A Thesis

entitled

High Resolution Structural Studies of vc-ASADH

By

Nina M. Potente

Submitted to the Graduate Faculty as partial fulfillment of the requirements for

The Masters of Science degree in Chemistry

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College of Graduate Studies

The University of Toledo

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An Abstract of
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The aspartate biosynthetic pathway is essential for many biological functions, including
the production of four naturally occurring amino acids. Since this pathway is only present
in plants and microbes, disruption of this pathway can potentially be fatal to these
organisms. L-aspartate-β-semialdehyde dehydrogenase (ASADH) plays an essential role
in the aspartate biosynthetic pathway and ASADH structures have been determined from
both gram positive and gram negative bacteria. In order to identify new selective
inhibitors of ASADH both soluble and organic fragment libraries are being screened. As
new inhibitors are identified, structural studies are being conducted with ASADHs from
representative bacterial species. Hanging drop crystallization experiments have produced
crystals of the ternary enzyme-inhibitor complexes, consisting of *Vibrio cholerae*
ASADH (vc-ASADH), the cofactor (NADP or ADP), and the respective inhibitor. X-ray
diffraction studies of the optimized crystals were run at the Ohio Crystallographic
Consortium (OCC) and at the Advanced Photon Source at Argonne National Laboratory,
to identify electron densities that will determine the mode of inhibitor binding to the
enzyme. This structural information will be used to design new inhibitors with higher potency and greater selectivity.
Acknowledgements

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>2’,5’-ADP</td>
<td>2’,5’-Adenosine diphosphate</td>
</tr>
<tr>
<td>ASADH</td>
<td>β-aspartate-semialdehyde-dehydrogenase</td>
</tr>
<tr>
<td>ASA</td>
<td>Aspartate semialdehyde</td>
</tr>
<tr>
<td>APS</td>
<td>Advanced Photon Source</td>
</tr>
<tr>
<td>Ca</td>
<td><em>Candida albicans</em></td>
</tr>
<tr>
<td>CHES</td>
<td>N-Cyclo-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>KPi</td>
<td>Inorganic Phosphate</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>NADP</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical Density at 600 nm</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethyleneglycol</td>
</tr>
<tr>
<td>SMCS</td>
<td>S-methyl-L-cysteine sulfoxide</td>
</tr>
</tbody>
</table>
SDS-PAGE  Sodium Dodecyl Sulfate - Polyacrylamide gel electrophoresis

Sp  Streptococcus pneumoniae

UV-VIS  Ultra violet and Visible

vc  Vibrio Cholerea
Chapter 1: Introduction

1.1 Aspartate Biosynthetic Pathway

The aspartate biosynthetic pathway is present in almost all plants and microbes. A number of enzymes are present within this pathway for the production of at least one-fourth of the essential amino acids needed in mammals. These amino acids include lysine, methionine, threonine and isoleucine. These enzymes have specific functions within the pathway so regulation of catalysis can occur. Below is the pathway scheme which shows the key enzymes and the role that they play in the overall pathway.

Figure 1: Aspartate biosynthetic pathway scheme. Each arrow indicates a step in the synthesis to produce the amino acids.
Aspartokinase is the initial enzyme used to catalyze the phosphorylation of aspartate. Depending on the organism, from one to three different isoforms of this enzyme are present, and these enzymes are either bifunctional or monofunctional. The second enzyme in the pathway is β-aspartate-semialdehyde-dehydrogenase (ASADH). This enzyme catalyzes the reductive dephosphorylation of β-aspartyl phosphate to aspartate-β-semialdehyde. This particular step produces a metabolite that can go in two different directions in the pathway. One route is the synthesis of dihydrodipicolinate which leads to the production of lysine while the other route leads to the synthesis of homoserine. The next enzymes in this branch are homoserine dehydrogenase and homoserine kinase which eventually lead to the production of methionine and isoleucine. The aspartate biosynthetic pathway is regulated by inhibition of these specific enzymes through the levels of the end product amino acids. Since the pathway is not present in mammals, the enzymes within it provide potential drug targets for the development of new herbicides and biocides. Inhibition of these enzymes will prevent the production of the essential amino acids. Our studies have focused on the enzyme ASADH since it is present in the first branch point within the aspartate biosynthetic pathway.

1.2 Aspartate-β-semialdehyde Dehydrogenase

As stated above, aspartate-β-semialdehyde dehydrogenase (ASADH) is the first branch point that leads to the synthesis of the four essential amino acids and also to diaminopimelate which is a cell wall precursor. ASADH catalyzes the conversion of β-
aspartyl phosphate to aspartate-β-semialdehyde. The reaction is described in Figure 2, and the proposed mechanism for the reaction is described in Figure 3.

![Figure 2: Catalyzed reaction of ASADH]

![Figure 3: Mechanism of ASADH in non-physiological direction]
In the reaction the cysteine nucleophile in ASADH attacks the β-aspartyl phosphate to produce an acyl intermediate. With the help of the cofactor, NADP, a hydride transfer takes place to produce aspartate-β-semialdehyde. In this mechanism, it was proposed that three residues of ASADH play main roles in catalysis of the reaction, Histidine 277, Cysteine 136, and Glutamine 243. The mechanism was proposed to have three specific steps. In step A, a hemithioacetal between the enzyme and the aspartyl phosphate is produced through the acid/base catalyst histidine and nucleophilic attack on the aspartyl phosphate by the cysteine. In step B, by way of the cofactor NADP, a hydride transfer occurs thus producing the thioester intermediate. In step C, nucleophilic attack of the phosphate ion occurs on the carbonyl carbon produces a second intermediate, stabilized by an active site water molecule. Collapse of this intermediate released the aspartyl phosphate product.

There are a number of residues which play a role in the binding of the substrate, ASA and the phosphate. These residues include, Cys 136, His 277, Arg 270, Asn 135, Arg 103, and Lys 246. As for stabilization within the active site, water molecules play a huge role as well as the residue Gly 357.

ASADH from *E. coli* and is a homodimer with each subunit having a molecular weight of 38 KDa and consisting of 367 residues. In the past our lab has studied and solved numerous species of ASADH and it has been determined that the active site is similar in all of these species.
1.2.1 \( vc\)-ASADH

The structure of the *Vibrio cholerae* species of ASADH was solved by Blanco et al. in 2002.\(^5\) *Vibrio cholerae* is the caustic agent of cholera, a severe diarrheal disease which can ultimately lead to death.\(^5\) The structure of this ASADH was solved as both the apo-enzyme structure and as the ternary complex which included the cofactor NADP and the covalently bound inhibitor S-methyl-L-cysteine sulfoxide (SMCS) which has been determined to inactivate the active site cysteine. The apo and ternary complex structures had a resolution of 2.77 Å and 1.84 Å, respectively. \( vc\)-ASADH is also a homodimer and each monomer has a molecular weight of 37.4 KDa.\(^5\) When the cofactor is bound, a conformational change in the active site occurs which induced tighter binding of the amino acid substrate and potential inhibitors within the active site.\(^3\)
Figure 4: Ribbon structure of vc-ASADH with both NADP and SMCS (boxed in green and circled in red respectively). (Regenerated in Pymol)

In Figure 5, the previously solved structure of vc-ASADH is shown with bound NADP and the covalently bound inhibitor, SMCS. It has been determined that the N-terminal provides the NADP binding site while the C-terminal is the substrate binding site. Electron density can be seen for both NADP and SMCS.
Figure 5: Active site of vc-ASADH with the covalent bound inhibitor SMCS.\textsuperscript{5}

In Figure 6 the active site of ASADH is shown along with the bound SMCS and the NADP cofactor. Residues within the active site provide the interactions needed for strong binding interactions for this inhibitor. Along with these previously mentioned, other residues are also prominent in the active site and play important binding roles. These active site amino acids are: Arg 267, Gln 161, Glu 240, Lys 243, and Arg 101. In previous studies mutations of these specific residues result in deactivation of the catalyst and reduced substrate binding thus proving that these residues are crucial for the catalytic activity.\textsuperscript{5,6} The residues within vc-ASADH active site are identical to those in the other
forms of ASADH. $Vc$-ASADH has a sequence identity of 66% and a similarity of 80% with $E. coli$ ASADH. By using different species of ASADH, we may be able to distinguish the different binding abilities between the different species of ASADH

1.3 Fragment Screening

Fragment screening is a viable way to determine binding sites of molecules to a target enzyme. Fragment screening gives a set of lower molecular weight compounds, typically with weaker binding affinities compared to other screening techniques. However, by using fragments it gives a greater probability of binding to the specific residues within the protein structure. Once the selected fragments have been well understood in their binding potential, buildup of these fragments may be achieved with the increased molecular weight and corresponding lower $K_i$ values needed for small molecule binding and to move on to the next step of drug discovery.

1.4 Structural and Kinetic Studies

In our studies we are using fragment screening libraries to find potential binding inhibitors for the enzyme ASADH. With our knowledge of the ASADH binding site, fragment screening can be done with the guidance of kinetic studies leading to structural studies. In the past, fragment studies by x-ray crystallography seemed like a daunting task because of the need to produce well diffracting crystals for a number of different fragment complexes. However, by using kinetic studies as a guide, crystal screening can
be more focused thus making the task more feasible. While kinetic studies will determine if the fragments bind and inhibit the enzyme, structural studies are needed to give a better idea of how these fragments bind and what interactions are occurring. Both techniques will be used for the production of stronger binding molecules.\textsuperscript{8,9}

In our studies, we are using the \textit{Vibrio cholerae} form of ASADH as a model for structural studies to determine the potential inhibitors for this enzyme. Our goal is to identify inhibitors that are specific to this form of this enzyme that can potentially be developed into lead compounds. We also would like to compare the differences and similarities certain fragments have in binding to \textit{vc}-ASADH with respect to other forms of the enzyme.
Chapter 2: Materials and Methods

2.1 Materials

The *Vibrio cholerae asd* gene was purchased from the American Type Culture Collection. The pet41a vector and the BL21(DE3) cell line was purchased from Novagen. The mini prep kits to produce the plasmid were purchased from Qiagen. LB media, EDTA, KPi, KCl, \((\text{NH}_4)_2\text{SO}_4\), NaCitrate, and NaOAc were purchased from Fisher Scientific. The antibiotic, kanamycin was purchased from usb. IPTG and DTT were from Gold Bio Technologies. Filter devices were purchased from Corning (0.8 µm), Amicron Bioseparations (10,000 MWCO), and Whatman (0.2 µm). As for crystallization studies, the ethylene glycol, PEG 3350, and 24 well Limbro plates were all purchased from Hampton Research. The SDS-PAGE materials (gels, sample buffer, and molecular weight marker) were purchased from Invitrogen.

The inhibitor compounds screened were obtained from two different fragment libraries, a water-soluble and a DMSO-soluble library. Each library consisted of 384 compounds that consisted of four different groups of 96 compounds. The water soluble library groups consisted of amino acids, sugars & bases, rings & functional groups, and metabolites & analogs. The DMSO library groups consisted of 5-membered heterocyclics, 6-membered heterocyclics, multiple rings & cyclic structures, and benzene derivatives.
2.2 Expression and Purification of vc-ASADH

As described below, the *asd*-containing plasmid was transformed into an *E. coli* cell line and grown in LB media. Enzyme purification required a two step purification process using an automated Akta Explorer chromatography system. An SDS-PAGE was run to determine purity.

2.2.1 Transformation and Cell Growth

The *vc*-ASADH plasmid transformation was done by using an *E. coli* cell line BL21 (DE3). Colonies were selected from an agar plate grown in the presence of 30 mg/ml of Kanamyacin. Cells were grown in LB broth inoculated with an overnight starter culture into four- one liter Tunair flasks in the presence of 30 mg/ml of kanamyacin. Cultures were grown in an incubator/ shaker at 37 °C until the OD\textsubscript{600} was approximately 0.7. 1 mM of IPTG was added and the growth continued for another 4 hours or until the cell density became stationary. The cells were then centrifuged and the cell paste harvested and stored at -80 °C for further use.

The cell paste was thawed in a buffer containing 10 mM HEPES, pH 7.0, 1 mM EDTA, and 1 mM DTT (Buffer A) at 20 mL per 1 g of cell paste. Once thawed, a sonication program was run for 5 min at 30 seconds on and 2 minutes off at 5 kpa. The cell paste was then centrifuged at 11,000 rpm for 25 minutes at 4 °C. The supernatant was extracted and filtered with a 0.8 µM syringe filter.
2.2.2 Purification

Enzyme purification consisted of a two step process. First, the protein was loaded on a Q-sepharose XL ion exchange using the lysis buffer (buffer A) and then eluted with a linear gradient from 0% to 100% buffer B containing 25 mM KPi with 1.0 M of KCl. After the first purification step, the protein fractions were pooled and ammonium sulfate was added incrementally up to 1.2M to prevent precipitation of the protein and allowed to stir for 1 hr on ice. The fractions were then centrifuged at 12,000 rpm for 20 minutes and filtered with a 0.2 µM syringe. The second purification step used a Phenyl Sepharose hydrophobic column in which buffer C consisted of buffer A plus 1.2 M ammonium sulfate. The column was eluted with a gradient from 0% to 100% Buffer A leading to a decreasing ammonium sulfate salt gradient and the resulting protein fractions were pooled. Previous purifications of the enzyme had required additional steps, however after the second purification step an SDS-PAGE analysis showed that the protein was pure enough for crystallization studies.

2.2.3 Specific Activity and Total Protein

Throughout the purification process the specific activity and total protein were determined from the crude sample and at each purification step. The protein concentration was determined by using a Bradford assay. The specific activity was measured by using a Cary 50 (Varian) UV-VIS spectrophotometer. The assay buffer consisted of 120 mM CHES, pH 8.6, 200 mM KCl, 40 mM KPi, aspartate semialdehyde
was present at 0.4 mM, and NADP was present at 0.25 mM. The activity was determined at 340 nm by measuring the appearance of NADPH.

2.2.4 Dialysis and Protein Concentration of vc-ASADH

The purified enzyme was dialyzed into a storage buffer composed of 10 mM HEPES, pH 7.0, 1 mM EDTA, and 1 mM DTT. After dialysis, the protein was concentrated by using a Micron centrifugal filter device with a 10,000 MWCO. The protein was then centrifuged at 4 °C and the final concentration was determined by a Bradford assay.

2.3 Crystallization of vc-ASADH

Once the protein was purified the protein complex solutions were prepared using a cofactor and inhibitor and buffer to make up the necessary volume. The solutions were then filtered and centrifuged. 24 well Limbro plates were prepared with grease so that a good seal was achieved between the plate and the siliconized glass slips. The protein was placed on the glass slips using an automated pipette to ensure the proper amount and no bubbles. The reservoir solution was then added to the drop. The glass slips were placed upside down in their respective wells and then sealed so that the drops would not dry out. The plates were then placed in a 20 °C incubator and left undisturbed to allow crystal growth.
2.3.1 Preparation of Protein Solutions

Both binary complexes and ternary complexes were assembled for soaking studies and for cocrystallization studies. The binary complex was made by using one of two cofactors, either 2',5'-ADP or NADP at 5 mM concentrations. The protein concentration was between 15-25 mg/ml. The solutions were made and then filtered with a 0.2 µm filter and a protein solutions micro filter system. The ternary complex was made with the protein at a specific concentration and 5 mM of the cofactor, either NADP or 2'5'-ADP, as well as the respective inhibitor at approximately ten times its determined Ki value.

2.3.2 Inhibitor Preparation

Prior to the availability of kinetic data the inhibitor concentration in the ternary complexes was set at the arbitrary value of 20 mM. The individual compounds that may serve as strong inhibitors were screened from both a water soluble and a DMSO soluble fragment library. These compounds were initially screened as part of a cocktail of four compounds, which was then narrowed down to the strongest binding compounds. Once the kinetic data was available, the strongest inhibitors for vc-ASADH were chosen and the Ki values were used as a guide for the final inhibitor concentrations in the protein solution. Inhibitor dilutions were made to the final complex concentrations by diluting stock solutions of 200 mM or 400 mM of the inhibitors.
2.3.3 Hanging Drop Crystal Tray Preparation

Hanging drop experiments for the crystallization \textit{vc}-ASADH were carried out in a 24 well limbro plate. The well solution consisted of PEG 3350, NaCitrate, pH 7.0, NaOAc, 5% ethylene glycol, DTT, and water. While a number of different PEG 3350 concentrations were examined, the optimal conditions were found in the range from 20-30 \% PEG 3350 (v/v). Previous crystallization studies were also carried out in NaCitrate but at a pH of 5.6. However, since the enzyme has optimal activity above pH of 7 and the inhibitor kinetic data was collected at higher pH, the pH of the crystallization buffer was increased from 5.6 to 7.0. DTT was present in the binary complex (cofactor + protein) crystallization, however was not added in the ternary complex to minimize interference with the binding of the inhibitors. Initially the size of the drop was varied to ensure optimal results. However, a 2 + 2 drop, with 2 µl of protein solution and 2 µl of reservoir solution gave the best results.

2.3.4 Microseeding Crystallization

\textit{Vc}-ASADH crystals generally grew over a period of two days, however under conditions where this did not occur a microseeding technique was used to initiate nucleation. Previously grown crystals from the same protein were crushed and added to the reservoir solution (approximately 20 µl). A fiber was dipped into the crystal solution and then dragged across the clear drops of the crystal solutions to introduce nuclei.
2.3.5 Crystal Soaking Studies

For crystal soaking experiments, crystals were grown in the presence of the cofactor. Prior to freezing, the crystal was then soaked in an inhibitor solution, initially at 20 mM concentration and later at 10 times the inhibitor Ki values, to ensure binding. The crystal was soaked for 10 minutes, and then frozen, as described below.

2.3.6 Cryo Protectant and Crystal Freezing

Crystals were frozen in the well solution at the same concentration of PEG 3350, salts and buffer. For vc-ASADH, the best cryo protectant that did not cause cracking or melting of the crystal was 25% ethylene glycol, since the crystals were grown in the presence of 5% ethylene glycol. The cofactor and inhibitor were also present at a concentration either equal to or greater than the original inhibitor concentrations. To ensure proper binding while freezing, each crystal was dipped in the cryo solution, and then plunged into liquid nitrogen and then stored for data collection.

2.4 Enzyme Structural Studies

Crystal screening, data collection and structural refinement of the vc-ASADH inhibitor complexes were carried out as described below.
2.4.1 Crystal Screening

Crystals were screened either in house on a Rigaku hi-brilliance diffractor or at the Advanced Photon Source (APS) at Argonne National Laboratory. The crystals were initially screened at two angles, 0º and 90º, with the detector distance set at 200 mm. After the frames were collected, the resolution and mosaicity were measured to determine the quality of the crystal for possible data collection. Initial indexing (usually done with HKL2000) and refinement were done to determine the space group of the unit cell. When crystals were found to be of sufficient quality an optimization strategy was done to determine the optimal number of frames needed to collect a complete data set.

2.4.2 Data Collection

During data collection, the data was scaled to ensure high quality. Once scaled the data was processed using CCP4 software package. First, the merged data was imported. This was done by using the scale file previously created from HKL2000 as the input file and producing an mtz file. Full data sets were typically collected at the GM/CA-CAT beamline at the APS.

2.4.3 Crystal Structure Determination and Refinement

Once imported, the refinement was done by using RefMac5. The first refinement was a rigid body refinement in which the previously created mtz file was the input file as well as a similar pdb file with the same space group. The second refinement was
restrained refinement in which the difference maps were created. The input file used was the output file from the rigid body refinement.

### 2.4.4 Model Building and Structure Analysis

Once the refinement was complete, the molecule was examined with the program COOT\textsuperscript{15,16}. Three files were open, the first being the protein and then both structure factor maps created by the restrained body refinement. This allowed the creation of the electron density map from the diffraction data. By creating both structure factor maps any unmodeled electron density could be examined to determine if inhibitor compound binding is present. Initially the active site was closely examined for unaccounted electron density since the inhibitor was shown to inhibit enzyme activity. If no unaccounted electron density was found at the active site then other potential binding sites within the protein was examined for additional electron density. When unmodeled blobs of electron density were located the structure of the inhibitor was modeled to determine if there was a fit to the electron density. To create the structure of each inhibitor the program DUNDEE prodrg 2 was used to draw the molecule and to produce a pdb file and cif file so that the molecule can be added to the protein model in COOT. When the compound was properly fit into its respective electron density and refined in REFMAC\textsuperscript{5}, the programs PYMOL\textsuperscript{13} and COOT\textsuperscript{15,16} are used to analyze types of ligand-protein interactions of the complex. Based on this ligand-protein complex structure, recommendations will be made for creating a new generation of improved inhibitors.
3.1 Preparation of Protein Complexes

3.1.1 Inhibitor Binding Measurements

The Ki values for the fragment library inhibitors were determined by Geng Gao and were used as a template for the inhibitor concentration for crystallization studies. Only the stronger inhibitors (with the Ki values less than 20 mM) were used to prepare the protein complexes. Compounds having weaker binding may cause precipitation of the protein when added at very high concentrations. Table 1 gives a representation listing of strong inhibitors for \( vc \)-ASADH along with the Ki values and the inhibitor concentration used in the complex.
**Table 1: Ki values of strong inhibitors for vc-ASADH.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ki mM*</th>
<th>[inhibitor] mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine ethyl ester</td>
<td>0.0043</td>
<td>0.04</td>
</tr>
<tr>
<td>S-carbamoyl-L-cysteine</td>
<td>0.13</td>
<td>1.0</td>
</tr>
<tr>
<td>L-homocysteine</td>
<td>0.47</td>
<td>5.0</td>
</tr>
<tr>
<td>L-cysteine sulfinate</td>
<td>0.25</td>
<td>3.0</td>
</tr>
<tr>
<td>L-cystine</td>
<td>0.0023</td>
<td>0.03</td>
</tr>
<tr>
<td>Propionic acid</td>
<td>0.57</td>
<td>6.0</td>
</tr>
<tr>
<td>DL-2,3-diaminopropionic acid</td>
<td>0.93</td>
<td>9.0</td>
</tr>
<tr>
<td>D-glutamate</td>
<td>0.13</td>
<td>1.5</td>
</tr>
<tr>
<td>D-glucosamine acid</td>
<td>3.2</td>
<td>30.0</td>
</tr>
<tr>
<td>4-aminohydroxybenzophenone</td>
<td>0.37</td>
<td>3.7</td>
</tr>
<tr>
<td>2,2',4,4'-tetrahydroxybenzophenone</td>
<td>0.041</td>
<td>4.1</td>
</tr>
<tr>
<td>Benzophenone imine</td>
<td>0.37</td>
<td>3.7</td>
</tr>
<tr>
<td>4-benzoyl benzoate</td>
<td>1.1</td>
<td>11.2</td>
</tr>
<tr>
<td>2-amino adipic acid</td>
<td>0.39</td>
<td>4.0</td>
</tr>
<tr>
<td>N-iodosuccinimide</td>
<td>0.016</td>
<td>0.16</td>
</tr>
<tr>
<td>2,6-dibromoquinone chlorimide</td>
<td>0.24</td>
<td>2.4</td>
</tr>
<tr>
<td>1-fluoro-2,4-dinitrobenzene</td>
<td>0.1</td>
<td>1.0</td>
</tr>
<tr>
<td>2-chloro-3'4'-dihydroxyacetophenone</td>
<td>0.16</td>
<td>1.6</td>
</tr>
<tr>
<td>5-chloro-2-nitrobenzaldehyde</td>
<td>0.33</td>
<td>5.3</td>
</tr>
</tbody>
</table>

*Ki values determined by Geng Gao

The protein complex was filtered with a 0.2 µm filter and then spun for 20 minutes. The protein complex should be a clear solution signifying that no precipitation occurs. If precipitant did occur, the protein solution would be cloudy. A Bradford assay was also done to determine if the inhibitor precipitated out the protein. Precipitation usually occurred with the complexes that had the inhibitors from the DMSO library. This was because the amount of DMSO was too high and would precipitate out the protein.
3.1.2 Binary Complexes

In addition to the ternary complexes produced, binary complexes were also produced. The binary complex included the protein and the cofactor. Once the crystals were grown under these conditions, they would then be soaked in their respective inhibitor solutions.

3.2 Crystallization of ASADH

3.2.1 Results of Crystal Growth

As stated, in the methods section the optimal crystal conditions for \( vc \)-ASADH crystal growth was approximately 20-30% PEG 3350 along with other additives and salts. For ternary complexes, the protein concentration ranged from 20-25 mg/ml and for binary complexes the protein concentration ranged from approximately 15-18 mg/ml. While a number of inhibitors were selected for \( vc \)-ASADH, crystal growth was difficult and in some cases crystals could not be produced. Examples of typical crystals grown under these conditions can be seen in Figure 7.
Figure 6: Crystal growth of ternary complexes for $\text{vc}$-ASADH. All images represent ternary complexes: A. cysteine sulfinite at 17.5% PEG 3350; B. S-carbamoyl-L-cysteine at 23.5% PEG 3350; C. S-carbamoyl-L-cysteine at 20% PEG 3350.

Depending on the inhibitor used, different morphologies were observed for the various crystals. Different dimensions of crystals were present, but it was a common trend that the smaller crystals were generally better diffracting crystals. The smaller crystals seemed to take a longer time to grow, but gave lower mosaicity, indicating better internal order. The larger crystals grew quickly causing more disorder and gave a higher mosaicity.

3.2.2 Soaking Studies

Binary crystal complexes were soaked in a solution with their respective inhibitors. However, for the $\text{vc}$ form of ASADH cracking occurred upon adding the crystal to the soaking solution. When the crystals were grown without the presence of the inhibitor, a specific enzyme conformation was preferred. However, once the crystals were
soaked the conformation and the morphology would change and thus cause cracking. While the crystals of the binary complex did frequently crack, screening was done to see if reasonable diffraction data could still be collected.

3.3 Data Collection

3.3.1 Complex Crystal Data

Each crystal was screened and strategically optimized for data collection. Most of the full datasets were collected at Argonne National Laboratory (APS) at the GM0CA-CAT beamline. Below in Table 2 are the different complexes that were collected at APS. While many crystals were screened, few were used for data collection. This was due to poor mosaicity, high resolution, or the crystal died too quickly in the beam. As each of the datasets were collected and the structures refined, binding was determined by using the programs previously described. The inhibitor complexes highlighted in red contained electron density that indicated binding. For the other complexes no convincing unassigned density was present to indicate the presence of a bound inhibitor.

3.4 Binding Results

As described in the materials and methods section, once unaccounted for density has been found, the inhibitor structure was then modeled in and interactions were drawn to the specific residues. Three inhibitor complexes that did show electron density in the active site of \( \nu_c \)-ASADH were S-carbamoyl-L-cysteine, and DL-2, 3-diaminopropionic
acid. (Table 2). All were bound in the active site in slightly different orientations, but with similar interactions with the active site residues. Water molecules were also inserted into the structure to make sure that the unmodeled electron density was not from water molecules binding close together. While all the inhibitors seem to bind in different orientations, three active site residues played key roles: Cys 134, His 274, Arg 267, Arg 101.

3.4.1 S-carbamoyl-L-cysteine Complex

S-carbamoyl-L-cysteine binds to vc-ASADH with a Ki value of 1.3 mM, so the inhibitor concentration was set to 13 mM. A fragment of the bound inhibitor can be seen in Figure 8. S-carbamoyl-L-cysteine shows covalent binding with the active site cysteine to form a disulfide bond. Unfortunately this finding indicates that S-carbamoyl-L-cysteine cannot be developed into a good inhibitor because a disulfide bond will not be selective. In addition to covalent binding with Cys134, this inhibitor also shows hydrogen binding with the other residues in the active site, His274, Arg267, and Glu240. A water molecule is also involved in the binding and stability. This finding is consistent with the SMCS ternary complex in a previously solved structure.\(^5\)
Table 2: Inhibitor Complex Diffraction Data

<table>
<thead>
<tr>
<th>Complex</th>
<th>Resolution Å</th>
<th>Rsym</th>
<th>ll sigma</th>
<th>Completeness %</th>
<th>Redundancy</th>
<th>Space Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine ethyl ester (NADP)</td>
<td>50.1-7(1.78-1.7)</td>
<td>0.14(0.32)</td>
<td>7.2(1.9)</td>
<td>93(81)</td>
<td>6.1(3.8)</td>
<td>P4_2_2</td>
</tr>
<tr>
<td>N-iodosuccinimide (NADP)</td>
<td>50-1.8(1.9-1.8)</td>
<td>0.088(0.337)</td>
<td>15.7(4.2)</td>
<td>99(98)</td>
<td>4.2(3.9)</td>
<td>P2_1_2_2_2</td>
</tr>
<tr>
<td>S-carbamoyl-L-cysteine (NADP)</td>
<td>50-1.8(1.8-1.75)</td>
<td>0.068(0.35)</td>
<td>19(3.6)</td>
<td>85(78)</td>
<td>4.1(3.5)</td>
<td>P4_2_2_2</td>
</tr>
<tr>
<td>S-carbamoyl-L-cysteine (ADP)</td>
<td>50-1.8(1.88-1.8)</td>
<td>0.102(0.4)</td>
<td>14.4(2.7)</td>
<td>99(98)</td>
<td>5.0(4.1)</td>
<td>P4_2_2_2</td>
</tr>
<tr>
<td>L-homocystine (NADP)</td>
<td>50-2.0(2.1-2.0)</td>
<td>0.052(0.11)</td>
<td>18(12.6)</td>
<td>90(95)</td>
<td>4.3(4.1)</td>
<td>P4_2_2_2</td>
</tr>
<tr>
<td>DL-2,3-diaminopropionate (NADP)</td>
<td>50-2.2(2.28-2.2)</td>
<td>0.153(0.56)</td>
<td>8.4(1.5)</td>
<td>97(88)</td>
<td>4.0(2.1)</td>
<td>P4_2_2_2</td>
</tr>
<tr>
<td>D-glutamate (NADP)</td>
<td>50-2.5(2.59-2.5)</td>
<td>0.08(0.2)</td>
<td>7.6(2.5)</td>
<td>82(55)</td>
<td>2.2(1.4)</td>
<td>P2_1</td>
</tr>
<tr>
<td>Benzophenone-imine (NADP)</td>
<td>50-2.5(2.59-2.5)</td>
<td>0.112(0.2)</td>
<td>10.3(3.6)</td>
<td>95(79)</td>
<td>3.4(2.1)</td>
<td>P2_1</td>
</tr>
<tr>
<td>5-chloro-2-nitrobenzaldehyde (NADP)</td>
<td>50-2.4(2.49-2.4)</td>
<td>0.08(0.488)</td>
<td>8.4(4.5)</td>
<td>93(76)</td>
<td>2.8(2.4)</td>
<td>P2_1</td>
</tr>
<tr>
<td>4-benzoyl-benzoate (NADP)</td>
<td>50-2.3(2.41-2.3)</td>
<td>0.06(0.2)</td>
<td>12(4.3)</td>
<td>71(25)</td>
<td>1.7(1.3)</td>
<td>P2_1</td>
</tr>
<tr>
<td>Tetrahydroxy benzophenone (NADP)</td>
<td>50-1.9(1.97-1.9)</td>
<td>0.06(0.2)</td>
<td>24(10.1)</td>
<td>99(95)</td>
<td>6.8(5.3)</td>
<td>P4_2_2_2</td>
</tr>
<tr>
<td>4-hydroxy-benzophenone (ADP)</td>
<td>50-2.4(2.49-2.4)</td>
<td>0.107(0.354)</td>
<td>10.8(2.1)</td>
<td>97(82)</td>
<td>3.6(2.4)</td>
<td>P4_3</td>
</tr>
<tr>
<td>DL-2,3-diaminopropionate (ADP)</td>
<td>50-2.2(2.28-2.2)</td>
<td>0.153(0.57)</td>
<td>8.4(1.51)</td>
<td>91(86)</td>
<td>4.0(2.1)</td>
<td>P4_2_2_2</td>
</tr>
<tr>
<td>2-amino adipic acid (APO)</td>
<td>50-2.3(2.23-2.3)</td>
<td>0.10(0.243)</td>
<td>8.6(3.5)</td>
<td>92(86)</td>
<td>3.8(3.1)</td>
<td>P4_2_2_2</td>
</tr>
<tr>
<td>Tetrahydroxy benzophenone (APO)</td>
<td>50-2.1(2.18-2.1)</td>
<td>0.06(0.369)</td>
<td>9.6(2.3)</td>
<td>96(98)</td>
<td>2.8(2.8)</td>
<td>P4_3</td>
</tr>
<tr>
<td>Benzophenone-imine (ADP)</td>
<td>50-2.5 (2.59-2.5)</td>
<td>0.112(0.223)</td>
<td>10.0(3.6)</td>
<td>95(79)</td>
<td>3.4(2.1)</td>
<td>P2_1</td>
</tr>
</tbody>
</table>

* Highest resolution shell
Figure 7: Active site of \textit{vc}-ASADH with bound S-carbamoyl-L-cysteine.

### 3.4.2 DL-2,3-diaminopropionate Complex

DL-2,3-diaminopropionate has a $K_i$ value of 0.93 mM and was examined at a concentration of 9.3 mM within the complex. This inhibitor was determined to be bound at the active site through hydrogen bonding interactions with several of the active site residues. The residue Asn 133 seems to play a role in binding for this inhibitor, the amide carbonyl group binding to the amino group of the inhibitor (Figure 9).
Both of these inhibitors were bound in the active site, but in slightly different orientations. Figure 10 shows that DL-2,3-diaminopropionic acid binds to active site residues by hydrogen bonding. However, S-carbamoyl-L-cysteine has a different orientation due to its covalent bond to Cys 134.
Figure 9: Overlap of the bound inhibitors for \( vc \)-ASADH. \( S \)-carbamoyl-\( L \)-cysteine complex structure (magenta); and the DL-2,3-diaminopropionic acid complex structure (blue)\(^{13} \).

### 3.4.4 Bound Inhibitors to Other Enzyme Forms

Several other forms of ASADH that have also been included in the fragment screening are those from \textit{streptococcus pneumoniae} (\textit{sp})\(^{11} \) and the recently solved structure from \textit{Candida albicans} (\textit{ca})\(^{12} \). Currently, no bound inhibitors have been found for \textit{ca}–ASADH. Crystals have been produced for \textit{ca}–ASADH inhibitor complexes, but they were thin rods and once screened they showed high mosaicity and low resolution. \textit{Sp}–ASADH has produced some complexes with the inhibitors DL-2,3-propionic acid and 2-amino adipic acid (Alexander Pavlovsky, unpublished results). What is interesting
regarding the 2-aminoadipic acid complex is that it was initially shown kinetically not to be an inhibitor of this enzyme, however structural studies showed that it is bound.

3.4.5 Comparison of \textit{vc}-ASADH and \textit{sp}-ASADH Inhibitor Complexes

One of the goals of this project is to compare the differences and similarities in the different forms of ASADH. Since both \textit{Vc} and \textit{Sp} have two of the same inhibitors bound, overlapping of the two active sites would be a good way to compare the two forms of the enzyme. In Figure 12, DL-2,4-diaminopropionic acid is shown for both enzyme forms. The two active site arginines can be seen to interact with the inhibitor in both enzyme forms, while Cys 128 also interacts in the \textit{sp}-ASADH inhibitor complex. This inhibitor is also seen to interact in two different conformations.
Figure 10: *Sp and Vc* forms of ASADH with bound DL-2,3-diaminopropionate. *Sp*-ASADH is in blue sticks and *vc*-ASADH is in the orange lines\(^\text{13}\). (*Sp*-ASADH structure determined by Dr. Alexander Pavlovsky)

In the *vc*-ASADH structure the inhibitor carboxyl group points towards Arg 101, while in the *sp*-ASADH complex the inhibitor functional group points towards Arg 245. This is an interesting observation for future inhibitor design since the active sites for both enzymes are essentially the same yet DL-2,3-diaminopropionate can bind in two different orientations.

Based on the inhibitors that have been determined to be in the active site of *vc*-ASADH, we see that the structures are similar. Drawn in a similar orientation, Figure 11 shows the structures of all the inhibitors and the substrate, ASA.
Figure 11: Structural Analysis of the bound inhibitors of \( v_c \)-ASADH. The red circles indicate that no convincing electron density was present for these specific atoms.
As shown in Figure 11, while the structures mimic the substrate, it is also worth noting that whenever a sulfur atom is present, a covalent bond occurs and any atom attached to the sulfur will be displaced. While a mechanism has not been determined as to what is going on, an inhibitor, such as cysteine analogs may not be the most optimal inhibitors due to sulfur atom producing noncovalent bonds with the active site cysteine. A more advantageous approach would be to replace the sulfur atom with an amine or carbonyl, so that a noncovalent bond may occur and produce overall stronger binding within the active site of the enzyme.

3.4.6 Difficulties with Structural Studies

While there has been some success with inhibitor binding with structural studies, it is not clear why so many of these inhibitors showed strong inhibition kinetically but were not bound when examined at concentrations well above the measured $K_i$ values. So, the conditions of the kinetic studies and the structural studies were examined more closely to identify differences that may be responsible for these divergent results.

3.5 Analysis of Crystallization Conditions

Between kinetic studies and structural studies, a number of obvious differences are present with regards to the conditions used for both techniques. Such as the concentration of protein used in both techniques. The concentration of protein in kinetic
studies is considerably lower than in the crystallization studies. Kinetic studies are done in the presence of the substrate, ASA and the cofactor, NADP. As for structural studies, a precipitant such as PEG or ammonium sulfate is needed for crystal growth. Crystals are soaked in a cryo solution containing ethylene glycol before freezing, which is not present in kinetic studies. The ASADH assay was done at a pH of 8.6 and crystals were grown at pH of between 5.8 and 6.5.

While some of these conditions cannot be controlled, such as the concentration of protein needed for both techniques, attempts were made to examine the other differences and minimize these differences.

3.5.1 Use of Cofactors in the Protein Complex

Different cofactors were examined to see if the binding of certain inhibitors would improve through the formation of a ternary complex. Also, it was hypothesized that some of the inhibitors may compete with the nicotinamide ring on NADP so an NADP analog, 2’,5’-ADP was used. For example, benzophenone and its derivatives possess an aromatic ring and thus has similar structural feature to the nicotinamide ring of NADP. Currently there are no conclusive results to confirm this hypothesis; however, it is also important to point out that data sets for all three different cofactors (ADP, NADP, and no cofactor) were difficult to obtain for the same inhibitor.
3.5.2 Examination of pH Effects

When examining the differences between the kinetic studies and the crystallization studies one condition that was obvious was the pH. As stated above, the pH of the ASADH assay is at 8.6. However, crystallization studies were being done at pH of 6.0 where the enzyme has significantly lower activity. Some crystals were grown at a pH of 7.0 in the presence of inhibitors, but any higher pH values did not produce crystals.

3.5.3 Examination of Precipitant Effects

A more recent condition that was looked into was the effect of the precipitant that was used in the well solution. In these structural studies PEG was used as the precipitant in the well solution. In order to determine if the presence of PEG had an effect on inhibitor binding, kinetic studies were done in specific precipitants. It was determined that while PEG 3350 did not interfere with the activity of the protein, it did cause binding of the inhibitors to become weaker, thus changing the amount needed to see binding in the structural studies. New Ki values were determined under conditions of PEG 3350 and the revised values are reported in Table 4 along with the new inhibitor concentrations used.
Table 3: Ki values of inhibitors at 20% PEG 3350

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Ki mM</th>
<th>Ki w/PEG</th>
<th>[Inhibitor] mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-2,3-diaminopropionate</td>
<td>0.46</td>
<td>n.i.</td>
<td>N/A</td>
</tr>
<tr>
<td>D-glutamate</td>
<td>1.3</td>
<td>n.i.</td>
<td>N/A</td>
</tr>
<tr>
<td>DL-2-aminoadipic acid</td>
<td>0.47</td>
<td>3.0-4.0</td>
<td>40</td>
</tr>
<tr>
<td>DL-5-aminocaproate</td>
<td>0.56</td>
<td>4.0-5.0</td>
<td>50</td>
</tr>
<tr>
<td>L-2,4-diaminobutyrate</td>
<td>0.14</td>
<td>1.5-2.0</td>
<td>20</td>
</tr>
<tr>
<td>L-2,5-diaminopentanoate</td>
<td>5.2</td>
<td>3.0-4.0</td>
<td>40</td>
</tr>
<tr>
<td>3-nitropropionate</td>
<td>0.24</td>
<td>1.5-2.5</td>
<td>26</td>
</tr>
<tr>
<td>2-amino-4-phosphonobutyrate</td>
<td>0.24</td>
<td>n.i.</td>
<td>N/A</td>
</tr>
</tbody>
</table>

\(^a\) Ki values provided by Geng Gao
\(^b\) no longer an inhibitor under PEG conditions

The inhibitors indicated in red represent inhibitors that no longer cause inhibition of \(vc\)-ASADH when examined from 20 mM after the addition of PEG 3350 in the kinetics studies. As for the other inhibitors, the Ki was increased by approximately ten fold the compound to the values in the absence of PEG. Due to the increased inhibitor concentration there was a concern that there higher values would cause precipitation. Since these are all water soluble inhibitors no precipitation occurred. However, when crystals were setup in the same conditions as the earlier studies, the increased inhibitor concentration led to phase separation in a number of wells. The protein solution was 25 mg/ml and NADP was the cofactor. The crystals grew quickly at lower concentrations of PEG, and then at higher concentrations of PEG, crystals grew over a period of 5 days. The wells that produced phase separation still grew crystals but there crystals had a different morphology. An example of attained crystal growth is shown in Figure 11.
Figure 12: Crystal growth of ternary complexes at 20% PEG.

The crystals were frozen using the previous cryo soaking techniques. There was some concern that the crystals would crack under the cryo conditions due to the increased amount of the inhibitor present, however this was not the case. The crystals were frozen by plunging into liquid nitrogen.

A number of these crystals were screened, however poor diffraction occurred. As was the case for other crystals, the smaller crystals seemed to diffract at a higher resolution and lower mosaicity. Two different inhibitor complexes were collected, L-2,5-
diaminopentanoate and DL-2,4-diaminocaproate. The data collection parameters for these complexes are recorded in Table 4.

**Table 4: Inhibitor complex crystal data.**

<table>
<thead>
<tr>
<th></th>
<th>L-2,4-diaminopentoate</th>
<th>DL-2,5-diamniocaproate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Resolution Å</strong></td>
<td>50.2.3 (2.38-2.3)</td>
<td>50.0.2.1(2.18-2.10)</td>
</tr>
<tr>
<td><strong>Rsym %</strong></td>
<td>0.11(0.42)</td>
<td>0.06(0.31)</td>
</tr>
<tr>
<td><strong>I/I sigma</strong></td>
<td>11.20(1.90)</td>
<td>17.3 (3.2)</td>
</tr>
<tr>
<td><strong>Completeness %</strong></td>
<td>97(76)</td>
<td>92.5(81.7)</td>
</tr>
<tr>
<td><strong>Redundancy</strong></td>
<td>2.1(1.5)</td>
<td>2.1(1.6)</td>
</tr>
<tr>
<td><strong>Space Group</strong></td>
<td>P4_2;2</td>
<td>P4_2;2</td>
</tr>
</tbody>
</table>

L-2,4-diaminopentanoate showed inhibitor density within the active site thus confirming that increased inhibitor concentration is needed to see binding. Figure 12 shows the electron density, however it indicates that the inhibitor is not bound in the same orientation as the other inhibitors. The carboxyl group of the diaminopentanoate is interacting with Asn 133 and Arg 101, but the additional amino group is pointing away from the positively charged Arg 101 side chain. This alternative orientation of the molecule may explain the weaker binding observed for this inhibitor. While a good dataset was collected for DL-2,5-diaminocaproate, no convincing evidence was apparent to conclude that the inhibitor was present in the active site.
The weaker binding of inhibitors in the presence of PEG suggested the need to screen other precipitants to identify new conditions for inhibitor screening. Based on previous crystal screening of \( \nu \text{-ASADH} \), ammonium sulfate was determined to be a good precipitant, with crystal optimizations in the range from 1.6 M to 2.1 M. In more recent studies three different complexes protein complexes were made with a new set of inhibitors: benzophenone imine, 4-hydroxy-benzophenone, and 2,2',4,4'-tetra-hydroxybenzophenone. A well solution containing ammonium sulfate (40%-50%), NaCitrate, pH 7.0, NaOAc, and 5% ethylene glycol was setup to screen the inhibitor complexes.
The crystal growth was minimal within the first few days and both the 4-hydroxy-benzophenone, and 2,2’,4,4’-tetra-hydroxybenzophenone precipitated. However, after a period of about seven days, rod crystals grew in the benzophenone imine complex. Figure 13 shows the crystal growth of this complex. While it took some time for crystal growth, once the solution reached the nucleation stage a number of crystals did grow.

![Crystal Growth](image)

**Figure 14:** vc-ASADH/Benzophenone imine crystals in 44% ammonium sulfate.

In order to control the production of so many crystals in the future microseeding will be a viable technique to use. This seeding will be done after a period of a few days so that the nucleation stage can be reached and the number of nuclei can be controlled. Optimization
is also needed for the ammonium sulfate growth conditions because kinetic studies cannot be carried out at these high salt conditions. An alternative approach is to use PEG 400 in the well solution for crystal growth. PEG 400 is a natural cryo protectant and the crystal can then be frozen in its own reservoir solution without additional additives. Alternatively needed to useful inhibitor, the reduction of ammonium sulfate within the cryo solution may be produce complex crystals.
Chapter 4: Conclusions

4.1 Summary

The goal of this project is to identify selective inhibitors of the ASADH family of enzymes that can be improved into lead compounds for the development of new antibiotics. Several complexes of initial inhibitors identified from kinetic screening of fragment libraries have been crystallized and structurally characterized. In these studies, we were able to screen numerous inhibitors of $\nu c$-ASADH using X-ray structural studies. We were able to determine that while certain inhibitors were found kinetically to bind with reasonable affinity, they did not seem to behave as well under protein crystallization conditions. Many of these selective inhibitors gave no convincing electron density when examined at levels in excess of their measured $K_i$ values. While this negative result showed that working with two different techniques make it necessary to carefully consider the differences in experimental conditions between these techniques. Analysis of these differences, such as the addition of the cofactor, pH values, and the effect that precipitant may have on the inhibitors has allowed us to understand some of the issues that may be causing the lack of noticeable binding in structural studies.

We have successfully identified three inhibitors that bind to the active site of $\nu c$-ASADH. The structures of two of these inhibitor complexes were determined prior to optimization of the crystallization conditions and one was determined after optimization.
of the differences in precipitant conditions. S-carbamoyl-L-cysteine is found bound to the active site of vc-ASADH where it forms a disulfide bond with the nucleophile Cys 134. While this causes enzyme inactivation it is unlikely that disulfide bond formation can be developed into a selective mode of inhibition for the target enzyme. From an analysis of the structures of the other three inhibitors we were able to determine that they are bound in different orientations while still having similar interactions with the active site residues. For each of these inhibitors the key active site residues: Arg 267, His 274, and on one two occasions Asn 133, contributed to orientation, binding and stability. With L-2,4-diaminopentanoate, the lack of binding under crystallization conditions was a consequence of the presence of the precipitant, PEG3350. Kinetic studies showed that the precipitant was causing the inhibitor to bind less strongly and an increase in the inhibitor concentration was needed to saturate the enzyme under these conditions. Once this was done sufficient electron density was apparent to allow modeling of this inhibitor into the active site of vc-ASADH.

4.2 Future Studies

With the weaker binding and the need to increase the inhibitor concentration to observe binding in the presence of PEG, it became apparent that more inhibitors must be tested kinetically to determine their Ki values under these crystallization conditions. This will uncover additional inhibitors that can interact with vc-ASADH at other binding sites such as the phosphate site or within the NADP site.
From the binding and structural studies that have been completed with several inhibitors we can conclude that the screening of thiol containing compounds would not be productive due to their nonselective character. With the other inhibitors studies, hydrogen bonding interactions between donors and acceptors on the inhibitors and the enzyme seems to be the most consistent mode of interaction. The addition of well-placed hydrogen bonding substituents on the inhibitor, guided by these structural studies, will lead to tighter binding within the active site. We are actively screening new analogs of these successful inhibitors, both kinetically and structurally. By using what we have learned from the conditions of the two techniques and combining these approaches with a second generation of inhibitors, we will be moving to the production of new potent and selective lead compounds and towards our goal of species-specific antibiotics.
References


