I. Structural and functional characterization of tartrate dehydrogenase
II. Characterization of proteins involved in Canavan disease

Radhika Malik

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A Dissertation

entitled

I. Structural and functional characterization of tartrate dehydrogenase

II. Characterization of proteins involved in Canavan disease

by

Radhika Malik

Submitted as partial fulfillment of the requirements for

the Doctor of Philosophy in Chemistry

________________________
Advisor: Ronald E. Viola, Ph.D.

________________________
College of Graduate Studies

University of Toledo

December 2009
An Abstract of

I. Structural and functional characterization of tartrate dehydrogenase

II. Characterization of proteins involved in Canavan disease

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In the first project, structural and functional studies of TDH are described. TDH is an unusual NAD-dependent enzyme that exhibits a multiplicity of catalytic activities at a single active site. These activities arise from the capacity of this enzyme to catalyze a reaction pathway in which different substrates undergo the same initial catalytic steps, but the subsequent intermediates can dissociate from the enzyme at different stages in the catalytic cycle thereby leading to different final products.

The first crystal structure of an NAD-dependent TDH has been solved to 2 Å resolution by single anomalous diffraction (SAD) phasing as a ternary complex with the
intermediate analogue oxalate, Mg$^{2+}$ and NADH, and also as a binary complex with Mg$^{2+}$ and NADH. The active site in these complexes is highly ordered, allowing identification of the substrate and cofactor binding groups and the ligands to the metal ions. Residues from the adjacent subunit are involved in both the substrate and the divalent metal ion binding sites, establishing a dimer as the functional unit and providing structural support for an alternating site reaction mechanism. Each substrate undergoes the initial hydride transfer, but differences in orientation of the substrate are proposed to account for the different reactions catalyzed by TDH.

For the second project two different approaches to treat Canavan disease are explored. Canavan disease is a fatal neurological disorder caused by mutations in the aspA gene leading to altered aspartoacylase with diminished catalytic activity. These clinical mutations are found at sites located throughout the enzyme suggesting that destabilization of the protein structure may be responsible for the loss of activity in many of these patients. In the first approach, mutations have been introduced at several of these sites and the purified enzymes were found to have low catalytic activity and, in some cases, diminished stability. Docking studies have suggested some small probe molecules that could bind at these sites. Optimization of these compounds, guided by kinetic and structural studies, will lead to pharmacological chaperones that have the potential to stabilize these defective enzyme forms and reverse the effect of these mutations in Canavan patients. As a second approach, enzyme replacement therapy for the treatment of Canavan disease is described. The structure of human aspartoacylase has allowed us to locate putative binding sites for polyethylene glycol (PEG) molecules. PEGylation has produced altered forms of the enzyme that could have decreased immunogenicity and
increased in vivo stability. In the study we have tested different PEGylation reactions and evaluated their effect on the catalytic activity of aspartoacylase. PEGylated forms of this enzyme have been injected into a mouse model of Canavan disease and the effects of enzyme replacement on the activity of aspartoacylase has been measured in the tissues. Enzyme activity levels in the mice brain have increased and dose dependent studies are currently performed. In conclusion, these studies will hopefully lead to a viable approach for the treatment of Canavan disease.
Acknowledgements

I would like to thank Dr. Viola for his guidance and constant encouragement. His vision and support has enabled me to undertake many challenging research problems during my Ph. D. I am extremely grateful to Dr. Viola for my development as a research scientist during these years.

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I would like to thank my parents (Dr. B. K. Malik and Mrs. Poonam Malik) and sister (Ms. Neha Malik) for their constant support.

I would like to dedicate this dissertation to the fond memory of my late grandfather, Dr. Dwarka N. Raheja (1919-2008).
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List of abbreviations

ASPA: aspartoacylase
ANAT: Aspartate N-acetyl transferase
ADP: Adenosine diphosphate
CD: Canavan disease
CE: C₈E₄
CSF: cerebrospinal space
CNS: central nervous system
Cymal 5: 5-Cyclohexyl-5-pentyl-β-D-maltoside
DDP: Dimethyldecylphosphine oxide
DLS: Dynamic light scattering
DM: n-decyl-β-D-glucopyranoside
DTM: n-dodecyl-β-D-thiomaltopyranoside
dT: n-decyl-β-D-thiomaltopyranoside
DTT: Dithiothreitol
ERT: Enzyme replacement therapy
E. Coli: Escherichia coli
ICDH: Isocitrate dehydrogenase
IPMDH: Isopropylmalate dehydrogenase
HEGA 10: decanoyl-N-hydroxyethylglucamide
HICDH: Homoisoisocitrate dehydrogenase
HIF: Host integration factor
MPD: 2-methyl-2,4-pentanediol
MEGA 9: nonanoyl-N-methyl-glucamide

NAA: N-acetyl-L-aspartate

NAD: Nicotinamide adenine dinucleotide

NAAG: N-acetylaspartylglutamate

OTM: n-octyl-β-D-thiomaltopyranoside

PDB: Protein data bank

OG: n-octyl-β-D-glucopyranoside

SDS: Sodium dodecylsulfate

SeMet: Selenomethionine

TDH: Tartrate dehydrogenase

TEV: Tobacco etch virus

UM: w-undecylenyl-β-D-maltopyranoside
Chapter 1: Background

1.1 Introduction

The first project presented involves the structural and functional studies of a highly versatile enzyme, tartrate dehydrogenase. The goal of this project is to obtain information about the active site architecture which will be used to guide engineering experiments to change the specificity of the enzyme. The remainder of the projects described in the dissertation are proteins that are directly or indirectly associated with Canavan disease (CD). CD is an autosomal recessive leukodystrophy which is primarily caused by mutation/s in the gene encoding for aspartoacylase. Aspartoacylase is responsible for hydrolyzing the second most abundant amino acid, N-acetyl-L-aspartate (NAA) in the oligodendrocytes. However, NAA is synthesized in the neurons by aspartate N-acetyl transferase and must be transported across the membrane by a sodium-coupled N-acetyl-L-aspartate transporter. A proper balance of NAA levels is essential for normal brain function and hence, the role of each protein described is extremely important. To date aspartoacylase has been the most extensively studied of these proteins in our lab. In this dissertation, two approaches, ‘enzyme replacement therapy’ and ‘pharmacological chaperones’ are described. These approaches are being examined as an attempt to identify a cure for CD.
1.2. Tartrate Dehydrogenase

Tartrate dehydrogenase (TDH) is an unusual NAD-dependent enzyme that exhibits a multiplicity of catalytic activities at a single active site\(^1\). These activities arise from the capacity of this enzyme to catalyze a reaction pathway in which different substrates undergo the same initial catalytic steps, but the subsequent intermediates can dissociate from the enzyme at different stages in the catalytic cycle thereby leading to different final products\(^2\). β-hydroxy acid dehydrogenases such as TDH generally produce a β-keto acid that may either be released from the enzyme or is retained and subsequently decarboxylated. TDH is unique from other hydroxy acid dehydrogenases in that the decarboxylation step it catalyzes depends on the structure and stereochemistry of the respective substrates\(^3\). TDH is found in a variety of microorganisms and functions as part of a pathway through which tartrate is converted into D-glycerate, thereby providing entry for these carbons into primary metabolic pathways\(^4\).

Bacterial TDH has the closest sequence homology to prokaryotic isopropylmalate dehydrogenases (IPMDH) (~35% identity), yeast homoisocitrate dehydrogenase (HIcDH) (34% identity) and to a lesser extent to Escherichia coli isocitrate dehydrogenase (ICDH) (25% identity) (Figure 1.1).

Interestingly, differences in the protein sequence within the hydroxy acid dehydrogenase family allow further classification based on the stereochemistry of the substrates, and also based on whether the enzyme can catalyze a subsequent decarboxylation of the reaction intermediate. Thus, S-hydroxy acid dehydrogenases show little homology to R-hydroxy acid dehydrogenases and are also quite distinct from the metal ion-dependent decarboxylating hydroxy acid dehydrogenases\(^5\). TDH is a member
of this latter metal ion-dependent R-hydroxy acid dehydrogenase family. This family has a distinct nucleotide binding domain that differs from the more traditional pyridine nucleotide-linked dehydrogenases.

Figure 1.1: Sequence Alignment of ppTDH with representative hydroxy acid dehydrogenases.

*Saccharomyces cerevisiae* Homoisocitrate dehydrogenase (*scHicDH*), 3-isopropylmalate dehydrogenase from *Thiobacillus ferooxidans* (*tfIPMMDH*), *Salmonella typhimurium* (*sIPMMDH*), *Thermus thermophilus* (*ttIPMMDH*) and *Escherichia coli* isocitrate dehydrogenase (*ecICDH*). Highly conserved residues in the nucleotide binding domain within this family are shaded in blue and the conserved residues involved in the dimerization domain are colored in pink. Conserved active site residues are marked with red triangles and the non-conserved substrate, catalytic and metal binding residues are labeled with red stars.
Among the characterized members of the R-hydroxy acid dehydrogenase family only homoisocitrate dehydrogenase (HICDH) is similar to TDH in its ability to be activated by K$^{+}$\textsuperscript{7}. However, HICDH has been shown to follow a random mechanism\textsuperscript{8}, while TDH has an ordered kinetic mechanism\textsuperscript{1}.

TDH catalyzes the oxidation of (+)-tartrate to form oxaloglycolate, and can also catalyze the oxidative decarboxylation of D-malate to produce pyruvate and CO\textsubscript{2} (Figure 1.2, Reactions 1 & 2).

**Reaction 1**

![Reaction 1 Diagram](image)

**Reaction 2**

![Reaction 2 Diagram](image)
A detailed kinetic investigation, including pH rate profile and isotope effect studies, supports an acid-base catalytic mechanism with participation of general acid catalysis in the oxidative decarboxylation of D-malate. The reaction in the forward direction commences with a hydride transfer from the 2-hydroxy acid substrate to NAD followed by decarboxylation. However, the reverse hydride transfer from NADH back to the oxaloacetate (OAA) intermediate is twice as fast as decarboxylation of OAA. But, since the re-carboxylation of pyruvate is negligible, the irreversibility of this step ensures that the reaction will proceed to the pyruvate product. Significantly, the rate constant for the dissociation of OAA intermediate from the enzyme active site is much slower than that for the dissociation of oxalylglycolate, the product of the enzyme-catalyzed oxidation of (+)-tartrate. TDH also catalyzes a third type of reaction, the decarboxylation of meso-tartrate to produce D-glycerate and CO₂ (Figure 1.2, Reaction 3). This reaction differs from the previously described reactions in that it is formally a decarboxylation with no net oxidation or reduction, however NAD is still required for the reaction to proceed.
TDH requires both a divalent metal ion (Mn$^{2+}$ or Mg$^{2+}$) and a monovalent (K$^+$) cation for activity. The cations have been proposed to function in substrate binding and in facilitating the decarboxylation of β-keto acid intermediates$^{11}$. Binding studies suggest a half-of-the-sites mechanism for TDH where NAD binds to the enzyme-metal ion complexes with a stoichiometry of two per enzyme dimer$^{11}$.

Previous studies have shown that each of the different substrates of TDH undergo the same initial oxidation to produce the corresponding α-keto acid with concomitant reduction of NAD$^1$. The ultimate fate of these intermediates, oxaloacetate, oxaloglycolate and hydroxypyruvate (Figure 1.2), seems to depend on their relative rates of dissociation from the enzyme. The oxaloglycolate obtained from the oxidation of (+)-tartrate is released and then rearranges non-enzymatically to yield dihydroxyfumarate. The failure of TDH to catalyze the decarboxylation of oxaloglycolate may simply be a consequence of unfavorable kinetics, caused by the more rapid dissociation of this product$^{10}$. The keto acids obtained from D-malate and meso-tartrate each undergo enzyme-catalyzed decarboxylation with assistance by the bound divalent metal ion yielding pyruvate and the enol form of hydroxypyruvate, respectively. Pyruvate is released from the enzyme, while hydroxypyruvate remains bound and is subsequently reduced to D-glycerate before being released$^1$. Oxalate acts as a time-dependent inhibitor of TDH through the formation of an abortive enzyme-NADH-oxalate complex$^{12}$. Oxalate resembles the intermediates formed during the catalytic cycle of the enzyme because of its sp$^2$-hybridized carbons.

TDH is an ideal system for the study of catalytic evolution since its catalytic capabilities have not been optimized for a single substrate. Our interest in this enzyme comes not only from the unusual multiple reactions that it catalyzes, but also from some
interesting protein engineering challenges. For the reaction in the reverse direction TDH produces a chiral center adjacent to another chiral carbon (Figure 1.2, Reaction 1). If the specificity of this enzyme can be expanded to include non-symmetric substrates then a series of chiral synthons can be produced with controlled stereochemistry at adjacent carbons. With a view to understand the mechanism of this unusual enzyme, structural and functional studies of TDH will be extensively discussed in Chapter 2 of this dissertation.

1.3 Canavan Disease

Canavan Disease (CD, OMIM #271900) is a recessive autosomal white matter leukodystrophy. It was first identified by brain pathology studies and was subsequently shown to be a medical disorder that is distinct from the previously identified leukodystrophies and other demyelinating diseases. In a genome-wide study performed on CD patients, it has been reported that 1,440 genes were significantly modulated, with 78% down-regulated and 22% up-regulated. In humans, the CD syndrome is marked by early onset, hydrocephalus, macroencephaly, head-lag, ataxia, psychomotor retardation, blindness, and spongiform myelin sheath vacuolization with progressive leukodystrophy. In addition to the hydrocephalus and spongiform vacuolation, the global osmotic nature of the disease is apparent in the swelling of the astrocytes, as well as in increased cerebrospinal space (CSF). Clinical analysis has shown that CD occurs most frequently in Ashkenazi Jews of European origin. In fact, genetic screening shows that 1 in 40 Ashkenazi Jews is a carrier of CD.

There are three recognized clinical forms of CD, a congenital form where the clinical symptoms become noticeable in first week of life and a juvenile form where symptoms appear after 4-5 years of life. However, the most common form of CD is the infantile
form in which the symptoms appear as early as six months of age. Metabolic hallmarks of the disease include an elevation in N-acetylaspartate (NAA) levels in brain, plasma and CSF, along with daily excretion of large amounts of NAA and its anabolic metabolite, N-acetylaspartylglutamate (NAAG)\textsuperscript{22,23}. Demyelination, hypertrophy and hyperplasia of astrocytes, and cortical as well as subcortical spongy degeneration are most common pathologies associated with CD\textsuperscript{18,19,24}. Of the observed neuropathies, the most important appears to be the extensive demyelination that interferes with normal neuronal signaling.

There are number of hypotheses that have been proposed to account for the demyelination associated with CD; one is a metabolic hypothesis, the acetate (Ac) transport hypothesis in which it is reasoned that myelinating oligodendrocytes normally require NAA-derived Ac in order to carry out the energy dependent process of myelin synthesis, and where a lack of the NAA-derived Ac is considered to inhibit the process\textsuperscript{25}. The second is a metabolic-mechanical hypothesis, the osmotic-hydrostatic hypothesis which suggests that the cause of the demyelination in CD is not due to a lack of Ac obtained from NAA, but that it is the presence of unhydrolyzed NAA itself which is the cause of the problem\textsuperscript{26}. In mice, enhanced levels of NAA significantly reduced total radical trapping antioxidant potential, both catalase and glucose 6-phosphate dehydrogenase activities, whereas protein carbonyl content superoxide dismutase activity was significantly enhanced. Hence, these studies indicate that accumulation of NAA impairs antioxidant defenses and induces oxidative damage to proteins, which could be involved in the neurotoxicity of NAA accumulation in CD patients\textsuperscript{27}.

The research in this dissertation will involve studies on two crucial membrane associated proteins, N-acetyl-L-aspartate transporter and aspartate N-acetyl transferase
(Chapter 3). These proteins are extremely important for maintaining a tight balance of NAA levels as well as its transport in the human brain (Figure 1.3). The studies addressed in this chapter will set the stage for detailed biochemical and structural characterization of these proteins.

Figure 1.3 Pathway for the synthesis and degradation of N-acetyl-L-aspartate in the human brain.

In addition, different approaches (Chapters 4 and 5) will be extensively tested to find a potential cure for CD by targeting the specific NAA hydrolytic enzyme, aspartoacylase (discussed below).

1.4 Aspartoacylase

Aspartoacylase (ASPA, also known as aminoacylase 2) is highly expressed in mature oligodendrocytes[^28-30], where it catalyzes the hydrolysis of the second most abundant
amino acid in the brain, N-acetylaspartate\textsuperscript{25} to acetate and aspartic acid. ASPA expression is limited to the central nervous system (CNS), where its activity peaks at the beginning of the second postnatal week in the mouse brain. The human aspartoacylase gene is localized on the short arm of chromosome 17 (17p13-ter) and consists of six exons and five introns. The cDNA is 1435 base pairs (bp) long with an open reading frame of 942 bp long that predicts a protein of 313 amino acids. Currently, there are at least 56 mutations in the ASPA gene that correlate to CD, including 44 missense and nonsense mutations (Figure 1.4).

![Figure 1.4](image)

**Figure 1.4: A map of some known mutations, each of which results in a defective aspartoacylase\textsuperscript{35}.** X indicates a mutation to a stop codon and Δ indicates frameshift mutations caused by base deletion.

Since CD was linked to ASPA gene mutations\textsuperscript{31}, a considerable effort has been made towards understanding the mechanism of disease pathology. Low acetate levels were detected in the brains of ASPA deficient knock-out mice, which likely results in the reduced synthesis of cerebrosides (galactocerebroside and its sulfated derivative sulfatide) and other myelin lipids observed in these animals\textsuperscript{32}. In this study, reduced cerebrosidase levels were also detected in the white matter from a human CD patient. These findings provide the foundation for the hypothesis that ASPA’s function is essential for proper CNS myelination because of its role in supplying NAA-derived acetate for myelin lipid synthesis\textsuperscript{33-34}. 


ASPA has been purified to homogeneity from bovine brain\textsuperscript{36}. Earlier, the enzyme has been proposed to act as an esterase by means of a catalytic Ser-His-Glu triad\textsuperscript{37}. However, sequence analysis has suggested that the enzyme belongs to the zinc-carboxypeptidase family\textsuperscript{38}. Biochemical evidence has established that ASPA binds zinc, which is essential for activity\textsuperscript{39}. The structure of aspartoacylase has been solved in the apo-form from two mammalian species, rat and human\textsuperscript{40}. Importantly, human ASPA structure has been solved in complex with an intermediate analog, N-phosphonomethyl-L-aspartate bound at its active site (Figure 1.5)\textsuperscript{41}. This complex identifies the active site architecture of this key enzyme.

\textbf{Figure 1.5: Active site of asparthoacylase.} Well defined difference density (Fo-Fc map contoured to 3 \( \sigma \)) allows accurate positioning of the intermediate analog into the active site. The heteroatoms of the intermediate are involved in multiple binding interactions with the active site functional groups, with dashed lines showing each interaction and the distances in angstrom listed. The active site mutants produced to examine the role of these functional groups are shown in parentheses\textsuperscript{41}. 
Mutations were made at these active site functional groups and these mutants were cloned, expressed, purified and kinetically characterized (Table 1.1).

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>% $k_{cat}$</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native</td>
<td>$12.7 \pm 0.05$</td>
<td>100</td>
<td>$0.12 \pm 0.03$</td>
<td>$1.0 \times 10^5$</td>
</tr>
<tr>
<td>Y288F</td>
<td>$0.130 \pm 0.001$</td>
<td>0.8</td>
<td>- - $^b$</td>
<td>- - $^b$</td>
</tr>
<tr>
<td>Y164F</td>
<td>$0.020 \pm 0.003$</td>
<td>0.2</td>
<td>$0.75 \pm 0.18$</td>
<td>$2.6 \times 10^2$</td>
</tr>
<tr>
<td>R168K</td>
<td>$0.13 \pm 0.05$</td>
<td>0.8</td>
<td>$0.10 \pm 0.03$</td>
<td>$1.3 \times 10^3$</td>
</tr>
<tr>
<td>R71K</td>
<td>$0.73 \pm 0.07$</td>
<td>5.7</td>
<td>$0.09 \pm 0.04$</td>
<td>$7.7 \times 10^3$</td>
</tr>
<tr>
<td>E178A</td>
<td>$0.46 \pm 0.05$</td>
<td>3.6</td>
<td>- - $^b$</td>
<td>- - $^b$</td>
</tr>
</tbody>
</table>

$^a$ taken from Le Coq et al., Biochemistry 45, 5878 (2006)

$^b$ we have been unable to obtain reproducible $K_m$ values with reasonable errors for these mutants

Hence, structural and functional studies have helped to establish the identity of the metal and the identity and importance of the active site functional groups. These results have been used to propose a plausible mechanism for ASPA$^{41}$.

1.5. N-acetyl-L-aspartate transporter

The N-acetyl-L-aspartate transporter is a high affinity Na$^+/$/dicarboxylate transporter, NaDC3, and is encoded by the SLC13A3 gene (Table 1.2). The SLC13 gene family consists of five members in humans, all of which are sodium-coupled transporters for anions such as sulfates or dicarboxylates. SLC13 transporters with related sequences and function are found in other vertebrates (Table 1.2), ranging from fish to mammals. These
transporters are secondary active transporters that couple multiple sodium ions to the movement of an anion substrate. The SLC13 family transporters are typically found on plasma membranes in mammalian cells.

The human NaDC3 is 43% identical in protein sequence to human NaDC1\textsuperscript{42}. NaDC3 (NAA transporter) has a broader tissue distribution than NaDC1 and is found in basolateral membranes in renal proximal tubule cells\textsuperscript{43}, as well as liver, brain and placenta\textsuperscript{44-46}.

<table>
<thead>
<tr>
<th><strong>Human gene</strong></th>
<th><strong>Protein names</strong></th>
<th><strong>Tissue Distribution</strong></th>
<th><strong>Substrates</strong></th>
<th><strong>Orthologs</strong></th>
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<tr>
<td>SLC13A1</td>
<td>NaS1 (NaSi-1)</td>
<td>Kidney (human, rat), intestine (rat)</td>
<td>Sulfate, low affinity, $K_m$ (sulfate): 0.4 mM</td>
<td>Human, rat, mouse, eel</td>
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<tr>
<td>SLC13A2</td>
<td>NaDC1 (NDC-1, NaC1)</td>
<td>Kidney (apical membrane), small intestine</td>
<td>Dicarboxylates, low affinity, $K_m$ (succinate): 0.8 mM</td>
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<tr>
<td>SLC13A3</td>
<td>NaDC3 (SDCT2, NaC3)</td>
<td>Brain, kidney (basolateral membrane), placenta, liver</td>
<td>Dicarboxylates, high affinity, $K_m$ (N-acetylaspartate): 60-250 µM</td>
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<tr>
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<td>NaS2 (SUT-1)</td>
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<td>Human</td>
</tr>
<tr>
<td>SLC13A5</td>
<td>NaCT (NaC2)</td>
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<td>Dicarboxylates, $K_m$ (citrate): 0.6 mM</td>
<td>Human, rat, mouse</td>
</tr>
</tbody>
</table>

NaDC3 handles substrates with substitutions on the third carbon, such as 2,3-dimethylsuccinate as well as the heavy metal chelators, meso-2,3-dimercaptosuccinate and dimercaptopropane-1-sulfonate\textsuperscript{47}. In the brain, NaDC3 mediates the transport of the
modified amino acid N-acetylaspartate, with a relatively high affinity (K\textsubscript{m} 60-250 \, \mu M)\textsuperscript{48}. NaDC3 may also interact with some monovalent organic anions, such as benzylpenicillin\textsuperscript{49}. The transport of substrates by NaDC3 is sodium-dependent, involves three Na\textsuperscript{+} ions with the substrate, and transport is electrogenic. These electrical properties have been examined by measuring whole cell currents using the two-electrode voltage clamp technique. The addition of substrate induces inward currents that are voltage-dependent. Recently, folate has been identified as a potential inhibitor for \textit{h}NaDC3 since it does not induce substrate-dependent currents\textsuperscript{50}. Also, a different type of cation current associated with NaDC3 has been characterized in the presence of flufenamate\textsuperscript{49}. Flufenamate is a non-steroidal anti-inflammatory drug that inhibits transport by NaDC3 and NaDC1\textsuperscript{49}. It has been suggested that the mechanism of flufenamate inhibition may be similar to that of palytoxin, which inhibits the Na\textsuperscript{+}/K\textsuperscript{+}-ATPase by converting it into a non-selective cation channel \textsuperscript{51}. The NaDC3 transporter from \textit{Xenopus laevis} exhibits a substrate dependent current that may be mediated by anions, such as chloride\textsuperscript{52}. The transport by NaDC3 is inhibited by millimolar concentrations of lithium\textsuperscript{53,54}.

Earlier studies have shown that carboxy terminal half of the SLC13 transport proteins is required for substrate recognition\textsuperscript{42,55}. Furthermore, residues involved in binding substrates and cations, either directly or indirectly, have also been identified in transmembrane helices (TM) 7-11\textsuperscript{55}. All of the vertebrate members of the SLC13 family contain one or two putative N-glycosylation sites at the C-terminal tail\textsuperscript{42,55}.

The current secondary structure models for these transporters are based on hydropathy analysis and contain between 11 and 14 predicted transmembrane helices. There is experimental evidence supporting odd number of TM, either 11 or 13, because
the N- and C-termini are on opposite sides of the membrane\textsuperscript{56}. No structural information for NaDC3 is known, and the mechanism of \textit{in vivo} regulation has not been determined. There has been one study showing that treatment with protein kinase C activators produces endocytosis of the transporter\textsuperscript{57}. Currently, no knockout mouse model has been produced to study the role of this transporter.

\textbf{1.6. Aspartate N-acetyl transferase}

N-acetylaspartate (NAA) is the second most abundant metabolite in the brain after glutamate. It is primarily presently in neurons, where its concentration reaches about 20 mM. NAA is formed from aspartate and acetyl Co-A (Figure 1.3) by aspartate N-acetyltransferase (Asp-NAT), a membrane bound enzyme\textsuperscript{58-60}. NAA also serves as a precursor for N-acetylaspartylglutamate\textsuperscript{61,62}, the most abundant dipeptide present in the brain and most likely a neurotransmitter\textsuperscript{63}. Its has been reported earlier that the enzyme is subcellularly localized in mitochondria\textsuperscript{64,65,67} as well as in microsomes\textsuperscript{60,66}. Attempts made to completely purify Asp-NAT from natural sources have been unsuccessful due to its inherent instability in various detergents\textsuperscript{68}.

Database searches indicated that among the putative N-acetyltransferases encoded by the human and mouse genomes, two, NAT8L and NAT14, were most exclusively expressed in brain. NAT8L, is overexpressed in brain after administration of metamphetamine to mice\textsuperscript{69}. All NAT8Ls are comprised of an N-terminal sequence of 15 residues, a proline- and alanine-rich region of variable length, and a highly hydrophobic region of about 30 amino acids that precedes the N-acetyltransferase domain. Recently, it was confirmed that NAT8L encodes for a protein with kinetic properties and specificities that are similar to Asp-NAT\textsuperscript{70}. The enzyme was inhibited by its reaction product NAA
and this inhibition was competitive with respect to aspartate ($K_i = 0.56$ mM) and uncompetitive versus acetyl-CoA. No inhibition was observed with any other amino acid, confirming its specific nature. Also, NAT8L has been shown to be associated with the endoplasmic reticulum based on labeling and immunostaining experiments\(^70\).

The NAT8L gene is mutated in the single known case of a disorder, called hypoacetylaspartia\(^71-73\), in which no NAA is detectable in the brain. This patient has truncal ataxia, marked developmental delay, seizures and secondary microcephaly. Absence of NAA in the patient indicates that this deficiency is due to the lack of synthesis of this compound in the brain and excludes other potential causes, such as an increased degradation by a hyperactive aspartoacylase, or a deficiency in the transport or the formation of aspartate and/or acetyl-CoA in the compartment in which aspartate N-acetyltransferase is present. This mutation, in fact, generates a null mutation. In addition, it has been shown that no NAA synthase activity could be detected in the cells transfected with the plasmid carrying the mutated allele\(^70\).
Chapter 2: Structural and Functional Characterization of Tartrate Dehydrogenase

No three dimensional structure has been determined for TDH from any species. The goal of the research was to carry out structural investigation of tartrate dehydrogenase in the absence or presence of metal, substrates/substrate analogs and cofactor. The interpretation of these structures will aid in characterizing the catalytic mechanism of this unusual enzyme, and set the stage for planned protein engineering studies.

2.1 Enzyme expression and purification

The host strain used for over-expression of this enzyme was Rosetta (DE3) cell line (Novagen). This host cell line includes transcripts for the tRNAs not typically used in \textit{E. coli}, therefore correcting for \textit{E. coli} codon bias. This codon bias is present in the TDH gene from \textit{Pseudomonas putida} and led to lower expression results using BL21(DE3) as the host strain. Protein expression was induced by the addition of IPTG into the minimal growth media, which turns on the host cell synthesis of the T7 RNA polymerase. Typical growth conditions for expressing ample amounts of \textit{pp}TDH are as follows: an overnight culture (50 mL) is used to inoculate a large scale culture (4 L), which is grown at 37 °C until an optical density (600 nm) of 0.7 is reached, the culture is cooled to 28 °C, and 1 mM IPTG is added to induce protein expression. The culture is shaken at 28 °C for an additional 8-9 hours and the cell paste is collected by centrifugation. It is found that the optimal growth temperature for soluble protein expression was 28 °C.
The cell paste obtained was resuspended in 25 mM MES (pH 5.8), 1 mM DTT, 10 mM KCl, containing a protease inhibitor cocktail [(4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64, bestatin, leupeptin, and aprotinin] and the supernatant was loaded onto an anion exchange column (Q Sepharose XL). A heat step used in previously published protocols was avoided since it resulted in non-homogeneous protein based on dynamic light scattering (DLS, Wyatt Technologies) and gave inconsistent results during crystallization trials. The protein eluted from the anion exchange column with a salt (KCl) gradient was then loaded onto a higher resolution ion-exchange column (Source 30Q). KCl was used for elution of the protein from this column (Figure 2.1). The protein was judged pure at this point, as there were no other protein contaminants as visualized on a Coomassie stained gel (Figure 2.1).

![Figure 2.1: FPLC chromatogram of ppTDH Source 30Q anion exchange chromatography with SDS-PAGE analysis](image)

*ppTDH elution is followed by monitoring the absorbance at 280 nm (red curve). The enzyme elutes over a linear gradient of low to high ionic strength (green curve). The SDS-PAGE shows ppTDH is free of contaminants following the Source 30Q column. (lane 1: Source 30Q, lane 2: marker) The molecular weight of ppTDH is 41 kDa.*
2.2 Native enzyme structural studies

2.2.1 Dynamic light scattering

Dynamic light scattering (DLS) was used as an additional indicator of conditions amenable to crystallization. Several studies have reported the use of DLS for predicting crystallization success \textit{a priori} \textsuperscript{98}. According to DLS, in the absence of substrate and presence of monovalent and divalent cation only, \textit{pp}TDH is a polydisperse distribution of protein molecules with the primary species having an average hydrodynamic radius ($R_h$) of 4.9 nm (Figure 2.2; 1). Upon addition of the cofactor, NAD, the enzyme assumes a radius of 4.4 nm (Figure 2.2; 2). The presence of the substrate (intermediate analog) leads to the convergence of the radius to a $R_h$ value of 3.8 nm (Figure 2.3; 3). Addition of the cofactor to the previous complex maintains the hydrodynamic radius to 3.8 nm (Figure 2.4; 4) with a molecular weight of 80 kDa establishing a dimer as the functional unit.

![Figure 2.2 Plot of the hydrodynamic radius ($R_h$) versus different complexes of TDH (1-4).](image)

**Figure 2.2** Plot of the hydrodynamic radius ($R_h$) versus different complexes of TDH (1-4).
1. TDH/K\textsuperscript{+}/Mn\textsuperscript{2+}; 2. TDH/K\textsuperscript{+}/Mn\textsuperscript{2+}/NAD; 3. TDH/K\textsuperscript{+}/Mn\textsuperscript{2+}/oxalate; 4. TDH/K\textsuperscript{+}/Mn\textsuperscript{2+}/oxalate/NAD.
2.2.2 Crystal screening and optimization

The enzyme was concentrated using a 10,000 molecular weight cutoff Amicon concentrator to a final concentration of 60 mg/ml before setting up crystal screens. Initial sparse matrix crystallization screens were set up for the apo-protein at a protein concentration of 15 mg/mL in 96-well sitting drop vapor diffusion format. Sparse matrix crystallization formulations were purchased from Hampton Research™, from which the PEG/Ion, Crystal Screen I, and Crystal Screen II were used to explore initial crystallization conditions. PACT™ and Ammonium sulfate screen purchased from Nextal™ were also used for the initial screening. The native crystals grew as extremely thin rod clusters from 18% PEG 3350, 0.2 M NH₄I as precipitants (Figure 2.3, panel A) at 20 °C. After repeated optimization attempts, these crystals did not grow to a reasonable size for X-ray data analysis. Simultaneously, substrate/cofactor/metal complexes of the enzyme were screened for crystallization hits. Cocrystallization of the enzyme with 5 mM NAD and 5 mM Mn (OAc)₂ gave crystalline material with 30% PEG 4000, 0.2 M NH₄(CH₃COO), 0.1 M Na₃citrate pH 5.6 as precipitants and were optimized to prism shaped crystals (Figure 2.3, panel B) in the presence of 25% PEG 4000, 0.1 M NH₄(CH₃COO), 0.2 M Na₃citrate, pH 5.5 at 20 °C.
Room temperature diffraction images of these crystals were collected on an Rigaku X-ray generator equipped with a Raxis IV++ image plate detector. These crystals diffracted to a maximum resolution of 5 Å at this temperature (Figure 2.4).
Further X-ray diffraction experiments were carried out at 100 K, thus suitable cryoprotection of the crystal mother liquor was necessary. A number of well established cryoprotectants (glycerol, ethylene glycol, MPD, PEG 400) were combined with the crystallization conditions at concentrations ranging from 20-30%. Direct and incremental exposure of \(pp\)TDH crystals into all combinations of these artificial mother liquor/cryoprotectant solutions were carried out and X-ray diffraction images collected on a Rigaku X-ray generator equipped with an Raxis IV\(\text{++}\) image plate detector. Ethylene glycol was the most suitable cryoprotectant for these crystals, however, a maximal resolution of only about 4 Å could be achieved. A second condition [1.8 M (NH\(_4\))\(_2\)SO\(_4\), 0.1 M Hepes pH 7.5, 10 mM DTT] was identified for this complex. These crystals (Figure 2.3 panel C) were extremely sensitive to any changes in the precipitant concentration in the equilibrated drop and would respond by dissolving immediately.

Figure 2.4 Room temperature diffraction image of TDH/Mn\(^{2+}/\)NAD complex. The crystal was mounted on a capillary and this image was collected at a 90° oscillation angle.
during their cryoprotection. After screening a number of crystals, the best artificial mother liquor for transferring the crystals was found to be 2.0 M (NH₄)₂SO₄ with 25% ethylene glycol. Importantly, this transfer had to be done as quickly as possible since a combination of (NH₄)₂SO₄ and ethylene glycol forms crystals of its own upon exposure to air. A complete X-ray diffraction data set was collected for the enzyme/metal/cofactor complex (ppTDH/Mn/NAD). The data were collected at the Advanced Photon Source at Argonne National Laboratory, on the GM/CA-CAT beamline 23ID-B. Using two initial diffraction images spaced 90° from each other, the unit cell and Bravais lattice symmetry were determined from the autoindexing routine within HKL2000. From the refined unit cell parameters and crystal orientation a data collection strategy was obtained. X-ray diffraction data was collected using a 3 second exposure time and 0.5° oscillations with a detector distance of 180 mm. The diffraction data were integrated with Denzo from within the HKL2000 interface where all reflections are assigned \( hkl \) indices and the reflection intensity is measured. After raw intensities are assigned to all measured reflections scale factors are applied to individual batches of reflections to bring all of the data to the same scale, after which symmetry equivalent reflections are merged and averaged using scalepack in HKL2000. The data were scaled in space group P622 where the intensities of systematic absences were consistent with this space group assignment. The reduced data from HKL2000 were 95.9% complete overall and 90.8% complete in the highest resolution shell (2.33-2.25 Å) with an overall \( R_{\text{merge}} \) of 0.089 and 0.339 in the highest resolution shell (Table 2.1). Initial attempts at determining the phase information necessary to compute the electron density distribution by the molecular replacement (MR) method failed using both full length and individual domains (nucleotide binding
and dimerization domains) of 3-isoproylmalate dehydrogenase (1IPD, 1HEX, 1WOD, 1CNZ, 1WPW, 1AO5) as well as isocitrate dehydrogenase (1AI2) as search models.

Simultaneously, the native enzyme was set up for crystallization with 5 mM NADH, 10 mM (+)-tartrate and 25 mM MgSO₄ in the protein buffer. Hexagonal shaped crystals (Figure 2.3, panel D) were optimized by hanging drop vapor diffusion at 20 °C with the optimum crystals obtained by mixing equal amounts of the protein (18 mg/ml) and the precipitant over a 600 µl reservoir solution. The drops were equilibrated against 1.65 M \((\text{NH}_4)_2\text{SO}_4\), 0.2 M ammonium tartrate and 10 mM DTT. The crystals were cryoprotected with freshly prepared mother liquor containing 2.0 M Li₂SO₄. A complete data set (Table 1.1) was collected at the Argonne National Laboratory on the SER-CAT beamline 22BM.

Another complex, TDH/ Mg\(^{2+}\)/pyruvate was crystallized in a rod shaped crystal shape (Figure 2.3 panel E) from 0.1 M Na₃citrate, pH 6.0, 0.2 M KCH₃COO and 1.75 M \((\text{NH}_4)_2\text{SO}_4\). The crystals were cryoprotected in artificial mother liquor containing 2.1 M Li₂SO₄ and a complete dataset (Table 2.1) was collected for this complex. The binary complex, TDH/Mg\(^{2+}\)/NADH has been solved by molecular replacement by Phaser using the monomer of the TDH ternary complex structure (discussed below) as the search model.

This data has been refined to modest R\(_{\text{work}}\) (26.9%) and R\(_{\text{free}}\) (29.9%) values using modules within the CNS and CCP4 crystallographic suites. The other complexes also yielded a molecular replacement solution with the same search model, however, the structures have not been completely refined. Further refinement of these structures was hampered by the presence of a large number of twinned reflections within the dataset.
Table 2.1 Data collection statistics of complexes of \textit{ppTDH}

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<th>Parameters</th>
<th>\textit{ppTDH- Mn}^{2+}-NAD</th>
<th>\textit{ppTDH-Mg}^{2+}-NADH</th>
<th>\textit{ppTDH}-Mg^{2+}-pyruvate</th>
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<td>1.03</td>
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<td>182.8, 182.6, 66.9</td>
<td>141.8, 141.8, 466.2</td>
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<td>90, 90, 120</td>
<td>90, 90, 120</td>
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<td>Resolution (Å) \textsuperscript{a}</td>
<td>50.0-2.25 (2.33-2.25)</td>
<td>50.0-2.6 (2.69-2.6)</td>
<td>50.0-3.2 (3.31-3.20)</td>
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<td>Redundancy</td>
<td>5.3 (2.5) \textsuperscript{a}</td>
<td>7.2 (2.1) \textsuperscript{a}</td>
<td>5.8 (3.8) \textsuperscript{a}</td>
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<td>Completeness (%) \textsuperscript{a}</td>
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<td>85.8 (25.9)</td>
<td>93.3 (82.9)</td>
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<tr>
<td>R sym (%) \textsuperscript{b}</td>
<td>8.9 (33.9) \textsuperscript{a}</td>
<td>7.3 (35.8) \textsuperscript{a}</td>
<td>18.5 (53.8) \textsuperscript{a}</td>
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<td>19.4 (2.01) \textsuperscript{a}</td>
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\textsuperscript{a}values in the parentheses are for the highest resolution shell

\textsuperscript{b} R_{sym} = \Sigma_h \Sigma_i |I_i(h)| - <I(h)>/ \Sigma_h \Sigma_i I_i(h), where I_i(h) is the intensity of individual measurement and <I(h)> is the mean intensity of the reflection.

2.2.3 Heavy atom soaking for phase determination

In the absence of structural information from the crystallization of the native enzyme, two methods were envisioned that would allow the structural determination of \textit{ppTDH}. The first one was to incorporate heavy atoms through soaks of native \textit{ppTDH} crystals for use in the multiple isomorphous replacement (MIR) method. The second was to incorporate selenomethionine into the enzyme (discussed below) and use the anomalous
scattering of selenium (Se). Firstly, a number of heavy metals were tried for soaking from (5-30) minutes at concentration range of (5-50) mM in the cryoprotectant solution. These metals included Platinum (Pt) salts [(NH₄)₂PtCl₄, K₂Pt(CN)₄, K₂PtBr₄], Cobalt (Co) salts [CoCl₂.6H₂O, Co(NH₃)₆Cl₂], Bromine (Br) salts [NaBr, NH₄Br], Samarium (Sm) acetate, Mercury salts [thiamersol (C₉H₉HgNaO₂S), Hg(CH₃CO₂)₂] and Ammonium rhenate (Re).

After screening the crystals, only three heavy metal soaks (concentration, soak time), NH₄ReO₄ (10 mM, 10 mins), NH₄Br (0.1 M, 10 mins) and C₉H₉HgNaO₂S (5 mM, 5 mins) resulted in diffraction quality data (Table 2.2).

<table>
<thead>
<tr>
<th>Table 2.2 Data collection statistics of heavy metal soaks for ppTDH</th>
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<td><strong>Parameters</strong></td>
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<td>R sym (%)</td>
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<tr>
<td>I/σ (I)</td>
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<tr>
<td>Total number of Reflections</td>
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<td>Total No. of unique reflections</td>
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*Values in the parentheses are for the highest resolution shell

R.sym = \sum h \sum i |I(h)| - \langle |I(h)|\rangle / \sum h \sum I(h), where I(h) is the intensity of individual measurement and \langle |I(h)|\rangle is the mean intensity of the reflection.
Increasing the concentration or extending the duration of the soak for each case resulted in higher mosaicity and lower diffraction limits for the crystal preventing any data collection. Upon analysis of the collected data in the SHARP software, none of the soaked crystals showed any heavy metal binding, with an exception of the mercury derivative. This derivative showed 19 possible Hg binding sites, however, each of the sites had very low occupancy. To prevent huge amount of non-specific binding, back soaking was performed, however, the resulting data did not yield any Hg binding this time. In conclusion, Hg derivatived crystals are possible candidates for obtaining de novo phase information for TDH, however, soaking times have to be optimized by screening a number of crystals to obtain the appropriate degree of binding to allow accurate structural determination.

In order to obtain the required phase information, derivatization of the crystals with I₂/KI by vapor diffusion was tried. In principle, this method allows the incorporation of the heavy metal, I₂, by derivatization of surface tyrosines of the protein. Derivatization was evident after a period of 30 minutes by change in color (Figure 2.5) of the crystals. The crystals were frozen immediately. The crystals completely lost diffraction, hence this method is not suitable for obtaining any phase information for TDH.

Figure 2.5 Derivatization of TDH crystals with Iodine solution (I₂/KI). Crystals changed color (red) indicative of derivatization of the protein.
2.2.4 Selenomethionine incorporation and crystallization

The selenomethionine (SeMet) form of the protein was produced by the methionine inhibition pathway method\textsuperscript{74}. Intact mass spectrometry (Ohio State University Mass Spectrometry Facility) was used to confirm the incorporation of selenomethionine in the protein. The spectrum of the derivatized protein sample indicated the presence of the parent peak of 40995 (m/z) Da. Bovine serum albumin was spotted in 2 wells adjacent to the sample, and default instrument calibration gave a average mass error of ±13 Da for the mass of the doubly charged ion of BSA, and ±6 Da for the MH\textsuperscript{+} for BSA. The enhancement in mass for derivatized TDH in comparison to the native enzyme (40630 Da) is indicative of the incorporation of 5 selenium atoms into the derivatized enzyme.

SeMet was purified in a similar fashion to the native enzyme, with the exception that a higher concentration of reducing agent was used in the SeMet protein buffer to minimize oxidation of the more sensitive selenomethionines. The resultant protein was shown to be >99% pure based on a SDS gel profile. The protein was finally transferred into 50 mM Hepes pH 7.5, 25 mM MgSO\textsubscript{4}, 10 mM DTT and 10 mM KCl by running through a gel filtration column.

Crystallization of the apo form of selenomethionine (SeMet) TDH was initially optimized (Figure 2.6 panel A) using 13-15\% 2-methyl-2,4-pentanediol (MPD) as the precipitant, with 0.05 M Na acetate, pH 5.0, 0.02 M CaCl\textsubscript{2}. Use of an additive screen (Hampton) showed some improvement in crystal quality, with D-glucose and D-xylitol as the best additives. Unfortunately these optimized crystals (Figure 2.6 panel B) did not diffract beyond 3.5 Å. Cross-linking the crystals with glutaraldehyde\textsuperscript{100} did not improve the diffraction quality of these crystals.
Crystals of the SeMet-substituted TDH complexed with oxalate, NADH and Mg$^{2+}$ were optimized at 20 °C from new starting conditions using the hanging drop vapor diffusion method. The optimum crystals (Figure 2.6 panel C) formed in a drop containing 2 µl of protein (15 mg/ml) in 50 mM Hepes (pH 7.5), 25 mM MgSO$_4$, 20 mM DTT, 10 mM KCl, 5 mM oxalate and 5 mM NADH and 2 µl of reservoir solution containing 1.75 M (NH$_4$)$_2$SO$_4$, 0.2 M K acetate, and 20 mM DTT.

Crystals of about 0.3 x 0.2 x 0.2 mm grew within 4-5 days. The crystals were flash frozen in liquid N$_2$ following equilibration in a stabilization solution containing 2.2 M Li$_2$SO$_4$, 2.5 mM oxalate, 2.5 mM NADH, 5 mM DTT, 0.05 M K acetate, 38 mM Hepes (pH 7.5), 19 mM MgSO$_4$, and 7.5 mM KCl. These crystals have tetragonal symmetry and the data best fits the P4$_3$2$_1$2 space group with cell dimensions of a = 117.27 Å, b = 117.27 Å, c = 291.32 Å, α = β = γ = 90° and a dimer of dimers in the asymmetric unit.

Figure 2.6 Optimized crystals of SeMet TDH (A) Crystal of apo-form of SeMet TDH diffracted to 9 Å (B) Addition of additive, D-glucose, improved the diffraction to 3.5 Å. (C) Ternary complex, TDH/oxalate/Mg$^{2+}$/NADH was crystallized and diffraction data collected at 100 K.
2.2.5 Structure determination and refinement

The complete diffraction data for the SeMet crystals were collected at Southeast Regional Collaborative Access team (SER-CAT), at beamline 22 ID at APS. The Se absorption edge was identified by an X-ray fluorescence energy scan from which the SAD data collection experiment was designed (Figure 2.7).

![X-ray absorption scan of Se-Met TDH crystal.](image)

**Figure 2.7 X-ray absorption scan of Se-Met TDH crystal.** Peak wavelength was observed at 0.9794 Å at which the data was collected.

The data set was indexed, integrated and scaled with HKL2000\textsuperscript{75}. Data collection statistics for the TDH intermediate analogue complex is reported in Table 2.3.
Table 2.3: Crystallographic and refinement statistics of Se-Met *pp*TDH

<table>
<thead>
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<tr>
<td>Resolution (Å)$^a$</td>
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<tr>
<td>Unique reflections</td>
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<tr>
<td>$R_{sym}$$^b$</td>
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<tr>
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<tr>
<td>Redundancy$^a$</td>
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<tr>
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<td>No. of Se atoms</td>
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<td>Figure of merit</td>
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<tr>
<td><strong>Refinement</strong></td>
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<tr>
<td>Wilson B factor (Å$^2$)</td>
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<tr>
<td>$R_{work}/R_{free}$ (%)$^c$</td>
<td>20.7/22.6</td>
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<tr>
<td>No. of atoms</td>
<td></td>
</tr>
<tr>
<td>(Protein/ligands/DTD/ H$_2$O)</td>
<td>11365/208/24/920</td>
</tr>
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<td>&lt;B-factors&gt; (Å$^2$)</td>
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</tr>
<tr>
<td>(Protein/ligands/H$_2$O)</td>
<td>20.4/16.1/30.3</td>
</tr>
<tr>
<td>rmsd</td>
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<tr>
<td>Bond length (Å)/angle (°)</td>
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</tr>
<tr>
<td>Ramachandran statistics (%)$^d$</td>
<td>89.3/9.5/0.7/0.4</td>
</tr>
</tbody>
</table>

$^a$ values in the parentheses are for the highest resolution shell

$^b$ $R_{sym} = \Sigma_h \Sigma_i |I_i(h)| - <I(h)> |\Sigma_h \Sigma_i I_i(h)$, where $I_i(h)$ is the intensity of individual measurement and $<I(h)>$ is the mean intensity of the reflection.

$^c$ $R_{work} = \Sigma_h ||F_{obs}|| - |F_{calc}||/ \Sigma_h |F_{obs}|$, where $F_{obs}$ and $F_{calc}$ are the observed and calculated structure factor amplitudes, respectively. $R_{free}$ was calculated for 5% of the randomly selected unique reflections omitted from structure refinement.

$^d$ most favored/favored/additionally allowed/disallowed
Experimental phases were initially obtained by inputting a 2.45 Å dataset obtained from SeMet-substituted TDH into the SGXPro Suite\textsuperscript{76} which was able to build over 1300 amino acids into the electron density maps of the dimer of dimers at the final stage. A higher resolution dataset was collected from a different crystal and \textit{SHARP}\textsuperscript{77} was used to find a solution. The maps obtained from this higher resolution data set (Table 2.3) were used as a guide to build the entire molecule. Manual correction of the model into the experimental electron density map was done by real space refinement with \textit{Coot}\textsuperscript{78}. The structure was refined by rigid body refinement followed by restrained refinement using the program \textit{Refmac5}\textsuperscript{79}. Finally, TLS refinement\textsuperscript{80} was performed by subdividing the monomer into three subdomains (amino acids 5-158, 159-295, and 296-363) with each monomer refined individually.

2.2.6 Overall structure

The highest quality crystals were obtained for a complex of SeMet-substituted TDH containing NADH, Mg\textsuperscript{2+} and the intermediate analogue oxalate. Higher levels of DTT (20 mM) were included in the crystallization conditions to minimize oxidation of the more sensitive selenomethionines. The presence of this antioxidant played a critical role in the improved crystal quality, resulting in a change from hexagonal to orthorhombic crystals. In the final structure three molecules of DTT are identified, bound at different sites. The DTT in site #1 (Figure 2.8B) bridges between two arginyl residues in one subunit and an asparagine in the adjacent subunit of the other dimer in the asymmetric unit, while DTT in site #2 (Figure 2.8C) is positioned near the dimer interface and interacts with a backbone amide group from monomer A and a glutamine side chain in
monomer B. The role of DTT in improving crystal quality is a consequence of its binding at these crucial interface regions within and between the dimers.

Figure 2.8 Overall structure of ppTDH. A. Ribbon drawing of the TDH dimer showing the domain organization and the position of the cofactor and intermediate analogue (brown sticks) and the divalent metal ion (pink spheres) bound at the active site in each monomer. The two monomers are colored green and blue, with the nucleotide binding domains shaded darker and the dimerization domains lighter. Bound DTT molecules are marked in light brown, with the three binding sites numbered and circled\(^{101}\). B. Site # 1 for DTT molecule C. Site # 2 for DTT molecule.
The asymmetric unit of the TDH complex with oxalate contains four molecules and the functional unit of TDH is a dimer composed of monomers with similar but not identical structures. Each monomer is organized into two domains and the conformation of these monomers is closed, in contrast to the more open structure found for the IPMDH substrate complex which shares a similar fold and topology with TDH. The nucleotide binding domain of the TDH monomer contains the N- and C-terminal segments, while the dimerization domain consists of four sets of α-helices and antiparallel β-sheets (residues 117-256) from each monomer (Figure 2.8). The active site is located in the cleft between the two domains and the substrate (or intermediate analogue), cofactor and divalent metal ion are each well-positioned into tight electron density in both active sites (Figure 2.9).

Figure 2.9 Fo-Fc electron density map of the active site of ppTDH. Mg$^{2+}$ ion (pink sphere), oxalate ion (green sticks) and NADH (yellow sticks) modeled into the density in monomer A. Map is contoured to 2.5 σ.
Each active site is constructed from residues derived from both subunits, thus establishing the dimer as the minimal functional unit. The first three residues of the N-terminus and the final C-terminal residue are disordered in the crystals, and their positions were not determined in the final structure.

2.3 Binding of the intermediate analog

The intermediate analogue oxalate (Figure 2.10) is well positioned in both the active sites of TDH through electrostatic interactions with several conserved active site arginines.

![Structures of R-hydroxy acid substrates](image)

**Figure 2.10 Structures of R-hydroxy acid substrates.** Comparison of the substrates for IPMDH, ICDH and HIcDH with the substrates and intermediate analogue for TDH.
The C1-carboxylate of oxalate interacts with R134 that is located 2.8-2.9 Å from one of the oxygens, and there is also a complementary bidentate interaction between the C1-carboxyl oxygens and the terminal guanidinium nitrogens of R108 at a distance of 2.9-3.2 Å (Figure 2.11).

![Figure 2.11 Divalent metal ion and substrate binding sites in monomer A.](image)

The residues from monomer A are shown in blue sticks and labels, while those from monomer B are in green sticks and labels. Mg$^{2+}$ (pink sphere) is coordinated by six ligands, two aspartate residues, two water molecules (blue spheres) and a bidentate interaction with carboxylate groups of oxalate, with the oxalate further positioned through interactions with several active site arginines.

An additional electrostatic interaction with the nearby R98 further anchors the position of this C1-carboxylate in the active site of each monomer. R98 is also located in close
proximity to the amide nitrogen of the nicotinamide moiety of NAD and forms an interaction through a water-mediated hydrogen bond, thereby bridging between the substrate and cofactor binding sites.

2.4 Characterization of active site mutants

To further characterize the residues, R98 and R108, mutations were made at these sites. A QuickChange™ site-directed mutagenesis kit (Stratagene) was used to make point mutations of these important active site residues, and each mutation was confirmed by nucleotide sequencing (MWG Biotech). The mutants were overexpressed in Rosetta (DE3) E. coli cells following induction with 1 mM IPTG and the cells were grown at 28 °C after induction. Because of the inherent instability of some of these mutants a reduction in growth temperature to 25 °C after induction was important to avoid expression of the mutants in inclusion bodies. The mutants were each purified using a similar protocol as was employed for the native enzyme. SDS-PAGE electrophoresis was used to confirm the presence of mutants which showed minimal catalytic activity.

Enzyme activity was measured in a SpectraMax 190 microplate reader at 25 °C using 96-well flat bottom plate (Fischer Scientific) in a final volume of 200 µl. All the reactions were carried out in 100 mM Hepes, pH 8.0. Enzymatic assays to determine kinetic parameters were carried out by varying one substrate with the other substrates held constant at saturating levels (>10 times $K_m$). Initial velocities were fitted to the hyperbolic curve by using an enzyme kinetics package adapted from previous kinetic programs.

Not surprisingly, replacement of the critical arginine at position 98 with a hydrophobic leucine causes a decrease in the stability of TDH, driving its expression into inclusion
bodies that cannot be properly refolded. A more subtle change to a glutamine at this position allows the mutant protein to retain a native structure and bind its substrates with $K_m$ values that are comparable to those of the native enzyme (Table 2.4).

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>% $k_{\text{cat}}$</th>
<th>$K_{\text{malate}}$ (µM)</th>
<th>$k_{\text{cat}}/K_{\text{malate}}$ (M$^{-1}$ s$^{-1}$)</th>
<th>$K_{\text{NAD}}$ (µM)</th>
<th>$k_{\text{cat}}/K_{\text{NAD}}$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>390 ± 4</td>
<td>100</td>
<td>79 ± 3</td>
<td>8.2 x 10$^3$</td>
<td>25.0 ± 0.4</td>
<td>2.6 x 10$^5$</td>
</tr>
<tr>
<td>R98L</td>
<td>----</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R98Q</td>
<td>10.5 ± 0.1</td>
<td>2.6</td>
<td>98 ± 2</td>
<td>1.8 x 10$^3$</td>
<td>96 ± 2</td>
<td>1.8 x 10$^3$</td>
</tr>
<tr>
<td>R108L</td>
<td>5.2 ± 0.9</td>
<td>1.3</td>
<td>180 ± 30</td>
<td>4.8 x 10$^2$</td>
<td>240 ± 40</td>
<td>3.6 x 10$^2$</td>
</tr>
<tr>
<td>R108Q</td>
<td>34.6 ± 1.8</td>
<td>8.7</td>
<td>93 ± 18</td>
<td>6.2 x 10$^3$</td>
<td>135 ± 26</td>
<td>4.3 x 10$^3$</td>
</tr>
</tbody>
</table>

$^a$ not expressed in stable form

However, the catalytic efficiency of this R98Q mutant is less than 3% that of the native enzyme. These studies support a critical role for R98 both in the stabilization of the structural integrity of the enzyme and in orienting the substrate for optimal catalytic activity. When the other substrate binding group R108 is mutated to a leucine the protein structure is also destabilized, but has been expressed in an active form at a lower temperature. The $K_m$ value for D-malate increases by a factor of two and that for NAD by a factor of ten in this active site mutant, leading to a decrease of over two orders of magnitude in $k_{\text{cat}}/K_m$ for the R108L mutant (Table 2.4). Changing the group at position 108 to a glutamine leads to some recovery of catalytic efficiency, with a 6-fold increase in $k_{\text{cat}}$ and a 13-fold increase in $k_{\text{cat}}/K_m$ relative to the leucine mutant. As with R98, this
R108 residue is also involved in a water-mediated hydrogen bond with the nicotinamide ring of the cofactor. It was shown by circular dichroism studies that the loss of activity at R108 is not related to the change in overall conformation of the protein (Figure 2.12).

![Figure 2.12](image)

**Figure 2.12 Plot of molar ellipticity versus scanned wavelength of TDH.** This CD data was collected at Bowling Green State University. The native enzyme (blue) curve is similar to the curve (pink) for the R108L mutant enzyme.

### 2.5 Divalent metal ion binding and its role in catalysis

Both of the carboxylate groups of the intermediate analogue oxalate interact with the divalent metal ion that is required for catalysis. The divalent metal ion binding site is hexacoordinate, with interactions to the side chain carboxylate group of D250 (at 2.16 Å), equatorial and axial water molecules (2.01-2.09 Å) and the two carboxylate oxygens from the oxalate intermediate analogue (2.34-2.39 Å) (Figure 2.11). A carboxylate side chain (D225) from the adjacent subunit (2.04 Å) completes the distorted octahedral binding site. The importance of D250 and D225 as metal ion binding ligands is verified by site-directed mutagenesis studies that showed a 10- and 20-fold decrease in metal ion binding.
affinity and a two order of magnitude decrease in catalysis when either of these ligands was removed (Table 2.5).

Table 2.5: Metal binding mutants of ppTDH

<table>
<thead>
<tr>
<th>Enzyme form</th>
<th>$k_{cat}$ (min$^{-1}$)</th>
<th>$% k_{cat}$</th>
<th>$K_{Mn}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>native</td>
<td>390 ± 4</td>
<td>100</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>D225A</td>
<td>0.91 ± 0.15</td>
<td>0.2</td>
<td>1.0 ± 0.4</td>
</tr>
<tr>
<td>D250A</td>
<td>5.3 ± 0.8</td>
<td>1.4</td>
<td>0.6 ± 0.1</td>
</tr>
</tbody>
</table>

Significant changes occur in the metal ion binding site once the keto acid product is released and the substrate carboxyl oxygens are no longer available to interact with the metal ion. In the binary complex with NADH the side chain of D250 is rotated approximately 90° from its previous position (Figure 2.11) to make a bidentate interaction with the divalent metal ion, with Mg$^{2+}$ to oxygen distances of 2.05 Å and 2.96 Å. The metal ion is still coordinated to the carboxylate group of D225 from subunit B but at an increased distance (3.0 Å), and the side chain hydroxyl of S253 has moved into position to make a long range interaction (3.47 Å). Two water molecules (at 2.76 Å and 2.42 Å) complete the distorted octahedral coordination. The metal ion position has shifted from its previous location by 1.06 Å towards the binding site vacated by the departure of oxalate, and the increased metal-ligand distances suggest a somewhat weaker interaction with the enzyme in this product complex.

**2.6. Cofactor binding**

NADH binds adjacent to the substrate binding site, with the position and orientation of each structural moiety stabilized by interactions with amino acid residues from both
subunits, as well as by hydrogen bond contacts mediated through active site water molecules. The adenine ring of the cofactor bound in subunit A is positioned by a hydrogen bond between N298 and the exocyclic amine at C6 along with several hydrophobic side chains (I16 and I291) (Figure 2.13).

![Diagram of NAD and monovalent cation binding sites in ppTDH](image)

**Figure 2.13 NAD and monovalent cation binding sites in ppTDH.** The active site residues from each subunit responsible for positioning the cofactor are labeled, with key water molecules indicated as blue spheres. The ammonium ion (grey sphere) is bound by backbone carbonyls and the carboxylate of E282 and interacts with the amide group of the nicotinamide ring.

hydrogen bond between N298 and the exocyclic amine at C6 along with several hydrophobic side chains (I16 and I291) (Figure 2.13).

The 2’- and 3’-hydroxyl groups of the adenine ribose are hydrogen bonded to the side chain carboxyl oxygens of D290. The 3’-hydroxyl also interacts with the guanidinium nitrogen of R230 from subunit B. The phosphate attached to the adenine ribose is stabilized by a direct interaction with the backbone NH of G286 and a slightly longer
interaction with the side chain hydroxyl of S287. The other phosphate of the pyrophosphate linker is stabilized by a water-mediated hydrogen bond to R230 from subunit B. The nicotinamide ribose hydroxyl groups form hydrogen bonds to the side chain carboxyl of D86 from subunit A and the amide of N194 from subunit B. The E282 carboxyl group stabilizes the orientation of the nicotinamide ring by interacting with the NH$_2$ group (Figure 2.13). These multiple interactions contributed by functional groups from both subunits result in a well defined binding pocket that orients the cofactor for efficient hydride transfer.

Interestingly, the orientation of the nicotinamide ring of NADH in the intermediate analogue complex of TDH is positioned in clearly defined density (Figure 2.9). This is in contrast to the absence of well ordered density for the nicotinamide of NAD bound to its closest homologue, IPMDH$^{85}$. In the absence of bound substrate two possible orientations have been proposed for the cofactor in IPMDH, neither of which are the same as that observed in TDH. The residues involved in stabilizing cofactor binding in IPMDH are similar to those found in TDH, with two major exceptions. The additional bonding interactions from the adjacent subunit that contribute to the binding of NADH in TDH are not present in IPMDH. The arginine from the adjacent subunit (R230) that interacts with the adenine ribose in TDH is replaced by a carboxyl group from D278 in the same subunit which plays this role in IPMDH. Hence, in the absence of the intermediate analogue there are fewer interactions to stabilize cofactor binding and orient the nicotinamide ring for efficient hydride transfer in IPMDH.
2.7 Monovalent cation and its role in cofactor binding

The presence of a monovalent potassium ion is essential for the catalytic activity of TDH\textsuperscript{95}, and monovalent ions such as potassium, ammonium and rubidium were found to activate the related enzyme HIcDH\textsuperscript{7}. However, the location of this monovalent cation and its exact role in the reaction has not been determined. On the basis of pH-rate profile studies the monovalent activator was proposed to bind to an ionized carboxyl side chain in HIcDH and function to increase the affinity of the enzyme for its cofactor through interactions with the pyrophosphate moiety of NAD\textsuperscript{7}. Initial velocity studies established the $K_a$ for $K^+$ binding to apo-TDH as 30 mM, and this value decreases to 4 mM in the presence of saturating NAD\textsuperscript{11}. However, potassium ions are not found in the active site in either TDH complex structure, despite the inclusion of millimolar levels of KCl in the crystallization buffers. Ammonium ions have previously been shown to also function as an activator of TDH\textsuperscript{95}, and the highly solvent exposed active site could allow facile exchange of $K^+$ ions with the molar concentration of NH$_4^+$ ions that were used as a crystallization precipitant. Upon careful examination one NH$_4^+$ ion was located within the active site in close proximity to the nicotinamide ring. This ion was identified by its high coordination number, the nearly tetrahedral bonding angle (111.6°, ideal: 109.5°), as well as its tendency to coordinate to only electronegative groups. This likely represents the monovalent cation activator site in TDH, and an ammonium ion is found at the same position in the NADH product complex. The monovalent ion is coordinated by the backbone carbonyl groups of P283 (2.85 Å bond distance), H285 (2.83 Å) and G76 (2.72 Å), along with a side chain carboxyl group of E282 (2.81 Å), thus confirming the proposed role of a carboxyl group in monovalent cation binding (Figure 2.13). Each of
these carbonyl groups are capable of acting only as hydrogen bond acceptors, thereby confirming the presence of an ammonium ion at this site and not a water molecule. However, the interaction of ammonium ions with the cofactor in TDH is through the amide oxygen atom of the nicotinamide rather than with the pyrophosphate moiety as was proposed\(^7\).

### 2.8 Subunit communications and alternating site reaction mechanism

Based upon isothermal titration calorimetry (ITC) studies that found a 7-fold difference in NAD binding affinity to each subunit in the dimer\(^{11}\), TDH has been proposed to use a ‘half-of-sites’ reaction mechanism to catalyze product formation. Each enzyme form was crystallized in the presence of saturating levels of NADH and a molecule of cofactor is bound in each active site with no evidence for asymmetry in binding. However, there are numerous routes by which the binding of substrates and the release of products in one subunit can be communicated to the adjacent subunit. Five residues that originate from the other subunit are involved in substrate, cofactor and divalent metal ion binding in the adjacent subunit of the dimer (Figures 2.11 & 2.15). In addition, H223 from one subunit interacts with D86, a residue involved in cofactor binding from the opposite subunit. In a similar manner, R230 from the adjacent subunit provides binding interactions to the ribose and phosphate groups of the NAD cofactor (Figure 2.15). D250, a metal binding residue, is engaged in a monodentate interaction with K192 from the other subunit and D225 from the adjacent subunit completes the metal ion coordination sphere (Figure 2.11). There are also a number of electrostatic and hydrogen bonding interactions across the subunit interface that serve to maintain the structural integrity of the functional dimer. From this ensemble of interactions each active
site could relay information to the other subunit via a number of different pathways, and structural comparisons of complexes with only a single substrate or cofactor bound to the dimer will be required to narrow these possibilities.

2.9 Structural comparison with R-hydroxy acid dehydrogenases

Homoisocitrate dehydrogenase (HICDH), 3-isopropylmalate dehydrogenase (IPMDH) and isocitrate dehydrogenase (ICDH) are the other structurally characterized members of the R-hydroxy acid dehydrogenase family, with a structure of the apo-form of HICDH and several structures of IPMDHs and ICDHs determined from different species. The IPMDH and ICDH structures depict the state of the enzyme in the presence of substrate and a metal ion or a cofactor. Because TDH shares the highest sequence homology with IPMDH from *Thermus thermophilus* (Figure 2.1), the structure and domain organization of these related hydroxy acid dehydrogenases were compared and found to be quite similar (Figure 2.14). However, despite the overall structural similarity significant differences are observed in several regions connecting the core secondary structure, as well as in the relative positioning of critical loops, helices and sheets. After optimization of the structural alignment a loop and helix (residues 80-90) in TDH are found to be rotated and shifted inwards in comparison to the related region in the complex structures of IPMDH (Figure 2.14). Importantly, this region participates in stabilizing the active site architecture of IPMDH, with several residues interacting directly with the isopropyl group of the substrate (Figure 2.10) as well as with the cofactor. In the IPMDH structure D87 forms a hydrogen bond with the nicotinamide ribose, while the residue at the corresponding position in TDH (L90) cannot form this interaction.
Instead this cofactor stabilization role is now played by the adjacent D86 in TDH (replacing a lysine at this position in IPMDH) and the loop carrying this residue shifts inward to support cofactor binding (Figure 2.14, inset). A second important structural difference involves the intersubunit antiparallel \( \beta \)-sheet located between the two domains of the enzyme, which is dramatically shifted inwards by as much as 10 Å in comparison to any of the IPMDH structures (Figure 2.14). As a consequence of this shift the closed conformation of the dimer is further stabilized upon binding of the intermediate analogue (or substrate). A comparison of the TDH structure with that of ICDH from \emph{E. coli} confirms the same overall fold and domain organization, but also identifies some additional structural variations. The intersubunit antiparallel \( \beta \)-sheet (Figure 2.14) that is present in both IPMDH and TDH is replaced by an \( \alpha \)-helix at the same position in bacterial ICDH. Also, part of the substrate binding helix in TDH has been replaced by a \( \beta \)-sheet in the ICDH structure, leading to a replacement of the hydrophobic side chain of L90 with S113 in ICDH. S113 interacts with the C3-carboxyl group of isocitrate (Figure 2.10) and phosphorylation of S113 in ICDH causes inactivation of the enzyme and prevents substrate binding. Because the TDH substrates do not contain this interior carboxylate group the presence of this serine is not required for substrate binding. Thus, positioning of this common loop adjacent to this substrate binding helix is divergent in all three enzymes. This loop is dramatically shifted inwards in the TDH ternary complex to support the cofactor binding \emph{via} D86, in contrast to its position in the ICDH ternary complex and the IPMDH cofactor or substrate complexes. There are also other structural features which are similar in ICDH and IPMDH, but differ in this TDH structure. There is an additional loop (residue 313-319) on the outer surface of TDH which is absent in the
other members of the family. In contrast, extra loops are present in the C-terminal region in IPMDH (residue 334-339) and ICDH (394-400) which are absent in TDH.

**Figure 2.14 Structural comparison of ppTDH and its closest homolog.** Overlay of the structures of ppTDH (green) and ttIPMDH (pink). Despite the similar overall fold there are substantial shifts in a number of important secondary structural elements. *Inset,* substrate binding helix showing the position of the cofactor binding groups D87 in IPMDH (in place of L90 in TDH) and D86 in TDH (in place
Despite these structural differences between these members of the hydroxy acid dehydrogenases many of the active site residues are fully conserved.

### 2.10 Substrate specificity

Sequence alignments among the hydroxy acid dehydrogenases and structural studies of ICDH and IPMDH indicate a high degree of conservation for the residues that interact with the C1-carboxylate group (Figure 2.10) of their respective substrates. However, the residues that interact with the functional group at the C4-position differ within this enzyme family, providing the necessary substrate specificity for each enzyme. For TDH the binding pocket that interacts with these diverse substrate groups is formed by an array of both hydrophilic and hydrophobic residues. The hydrophilic side chain of N194 that interacts with the ribose ring of the cofactor is also in position to hydrogen bond with the carboxyl functional group at the C4-position of the TDH substrates (Figure 2.10) through an intervening water molecule. A comparison of the active sites of TDH and IPMDH reveal that the amino acid side chains surrounding this region of the substrate are shifted inward in comparison to their conserved counterparts that stabilize the C1-position in IPMDH.

Several models have been proposed to account for the conformational selectivity of substrate binding that can either promote or disfavor the decarboxylation of different substrates. However, a detailed understanding of the unusual catalytic diversity of TDH has been hindered by the absence of structural information to examine these proposed binding models. An examination of the possible substrate conformers suggested a model in which bidentate metal ion coordination to the adjacent hydroxyl groups would orient the substrate carboxyl groups to account for the products observed\textsuperscript{131}. However, there
was no direct evidence available to show metal ion coordination to the enzyme-bound substrate. While structures of TDH with its different substrates bound are not yet available, the positioning of the active site groups in the intermediate analogue complex gives some insights into the diversity of reactions catalyzed by this enzyme. The TDH structure with the intermediate analogue oxalate shows that the divalent metal ion is within coordination distance to both carboxylate groups, consistent with this metal ion coordination model for substrate binding. However, the divalent metal ion would have to shift position in order to coordinate to the C3-hydroxyl group and play the role in substrate conformer selectivity that was proposed \textsuperscript{131}.

Based on results with several malate analogues an alternative model was proposed in which a putative hydrophobic binding pocket could provide the necessary conformer selectivity\textsuperscript{131}. The active site architecture of TDH has a predominant hydrophobic face that interacts with the carbon backbone of the substrate and a polar face that interacts with the functional groups. The residues shown above and below the substrate in Figure 2.15 consist of the catalytic residues as well as the positively charged binding residues that interact with the functional groups of the substrate. The binding of meso-tartrate (modeled in Figure 2.15A) places the C1-carboxyl group in the same position as the oxalate carboxyl group, with the C4-functional group making interactions with charge groups on the polar surface.
This orientation places meso-tartrate in a favorable position for metal ion-supported decarboxylation after hydride transfer. In contrast, the modeling of (2R,3S)-3-methyltartrate into this binding pocket (not shown) requires a reorientation in order to avoid unfavorable interactions between either of the hydroxyl groups and this hydrophobic surface. It is this necessary repositioning that presumably does not allow the decarboxylation of (+)-tartrate after TDH catalyzes its oxidation to oxaloglycolate (Scheme 1, Reaction 1).
(2R,3S)-3-methyl tartrate (equivalent to meso-tartrate) was found to undergo oxidation but not decarboxylation\textsuperscript{131}, and this failure to decarboxylate was attributed to the accommodation of the methyl group into a hydrophobic pocket that would force the carboxylate group to become coplanar. When (2R,3S)-3-methyl tartrate is modeled into the active site cavity of the TDH structure (Figure 2.15B) the methyl group is oriented towards a large hydrophobic cavity formed by L90 and W91, consistent with this proposal. This rotomer places the carboxylate group in the plane of the molecule and would disfavor decarboxylation.

2.11 Examination of alternative substrates and analogs

Substrate analogs of D-malate such as 2-hydroxybutyric and isocitric acid were examined and showed weak inhibition with $K_i$ values of 5.5 ± 0.4 mM and 2.5 ± 0.3 mM, respectively. Assays were carried out in a similar fashion as described earlier in the presence of competitive levels of D-malate and the kinetic parameters were obtained by the Dixon plot. Components of the cofactor NAD$^+$ such as ADP show stronger inhibition with a $K_i$ value of 10.8 ± 2.9 µM. Nicotinamide adenine mononucleotide and AMP were also analyzed kinetically and showed no inhibition in the presence of comparable levels of NAD$^+$. These results indicate the importance of the ADP moiety for coenzyme binding.

2.12 Catalytic mechanism of TDH

The initial step in the catalytic cycle with D-malate as the substrate is a proton abstraction accompanied by hydride transfer to NAD to form the oxaloacetate intermediate (Figure 2.16, panel A). K192 is the likely base involved in the initial proton abstraction through a water mediated interaction from the C2 hydroxyl oxygen of the
substrate. The ε-amino group of K192 must be neutral to act as a general base and the presence of a nearby protonated histidine (H223) is sufficient to lower the pK of this functional group. This histidine is substituted by a neutral amino acid in both ICDH and IPMDH, requiring some other interactions to allow this lysine to serve as a general base in these enzymes. The lysyl amino group likely acts via a proton relay through a nearby active site water molecule which initially accepts the proton. This water molecule is positioned through hydrogen bonding interactions with K192 and N194. The hydride transfer to the cofactor will be stabilized by the monovalent metal ion near the nicotinamide ring. Decarboxylation of the intermediate proceeds with Lewis acid assistance from the divalent metal ion to give enolpyruvate and CO₂ products (Figure 2.16, panel B). Oxalate is an analogue of the enolpyruvate intermediate that is formed in the TDH-catalyzed oxidative decarboxylation of D-malate, and the oxalate complex structure represents this enzyme intermediate. Dissociation of carbon dioxide and tautomerization of enolpyruvate assisted by Y141 acting as a general acid (panel C) leads to the final product. A tyrosine at the corresponding position has been proposed to play this role in the other members of the β-hydroxy acid decarboxylase family. Dissociation of pyruvate from the enzyme leaves the NADH product complex (panel D) that has now been structural characterized. Departure of the reduced cofactor and a proton transfer from K192 to Y141, followed by binding of the oxidized cofactor and substrate completes the catalytic cycle (Figure 2.16).
Figure 2.16 Catalytic mechanism of TDH
Chapter 3: Biochemical evaluation of membrane associated proteins, N-acetyl-L-aspartate (NAA) transporter and Aspartate N-acetyl transferase

3.1. N-acetyl-L-aspartate (NAA) transporter

Cloning, expression and purification of various constructs of the NAA transporter have been performed with the aim of obtaining a stable and soluble form of the protein for crystallization studies.

3.1.1 Molecular cloning

Full length as well as truncated constructs of the human NAA transporter were cloned using Gateway technology\textsuperscript{102} (Invitrogen). Gateway technology is a cloning method that provides a highly efficient route to cloning/subcloning of DNA segments. The gateway method allows transfer of DNA segments between different cloning vectors while maintaining orientation and reading frame, effectively replacing the use of restriction endonucleases and ligase.

In phage I, the integration site is known as \textit{attP}, while gene to be inserted in the site is called \textit{attB}. The \textit{att} sites contain the binding sites for the proteins that mediate the specific recombination. The integration reaction (\textit{attB} x \textit{attP}) is mediated by the proteins integrase (Int) and host integration factor (IHF). When integration occurs, two new sites are created, \textit{attL} and \textit{attR}, flanking the integrated prophage, with no loss of DNA.
sequence (Figure 3.1 a).

![Diagram of Gateway reactions]

**Figure 3.1 Gateway reactions** a. BP reaction b. LR reaction

All of the att sites are alike in that they contain a 15-base pair recognition sequence for the recombinase (integrase). Transfer of the open reading frame from the entry vector into the destination vectors takes place through an LR reaction (Figure 3.1 b). Destination vectors contain all the sequence information required for expression. This plasmid also contains two recombination sites (attR1 and attR2) that flank a gene for negative selection, ccdB. With the help of IHF, Int and excisionase [Xis], two recombination events will lead to the formation of final expression plasmid.

Primers were designed for cloning the full length NAA transporter. A two-step polymerase chain reaction (PCR) strategy was utilized to clone the construct (attB1-His – MBP-TEV-full length NAA transporter-attB2) from the cDNA.

**Forward Primer**

55
Forward Primer II (Universal Primer for constructs with attB1 site and TEV cleavage site):
G GGG ACA AGT TTG TAC AAA AAA GCA GGC TTC GAA AAT CTT TAT TTT CAA GGA
Reverse Primer I
GAA AGC TGG GTT TCA GAG GGT CCG AAA TGT GTC ATT GGC CAA GGT GGG
Reverse Primer II
GGGG AC CAC TTT GTA CAA GAA AGC TGG GTT TCA GAG GGT CCG

A number of parameters including annealing temperature, cDNA and primer concentration, number of cycles and the type of DNA polymerase were optimized. After running a sufficient number of cycles, it was clear that an optimal annealing temperature of 68 °C was essential to obtain the final PCR product (Figure 3.2, Lanes 2-4).

Figure 3.2. DNA gel electrophoresis for PCR products of NAA transporter. Lane 1: Molecular weight markers; Lane 2-4: Full length NAA transporter; Lane 5-6: Truncated (1-906 bps) NAA transporter.
PCR products were sequenced (MWG Biotech.) to ensure the correct identity of the amplified gene sequence. This PCR product was then subjected to BP and LR reactions (described above) to obtain the final expression construct. Shorter constructs including an N-terminal (aa 1-302; Figure 3.2, Lanes 5-6) and a C-terminal construct (aa 356-586) were relatively straightforward to clone. These constructs were designed by identifying the regions (Figure 3.3) which were predicted to be disordered based on computational algorithms\textsuperscript{103}. This software predicted a high probability of a disordered region for two 30-40 amino acid segments near the middle of the sequence.

![Stability prediction of human NAA transporter](image)

**Figure 3.3. Stability prediction of human NAA transporter.** Residue number is plotted versus probability disorder. Secondary structural elements: α helix (blue); β sheet (red) and coil (purple) are colored accordingly. Region marked (purple) indicates the most stable region predicted for the transporter. Stability predictions have been made on [http://www.strubi.ox.ac.uk/RONN](http://www.strubi.ox.ac.uk/RONN) server.
3.1.2 Purification and detergent screening of the amino terminal region

A number of different cell lines including C41, C43, LMG194, XL1 blue cells were tested for protein expression. Both the amino terminal as well as the carboxy-terminal constructs expressed well as fusion proteins with the maltose binding protein (MBP) in C41 and C43 cell lines, respectively. In the case of the N-terminal transporter, a portion of the desired protein was observed in the supernatant, and was bound and then eluted from an affinity column (GE healthcare) using an imidazole gradient (Figure 3.4 A). The protein was then treated with TEV protease (purified in the laboratory using a published protocol) to remove the tags. The reaction was carried out by mixing the protein fusion with TEV protease in a 1:5 ratio. The reaction was carried out 4 °C with gentle stirring for 12 hours.

Figure 3.4. Affinity chromatography and SDS gel profile for soluble portion of amino-terminal NAA transporter A. Ni histrap column with the protein (arrow) bound. B. Subtractive affinity chromatography after removal of the histidine tag. Protein (arrow) of interest is eluted as the flow through.
The treated protein was re-loaded on to the affinity column and the cleaved truncated transporter was collected in the flow through (Figure 3.4 B). This protein is unstable and precipitates within few hours after removal of the tag.

The majority of this fusion protein was found in the pellet after disruption of the cells and was solubilized by treatment with a detergent (40 mM CHAPS). A similar purification procedure was utilized for the removal of impurities from this detergent solubilized protein fraction (Figure 3.5 A and B).

![Figure 3.5. Affinity chromatography and SDS gel profiles for detergent solubilized portion of amino-terminal NAA transporter](image)

A. Ni histrap column with the protein (arrow) bound. B. Subtractive affinity chromatography. Protein (arrows) of interest is eluted as the flow through.

SDS and western gel analysis were used to identify the bands of interest. In addition to the purification trials, experiments were simultaneously performed to find the best detergent for extraction of the protein. The samples were treated with detergents at a
concentration of two times their CMC (critical micelle concentration). It was found that ANAMEG-7 (Figure 3.6) a sugar derivative [6-O-(N-heptylcarbamoyl)-methyl-α-D-glucopyranoside] was one of the better detergents for efficient extraction from a total of 17 detergents screened.

Figure 3.6. Plot of detergent type versus the % pixel count* for the amino-terminal NAA transporter. Different classes of detergents were evaluated for solubilization of the membrane associated protein. * [% pixel count (% PC); UN-SCAN-IT gel version 6.1; Silk Scientific; Utah].

3.1.3 Purification and detergent screening of the carboxy-terminal region

The carboxy-terminal transporter was purified in a similar fashion to the N-terminal transporter. As before, the soluble portion was purified by using affinity chromatography. However, upon treatment with TEV protease, a heavy precipitation of the protein was observed which could not be brought back into solution. The time of the cleavage
reaction as well as the ratio between the transporter and protease were varied to overcome this issue, however, no success has yet been achieved. A detergent solubilized portion of the C-terminal region (8 mM dodecylmaltoside) was also loaded on to the affinity column, treated with TEV protease and loaded on to the affinity column again (Figure 3.7 A and B).

![Figure 3.7. Affinity chromatography and SDS gel profiles for detergent solubilized portion of carboxy-terminal NAA transporter A. Ni histag column with the protein (arrow) bound. B. Subtractive affinity chromatography. Protein (arrow) of interest is eluted as the flow through.](image)

The protein eluted from the column was >95% pure and was subjected to crystallization experiments, however, no crystal hits have yet been obtained. Simultaneously, detergent screening was conducted to optimize the extraction of the carboxy terminal part of the transporter, and n-octyl-β-D-glucopyranoside (OG) was found to be among the better detergents (Figure 3.8) for the solubilization of this region of the membrane protein.
3.2 Aspartate N-acetyl transferase (ANAT)

A partially purified form of aspartate N-acetyl transferase, the brain enzyme that synthesizes N-acetyl-L-aspartate was obtained from our collaborators (Dr. Namboodiri, Uniform Military Services Hospital, Bethesda, MD). Based on dynamic light scattering (Figure 3.9 A and B), the primary protein bands in the mixture were centered near 200 kDa, with some higher molecular weight aggregation.
This protein sample was subjected to an anion exchange column (Q sepharose XL) (Figure 3.10) and the fractions obtained were run on a SDS gel (Figure 3.11).
Figure 3.10 Anion exchange chromatography profile of putative ANAT. 280 nm (blue) and 260 (pink) curves are highlighted. The increasing salt gradient is indicated by the green line. P1 is a mixture of proteins separated from the crude ANAT sample. P2 was identified as nucleic acid.

Figure 3.11 SDS gel profiles of putative ANAT fractions. Peak 1 consists of a mixture of proteins 1. the leading edge (F2-F4) contains a major component at ~160 kDa (*) 2. the middle of this peak (F8-F9) contains a major component at ~130 kDa (*) 3. the trailing edge (F12-F18) contains a single protein at ~145 kDa (*).
In conclusion, Peak 1 had been fractionated into several major protein components. The major components in peak 1 each show some transferase activity as measured by a crude thioester assay. These peaks were further fractionated by using HPLC to obtain protein of higher purity for mass spectrometry. While the separation was successful, the amounts obtained were insufficient for determination of the sequence using mass spectrometry. Scale up and further purification will be required to obtain sequence information about ANAT. Meanwhile, focus has been shifted to the cloning of a novel protein with transferase-like activity (NAT-18) which seems to be an extremely promising candidate involved in the formation of NAA.
Chapter 4: Enzyme replacement therapy for Canavan Disease

4.1 Introduction

Several gene replacement therapy trials have been approved for the treatment of infants with defective aspartoacylase\textsuperscript{106,107} and some success has been seen in slowing the relentless progress of this disease. The primary barriers that must be overcome in gene therapy are the successful incorporation of the replacement gene into the patient’s genomic DNA and the expression and control of the protein product. A potential alternative approach that would bypass these issues is direct administration of the active enzyme. Enzyme replacement therapy (ERT) has been approved for the treatment of a number of metabolic disorders as well as some neurological disorders. However, for enzyme replacement therapy to succeed a different set of barriers must be surmounted. Injection of foreign proteins into a patient will cause an immune response, the half-life of injected proteins can be quite short, and the protein must become localized in the cells where the deficiency is manifest to be most effective. In order to overcome the existing challenges and make ERT a viable therapy for the treatment of neurological disorders we must be able to demonstrate that:

1. Surface properties of our target enzymes can be selectively and systematically altered to overcome an immune response
2. Modifications do not adversely affect catalytic activity or enzyme stability
3. Modified enzymes are accumulated to therapeutic levels in neuronal cells.
Surface modifications of proteins can lead to decreased immunogenicity, and the introduction of polyethylene glycol (PEG) polymers through covalent modification of exposed protein functional groups has been shown to decrease the immune response to these modified proteins and increase their circulation half-life\textsuperscript{108}. Recent studies have demonstrated the efficacy of enzyme replacement therapy in an animal model for phenylketonuria, a metabolic disorder caused by genetic mutations. In this disease, phenylalanine does not get metabolized due to mutations in the gene for the key enzyme, phenylalanine hydroxylase. Treatment of recombinant phenylalanine hydroxylases with several PEGs lead to modified forms of these enzymes that retain full catalytic activity and achieve increased stability\textsuperscript{109}. Injection of these PEGylated enzymes into a mouse model with defective phenylalanine hydroxylase results in enhanced \textit{in vivo} catalytic activity, decreased serum phenylalanine levels, and significantly reduced immunogenicity when compared to control studies with the untreated enzyme\textsuperscript{110}. Achieving similar success with aspartoacylase will be required to consider enzyme replacement therapy as a viable approach for the treatment of Canavan disease.

Charged and polar amino acids found on the surface of all soluble proteins provide many of the binding epitopes that are recognized during an immune response to a foreign protein. These functional groups also provide reactive sites that can be selectively modified to alter protein surfaces. Human aspartoacylase contains 23 lysines per monomer, all of which are solvent exposed to varying degrees (Figure 4.1). Modification of these lysines will alter the surface properties of ASPA thereby producing altered forms of the enzyme that should have decreased immunogenicity and increased \textit{in vivo} lifetimes.
In order to carry out an extensive number of PEGylation reactions, large quantities of purified protein are required. To meet these requirements, some fermentation procedures were tested in addition to the traditional ‘shaker flask’ growth strategy used in the laboratory.

4.2 Large scale cell growth

*Pichia pastoris*, a methylotrophic yeast, has been developed into a heterologous protein expression system. *Pichia pastoris* combines many of the benefits of *E. coli* expression with the advantages of expression in a eukaryotic system (e.g. protein processing, folding, and posttranslational modifications). *Pichia pastoris* is capable of metabolizing methanol as the sole carbon source. The first step in the metabolism of methanol is the oxidation of methanol to formaldehyde by the enzyme, alcohol oxidase.

Figure 4.1 Surface representation of the structure of human aspartoacylase (PDB ID: 1O4H). The positively charged surface lysyl side chains are shown in blue.
Expression of this enzyme, coded for by the AOX1 gene, is tightly regulated and induced by methanol to very high levels of the total soluble protein in the cells. To achieve higher levels of protein production, an older model of the fermentation equipment (VIRTI Culture, Figure 4.2 A) was tested. A growth procedure (Invitrogen) for 10 L culture was followed and antifoam C (Sigma) was used to control the overproduction of foam due to agitation. Methanol was added at a minimum allowed rate of 1.5 ml/minute for overexpression of protein. The amount of dissolved oxygen is a key indicator necessary to control the transition from the glycerol to the methanol media. Unfortunately, no protein expression was observed using this equipment. A primary reason for the failure of the experiment is attributed to the lack of assessment of the amount of dissolved oxygen during fermentation. Hence, another fermentation apparatus (Figure 4.2 B) with superior controls for solvent addition as well as pH and dissolved oxygen levels was used.

Figure 4.2 Fermentation apparatus A: Virti culture (maximum capacity of 12 L) B: Bioflo 110 with probes for pH and finer solvent delivery system (maximum capacity of 5 L).
Various parameters including pH, cell density and dissolved oxygen (Table 4.1) were monitored

**Table 4.1: Change in parameters during fermentation.**
Dissolved oxygen levels to 100% indicated the start of induction with methanol

<table>
<thead>
<tr>
<th>Days</th>
<th>pH</th>
<th>OD 600</th>
<th>Dissolved oxygen (DO)</th>
<th>Supplies (rate of addition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.02</td>
<td>1.0</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3.28</td>
<td>2.0</td>
<td>100</td>
<td>100% Glycerol (0.7 ml/min)</td>
</tr>
<tr>
<td>3</td>
<td>3.11</td>
<td>5.5</td>
<td>21</td>
<td>100% Glycerol (0.7 ml/min)</td>
</tr>
<tr>
<td>4</td>
<td>3.07</td>
<td>8.5</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>3.1</td>
<td>9</td>
<td>80</td>
<td>a.100% methanol (0.007 ml/min)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>b. 12 hrs + 100 % methanol (0.28 ml/min)</td>
</tr>
<tr>
<td>6</td>
<td>3.1</td>
<td>9</td>
<td>30</td>
<td>100% methanol (0.56 ml/min)</td>
</tr>
<tr>
<td>8</td>
<td>3.2</td>
<td>9.2</td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

Cell paste yield of 75 g per 5L was observed. Protein was overexpressed (Figure 4.3) however the yields were comparable to the ‘shaker flask’ procedure [Protein yields (shaker flask): 3-4 mg/15 g cell paste; Protein yields (fermentation): 3.2 mg/15 mg cell paste]. Upon analysis, the fermentation procedure has only one advantage over the ‘shaker flask’ procedure. It is a ‘single pot’ expression system and dissolved oxygen levels will indicate the time of addition of methanol to the growth media. In the shaker flask method, glycerol has to be completely removed from the media before addition of methanol media typically after a set number of hours (Invitrogen).
4.3 PEGylation of aspartoacylase

In this study, we have examined the effect of PEGylation on the catalytic activity and the protein stability of aspartoacylase. The following parameters have been tested in different permutation and combinations:

1. Type of PEG (Variation in the type of linker and activating group)
2. Molecular weight of PEG
3. Incubation time
4. Ratio of PEG to enzyme
5. Temperature

PEGs of highest purity were purchased from NOF Corporation (Japan). These PEGs varied in the type of linker (Figure 4.4; A and B) as well as the nature of the activating group (Figure 4.4; C). The molecular weight of the PEGs ranged from 2 kDa to 40 kDa.

![Figure 4.4 Structures of activated PEGs](image)

**Figure 4.4 Structures of activated PEGs** A. PEG with N-succinimidyl as the activating group (black) and carboxymethyl linker (blue) B. PEG with succinimidyl activating group (black) and succinyl linker (blue). C. PEG with an aldehyde (black) activating group.

The reactions were set up at temperatures from 4 ºC to 37 ºC for a duration of 0 min to 7 days. In order to achieve a reasonable degree of lysine modifications for the PEGylation reactions, ratios of PEGs to the enzyme were varied anywhere from 1:2 to 1:64 mole ratio. The treated samples are concentrated using a 30 kDa concentrator to remove the excess PEG. 75 kDa cutoff concentrators were used for removing excess PEG in case of 40 kDa PEGs. After completion of the reaction, followed by several buffer exchanges, the modified sample is concentrated to 1 mg/mL in a buffer containing 50 mM Hepes, pH 7.5, 1 mM DTT, 0.1 M NaCl.
The best temperature for all the PEGylation reactions was found to be 20 °C. Extremes temperatures such as 37 °C resulted in heavy precipitation of the protein within a few hours. Lower temperatures including 4 °C resulted in very slow reaction rates. The catalytic activity of the PEGylated forms of aspartoacylase was measured by using the coupled assay, and the values obtained have been compared with the untreated native enzyme. ASPA is quite sensitive to oxidation and some variability in specific activity is observed depending on the age of the purified enzyme. Despite this variability many of the PEGylated forms of ASPA tested have shown activities similar to the native enzyme (Table 4.2). Hence, the modification of these surface lysyl residues does not have any significant effect on the catalytic activity of the enzyme. The enzyme activity of all the PEGylated ASPA samples was monitored for 72 hours without substantial loss in activity. PEGs with aldehyde as the activating group were preferably used for the studies in comparison to the PEG with an N-succinimide activating group especially in the case of 5 kDa-PEG. An aldehyde activating group conserved the catalytic activity to a greater extent and is presumably less disruptive. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated the successful PEGylation of ASPA, with the different ASPA: PEG reaction ratios generating different distributions of the PEGylated species.
In most cases reactions with these PEG reagents have been shown to modify multiple surface lysine residues of ASPA. Initial experiments have shown that treatment with linear 5 kDa PEG gave a heterogeneous protein mixture where distinct bands (denoted by arrows) can be observed corresponding to the modification of approximately
3, 5 and 7 lysines per monomer, with the higher molecular weight (MW) bands suggesting the modification of as many as 10 lysines per monomer (Figure 4.5, lane 3). Treatment with the higher MW branched PEG caused a significant increase in the mass of ASPA due to the modification of several lysines, such that the enzyme is observed primarily in the loading wells of the gel (Figure 4.5, lanes 4 & 5).

![Native gel profile of PEGylated samples of aspartoacylase.](image)

**Figure 4.5.** Native gel profile of PEGylated samples of aspartoacylase. Lane 1. Molecular weight marker Lane 2. Aspartoacylase (ASPA), monomer (M) and dimer (D) Lane 3. ASPA: PEG-5 kDa (1:4 ratio) Lane 4. ASPA: PEG-40 kDa (1:2); Lane 5. ASPA: PEG-40 kDa (1:8).

Treatment with 2 kDa PEG resulted in a heterogeneous species where distinct bands can be observed for the modification of 5 and 10 lysines per monomer as well as approximately 10 lysines per dimer (Figure 4.6, Lane 2). A completely modified single
band can also be observed at lower ASPA to PEG ratio corresponding to at least 20 lysines per dimer of ASPA (Figure 4.6, Lane 3). Addition of excess lysine to the protein prior to the addition of the activated PEG helped in quenching the reaction at zero time and enabled a time dependent modification study (Figure 4.6, Lane 4-8) of ASPA with 5 kDa PEG. Treatment with linear 5 kDa PEG gave a mixture along with some unmodified ASPA (Figure 4.6, Lane 8). Bands correspond to the modification of approximately 4 and 8 lysines per ASPA monomer, with a higher MW band suggesting

Figure 4.6: SDS gel profile of PEGylated forms of aspartoacylase.
1. Molecular weight marker 2. 8 hr ASPA: PEG-2 kDa (1:24) 3. 24 hr ASPA: PEG-2kDa (1: 12) 4. 0 hr ASPA: PEG-5 kDa (1: 24) 5. 3 hr ASPA: PEG-5 kDa (1: 24) 6. 6 hr ASPA: PEG-5 kDa (1: 24) 7. 9 hr ASPA: PEG-5 kDa (1: 24) 8. 12 hr ASPA: PEG-5 kDa (1: 24) 9. 32 hr ASPA; PEG-5 kDa (1: 24)
the modification of as many as 10 lysines per monomer (Figure 4.6, Lane 8). Low resolution anion exchange chromatography was performed for separation of the individual PEGylated species into separate fractions based on charge. Samples were separated from the excess PEG and none of the PEGylated species were separated from each other. A higher resolution column will be tested to carry out the separation. In addition, the reactions carried out for longer duration for 5 kDa-PEG resulted in removal of >98% (Figure 4.6, Lane 9) of the unmodified species.

4.4 Treatment of Canavan mice with modified aspartoacylases

4.4.1 Tissue preparation

Canavan mice in which the ASPA gene was knocked out\(^\text{112}\) were treated with several forms of aspartoacylase to measure the \textit{in vivo} levels of catalytic activity achieved in target tissues after injection. The mice kidney tissues were cut and homogenized in 50 mM Tris, pH 8.0 using a tissue grinder (Kontes Glass Co.). The homogenate was sonicated and centrifuged to obtain a clear supernatant. Analysis of the brain tissues from the mice was performed by making an acetone powder after sonication of the tissues. 8 volumes of cold acetone on dry ice (with alcohol) mixed with the homogenate and allowed to stand for 30 minutes. The supernatant was removed and the pellet was dried to remove traces of acetone. Water was then added to the pellet and the pH of the solution was adjusted to value of 8.0. Activity was measured by using 10 \(\mu\)g of total protein in the coupled assay.

4.4.2 Tissue enzyme activity levels

Enhancement in catalytic activity was observed in both kidney and brain tissue samples compared to the level observed in the untreated mice (Table 4.3). Tissues from
the mice treated with PEGylated enzyme showed a several-fold increase in catalytic activity, indicative of the modified aspartoacylase reaching the target tissues and also the accumulation of the enzyme in these tissues after intraperitoneal (i. p.) injection. These preliminary studies show that ASPA modified with branched, higher molecular weight PEG caused the greatest increase (3-fold) in aspartoacylase activity in brain tissue, which is the primary site that is affected in Canavan disease.

<table>
<thead>
<tr>
<th>treatment</th>
<th>kidney tissue</th>
<th>brain tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity (U/mg)</td>
<td>fold increase</td>
</tr>
<tr>
<td>control</td>
<td>0.065</td>
<td>---</td>
</tr>
<tr>
<td>native ASPA</td>
<td>0.156</td>
<td>2.4</td>
</tr>
<tr>
<td>PEGylated-ASPA (40 kDa)</td>
<td>0.181</td>
<td>2.8</td>
</tr>
<tr>
<td>PEGylated-ASPA (5 kDa)</td>
<td>0.166</td>
<td>2.6</td>
</tr>
</tbody>
</table>

These results are encouraging, however, they have to be confirmed by direct evidence of the presence of the PEGylated aspartoacylase in the mice brains. Hence, fluorescein-tagged activated 5 kDa PEG has been used to PEGylate aspartoacylase and the modified enzyme will be shipped to our collaborators (Dr. Namboodiri, Bethesda) for brain imaging studies. Simultaneously, dose response studies are currently being carried out using 5 kDa and 2 kDa modified aspartoacylase by our collaborators (Dr. Matalon, University of Texas Medical branch, Galveston) using their mouse model of Canavan disease.
4.5 Conclusions

In conclusion, we have established the optimal conditions to produce modified forms of aspartoacylase with 2 kDa and 5 kDa PEGs. Preliminary results have shown that treatment of a mouse model of Canavan disease with PEGylated aspartoacylase resulted in enhanced enzyme activity levels in both kidney and brain tissues. At the end of the current mouse studies, we should be able to get dose response curves for administration of functional aspartoacylase as well as answers to the effects of the modifications on the phenotype and protein immunogenicity in the mouse model for Canavan disease.
Chapter 5: Development of pharmacological chaperones for the treatment of Canavan disease

5.1. Introduction

Canavan disease is caused by defects in the *aspA* gene that encodes for the enzyme aspartoacylase. These mutations result in a loss of catalytic activity, leading to an accumulation of N-acetyl-L-aspartate and a decrease in the production of acetate that is required for fatty acid biosynthesis in neuronal cells. Subsequent demyelination leads to the hallmark symptoms of this fatal neurological disorder.

![Figure 5.1 Monomer of aspartoacylase with clinical sites highlighted.](image) Total of 25 most prevalent clinical sites are highlighted. 5 sites/residues (blue) are directly involved in catalysis and substrate binding. The rest of the sites (red) are distributed all over the protein surface.
Only a few of these mutations have been mapped to amino acid positions that play essential roles in either substrate or metal ion binding in aspartoacylase (Figure 5.1; blue surface). Most of the commonly observed clinical mutations are distributed throughout the protein structure (Figure 5.1; red surface), with many at sites that are well removed from the active site of this enzyme. It is likely that the loss of activity seen in aspartoacylase from Canavan patients is due to a disruption of the structural integrity of this enzyme caused by these mutations. It is hypothesized that mutations that disrupt important structural interactions will cause destabilization of the protein leading to misfolding, diminished activity and shortened lifetimes.

Several other neurological disorders, including Alzheimer's disease, occur due to misfolding of proteins such as microtubule associated protein tau as well as amyloid β peptide. In genetic diseases such as Gaucher disease the onset of the disease is caused by the loss of catalytic activity as a consequence of the misfolding of mutant forms of a critical enzyme, acid-β-glucosidase. Pharmacological chaperones constitute a promising new treatment strategy for diseases of protein misfolding and mistrafficking. The mechanism by which pharmacological chaperones function is not known, but two hypotheses can be proposed. The structure of the folded mutant enzyme that retains partial catalytic activity is likely to be very similar to the native, wild-type structure. Thus, pharmacological chaperones might bind to a fully folded mutant protein target to stabilize a native-like conformation. Alternatively, mutations may affect the folding pathway by stabilizing the non-native less stable intermediate state. In this case, pharmacological chaperone may bind to a non-native conformation and accelerate the transition to the native state.
Both experimental and computational approaches have been used to identify potential binding sites for pharmacological chaperones on target proteins. High resolution NMR and X-ray crystallography of β-glucosidase\textsuperscript{121} and the tumor suppressor P53\textsuperscript{122} have shown stabilization of mutant forms of these proteins in the presence of potential small molecule chaperones. These experimental approaches have also been used with probe molecules to locate new binding sites on various proteins\textsuperscript{123}.

5.2. Computational studies

The surface of the apo form of aspartoacylase was examined by using the FTMAP algorithm (www.ftmap.bu.edu\textsuperscript{124}) to locate the 12 most likely cluster sites (Figure 5.2) for the binding of small probe molecules.

![Figure 5.2. Binding sites of probes molecules with aspartoacylase. Small probe molecules (acetaldehyde, urea, acetamide, acetonitrile, dimethylether, cyclohexane, ethanol, acetone, benzene, isobutanol, ethane, phenol, isopropanol, methylamine, N,N-dimethylformamide, benzaldehyde) are docked at different sites (sticks) of human aspartoacylase.](image)

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A set of 16 different probe molecules was used to identify sites for the binding of small molecules that can potentially stabilize the structure of clinical aspartoacylase mutants. Not surprisingly, the highest ranked site is in the active site cleft. This is a potentially useful site since the binding of a designed high affinity pharmacological chaperone at the active site could stabilize mutant forms of the enzyme in Canavan patients. However, occupying this site for protein stabilization would require the substrate to displace the chaperone before catalysis could occur. The next highest ranked binding site is located about 4.5 Å from the active site and is predicted to bind 13 of the set of probe molecules (Figure 5.3). This site contains three amino acid positions that are found to be mutated in Canavan patients, leading to enzyme forms with little or no expressed catalytic activity\textsuperscript{125-128}.

\textbf{Figure 5.3 Binding site of probe molecules at a distance of 4.5 Å from the active site} 13 small molecules (acetaldehyde, acetamide, acetonitrile, dimethylether, cyclohexane, ethanol, acetone, benzene, isobutanol, phenol, isopropanol, N, N-dimethylformamide, benzaldehyde) are docked at this site with three clinical mutation (R168C, D68A, H21P) identified.
However, His21 in this site has been shown to function as a binding ligand for the catalytically essential zinc and Arg168 was found to participate in binding the carboxylate group of the substrate$^{41}$. So, as with active site binding, it is likely that the binding of

![Figure 5.4 Binding pocket for the docked probe molecules with the clinically relevant residues](image)

84 probe molecules (acetaldehyde, acetamide, acetonitrile, urea, methylamine, ethanol, dimethylether, ethane) were bound at the site. All possible interactions have been highlighted (black) along with the corresponding residues.
stabilizing molecules at this site would also interfere with catalytic function. The third ranked site is predicted to bind 8 of the 16 probe molecules, with several of these binding in multiple orientations (Figure 5.4).

This probe cluster is located in a predominantly hydrophobic pocket. The only hydrophilic residues that directly interact with the probe cluster are through backbone carbonyl and amide groups. More importantly, this site is found in a region of the protein structure that also contains two positions that are mutated in Canavan patients. The side chain sulfhydryl group of Cys152 has been proposed to play a significant role in maintaining the enzyme in a conformationally active state\textsuperscript{129} and several different clinical mutations at this position each lead to a defective enzyme\textsuperscript{130}.

![Figure 5.5 C152 binding pocket of aspartoacylase.](image)
Structural evidence indicates a number of key electrostatic and hydrogen bond interactions between Cys 152 and neighboring functional groups (Figure 5.5 A) that would be disrupted upon mutation of this critical residue. One of the clinical mutants at this position, C152W, causes rapid onset of symptoms at an unusually early age\textsuperscript{127} suggesting a more aggressive form of Canavan disease and verifying the importance of this position in maintaining a functional enzyme.

An I143F mutant identified in Guatemalian patients\textsuperscript{127} and an I143T mutation found in patients of Japanese origin is also located at a position within this putative binding site. Most of the residues in close proximity to I143 are hydrophobic in nature, with exception of the side chain of C152 (Figure 5.5 B). Changing I143 either by introduction of a bulky

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**Figure 5.6 I143 binding pocket of aspartoacylase.**

An I143F mutant identified in Guatemalian patients\textsuperscript{127} and an I143T mutation found in patients of Japanese origin is also located at a position within this putative binding site. Most of the residues in close proximity to I143 are hydrophobic in nature, with exception of the side chain of C152 (Figure 5.5 B). Changing I143 either by introduction of a bulky
residue (phenylalanine) or a polar residue (threonine), will perturb the necessary stabilizing interactions within this pocket.

While these mutations at this site lead to impairments in the catalytic function of aspartoacylase neither of these amino acids have been shown to play direct roles either in substrate binding or in catalysis. It is therefore likely that these mutations affect the structural integrity of the enzyme, an effect that could potentially be overcome through the use of appropriately designed stabilizing molecules that can bind at this site.

5.3 Biochemical characterization of clinical mutants at these sites

5.3.1 Site directed mutagenesis

Quick change II site directed mutagenesis kit (Stratagene) was used to generate point mutations for I143 (I143T, I143A) and C152 (C152W, C152Y, C152S, C152A). Mutations were confirmed by DNA sequencing (MWG Biotech.). Two additional mutants, C152R and I143F, are currently being generated and will be subsequently characterized. Each mutant was linearized with SacI in order to insert the gene into the yeast (Pichia pastoris; KM71H) genomic DNA by homologous recombination. 1% methanol was used for overexpressing the protein. Each mutant was purified for the thermodynamic analysis using affinity as well as anion exchange chromatography. Thermodynamic data as well as preliminary kinetic data were recorded for each mutant enzyme.

5.3.2 Differential scanning calorimetry

The stability of native and mutant forms of aspartoacylase was measured by differential scanning calorimetry (DSC) using a Microcal model VP-DSC instrument. The protein was dialyzed overnight into 50 mM Hepes pH 7.5 and 1 mM TCEP. The
protein and the buffer solution were degassed for 30 minutes at 20 °C. Buffer was initially placed in both the control as well as the sample cell to achieve a stable baseline and labelled as the control curve. 0.5 mL of 1.5 mg/mL protein solution was then placed in the sample cell with a similar volume of buffer in the control cell. The scan was run from 20 °C to 90 °C. The curve obtained for the sample run are subtracted from the control curve followed by normalization of the resultant data for protein concentration. All the data are processed in the Origin Version 5.0 software to obtain the unfolding temperature.

Preliminary kinetic analysis was recorded and data is summarized (Table 5.1).

**Table 5.1 Survey of kinetic data**

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Specific Activity (U/mg)</th>
<th>% Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>5-10</td>
<td>100</td>
</tr>
<tr>
<td>I143T</td>
<td>1.2</td>
<td>20</td>
</tr>
<tr>
<td>I143A</td>
<td>1.6</td>
<td>32</td>
</tr>
<tr>
<td>C152W</td>
<td>0.07</td>
<td>2</td>
</tr>
<tr>
<td>C152Y</td>
<td>0.04</td>
<td>1</td>
</tr>
<tr>
<td>C152S</td>
<td>2.3</td>
<td>45</td>
</tr>
<tr>
<td>C152A</td>
<td>0.5</td>
<td>10</td>
</tr>
</tbody>
</table>

As expected these mutants were each found to have lower catalytic activity, with the most active possessing less than 50% of the native aspartoacylase activity (Table 5.1).

Differential scanning calorimetric data for all the mutants has been collected and it is being analyzed currently. In addition, urea unfolding experiments are carried out to provide additional support for this hypothesis.
5.4 Identification of pharmacological chaperones

Two different approaches were used to identify potential chaperones for the unstable mutants.

1. Mutants were treated with the theoretically (docked) identified chaperones
2. High-throughput screening of a library of small molecule compound

In the first approach, cell paste of the mutants has been aliquoted to small amounts (typically 5 g) and opened using a vortexing procedure in the presence of the theoretically identified chaperones. The presence of the chaperone in the lysis buffer should ensure that the presumably non-native conformation of the protein is immediately stabilized by these chaperones. The chaperone concentration is kept constant for subsequent steps of purification in order to ensure that the protein remains in its properly folded state. In the second approach, the mutants are opened in a ‘non-chaperone stabilized’ environment and then purified. The mutant is subsequently screened for potential activators using the coupled kinetic assay. The screens tested in this approach consist of small molecules with a variety of functionalities (Heterocycles, sugars, nucleobases, benzene derivatives, amino acids) and has been assembled by the principal investigator (Dr. R. E. Viola) of the laboratory.

Based on preliminary screening of 384 compounds, one compound, 4-methylcyclohexanol enhanced the activity of the I143T by more than 3 times [I143T: 1.1*10^{-3} units/ml; I143T+chaperone: 3.6*10^{-3} units/ml; Figure 5.8, A). The time course of these reactions were followed at 240 nm.
These studies are very promising and further experiments are underway to find additional chaperones to stabilize the clinical mutants. Through differential scanning calorimetric and urea folding studies we hope to demonstrate that a non-native conformation of the clinical mutants of aspartoacylase is one of the key causes of the loss of activity of this enzyme. Since the natural protein chaperones found in the human body are not apparently able to correct this misfolded state, there is a need for external intervention by addition of chemical agents (pharmacological chaperones) that have the potential to recover catalytic activity, which can hopefully slow or even reverse the symptoms of Canavan disease.

Figure 5.8 Kinetic graph for clinical mutant of aspartoacylase with chemical chaperone. Absorbance is plotted versus time (seconds): (1) Clinical mutant, I143T with 5 mM 4-methylcyclohexanol (2) I143T only.
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