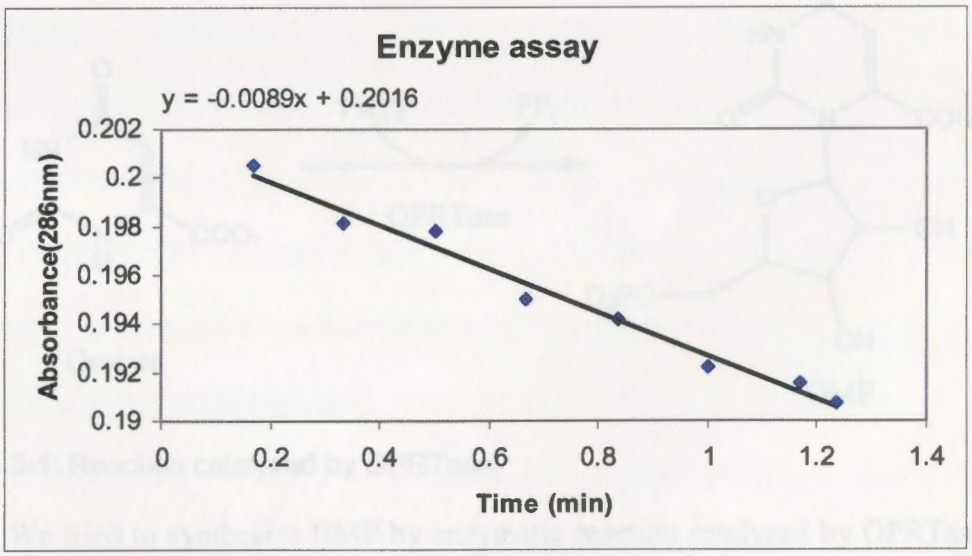


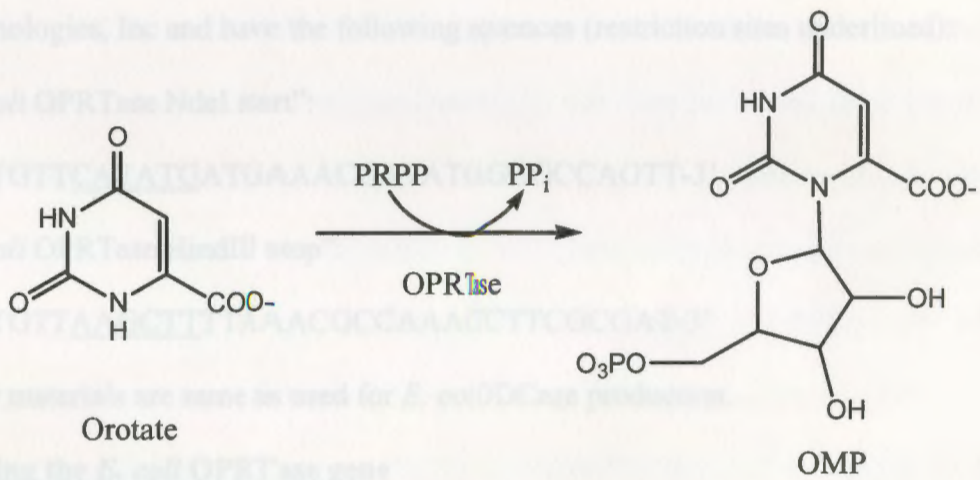
Figure 2-7: Typical data from an ODCase enzyme assay. The negative slope is divided by –2250 M^{-1} to calculate the change in OMP concentration per minute.

Chapter 3

Cloning of *E. coli* OPRTase gene and Production and Purification of *E. coli* OPRTase: Attempted Use of OPRTase for Enzymatic Synthesis of ODCase Inhibitors

Introduction

A crystal structure of ODCase in complex with different inhibitors is one of the best ways used to reveal the binding of substrate in the enzyme active site. We made an attempt to synthesize BMP, a potent ODCase inhibitor, by an enzymatic reaction. Orotate phosphoribosyltransferase (OPRTase) plays a role in *de novo* synthesis of pyrimidine nucleotides and transfers orotate to 5-phosphoribosyl-1-pyrophosphate (PRPP) to form OMP) [26].



Orotate OMP

Figure 3-1: Reaction catalyzed by OPRTase.

We tried to synthesize BMP by enzymatic reaction catalyzed by OPRTase using barbituric acid as a substrate. Barbituric acid can be purchased from commercial sources or easily synthesized from urea and diethyl malonate; it is easy to obtain the isotope labeled barbituric acid from these compounds having isotope labeling. To proceed for

this reaction considerable amount of protein was required, so we cloned the gene for over production of protein. Berges and Perrot group [27] isolated the nucleotide sequence of the *ura5* gene which encodes the OPRTase gene. Previously the OPRTase enzyme was expressed in *E. coli* and purified [28]. They introduced the gene encoding OPRTase into a plasmid with a tac promoter and overexpressed the protein by inducing with IPTG. Purified protein was obtained by following purification steps including ammonium sulfate fractionation, and KCl gradient elution from DEAE-Sephadex column. We followed the same method used for *E. coli* ODCase cloning and attempted purification using Affi-Gel Blue.

Materials and Methods

PCR Primers

Primers for the cloning of OPRTase gene are obtained from Integrated DNA Technologies, Inc and have the following sequences (restriction sites underlined):

"*E. coli* OPRTase NdeI start":

5'-ATGTTCATATGATGAAACCATATCAGCGCCAGTT-3'

"*E. coli* OPRTase HindIII stop":

5'-ATGTTAAGCTTTTAAACGCCAAACTCTTCGCGAT-3'

Other materials are same as used for *E. coli* ODCase production.

Cloning the *E. coli* OPRTase gene

The gene for OPRTase was cloned into plasmid pCal-n (Figure 2-1) using restriction sites NdeI and HindIII. Polymerase chain reaction (PCR) was run with *E. coli* genomic DNA as template (similar to ODCase gene isolation, Chapter 2) to amplify the OPRTase gene. The PCR product and the plasmid pCal-n were digested with restriction

enzymes NdeI and Hind III. The restriction enzyme digested PCR product and plasmid pCal-n were used in a ligation reaction with DNA ligase. The ligated product was transferred into *E. coli* cells. These cells are grown on LB-Amp medium allowing only the cells having a plasmid with ampicillin resistant gene to grow. The colonies from this plate were selected and then the plasmid was extracted and checked on the agarose gel. The cells having the plasmid with insert were grown and plasmid from these cells was purified using Qiagen HiSpeed Plasmid Midi Kit. This purified plasmid was checked on an agarose gel and the insertion of OPRTase gene into the plasmid pCal-n was examined. Appropriately constructed plasmid was inserted into BL21 E. coli cells to overexpress the protein.

Overexpression and Purification of *E. coli* OPRTase

The *E. coli* cells with the plasmid for OPRTase were grown in LB-Amp medium overnight at 37°C with continuous shaking. This overnight culture was used to inoculate a secondary culture of LB-Amp medium, which was then incubated for 3 hrs at 37°C under continuous shaking and then induced with 50 µM IPTG. Portions of cells from this culture were collected for every 2 hrs up to 6 hrs. These collected samples are lysed using glass bead disruption in lysate buffer (same as in Chapter 2). The lysates of cells collected at 2 hr intervals are tested for protein on an SDS-polyacrylamide gel.

Large scale production of OPRTase was started by growing the cells in 4L culture of LB-Amp. 4 L of LB-Amp medium was inoculated by an overnight culture and allowed incubated for 3 hrs at 37°C with continuous shaking. Then the culture was induced with 50 µM IPTG and incubated for another 4 hrs. The cells were lysed and the lysate subjected to 40-70% of ammonium sulfate fractionation. The redissolved 70%

ammonium sulfate pellet was applied to Affi-Gel blue column and the column washed with dialysis buffer (same as in Chapter 2) to remove non-specific proteins. To check whether the OPRTase was sticking to column or not, SDS gel was run to examine different samples collected with washing buffer. Then the column was washed with dialysis buffer plus 1 mM orotic acid. Finally dialysis buffer plus 1 M NaCl was run through the column (Table 3-1). Different fractions eluted with the high salt buffer were electrophoresed on an SDS polyacrylamide gel to test the purity of the protein. The protein was dialyzed overnight to remove the salt (NaCl). Protein concentration was determined by Bradford assay.

The activity of purified OPRTase was checked using an assay containing orotic acid and PRPP, and analyzing the products on HPLC. Standard orotic acid and OMP can easily be separated using Waters 1525 HPLC; Hamilton column, PRP-X100 with length 250 mm and diameter of 4.6 mm using the following elution conditions: solvent A: water; solvent B: 0.8 M ammonium bicarbonate; gradient: 100% A to 100% B over 20 min (Figure 3-7). The formation of OMP from orotic acid could then be determined in the reaction mixtures by running the samples every 20 min.

Attempt to synthesize BMP by enzymatic reaction using purified OPRTase

BMP, 1-(5'-phospho-β-D-ribofuranosyl) barbituric acid is an tightly binding reversible ODCase inhibitor, which can be prepared chemically using barbituric acid. But this is a tedious process, so we attempted to prepare BMP by OPRTase catalysis using barbituric acid as substrate instead of orotic acid and PRPP.

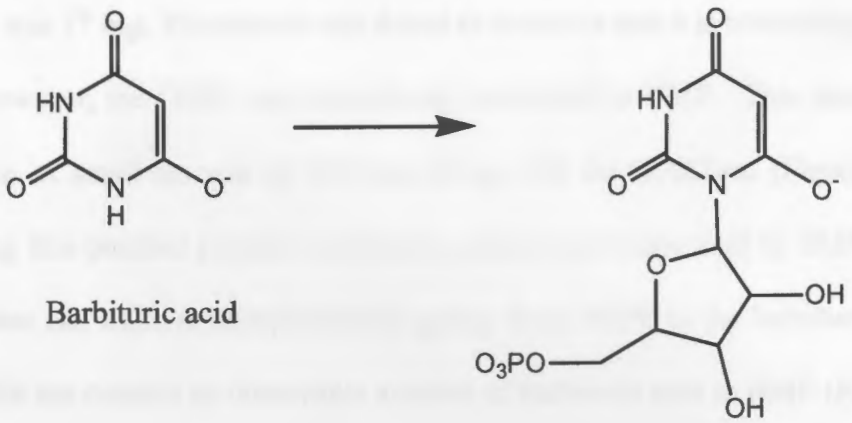


Figure 3-2: Conversion of barbituric acid to BMP.

Barbituric acid and BMP can be detected separately on HPLC. The peaks of barbituric acid and BMP are determined by running them separately on HPLC under the same conditions described above. We prepared the reaction sample of barbituric acid, PRPP and OPRTase and checked this sample for every 20 min on HPLC to detect the conversion of barbituric acid to BMP by OPRTase.

Results and Discussion

In an attempt to synthesize BMP by enzymatic catalysis using OPRTase, we cloned the gene for *E. coli* OPRTase and purified the produced protein. The OPRTase gene was cloned using plasmid pCal-n using restriction enzymes NdeI and HindIII (Figure 3-3). Optimum conditions for overproduction of OPRTase were found to be growing *E. coli* cells with inserted plasmid in LB-Amp medium incubated at 37°C with continuous shaking and induction with 50 µM of IPTG for 4 hrs (Figure 3-4). After confirming that OPRTase was binding to Affi-Gel Blue column (Figure 3-5), the protein was purified by ammonium sulfate fractionation and using Affi-Gel Blue column by eluting the protein with 1 M NaCl-dialysis buffer (Figure 3-6). The protein was found to be about 95% pure by running on the SDS PAGE gel. The maximum yield obtained from

4 L culture was 77 mg. The protein was found to be active and it is converting orotic acid to OMP; however, the OMP was immediately converted to UMP. This must be due to the presence of small amount of ODCase along with the OPRTase (Figures 3-8, 3-9, 3-10). Using this purified protein we tried to convert barbituric acid to BMP, in a hope that OPRTase can transfer phosphoribosyl group from PRPP to the barbituric acid. But OPRTase did not convert an observable amount of barbituric acid to BMP (Figures 3-11, 3-12).

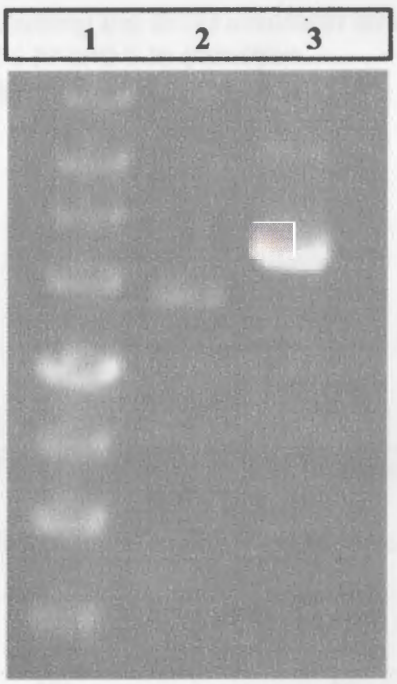


Figure 3-3: Agarose gel picture showing the inserted plasmid with OPRTase gene.
Lane 1: DNA ladder
Lane 2: plasmid pCal-n
Lane 3: plasmid pCal-n with inserted gene.

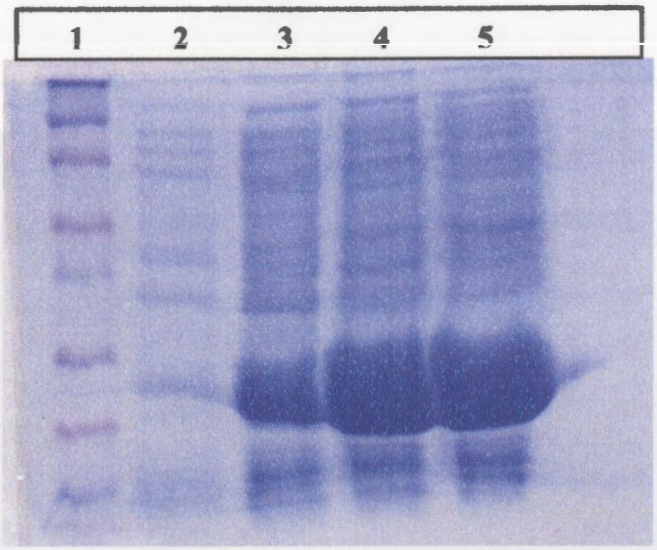


Figure 3-4: SDS-PAGE gel showing the IPTG induction time points: OPRTase was produced in large amounts at 4 hr and 6 hr induction.
Lane 1: protein molecular weight marker
Lane 2: 0 hr induction
Lane 3: 2 hr induction
Lane 4: 4 hr induction
Lane 5: 6 hr induction

Dialysis buffer with elutant	No of non specific fractions	OPRTase start coming from	No. fractions of OPRTase
1 mM orotic acid	36	OPRTase not eluted	0
1 M NaCl	36	18	31

Table 3-1: Affinity chromatography results

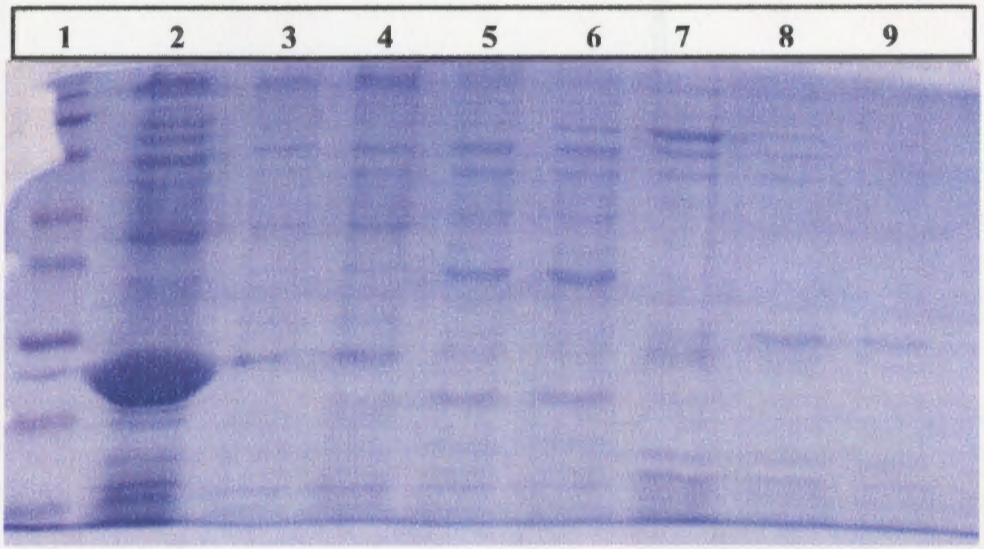


Figure 3-5: SDS-PAGE gel showing wash out fractions of Affi-Gel Blue column to confirm the binding of OPRTase to the column material.
Lane 1: protein molecular weight marker
Lane 2: lysate with OPRTase
Lanes 3, 4, 5, 6, 7, 8, 9: different wash fractions showing less or no OPRTase.

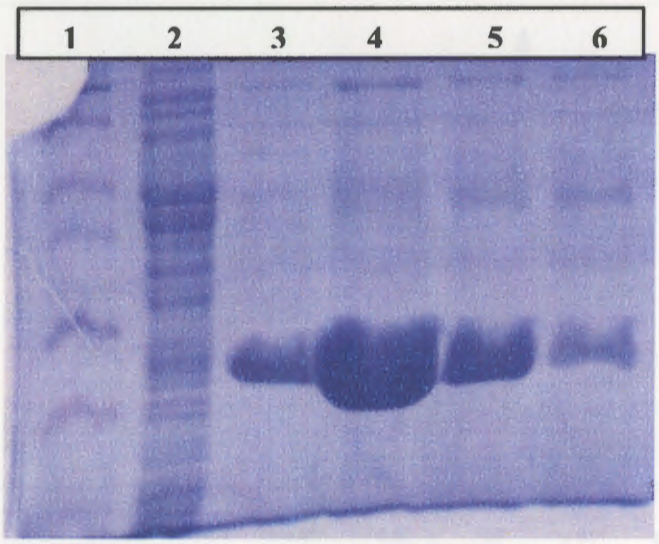


Figure 3-6: SDS-PAGE gel showing purified OPRTase.
Lane 1: protein molecular weight marker
Lane 2: Affi-Gel Blue column wash fraction with less OPRTase
Lanes 3, 4, 5, 6: fractions eluted with 1 M NaCl, representing purified OPRTase.

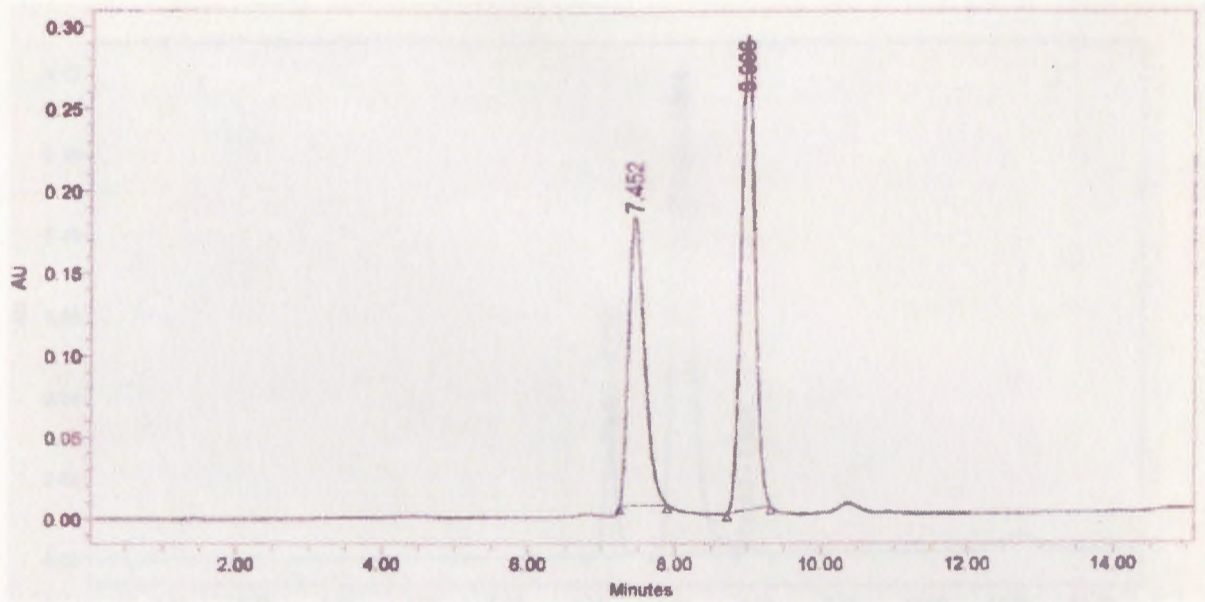


Figure 3-7: HPLC chromatogram showing separation of orotate and OMP in a mixture of orotate plus OMP.
Peak 1: orotate (7.4 min)
Peak 2: OMP (8.9 min).

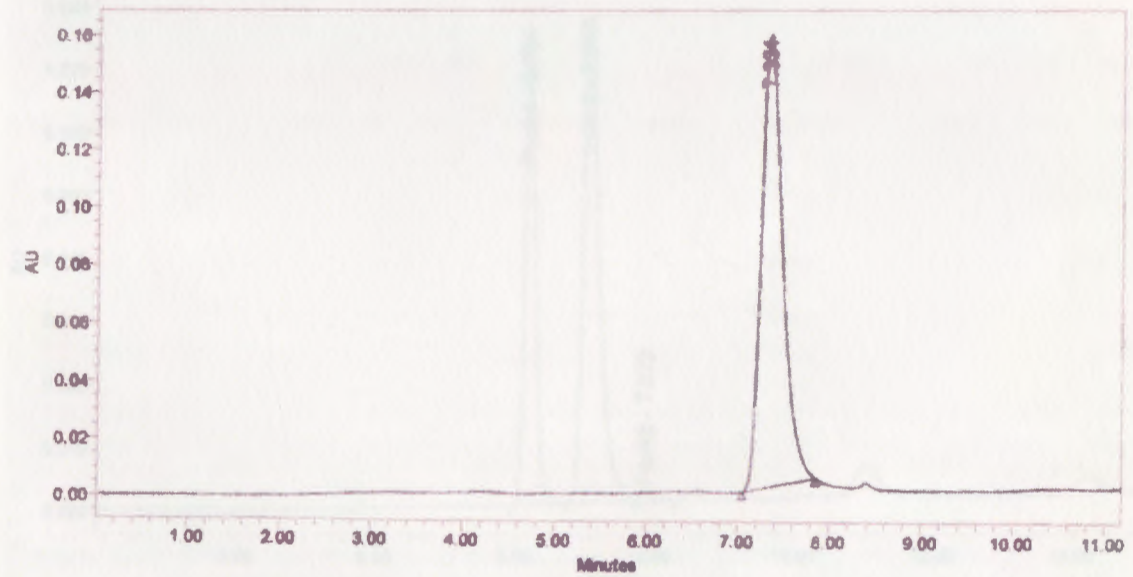


Figure 3-8: HPLC chromatogram for the beginning of the reaction of orotate, PRPP and OPRTase.
Peak 1: orotate (7.3 min)

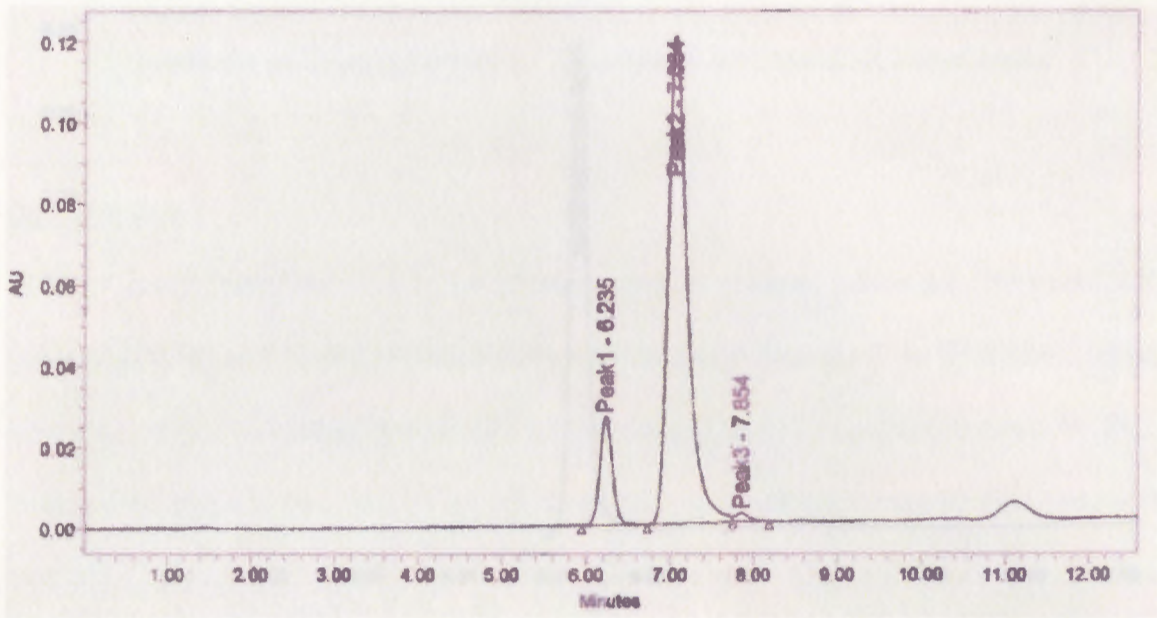


Figure 3-9: HPLC chromatogram for 20 min reaction showing the conversion of orotate to OMP which was immediately converted to UMP
Peak 1: UMP (6.2 min)
Peak 2: orotate (7.0 min).

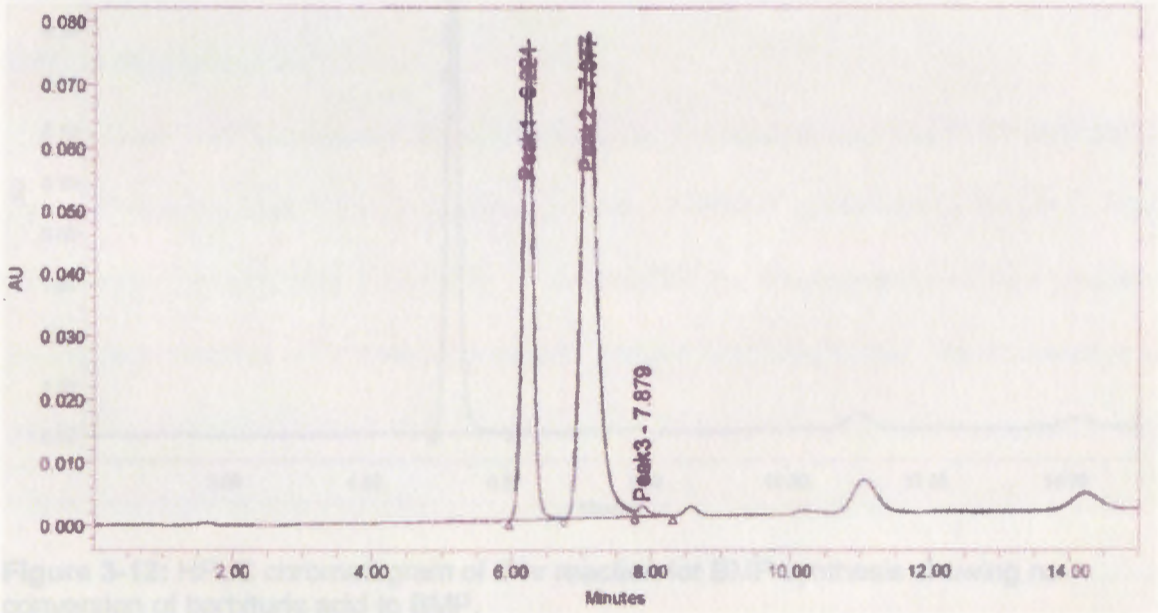


Figure 3-10: HPLC chromatogram for 60 min reaction showing increased UMP peak.
Peak 1: UMP (6.2 min)
Peak 2: orotate (7.0 min)

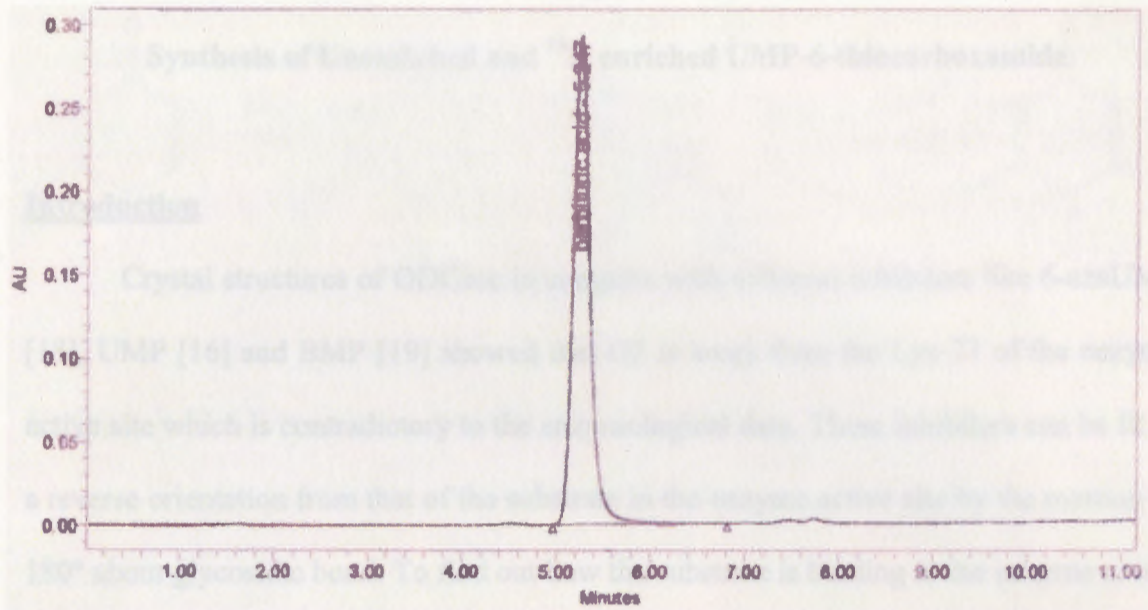


Figure 3-11: HPLC chromatogram for the beginning of the attempted reaction for BMP synthesis.
Peak 1: barbituric acid (5.2 min)

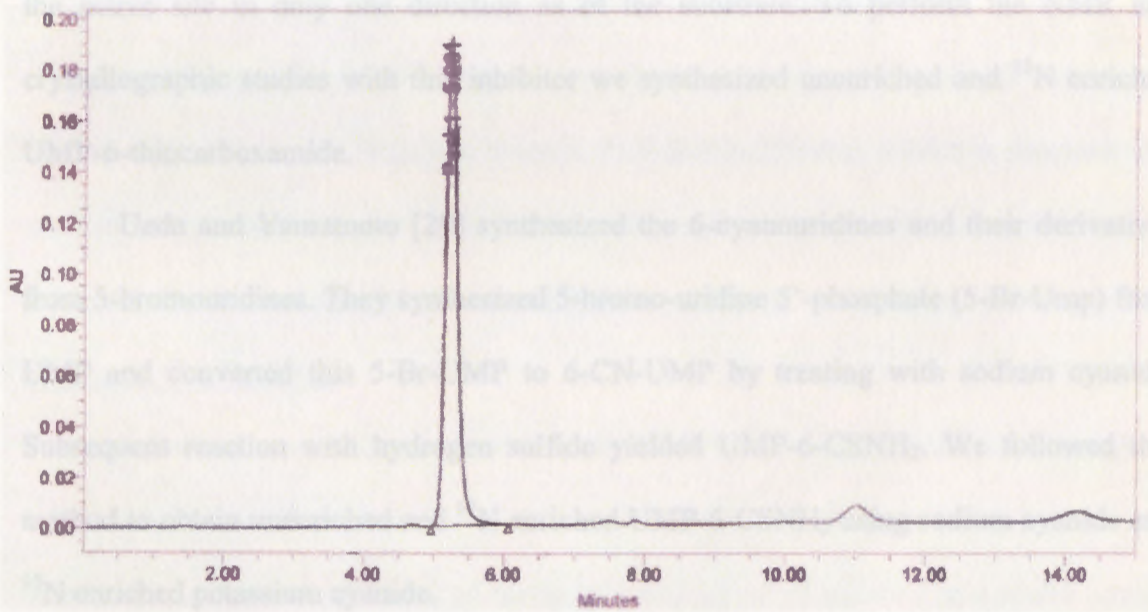


Figure 3-12: HPLC chromatogram of 2-hr reaction for BMP synthesis showing no conversion of barbituric acid to BMP.
Peak 1: barbituric acid (5.2 min)
BMP is expected to elute from the HPLC system before 10 min.

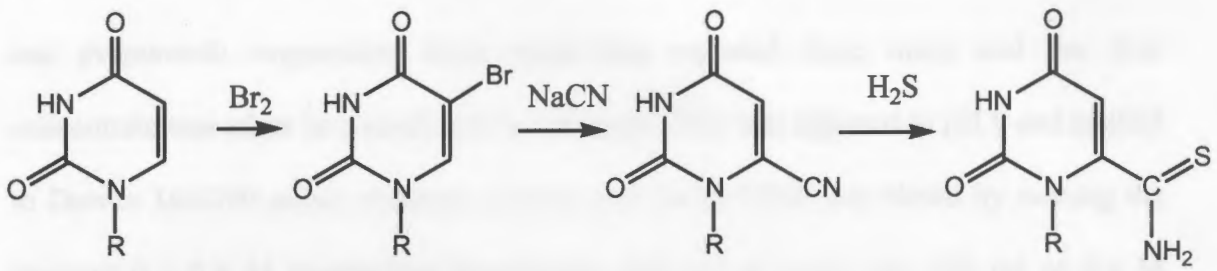
Chapter 4

Synthesis of Unenriched and ^{15}N enriched UMP-6-thiocarboxamide

Introduction

Crystal structures of ODCase in complex with different inhibitors like 6-azaUMP [18], UMP [16] and BMP [19] showed that O2 is away from the Lys-73 of the enzyme active site which is contradictory to the enzymological data. These inhibitors can be fit in a reverse orientation from that of the substrate in the enzyme active site by the rotation of 180° about glycosidic bond. To find out how the substrate is binding in the enzyme active site, it is appropriate to get the crystal structures of ODCase in complex with inhibitor, UMP-6-thiocarboxamide (UMP-6-$CSNH_2$), a substrate isostere. This inhibitor can fit into the active site in only one direction as of the substrate. To perform the NMR and crystallographic studies with this inhibitor we synthesized unenriched and ^{15}N enriched UMP-6-thiocarboxamide.

Ueda and Yamamoto [29] synthesized the 6-cyanouridines and their derivatives from 5-bromouridines. They synthesized 5-bromo-uridine 5'-phosphate (5-Br-Ump) from UMP and converted this 5-Br-UMP to 6-CN-UMP by treating with sodium cyanide. Subsequent reaction with hydrogen sulfide yielded UMP-6-$CSNH_2$. We followed this method to obtain unenriched and ^{15}N enriched UMP-6-$CSNH_2$ using sodium cyanide and ^{15}N enriched potassium cyanide.



UMP Br-UMP CN- UMP $UMP\text{-}CSNH_2$

Figure 4-1: Reaction pathway for $UMP\text{-}CSNH_2$ synthesis.

Materials and Methods

Materials

UMP was obtained from Sigma; pyridine from Aldrich; bromine and sodium sulfide from Fisher; acetic acid and hydrochloric acid from Pharmaco products Inc.; sodium cyanide from Matheson Coleman & Bell manufacturing chemists and ^{15}N-enriched potassium cyanide was obtained from Cambridge Isotope Laboratories, Inc. Waters 1525 HPLC with Hamilton column, PRP-X100 (250 mm × 4.6 mm diameter was used to analyze the samples with the following solvent conditions: solvent A: water; solvent B: 0.8 M ammonium bicarbonate; gradient: 100% A to 100% B over 20 min.

Synthesis of 5-Br-UMP

5-Br-UMP can be synthesized from the UMP by keeping the reaction overnight at room temperature. To synthesize 6 mM of Br-UMP, 3 g (8.13 mmol) of UMP was dissolved in a mixture of 15 ml of acetic acid and 30 ml of pyridine in a round bottom flask. To this 0.99 g (8.13 mmol) of bromine was added with stirring, then this reaction mixture was allowed to stay overnight at room temperature. The completion of the reaction was known by checking the sample on HPLC. The orange colored reaction

mixture was evaporated to remove solvent and the resulting syrup was dissolved in water and evaporated; evaporation from water was repeated three times and the final concentrate was taken in a small amount of water. This was adjusted to pH 9 and applied to Dowex 1x8-200 anion exchange column and the Br-UMP was eluted by running the gradient 0 - 0.8 M ammonium bicarbonate (500 ml of water and 500 ml of 0.8 M NH_4HCO_3). The fractions with 5-Br-UMP are selected by checking their UV absorbance at 278 nm and the fractions with absorbance more than one are pooled. Pooled fractions are washed with acid-washed resin to remove ammonium bicarbonate. This sample was concentrated by evaporation and the concentration was determined by measuring the UV absorbance at 278 nm (ε_{278} = 10,000 M^{-1}).

Synthesis of unenriched CN-UMP and ^{15}N enriched $C^{15}N$ -UMP

Unenriched and ^{15}N enriched 6-CN-UMP was prepared from the synthesized 5-Br-UMP by using unenriched sodium cyanide (NaCN) and ^{15}N enriched potassium cyanide ($KC^{15}N$). Two reactions are set up separately for labeled and unlabeled 6-CN-UMP each with 1 mmol 5-Br-UMP. 5-Br-UMP was dissolved in a small amount of water and evaporated; this was repeated three times to leave syrupy 5-Br-UMP. This was dissolved in 10 ml of dimethylsulfoxide (DMSO) and 98 mg (2 mmol) of NaCN for unlabeled reaction, 134 mg (2 mmol) of $KC^{15}N$ for labeled reaction were added. These reactions are left for 2 days at room temperature under stirring. After 2 days these reactions are checked on HPLC. To push the reaction toward completion, another 0.5 mmol of each NaCN and $KC^{15}N$ were added to the unlabeled and labeled reactions and left overnight and checked on HPLC. To the labeled reaction more DMSO was added in order to better dissolve the reactants; the reaction was continued for another day.

Both labeled and unlabeled 6-CN-UMP samples are evaporated to remove DMSO and diluted with three volumes of water and the pH was adjusted to 7.5 and applied to anion exchange column. The 6-CN-UMP was eluted by running the column with gradient of 0 - 0.4 M ammonium bicarbonate (500ml of water and 500ml of 0.4 M NH_4HCO_3, after that only 0.8 M NH_4HCO_3). The absorbances of the collected samples are measured at 280 nm and the samples of absorbance above 1.0 are checked on the HPLC. A graph of absorbance versus fraction number was generated (Figure 4-4, 4-6). Selected fractions were analyzed by the HPLC; those having more than 97% peak area for CN-UMP were pooled. The pooled fractions were washed with acid-washed cation exchange resin to remove the ammonium bicarbonate, concentrated by evaporation and the concentration was estimated by measuring the UV absorbance at 280 nm ($\varepsilon_{280} = 8570\ M^{-1}$).

Synthesis of ^{15}N enriched and Unenriched UMP-thiocarboxamide

^{15}N enriched and unenriched UMP-thiocarboxamide (UMP-$CSNH_2$) were prepared by bubbling hydrogen sulfide (H_2S) gas through 6-CN-UMP. 0.32 mmol of unlabeled 6-CN-UMP was dissolved in 5.6 ml of pyridine and H_2S gas, which was produced by reacting hydrochloric acid with sodium sulfide, was bubbled through it. This reaction was continued for 120 minutes until all the 6-CN-UMP was converted to thiocarboxamide, which was confirmed by testing the samples on HPLC for every 20 minutes (retention time of 11.8 min). Synthesis of ^{15}N enriched UMP-6-$CSNH_2$ was carried in the same way as the unenriched nucleotide. 0.21 mmol of 6-$C^{15}N$ –UMP was dissolved in 3.7 ml of pyridine and the H_2S gas was bubbled until all the 6-$C^{15}N$ –UMP was converted to UMP-$CS^{15}NH_2$. The reaction proceeded for 140 minutes. The both samples were evaporated to remove pyridine; water was added and evaporated to dryness

and this evaporation from water was repeated three times. Both the samples were tested by mass spectrometry.

Labeled and unlabeled UMP-6-$CSNH_2$ were purified by chromatography using the Dowex 1x8-200 anion exchange column by elution with a 0 - 0.4 M ammonium bicarbonate gradient. The fractions collected were checked on UV spectrophotometer at 270 nm; fractions (Figure 4-13, 4-14) having absorbance greater than 1.0 were analyzed by HPLC to estimate the percentage purity. The samples with > 95% pure UMP-$CSNH_2$ were pooled and washed with acid-washed cation exchange resin to remove ammonium bicarbonate and concentrated by evaporation. The concentrations of labeled and unlabeled UMP-6-$CSNH_2$ were estimated at 270 nm (ε_{270} = 11,300 M^{-1}).

Results and Discussion

The synthesis of 5-Br-UMP was confirmed by observing the peak for 5-Br-UMP by HPLC analysis (retention time = 8 min) showing the reaction was complete (Figure 4-2). The amount of pure 5-Br-UMP was found to be 5 mmol. From this purified 5-Br-UMP, unenriched and ^{15}N enriched 6-CN-UMP were synthesized using NaCN and $KC^{15}N$. The peak for 6-CN-UMP was observed (Figure 4-3, 4-5) with all of the 5-Br-UMP comsumed in the unlabeled reaction but only a partial reaction was observed in the ^{15}N reaction. The amounts of unenriched and ^{15}N enriched 6-CN-UMP were found to be 0.32 mmol and 0.21 mmol. Mass spectrometry showed the molecular weights for the two nucleotides are observed to be 347.9 (Figure 4-7) and 348.9 (Figure 4-8) confirming the insertion of labeled ^{15}N into the 6-$C^{15}N$ –UMP.

We successfully synthesized the ^{15}N labeled and unlabeled UMP-thiocarboxamide to carry out NMR and crystallographic studies with this inhibitor in complex with

ODCase (Figure 4-9, 4-10). From mass spectrometry results, the products were confirmed to have formula weights of 381.9 (Figure 4-11) and 382.9 (Figure 4-12) for unlabeled UMP-6-$CSNH_2$ and labeled UMP-6-$CS^{15}NH_2$ respectively. The amounts of labeled and unlabeled UMP-6-$CSNH_2$ were estimated to be 96 µmol and 113 µmol. The ^{15}N labeling was confirmed by the mass spectroscopy results and NMR results. Enrichment of UMP-6-$CSNH_2$ with ^{15}N was further confirmed by NMR results by observing the major signal for ^{15}N (Figure 4-15).

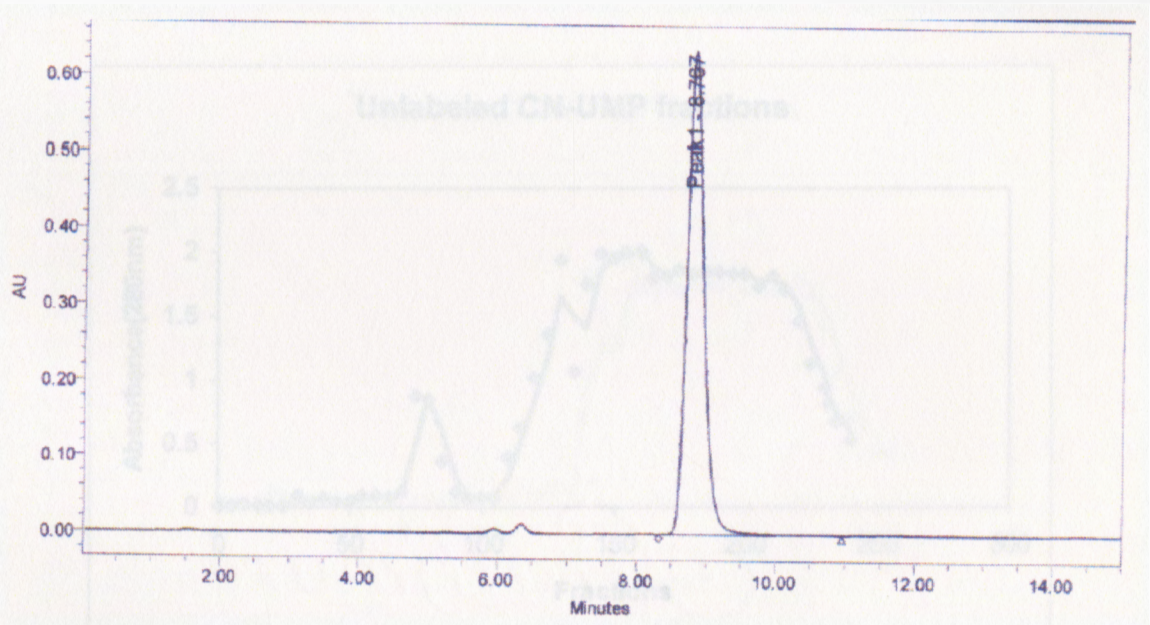


Figure 4-2: HPLC chromatogram showing near complete conversion of UMP (retention time ≈ 6 min) to 5-Br-UMP (retention time ≈ 9 min).

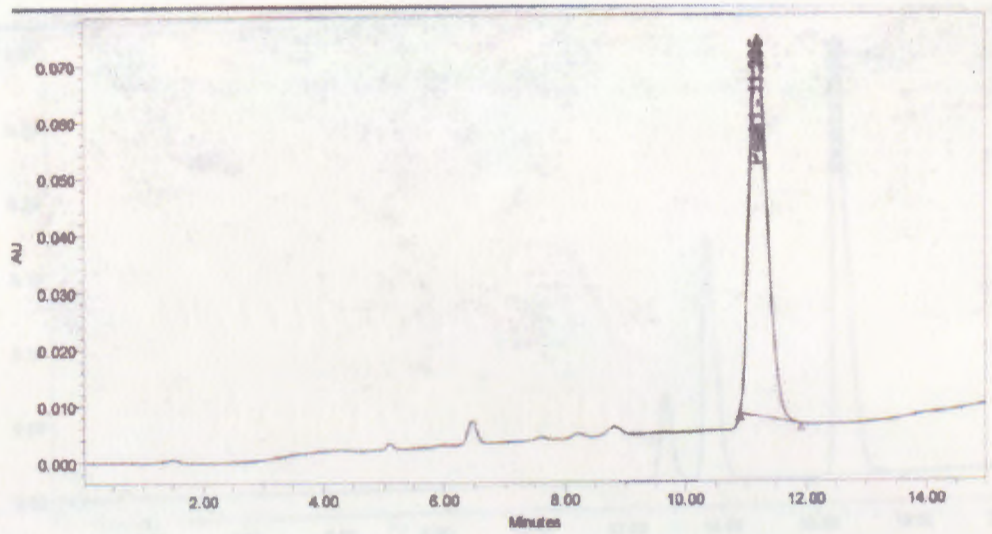


Figure 4-3: HPLC chromatogram showing the near-complete conversion of 5-Br-UMP to unenriched 6-CN-UMP.

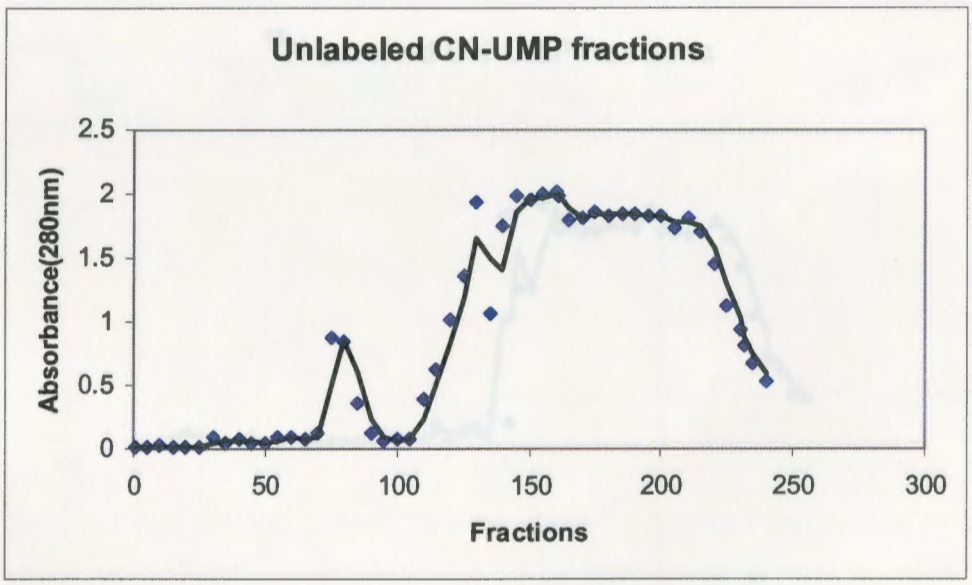


Figure 4-4: Chromatogram used for the selection of unenriched 6-CN-UMP fractions to test on the HPLC.

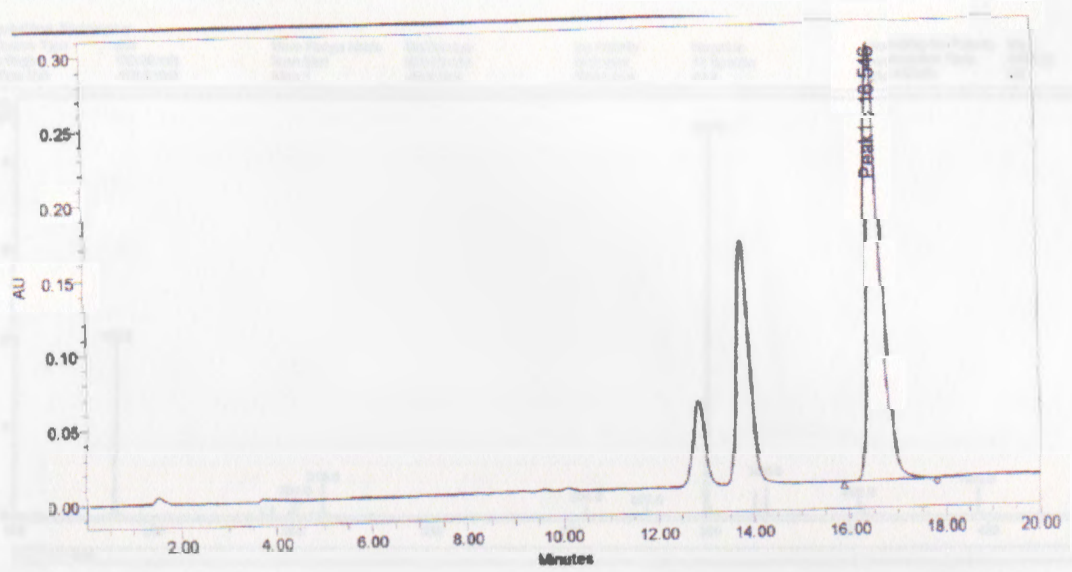


Figure 4-5: HPLC chromatogram showing the partial conversion of 5-Br-UMP to ^{15}N enriched CN-UMP.

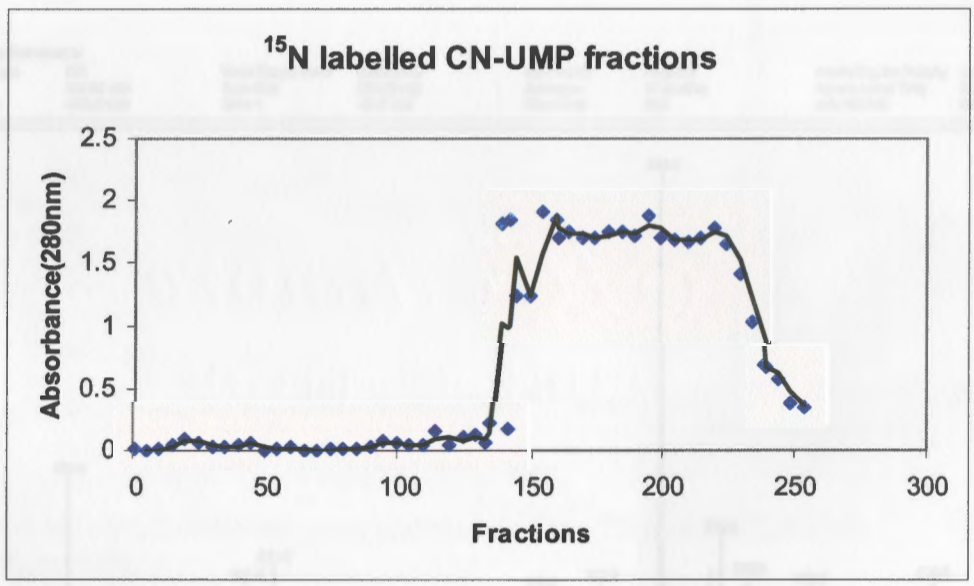


Figure 4-6: Chromatogram used for the selection of ^{15}N enriched 6-CN-UMP fractions to test on the HPLC.

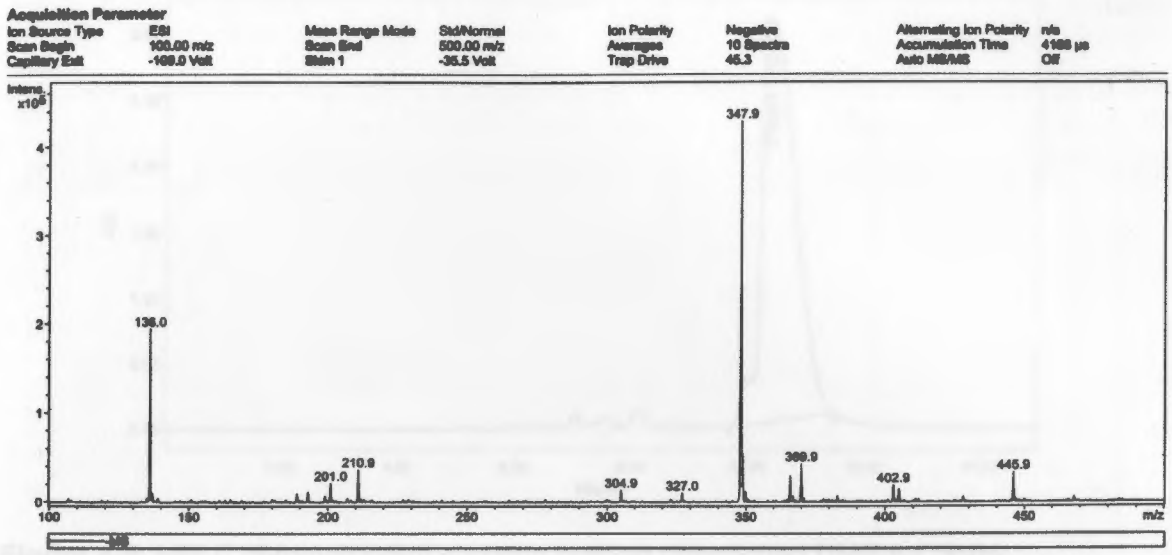


Figure 4-7: Mass spectroscopic results of unenriched 6-CN-UMP.

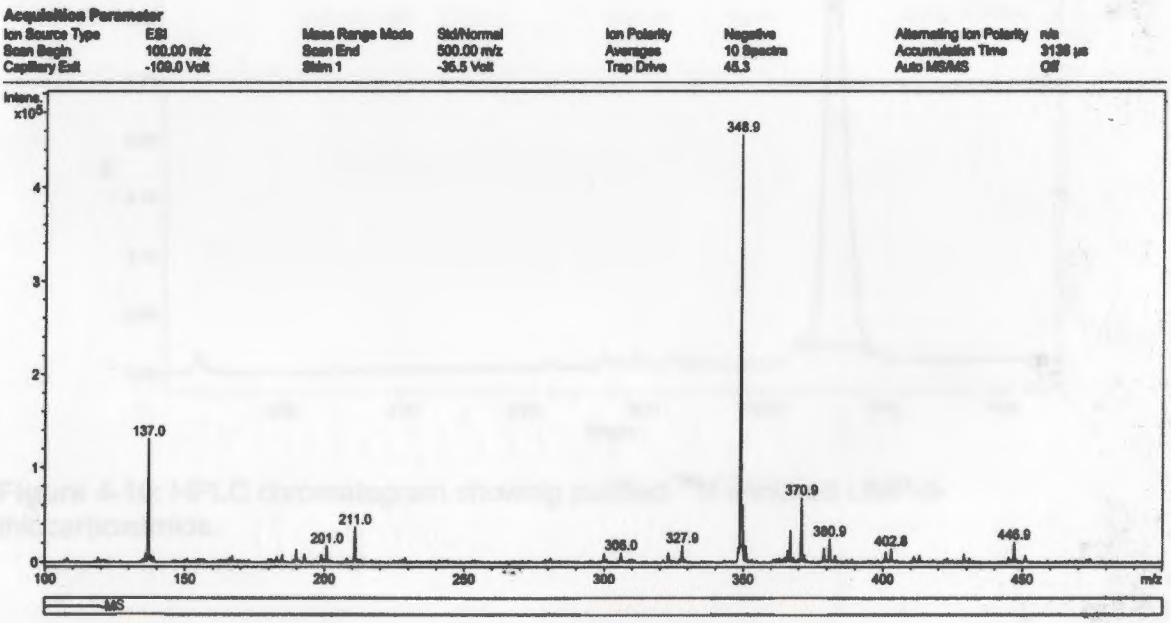


Figure 4-8: Mass spectroscopic results of ^{15}N enriched 6-C^{15}N-UMP.

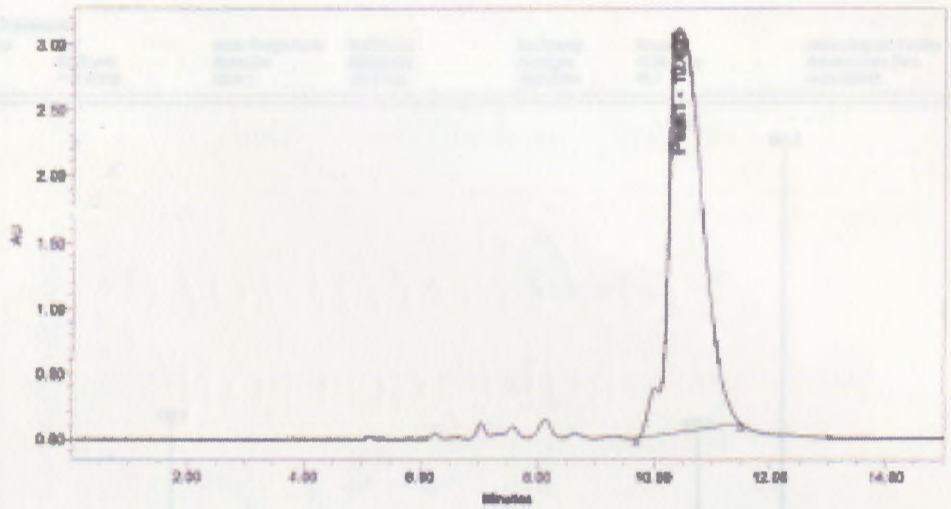


Figure 4-9: HPLC chromatogram showing purified unenriched UMP-6-$CSNH_2$.

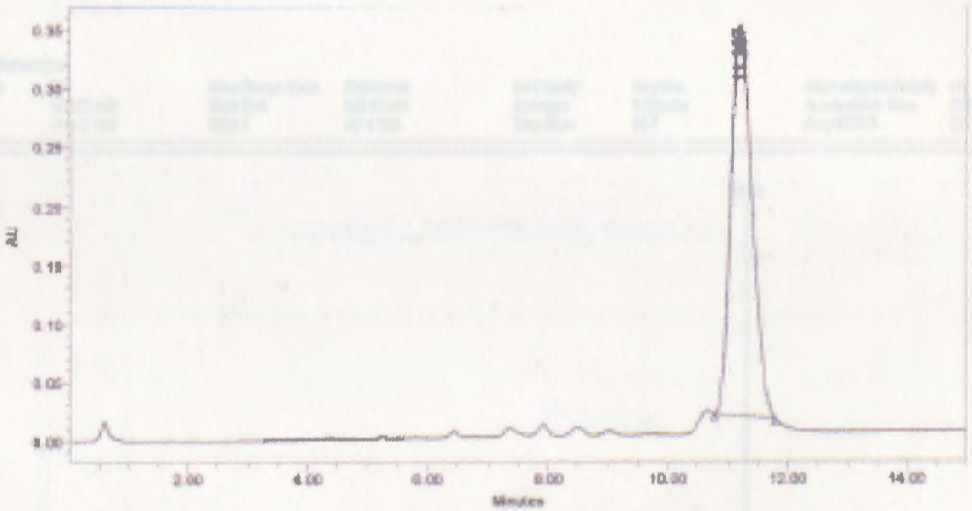


Figure 4-10: HPLC chromatogram showing purified ^{15}N enriched UMP-6-thiocarboxamide.

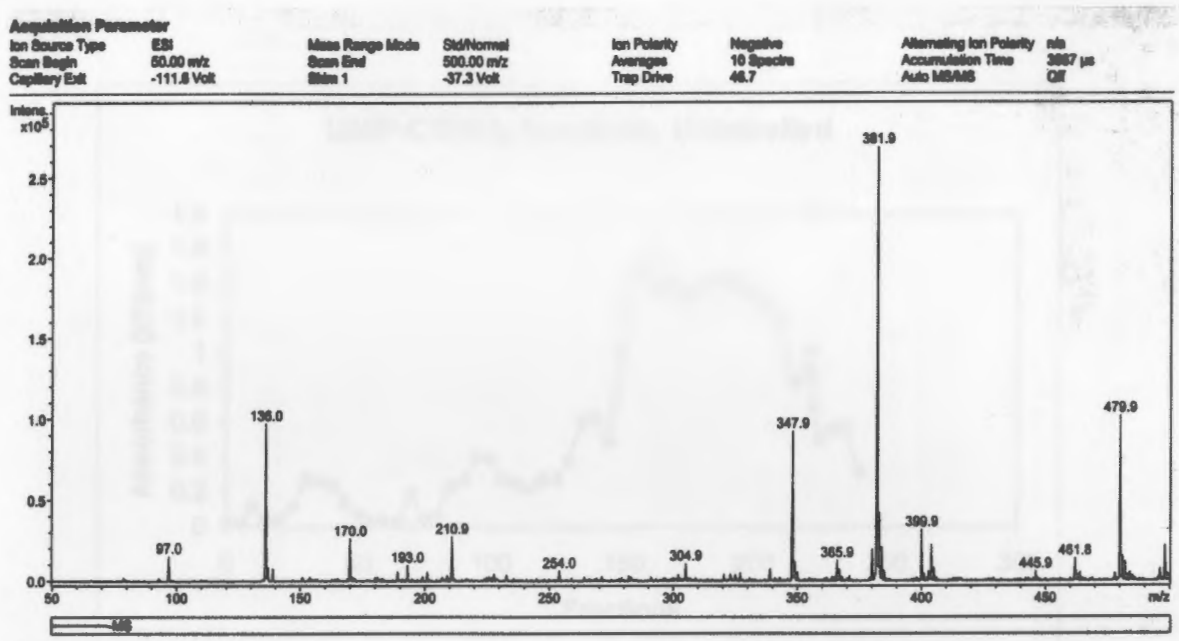


Figure 4-11: Mass spectroscopic results of unenriched UMP-6-$CSNH_2$.

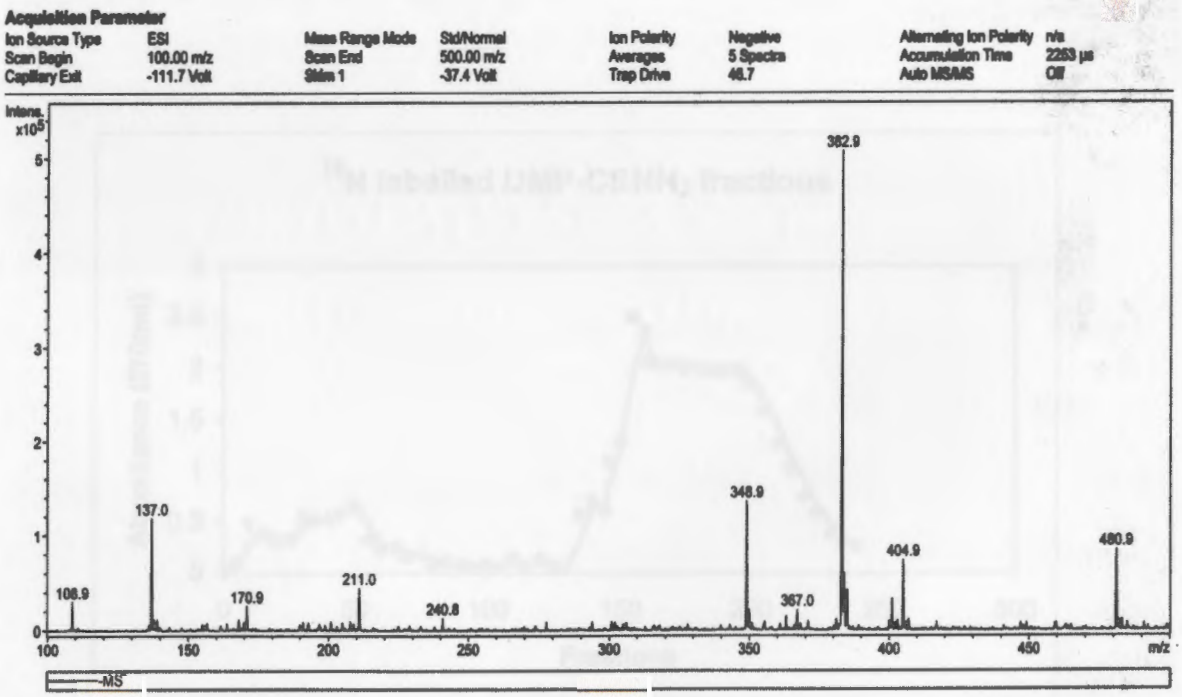


Figure 4-12: Mass spectroscopic results of ^{15}N enriched UMP-6-$CS^{15}NH_2$.

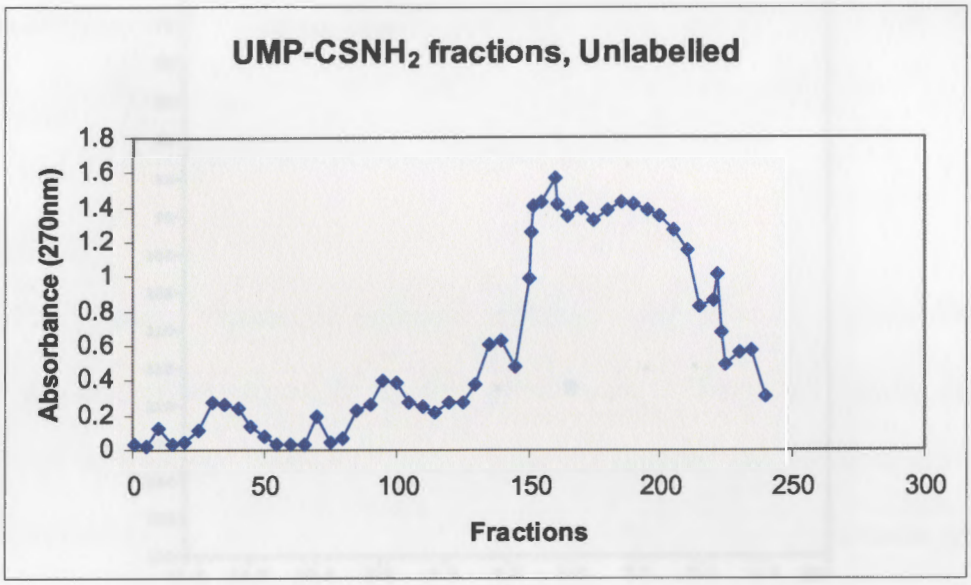


Figure 4-13: Chromatogram used for selection of unenriched UMP-6-$CSNH_2$ fractions to test on the HPLC.

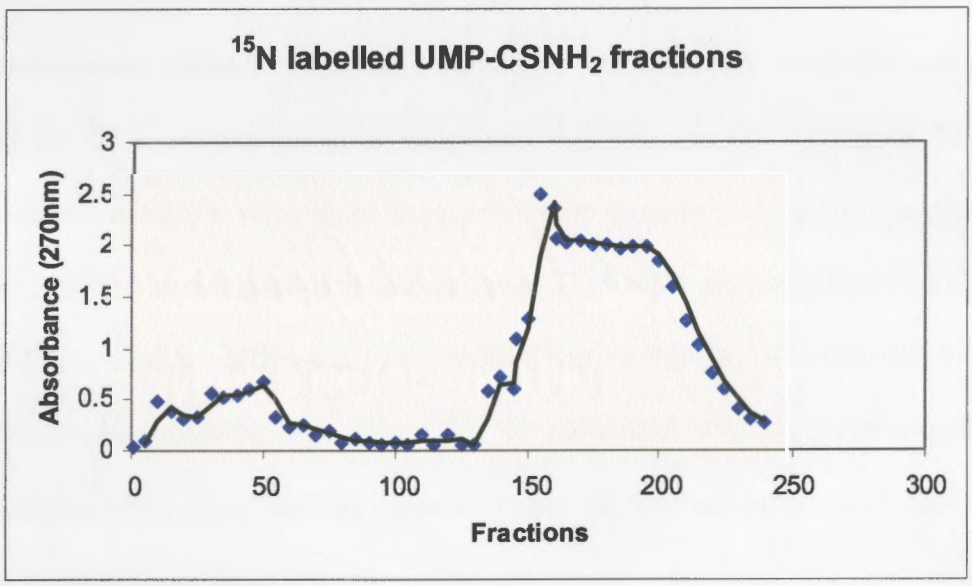


Figure 4-14: The plot used for the selection of ^{15}N enriched UMP-6-$CSNH_2$ fractions to test on the HPLC.

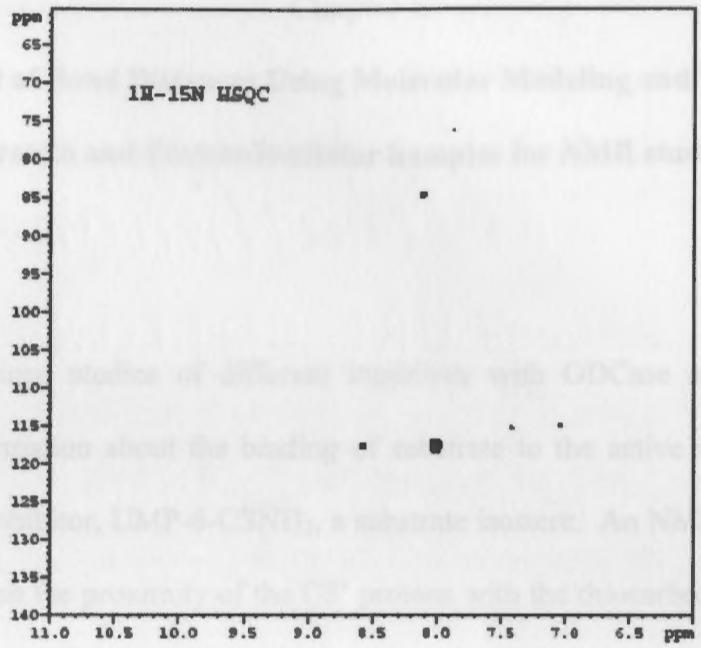


Figure 4-15: The NMR spectra from Ohio state university CCIC for ^{15}N enriched UMP-6-$CSNH_2$ (1 mM) showing major signal for ^{15}N.

Chapter 5

Measurement of Bond Distances Using Molecular Modeling and Preparation of Protein and Protein/Inhibitor Samples for NMR studies

Introduction

The previous studies of different inhibitors with ODCase are insufficient to provide the information about the binding of substrate to the active site, so our group synthesized the inhibitor, UMP-6-$CSNH_2$, a substrate isostere. An NMR experiment was suggested in which the proximity of the C5' protons with the thiocarboxamide protons in UMP-6-$CS^{15}NH_2$ could be examined. To know whether the structure of this inhibitor allows possible conformations where the thiocarboxamide protons are close to the C5' protons, it is important to know the possible bond distances between the hydrogens when the UMP-6-$CSNH_2$ is in *syn* or *anti* conformations. To get this information, we opted for the computational method, molecular modeling. From this experiment we want to know whether the ^{15}N hydrogens are in the range of 5-6 Å from the C5' hydrogens. Previously computational methods were used successfully to develop the active site models of ODCase [30] and to calculate the bond energies [31]. Lundberg group developed the QM models of the ODCase active site to investigate the mechanism of action of ODCase by using density functional theory with the B3LYP functional. Warshel group calculated the bond energies using computational method to show the rate enhancement of ODCase was due the transition state stabilization.

We used PC Spartan Pro program to model the UMP-6-$CSNH_2$ structure. Bond distances of hydrogens in thiocarboxamide (NHα, NHβ) to the hydrogens in ribose ring

(C5'α, C5'β) are measured by changing the C-C dihedral angle (C6 in the pyrimidine ring and the other carbon in thiocarboxamide) and the C-N dihedral angle (carbon and nitrogen in thiocarboxamide ring) using fixed angles of rotation of the N-glycosidic bond (N1-C1') (Figure 5-1).

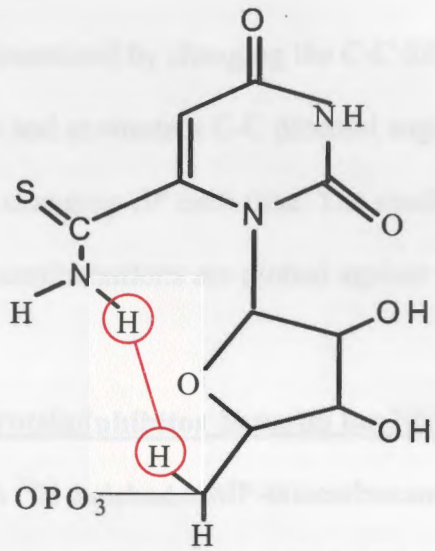


Figure 5-1: Structure of UMP-thiocarboxamide showing the NHα, NHβ and C5'α, C5'β. One of the possible H – H distances is highlighted in red.

Materials and Methods

Materials

PC Spartan Pro program was used for modeling the UMP-6-thiocarboxamide in the enzyme active site. BMP structure has been imported from the protein data bank.

Modeling of UMP-thiocarboxamide in PC Spartan Pro

The BMP which was complexed with the ODCase active site was taken from the protein data bank and was imported into the Spartan program (Figure 5-2) so that the configuration of the nucleotide will be similar to that in the enzyme's active site. Then the BMP structure was modified to UMP-6-$CSNH_2$ by replacing O6 with the thiocarboxamide group. The *syn* conformation was made by placing the thiocarboxamide

in the same side as that of the phosphoryl group (Figure 5-3) and the *anti* conformation is made by placing the thiocarboxamide group in the opposite direction to the phosphoryl group (Figure 5-4)

Measurement of bond distances

The bond distances are measured by changing the C-C dihedral angle from 0° to 180° by changing 10° each time and at constant C-C dihedral angle changing the C-N dihedral angle from 0°-180° by changing 10° each time. The smallest and the largest H - H distances in *syn* and *anti* conformations are plotted against the C-C dihedral angle (Figure 5-5).

Preparation of Protein and Protein/Inhibitor Samples for NMR studies

The protein sample with ^{15}N enriched UMP-thiocarboxamide was prepared in the same way as in Chapter 2 by eluting ODCase from Affi-Gel Blue column using the dialysis buffer (50 mM Tris, 5 mM BME and 10% glycerol) with UMP. Then the protein was dialyzed to remove UMP, and concentration of ^{15}N enriched UMP-6-$CSNH_2$ equal to that of the ODCase was added. The sample was dialyzed for a final time to remove any unbound UMP and UMP-6-$CSNH_2$.

To check whether the inhibitor was lost by the dialysis, we checked the protein sample with inhibitor on HPLC after dialyzing against the dialysis buffer. Ethanol was added to denature the protein and the sample was vacuum dried after removing the precipitated protein pellet by centrifugation. The dried sample was dissolved in water and analyzed on HPLC. A standard curve for UMP-6-$CSNH_2$ (Figure 5-6) was constructed by running 0-100 nmol of UMP-6-$CSNH_2$ samples and plotting the graph with concentration versus peak area (Table 5-2). A fresh batch of protein without inhibitor

was send to the NMR lab at Ohio State University Campus Chemical Instrumentation Center (CCIC, Dr. Ming-Daw Tsai, principal investigator, and Dr. Chunhua Yuan, NMR spectrometrist) along with samples of inhibitor. Other batches of protein samples (0.2 mM and 0.5 mM) with and without inhibitor were prepared by changing the elution and dialysis buffers to contain no glycerol and 20 mM Tris, pH 7.4, 1 mM βME.

Results and Discussion

We succeeded in modeling the UMP-6-$CSNH_2$ using PC Spartan Pro program by importing the BMP structure as in the enzyme active site. The smallest bond distances of hydrogens in the thiocarboxamide (NHα, NHβ) to the C5' hydrogens in *syn* and *anti* configuration are measured as 2.2 - 5.3 Å and 4.3 - 8.0 Å, respectively (Table 5-1, Figure 5-5). Therefore, if NMR data showed that one or more combinations of the hydrogen distances between the thiocarboxamide and the C5' hydrogens was less than 4.0 Å, it could be concluded that the UMP-6-$CSNH_2$ is bound in the *syn* conformation, different from the other inhibitors in the crystal structures.

The protein/^{15}N enriched UMP-6-$CSNH_2$ sample prepared with dialysis buffer containing glycerol showed faint signals on NMR analysis. The presence of inhibitor in the protein sample was verified by comparing samples of protein + inhibitor with the standard curve. The peak area of the inhibitor in the protein/inhibitor sample after dialyzing is in correspondence with the concentration of the inhibitor that was used before for the protein preparation. This confirms that the inhibitor was remaining bound to the protein even after the dialysis.

The NMR spectra of ODCase with ^{15}N enriched UMP-$CSNH_2$ prepared by changing the buffer to contain no glycerol again showed only faint signals. The NMR

spectra showed that the protein was well folded (Figure 5-7) but the ^{15}N chemical shift was not observed (Figures 5-8 – 5-10).

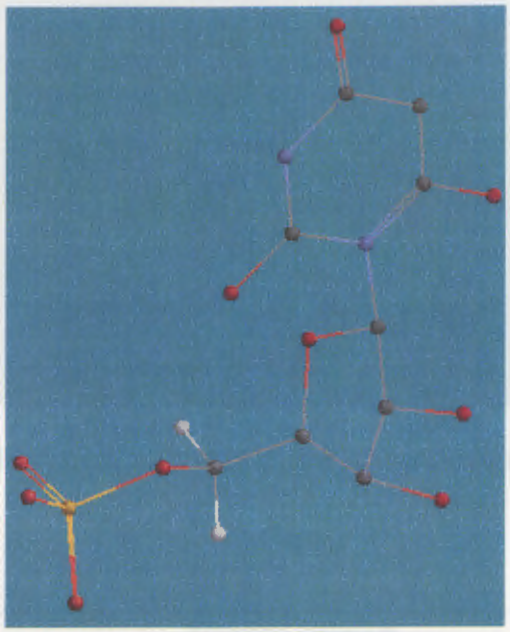


Figure 5-2: Model of BMP in PC Spartan Pro, Imported from the protein data bank.

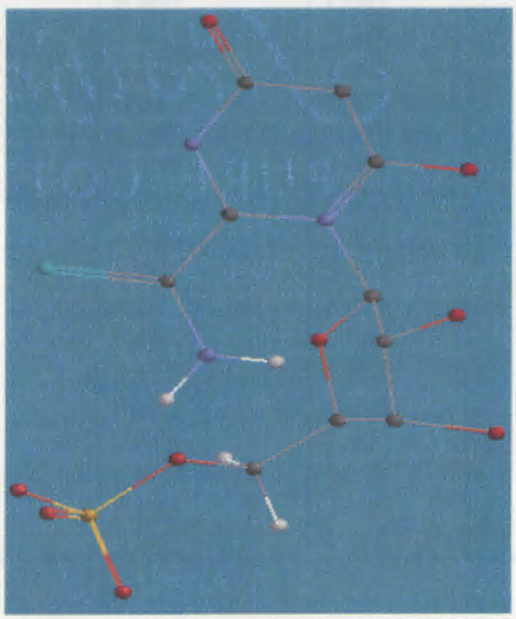


Figure 5-3: Model of UMP-thiocarboxamide in syn conformation.

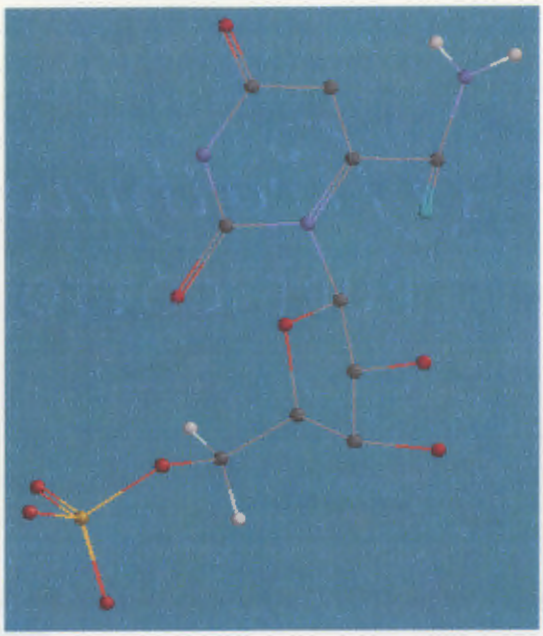


Figure 5-4: Model of UMP-thiocarboxamide in anti conformation.

Smallest distance		
C-C dihedral angle	Distance(syn) (Å)	Distance(anti) (Å)
180°	5.033	8.042
170°	5.2	7.821
160°	5.305	7.567
150°	5.372	7.285
140°	5.376	7.008
130°	5.33	6.661
120°	5.236	6.333
110°	5.077	6.001
100°	4.823	5.668
90°	4.546	5.356
80°	4.262	5.079
70°	4.09	4.851
60°	3.691	4.647
50°	3.415	4.532
40°	3.179	4.419
30°	2.916	4.385
20°	2.652	4.377
10°	2.427	4.407
0°	2.203	4.496

Table 5-1: Table showing smallest distances in *syn* and *anti* confirmations at different C-C dihedral angles.

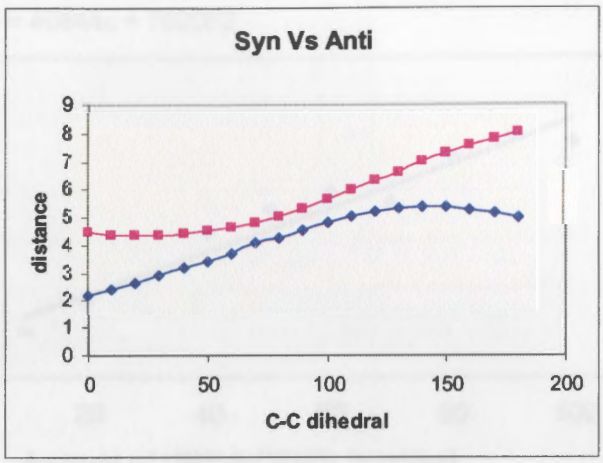


Figure 5-5: Plot showing smallest distance Vs C-C dihedral angle in *syn* (in blue) and *anti* (in pink) conformations.

Amount of UMP-6-$CSNH_2$ (nmoles)	Area(μV sec)
10	861013
20	1418668
30	1920177
40	2737676
50	3174089
60	3525272
70	3290765
80	4157228
90	4560965
100	4438840

Table 5-2: Peak areas measured on HPLC for 0-100 nmol samples of UMP-6-$CSNH_2$.

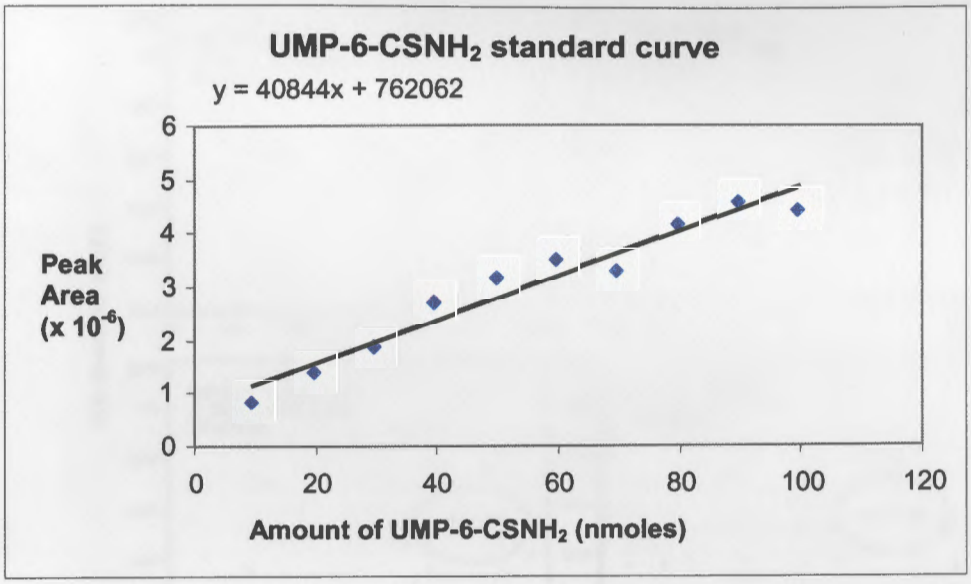


Figure 5-6: Standard concentration curve for UMP-6-$CSNH_2$.

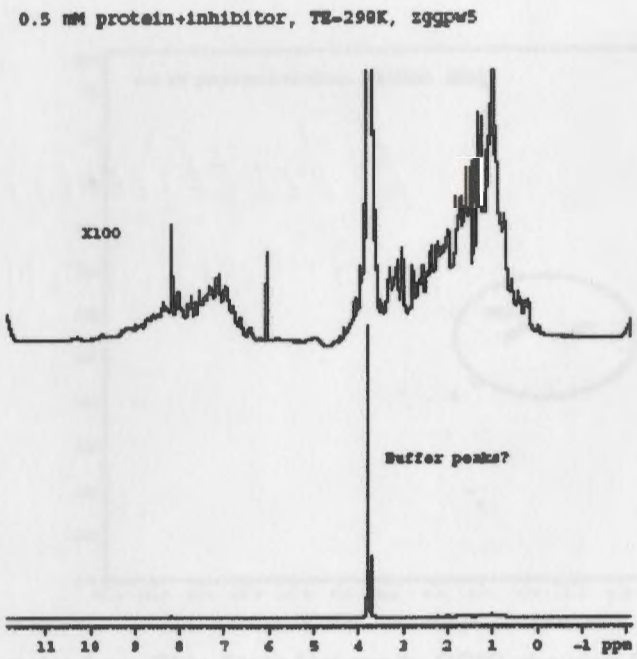


Figure 5-7: 1D ^1H NMR spectra from Ohio State University for protein samples. The presence of sharp peaks indicates that the protein was folded well.

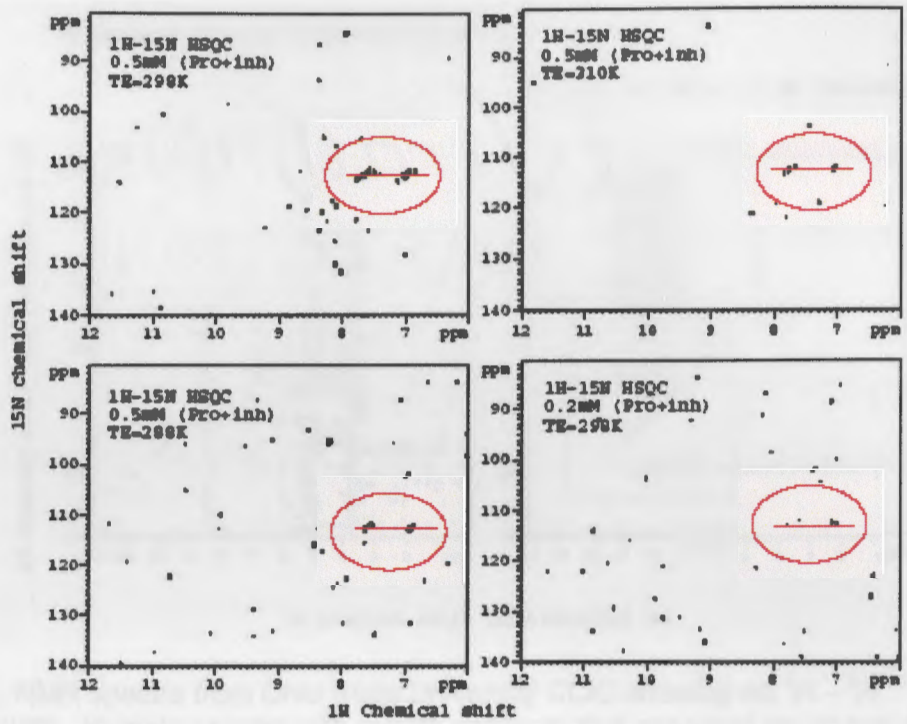


Figure 5-8: NMR spectra from Ohio State University CCIC showing 2D $^{1}H - ^{15}N$ correlation, indicating the integrity of the ^{15}N-enriched nucleotide.

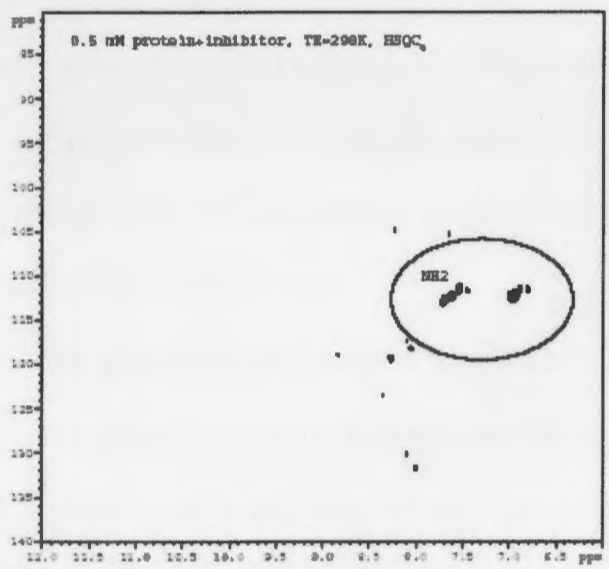


Figure 5-9: NMR spectra from Ohio State University CCIC showing 2D $^{1}H - ^{15}N$ correlation, indicating the integrity of the ^{15}N-enriched nucleotide.

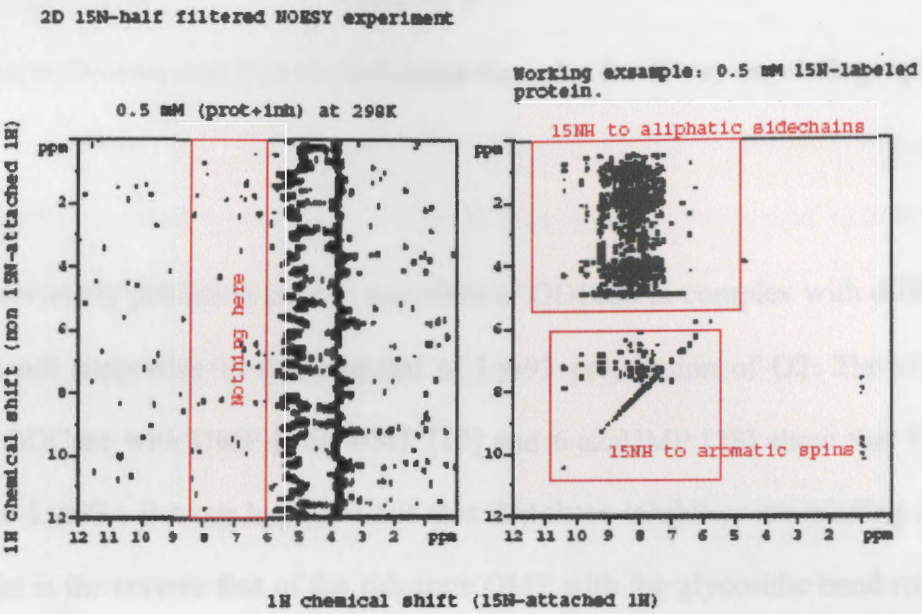


Figure 5-10: NMR spectra from Ohio State University CCIC showing no $^{1}H - ^{1}H$ interactions (left). Sample spectra with results similar to that expected are shown on the right.

Chapter 6

Preparation of Protein and Protein/Inhibitor Samples for X-ray crystallography

Introduction

The previously published crystal structures of ODCase in complex with different inhibitors are not supportive to the proposal of Lys93 protonation of O2. The crystal structures of ODCase with UMP [16], BMP [17] and 6-azaUMP [18] show that O2 is away from the Lys93. But our hypothesis is that that these inhibitors are binding in an orientation that is the reverse that of the substrate OMP with the glycosidic bond rotated from the structure by about 180°. As it is not possible to get the crystal structure of ODCase in complex with the substrate, OMP, due to the conversion of OMP to UMP immediately in the enzyme's active site, we planned to get the crystal structure of ODCase in complex with UMP-6-$CSNH_2$, a substrate isostere. UMP-6- $CSNH_2$ was assumed to bind in the same orientation as the substrate, OMP, so it can be possible to get an idea of binding of substrate, OMP in the enzyme active site. We attempted to crystallize ODCase in complex with UMP-6- $CSNH_2$ with the hope that the binding of substrate in the enzyme active site can be revealed.

Different protein and protein/inhibitor samples suitable to the crystallographic studies are prepared using the purified ODCase (Chapter 2) and the synthesized inhibitor, UMP-6-thiocarboxamide (Chapter 4). To get better results the protein preparation was changed by using the different dialysis buffer without glycerol and 20 mM Tris at pH 7.4 and 1 mM βME [32]. To investigate the binding of inhibitor in the enzyme active site the UV absorbance of protein, inhibitor and protein/inhibitor samples are measured.

Materials and Methods

Materials

All the materials used for preparing protein and protein/inhibitor samples are same as in Chapter 2. The protein crystallography lab at the University of Toledo (Dr. Ron Viola, principal investigator) carried out crystallization trials.

Preparation of Protein and Protein/Inhibitor Samples for X-ray crystallography

We prepared the protein and protein/inhibitor samples that are appropriate for the crystallographic studies. Maximum amount of ODCase was produced by growing the *E. coli* cells with inserted gene in the method described in Chapter 2. Nearly 70 mg of ODCase was produced from 4 L of culture by eluting the protein from the Affi Gel Blue column with 1 mM UMP. The protein was concentrated to 5 mg/ml, and the UMP was removed by dialyzing three times (Figure 2-6) with the dialysis buffer (50 mM Tris, 5 mM βME and 10% glycerol). The protein sample was stored at -20°C, and checked for activity periodically. Samples of synthesized UMP-6-$CSNH_2$ were vacuum dried and send to the crystallographer along with the stored inhibitor-free protein sample (5 mg/ml) on dry ice. Afterwards, fresh samples of protein were sent to the X-ray crystallography lab on ice.

To avoid the aggregation we changed the dialysis buffer according to the published procedure [32], in which ODCase was crystallized with BMP following dialysis with buffer containing 20 mM Tris at pH 7.4 and 1 mM βME without glycerol. We prepared the protein sample in the same way as before except changing the dialysis buffer to the one with out glycerol and shipping to the crystallographer on ice. We also sent a batch of protein (1.9 mM) with double the concentration of inhibitor (3.8 mM)

added to it by preparing the protein preparation in the same way as with the changed dialysis buffer. Then to the UMP-free protein sample UMP-6-$CSNH_2$ was added and dialyzed against glycerol-free buffer without glycerol for 2 hrs to remove any unbound inhibitor. This fresh batch of protein with inhibitor was send to the crystallographer on ice.

Checking the possibility of covalent binding of UMP-6-$CSNH_2$ to ODCase by observing the UV absorbance

The type of interaction of UMP-6-$CSNH_2$ with the enzyme's active site was investigated by observing any possible change in absorbance when the inhibitor is added to the protein. In the procedure, the absorbance of the inhibitor is measured in phosphate buffer only, then measured in solution containing protein, after the instrument is blanked with the protein present. If the two spectra are virtually identical, then no covalent attachment of the enzyme to the inhibitor is likely.

The spectrophotometer was blanked at 270 nm using phosphate buffer and the absorbences were measured for inhibitor only sample (30 µM of inhibitor), protein only sample (30 µM of protein) and the protein with inhibitor sample (30 µM each). Then the absorbances of the sample with inhibitor plus protein (30 µM each) are measured after blanking the spectrophotometer at 270 nm with protein in phosphate buffer.

Results and Discussion

By checking the activity of stored protein after dialysis at -20° C it was confirmed that the protein remains stable by storing at -20° C. After checking the protein sample prepared using dialysis buffer with glycerol, the crystallography lab reported that the ODCase exhibited aggregation, based on dynamic light scattering data (not shown). It

was assumed that the protein was forming aggregates because of freezing it at -20° C. The fresh batch of protein also showed the same results. The protein preparation with changed dialysis buffer without glycerol giving better results on the dynamic light scattering. Different protein and protein/inhibitor samples send the crystallography lab are summarized in the table 6-1. Crystallization of ODCase with UMP-$CSNH_2$ appears promising with some good crystals (Figure 6-1). Further crystallography trials will be necessary in order to obtain crystals that are the high quality necessary for obtaining diffraction data.

The UV absorbances of samples with inhibitor and the sample with protein and inhibitor (after blanking the spectrophotometer with protein sample) are found to be 0.45 and 0.41. If the inhibitor was bound covalently from the enzyme to the pyrimidine ring, then a change in UV absorbance would be expected. But not much change in the absorbance was observed with the presence of inhibitor in the protein sample, indicating that the inhibitor is not binding covalently to the protein.

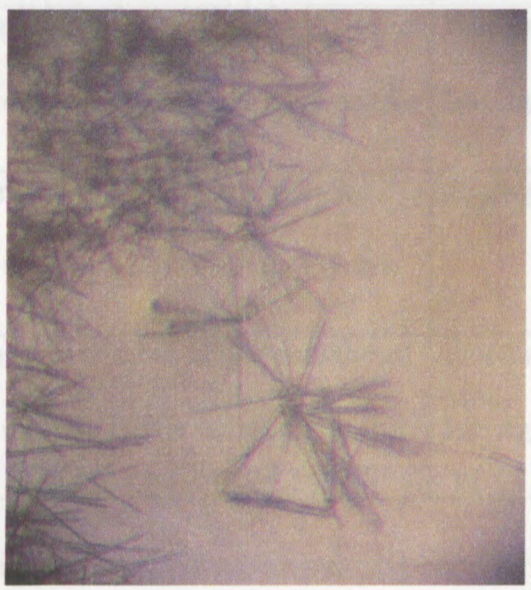


Figure 6-1: Crystals of ODCase/UMP-6-$CSNH_2$ complex

Amount of protein (mg)	Conditions	Results with crystallography
34	Fresh batch of protein with inhibitor dialyzed against dialysis buffer with glycerol	Asked for more protein
24	Frozen protein dialyzed with inhibitor against dialysis buffer with glycerol	Asked for more protein
35	Fresh batch of protein with inhibitor dialyzed against dialysis buffer with glycerol	Asked for more protein
48	Frozen protein dialyzed against dialysis buffer with glycerol and dried inhibitor separately	Observed aggregates on DLS.
56	Frozen protein without inhibitor dialyzed against changed dialysis buffer without glycerol	Better results but still some aggregates
51	Fresh batch of protein without inhibitor dialyzed against changed dialysis buffer without glycerol	Good results on DLS, less aggregation
76	Fresh batch of protein with inhibitor dialyzed against changed dialysis buffer without glycerol	Aggregates observed
70	Fresh batch of protein without inhibitor dialyzed against changed dialysis buffer without glycerol	Good results on DLS, less aggregation

Table 6.1: The different protein and protein/inhibitor samples send for the crystallographic studies.

Chapter 7

Conclusions

Large amounts of *E. coli* ODCase were produced by growing the cells having cloned ODCase gene in LB-Amp medium at 37° C with continuous shaking and induction with IPTG for 6 hr. Purified ODCase was obtained by applying to Affi-Gel Blue column after ammonium sulfate fractionation and eluting with the inhibitors 6-azaUMP, UMP and UMP-thiocarboxamide. The purity of the protein was confirmed by checking on 12% SDS-polyacrylamide gel. The inhibitor-free protein was obtained by eluting the protein with UMP and dialyzing it three times to remove UMP. Approximately 60-70 mg of protein was obtained from 4 L culture of *E. coli* with the specific activity of 54.8 nmol $min^{-1}\mu g^{-1}$.

The *E. coli* OPRTase was produced and purified in an attempt to synthesize BMP by enzymatic catalysis of OPRTase. The *E. coli* OPRTase was produced in the same way as *E. coli* ODCase by cloning the gene and growing the cells in LB-Amp medium at 37° C with continuous shaking and induction with IPTG for 4 hr. The produced protein was purified by applying to Affi-Gel Blue column and eluting with 1 M NaCl. The maximum yield obtained from 4 L culture was 77 mg and the protein was found to be active by HPLC analysis. But the purified OPRTase failed to convert barbituric acid to BMP.

To prepare protein samples with UMP-6-thiocarboxamide for crystallographic and NMR studies, unenriched and ^{15}N enriched UMP-6-$CSNH_2$ were prepared. The nucleotides were found to be stable by not degrading during storage in refrigerator. The bond distances of hydrogens attached to ^{15}N and hydrogens attached to C5' were measured using molecular modeling, in order to determine whether or not NMR signals

resulting from close proximity of these hydrogens is possible. Different protein and protein inhibitor samples were prepared for crystallographic and NMR studies. The best results were obtained using buffers without glycerol. The NMR spectra of ODCase with ^{15}N labeled UMP-$CSNH_2$, carried out at the Ohio State University, showed only faint signals. Crystallization of ODCase with UMP-6-$CSNH_2$, carried out at the University of Toledo, appears promising.

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