The Search for the active site configuration of glutamate dehydrogenase i) Reactivity of LYS-126 ii) Preparation of O-Se-NADP+

Deborah Judd

Follow this and additional works at: http://scholarworks.rit.edu/theses

Recommended Citation

This Thesis is brought to you for free and open access by the Thesis/Dissertation Collections at RIT Scholar Works. It has been accepted for inclusion in Theses by an authorized administrator of RIT Scholar Works. For more information, please contact ritscholarworks@rit.edu.
THE SEARCH FOR THE ACTIVE SITE CONFIGURATION
OF GLUTAMATE DEHYDROGENASE
i) REACTIVITY OF LYS-126
ii) PREPARATION OF O-Se-NADP+

DEBORAH JUDD

AUGUST, 1991

THESIS
SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF THE MASTER OF SCIENCE
APPROVED:

J. E. Bell
PROJECT ADVISOR

Name Illegible
DEPARTMENT HEAD

Name Illegible
LIBRARY

Rochester Institute of Technology
Rochester, New York 14623
Department of Chemistry
THE SEARCH FOR THE ACTIVE SITE CONFIGURATION OF GLUTAMATE DEHYDROGENASE

i) REACTIVITY OF LYS-126

ii) PREPARATION OF O-Se-NADP+

I Deborah Judd hereby grant permission to the Wallace Memorial Library of RIT to reproduce my thesis in whole or in part. Any reproduction will not be for commercial use or profit.

August, 1991
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>i</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>ii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>iii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>1</td>
</tr>
<tr>
<td><strong>INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>Reactivity of Lys-126</td>
<td>2</td>
</tr>
<tr>
<td>Preparation of a Selenium NADP$^+$ Analog</td>
<td>7</td>
</tr>
<tr>
<td><strong>EXPERIMENTAL</strong></td>
<td></td>
</tr>
<tr>
<td>Reactivity of Lys-126</td>
<td></td>
</tr>
<tr>
<td>Inactivation</td>
<td>26</td>
</tr>
<tr>
<td>Reactivation</td>
<td>26</td>
</tr>
<tr>
<td>Kinetic Calculations</td>
<td>28</td>
</tr>
<tr>
<td>Criteria for Significance</td>
<td>29</td>
</tr>
<tr>
<td>Preparation of a Selenium NADP$^+$ Analog</td>
<td></td>
</tr>
<tr>
<td>Ion Exchange Chromatography of NAD$^+$ and NADP$^+$</td>
<td>29</td>
</tr>
<tr>
<td>Determination of Sodium Chloride Gradient</td>
<td>30</td>
</tr>
<tr>
<td>Ion Exchange Chromatography of NAD$^+$ and NADP$^+$</td>
<td>35</td>
</tr>
<tr>
<td>New Protocol</td>
<td></td>
</tr>
<tr>
<td>Enzymatic Synthesis of NADP$^+$ using NAD$^+$-Kinase</td>
<td>37</td>
</tr>
<tr>
<td>Ion Exchange Chromatography of ADP and ATP</td>
<td>39</td>
</tr>
<tr>
<td><strong>RESULTS</strong></td>
<td></td>
</tr>
<tr>
<td>Reactivity of Lys-126</td>
<td></td>
</tr>
<tr>
<td>Determination of Pyridoxyl 5' Phosphate Concentration to Study Rate of Inactivation</td>
<td>44</td>
</tr>
<tr>
<td>Determination of $k_{\text{inact}}$ of GDH by PLP</td>
<td>44</td>
</tr>
<tr>
<td>Determination of $k_{\text{inact}}$ of GDH by PLP in Presence of Various Ligands</td>
<td>46</td>
</tr>
<tr>
<td>Determination of $k_{\text{react}}$ of Pyridoxylated GDH</td>
<td>55</td>
</tr>
<tr>
<td>Determination of $k_{\text{react}}$ of Pyridoxylated GDH in Presence of Various Ligands</td>
<td></td>
</tr>
</tbody>
</table>
Preparation of a Selenium NADP⁺ Analog
   Ion Exchange Chromatography of NAD⁺ and NADP⁺ 59
   Enzymatic Synthesis of NADP⁺ using NAD⁺-kinase 59

DISCUSSION

Reactivity of Lys-126
   Effects of Ligands on the Rate Constant of Modification 60
   Effects of the Ligands on the Extent of Modification 65
   Conclusions on the Purine Nucleotides, ADP and GTP. 66
   Conclusions on the 5 Carbon Dicarboxylic Acid Substrate Analogs 67
   Conclusions on Succinate 69
   Summary of Results 69

Preparation of a Selenium NADP⁺ Analog 72

REFERENCES 73
Acknowledgements

I would like to thank the administration at Rochester Institute of Technology for supporting my research these past two years.

I would like to thank Dr. Bell for both his help with my research and his friendship. Thank you for all the opportunities you have provided for me especially the chance to speak at the Northeast Enzymes and Proteins Group Meeting and the opportunity to go to St. Peter's College, Oxford, England for the summer to study.

I would also like to thank the members of my committee, Dr. Waud, Dr. Reinhardt, Dr. Neenan and Dr. Tubbs for their support. I would like to thank Dr. Waud for his comments on my thesis, without which I would have had a lot more awkward sentences than I do. Also I would like to especially thank Dr. Tubbs for taking on the burden of being in charge of the final draft of my thesis. I appreciate your help more than I can express in words.

Thank you Dr. Gennett for filling in for one of my committee member for my thesis defense. Also I owe you my Master's degree for allowing me to take over your office and computer to get it done.

To all the faculty involved with my research and Dr. Bell, I would like to dedicate this poem:

We seek him here.
We seek him there.
We seek him everywhere.

Is he in Heaven,
Or is he in Hell?
That damned elusive
Ellis Bell.
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Schiff's base formation between lys-126 and pyridoxyl 5' phosphate.</td>
<td>6</td>
</tr>
<tr>
<td>Figure 2</td>
<td>An outline of the steps in the X-ray crystallographic determination of protein structures.</td>
<td>10</td>
</tr>
<tr>
<td>Figure 3</td>
<td>Model of the NAD+ binding domain common to dehydrogenases.</td>
<td>13</td>
</tr>
<tr>
<td>Figure 4</td>
<td>Structure of NAD+ and NADP+.</td>
<td>16</td>
</tr>
<tr>
<td>Figure 5</td>
<td>Structure of O-Se-NADP+.</td>
<td>18</td>
</tr>
<tr>
<td>Figure 6</td>
<td>Reduction of NAD(P)+.</td>
<td>19</td>
</tr>
<tr>
<td>Figure 7</td>
<td>Structure of S-NAD(P)+.</td>
<td>20</td>
</tr>
<tr>
<td>Figure 8</td>
<td>Strategy for covalent attachment of heavy atom probe.</td>
<td>22</td>
</tr>
<tr>
<td>Figure 9</td>
<td>Strategy for preparing O-Se-NADP+.</td>
<td>24</td>
</tr>
<tr>
<td>Figure 10</td>
<td>Separation of pyridoxylated glutamate dehydrogenase from excess pyridoxyl 5' phosphate.</td>
<td>27</td>
</tr>
</tbody>
</table>
Figure 11 - Ion exchange chromatography of NAD\(^+\) and NADP\(^+\).

Figure 12 - Structure of NAD\(^+\) and NADP\(^+\) showing charges.

Figure 13 - Sodium chloride gradient used to elute NAD\(^+\) and NADP\(^+\).

Figure 14 - Ion exchange chromatography of NAD\(^+\) and NADP\(^+\) using a new protocol.

Figure 15 - Enzymatic synthesis of NADP\(^+\) using NAD\(^+\)-kinase.

Figure 16 - Structures of ADP and ATP showing charges.

Figure 17 - Ion exchange chromatography of ADP and ATP.

Figure 18 - Enzymatic synthesis of NADP\(^+\) using NAD\(^+\)-kinase. Trial 2.

Figure 19 - Effects of pyridoxyl 5' phosphate concentration on the inactivation of glutamate dehydrogenase.

Figure 20 - Time course of inactivation of glutamate dehydrogenase by pyridoxyl 5' phosphate.

Figure 21 - Rate of glutamate dehydrogenase activity vs time.

Figure 22 - Spectrum of pyridoxyl 5' phosphate from 300 to 500 nm.
Figure 23 - OD at 360 nm vs time of the blank.

Figure 24 - Time course of reactivation of pyridoxylated glutamate dehydrogenase.

Figure 25 - Structures of substrate analogs.

Figure 26 - Structures of ADP and GTP.

Figure 27 - Conformational effects of ADP and GTP.

Figure 28 - A model of the active site of glutamate dehydrogenase with glutamate bound.

Figure 29 - A model of the active site of glutamate dehydrogenase with succinate bound.

Figure 30 - A model of the active site of glutamate dehydrogenase with the pyrophosphate of cofactor bound.
List of Tables

Table 1 - Effects of ligands on the time course of inactivation of glutamate dehydrogenase by pyridoxyl 5' phosphate.

Table 2 - Time course of inactivation of the pyridoxyl 5' phosphate blank assayed into a solution containing 250 μM NAD+ and 20 mM glutamate.

Table 3 - Effects of ligands on the time course of inactivation of glutamate dehydrogenase by pyridoxyl 5' phosphate after correction for decrease in absorbance due to pyridoxyl 5' phosphate.

Table 4 - Effects of ligands on the time course of reactivation of pyridoxylated glutamate dehydrogenase.
Abstract

The reactivity of lys-126 in glutamate dehydrogenase was investigated by monitoring the loss of enzymatic activity when lys-126 is modified by pyridoxyl 5' phosphate. The effect of various ligands on the rate constant of inactivation ($k_{inact}$) was determined. The purine nucleotides ADP and GTP increased the $k_{inact}$, glutarate slightly increased the $k_{inact}$, 2-oxoglutarate and succinate were determined to have no effect on $k_{inact}$ and no conclusions could be drawn on L-2-hydroxyglutarate. After the modification of glutamate dehydrogenase with pyridoxyl 5' phosphate, the percent residual activity (%R.A.) was determined and evaluated. ADP, GTP and succinate had no effect on the %R.A., glutarate and 2-oxoglutarate significantly increased the %R.A. No conclusions could be drawn about the effect of L-2-hydroxyglutarate. Modification of lys-126 by pyridoxyl 5' phosphate is reversible, so the effects of the various ligands on the rate constant of reactivation ($k_{react}$) was also investigated. Both the purine nucleotides, ADP and GTP, as well as the substrate analogs, glutarate, succinate, L-2-hydroxyglutarate, and 2-oxoglutarate, had no effect on the rate constant of reactivation.

Using ion exchange chromatography, a protocol for separating NAD$^+$ from NADP$^+$ was determined. This separation is a necessary step for the enzymatic synthesis of a selenium NADP$^+$ analog for X-ray crystallography.
INTRODUCTION

Reactivity of Lys-126

Glutamate dehydrogenase (E.C. 1.4.1.3) is a complex allosteric enzyme that catalyzes both the oxidative deamination of glutamate and the reductive amination of alphaketoglutarate thus providing a link between the citric acid and urea cycles.

Glutamate + NAD(P)⁺ + H₂O ⇌ α-Ketoglutarate + NAD(P)H + NH₄⁺

Due to its pivotal role and unique ability to use either NAD⁺ or NADP⁺ (the use of either NAD⁺ or NADP⁺ will be abbreviated NAD(P)⁺) with an equal affinity, glutamate dehydrogenase has been extensively studied.¹,² Glutamate dehydrogenase is thought to play an important role in ammonia metabolism. Carbamoylphosphate synthase, N-acetylglutamate synthase and glutamate dehydrogenase are all mitochondrial enzymes involved in the regulation of ammonia metabolism.³ Through the concerted action of these enzymes, the urea cycle is indirectly controlled. Because glutamate dehydrogenase catalyzes a reaction at, or near equilibrium, in vivo, any increase in the concentration of ammonia results in the activation of the reductive amination reaction of glutamate dehydrogenase causing an increase in the concentration of glutamate.³ N-acetylglutamate synthase then utilizes glutamate to form N-acetylglutamate.³ N-acetylglutamate is an absolutely required allosteric regulator of, and stimulates carbamoylphosphate synthase activity resulting in carbamoylphosphate being produced, which in turns activates the urea cycle.³ Therefore the major role of glutamate dehydrogenase seems to be the rapid removal of ammonia in the mitochondrial matrix.³ Elevated
levels of ammonia have been shown to inhibit the ability of the mitochondrion to produce ATP.\(^4\) Thus glutamate dehydrogenase serves to maintain the ability of the mitochondrion to produce ATP.\(^3\)

Glutamate dehydrogenase consists of six chemically identical polypeptide chains consisting of 501 residues per chain.\(^5\) By electron microscopy, sedimentation and viscosity studies as well as guanidine chloride denaturation, glutamate dehydrogenase has been found to be arranged as a dimer of trimers.\(^6\) Chemical crosslinking confirmed the hexameric structure with D3 symmetry \(^7\) of molecular weight 336 KDa.\(^8,9\) The hexamer form of the enzyme can be reversibly dissociated into trimers with guanidine hydrochloride.\(^8\) However from the construction of heterohexamers which consist of one native trimer and one modified, inactive trimer, it was discovered that the hexamer is required for catalytic activity.\(^9\) A concentration dependent polymerization is observed although this polymerization does not affect the activity or regulatory properties of the enzyme.\(^10-18\)

The regulation of glutamate dehydrogenase is complex and, as of yet, not fully understood. From initial rate studies of the oxidative deamination reaction of glutamate dehydrogenase, non-linear Lineweaver-Burke plots were obtained when the concentration of the glutamate was fixed with the concentration of either NAD\(^+\) or NADP\(^+\) varied.\(^19\) To explain this phenomena, Dalziel and Engel proposed that it was a result of negative cooperativity between the presumably six identical subunits.\(^20,21\) Upon re-examination of the initial rate data, Dalziel and Engel suggested that the negative cooperativity must be a result of both negative and positive interactions between subunits.\(^22\) They also proposed that this negative cooperativity must involve both the catalytic step and the coenzyme binding step.\(^22\) Evidence supporting the assumption that the binding of the
coenzyme was involved was reported by Bell and Dalziel. They found that upon binding either the oxidized or reduced coenzyme to half of its subunits in the presence of glutarate, a catalytically inactive substrate analog of glutamate, a conformational change on the second half of the hexamer resulted.\(^{(23)}\)

Involvement of the catalytic step was determined using the thionicotinamide analogs of NAD\(^+\) and NADP\(^+\). Thio-NAD\(^+\) and thio-NADP\(^+\) have been proven to accurately mimic the behavior of the natural coenzymes NAD\(^+\) and NADP\(^+\).\(^{(24)}\) The reduced form of thio-NAD(P)\(^+\) has an absorption maxima at 400 nm with very little absorbance at 340 nm while NAD(P)\(^+\) has an absorption maxima at 340 nm with insignificant absorption at 400 nm. This difference in the absorption spectra allows for the use of dual wavelength spectroscopy to monitor the utilization of both the natural cofactor and the thionicotinamide analog of the cofactor at the same time. When glutamate dehydrogenase is presented with both NAD(P)\(^+\) and thio-NAD(P)\(^+\) simultaneously the Lineweaver-Burke plot is linear. At low concentrations of the cofactor whose activity is being monitored, its utilization is decreased compared to when it is presented to glutamate dehydrogenase alone. At high concentrations, the utilization of the cofactor whose reduction was being monitored was increased in comparison to the presence of only a single cofactor. Since the two cofactors can not be in the active site at the same time, this indicates that upon binding of the second coenzyme to a different subunit, a conformational change must occur across subunit interfaces of the hexamer of glutamate dehydrogenase affecting the utilization of the other coenzyme. This demonstrates that within the catalytically functioning enzyme, there are negatively cooperative interactions. Subsequent studies using substrate analogs suggested that the 2-position in glutamate was responsible for the induction of the conformation change attributed to the negative cooperativity of glutamate dehydrogenase.\(^{(25)}\)
Regulation of glutamate dehydrogenase by the purine nucleotides GTP and ADP seem to have opposing effects on the activity of glutamate dehydrogenase. GTP is a potent inhibitor of the enzyme.\(^{26}\) The inhibition by GTP seems to be a consequence of stabilizing a glutamate dehydrogenase-NADH-glutamate abortive complex. This abortive complex appears to play a role in the normal catalytic cycle at pH values above 7.0. ADP alone acts as an activator by destabilizing this complex at pH values above 7.0. ADP can also relieve the inhibition of GTP at all pH values by displacing it from its binding site and destabilizing this abortive complex, even though at low pH values, ADP, in the absence of other regulators, acts as an inhibitor.\(^{3}\) This inhibition appears to be a result of competition between ADP and NAD(P)\(^+\) for the coenzyme binding site.\(^{3}\) Competitive inhibition with respect to the coenzyme of glutamate dehydrogenase by ADP is also observed at high concentrations of ADP.\(^{3}\) At either high or low pH values in the presence of ADP, the non-linear Lineweaver-Burke plots obtained with NAD(P)\(^+\) as the varied substrate are linear suggesting that ADP removes the negative cooperativity of glutamate dehydrogenase.\(^{3}\)

In order to understand the complex, allosteric regulation of glutamate dehydrogenase, chemical modification studies have been used to investigate the local structure around substrate and regulatory binding sites. These studies also assessed the accompanying changes in the conformation of the enzyme upon binding of these ligands. Talbot, et. al.\(^{27}\) identified two lysine residues, lys-126 and lys-333, that are specifically modified by pyridoxal 5' phosphate. Pyridoxal 5' phosphate reacts specifically with lysine residues forming a reversible Schiff's base (figure 1). Using protecting ligands, selective modification of one of the two lysines showed that lys-333 is involved in the ability of the enzyme to further polymerize into long polymers, while modification of lys-126 was shown to result
Figure 1: Formation of Schiff's base between pyridoxyl 5' phosphate and lysine-126 in the active site of glutamate dehydrogenase. Top picture done on Alchemy.
in the loss of catalytic activity.\(^{(27)}\)

Lys-126 was originally proposed to interact with the one position carboxyl group of dicarboxylic acid substrates.\(^{(25)}\) Recent studies in our lab however, have suggested that lys-126 interacts with the 5' carboxyl of glutamate or a-ketoglutarate. The reactivity of this lysine however has not been extensively investigated. The goal of the current work was to characterize the stability of the Schiff's base formed between pyridoxal 5' phosphate and lys-126. The effects of various substrate analogs and regulators on its stability were investigated.

**Preparation of Selenium NADP\(^+\) Analog**

With the question, "What would happen if the wavelength of the X-ray is smaller than the repeat distance in a crystal?", Laue founded the science of X-ray crystallography.\(^{(28)}\) Friedrich and Knipping answered this question when they successfully recorded the diffraction pattern of CuSO\(_4\)·5H\(_2\)O in 1912.\(^{(28)}\) W.L. Bragg provided an explanation for the diffraction pattern of an X-ray caused by a crystal lattice at about the same time.\(^{(28)}\) Since these early days in the history of X-ray crystallography, many advances both in this field and in the fields of mathematics and computer science have made the structure determination of small crystals by X-ray crystallography an almost trivial task. Crystallography gives a very detailed structure of small molecules with resolutions up until 0.01 angstroms.\(^{(29)}\) Because of this and due to the relative ease of solving structures with the aid of the computer program packages now available, chemists are increasingly bypassing traditional methods of characterization such as NMR and IR, for X-ray crystallography when the product is easily crystallized.
The recent advances in X-ray crystallography makes it one of the most powerful tools available for elucidating the structure of molecules. With the realization that proteins are ordered in a crystal lattice, came the possibility that protein structures could be solved using X-ray diffraction.\(^{(28)}\) X-ray crystallography has the possibility of giving a very detailed three-dimensional picture of the structure of a protein, while other commonly used methods for getting structural information give just pieces of the puzzle. These pieces then have to be fit together in order to get only a rough sketch of the overall structure of the molecule.

Despite the promise of getting a very detailed picture of the placement of the atoms of a protein in space by X-ray crystallography, proteins introduce many complications into the experiment. Proteins typically are composed of about fifty percent water and lose their crystallinity when dehydrated.\(^{(28)}\) This results in proteins crystals being generally less ordered than small molecules so that atomic resolution is often not possible.\(^{(28)}\) On top of poor resolution, the phase problem of all X-ray crystallography experiments is even more of a challenge with proteins because the methods used for small molecules such as the direct method and Patterson methods get impossibly complicated for proteins.\(^{(28)}\) Also, because protein molecules contain a large number of atoms, there is a large number of X-ray diffraction measurements that have to be made. The intensities of these diffracted beams being less than that of beams diffracted from small molecules further complicating data collection.\(^{(28)}\)

In spite of the many obstacles that need to be overcome, protein chemists have been able to solve the structures of proteins using X-ray crystallography. However, the use of this technique is limited by two factors: 1) the need for a
crystal and 2) the fact that the X-ray method is essentially static where as biological processes are dynamic.\textsuperscript{(28)}

In order to perform a crystallography experiment, a reasonably sized crystal of very high purity has to be available. The necessity of a crystal is a major constraint on the usage of this technique since many large macromolecules can not be crystallized. Also the conditions used to crystallize proteins are usually very harsh which raises questions as to the validity of the structure obtained.\textsuperscript{(29)} First does the structure represent the structure of the native protein? Secondly do the conditions used to crystallize the protein affect the three dimensional structure? Studies done on the activity of ribonuclease, carboxypeptidase, chymotrypsin, papain, and alcohol dehydrogenase in the crystalline form have shown that the crystal structure represents the structure of the functioning enzyme.\textsuperscript{(29)} and chymotrypsin, despite their nearly identical amino acid sequence crystallize under quite different conditions but have been found to have essentially the same tertiary structure suggesting that crystallization conditions have little if any effect on the three dimensional structure of these molecules.\textsuperscript{(29)}

The static nature of X-ray diffraction puts limitations on the type of information that may be obtained. In order to perform an X-ray crystallography experiment, several days are needed to record the data.\textsuperscript{(28)} Because of this, the structure obtained is only an average structure over the time period of data collection. Due to the nature of the X-ray experiment, different conformations an enzyme may assume during its function could not be investigated using this technique.\textsuperscript{(29)}

The X-ray analysis of a protein usually consists of eight steps outlined in figure 2.
Figure 2: Steps in the structure determination of a protein by X-ray crystallography.
The major problem in an X-ray diffraction experiment is that only the intensity of the diffracted rays are recorded. However in order to determine the structure from the diffraction pattern both the intensity and the phase of the diffracted rays have to be known.\(^{(28)}\) Possibly one of the most frustrating and challenging aspect of an X-ray analysis is the determination of the phases of the diffracted rays. Four methods are available to overcome the phase problem for small crystals. However two of these methods, the direct method and the Patterson summation, are not suitable for determining the phases of diffracted rays in the analysis of protein because they get too complicated. The two other methods, heavy atom isomorphous replacement and anomalous scattering, have been successfully used in protein structure determination by X-ray crystallography.

Heavy atom isomorphous replacement involves introducing a heavy atom into the crystal. A perfect isomorphous derivative is one where the only change in the electron density between the derivative and the crystal is at the site of the heavy atom substitution.\(^{(28)}\) In small molecules this is easily achieved by replacing an atom with a similar but more electron dense atom such as replacing sulfur with a selenium, or bromine with an iodine.\(^{(28)}\) Usually a different approach has to be taken with proteins because the atoms are covalently attached which makes it difficult to replace just one atom. A more common technique that is used to introduce heavy atoms into the protein is to diffuse a heavy atom salt into the protein and hope that the salt chelates to a unique amino acid side chain.\(^{(28)}\) This method is obviously a hit and miss approach and may also further complicate the phasing of the diffracted rays if the heavy atom binds at many different points in the protein.\(^{(28)}\)
Enzymes and carrier proteins offer alternative methods of introducing heavy atom probes into the crystal.\(^{(28)}\) If the specificity of the active site of an enzyme or carrier protein is known, a heavy atom analog of the substrate can often be used in the determination of the phases.\(^{(28)}\) Frequently proteins and enzymes have metal ions associated with them. If these metal ions can be removed and replaced with a heavier metal ion, then the phases can be solved from these heavy atom derivatives.\(^{(28)}\) Recently site directed mutagenesis was used to specifically change a sulfur in methionine to selenium giving a selenomethione residue.\(^{(30)}\) However, isomorphous replacement of a sulfur to selenium in the protein does not result in enough of a difference in the electron density to allow its routine use as an adequate heavy atom derivative for phase determination.\(^{(30)}\)

On account of the difficulties encountered in the determination of phases in the elucidation of the structure of a protein by X-ray crystallography, new techniques are constantly being sought to solve the 3-dimensional structure of a protein. Dehydrogenases are an important class of enzymes that have been extensively studied. The dehydrogenases provide a source of high potential hydrogen atoms that are essential in biosynthetic pathways for the reduction of double bonds.\(^{(31)}\) Until this point in time only the crystal structures of NAD\(^+\)-dependent dehydrogenases have been solved. From the structures of these NAD\(^+\)-dependent dehydrogenases, all have been determined to have a common motif in the binding site for NAD\(^+\). The pyridine nucleotide binding domain consists of a series of beta-pleated sheets that are connected by helical regions or loops of polypeptide chains with an angle of twist between sheets of 100 degrees.\(^{(29)}\) This is illustrated in figure 3. Root mean square deviations between equivalent atoms
Figure 3: Diagram of pyridine nucleotide binding domain in NAD$^+$ dependent dehydrogenases.\textsuperscript{(29)}
indicate that there is structural homology between equivalent atoms in the NAD$^+$ binding domain.\(^{(29)}\) In spite of this marked structural homology, there is very little sequence homology in this region.\(^{(29)}\) Only five out of the twenty residues that make up the Rossman fold are conserved.\(^{(29)}\) Three of these residues, gly-25, gly-33 and asp-53 (from the lactate dehydrogenase sequence), have a specific function.\(^{(29)}\) Gly-25 and gly-33 are required in order to let the -A sheet lie in the proper orientation.\(^{(29)}\) Gly-25 is required because if any other amino acid was at position 25 the carbon of the side chain would overlap with the C2 carbon of the adenine moiety of NAD$^+$ and prevent it from binding in the orientation necessary for catalytic activity.\(^{(29)}\) The carboxyl group of asp-53 has been determined to help facilitate the binding of NAD$^+$ by forming a hydrogen bond with the oxygen on the adenine ribose moiety.\(^{(29)}\) Asp-53 may also be important in the specificity of an enzyme for NAD$^+$ over NADP$^+$.

Due to the marked structural homology between the binding sites for NAD$^+$ in NAD$^+$-dependent dehydrogenases and many other NAD$^+$ utilizing enzymes whose structures have been solved, it is of great interest to determine if an NADP$^+$ equivalent of the Rossman fold exists. Additionally, another question that needs to be answered is whether an enzyme is able to preferentially use NAD$^+$ over NADP$^+$ or vice versa or better yet how can an enzyme utilize either NAD$^+$ or NADP$^+$ but with differing selectivity?

The only difference in the structure of NAD$^+$ and NADP$^+$ is that NADP$^+$ has a phosphate group on the C2 carbon of the adenine ribose moiety whereas NAD$^+$ has a hydroxyl group (see figure 4). In the binding domain for NAD$^+$ an aspartate residue is oriented in such a way as to be able to form a hydrogen bond.
Figure 4: Structures of NAD$^+$ and NADP$^+$.
with the hydroxyl group on the C2 of NAD$^+$ which has been shown to help facilitate the binding of NAD$^+$. Clearly NADP$^+$ can not form this hydrogen bond with the aspartate residue due to the fact that this hydroxyl group is phosphorylated. This aspartate residue suggests a potential site responsible for forming an interaction that confers specificity. The question that now needs to be addressed is whether just one residue forming one interaction is enough to give an enzyme the ability to use one cofactor over the other or are there other structural features that determine the specificity for co-enzyme?

In order to answer this question the crystal structure of NADP$^+$-dependent enzymes and those that can use either NAD$^+$ or NADP$^+$ need to be solved. Glutamate dehydrogenase and glucose-6-phosphate dehydrogenase appear to be good starting points to answer these questions. Glutamate dehydrogenase can utilize either NAD$^+$ or NADP$^+$ with an equal affinity while glucose-6-phosphate dehydrogenase can utilize either NAD$^+$ or NADP, but binds to NADP$^+$ with an affinity one thousand times greater than NAD$^+$. Both these enzymes have recently been crystallized after many decades of unsuccessful trials. Despite the fact that crystals are now available, the crystal structures have not been solved due to the lack of a heavy atom derivative needed to solve for the phases of the diffracted beams.

Heavy atom derivatives of substrates and inhibitors of enzymes are commonly used as probes for their structure in an X-ray crystallography experiment. Problems however often accompany this approach. Frequently the inhibitor-enzyme complex is not completely isomorphous with the native enzyme. Usually this is a result of an induced conformational change in the enzyme upon binding of the substrate or inhibitor. When a conformational change occurs and the
data from the derivative is combined with that of the native enzyme, errors occur in the phase determination resulting in an inaccurate picture of the enzyme.

For example, Chymotrypsin has an active site serine residue which was modified to give a series of inhibited-enzyme complexes, the tosyl, BSF and pipsyl\(^{(28)}\) derivatives. These derivatives proved to be isomorphous with each other but not with the native enzyme. To overcome this problem, the tosyl derivative was used as the parent protein in the phase determination step. However using an inhibitor-enzyme complex as the parent compound in the phase determination leads to problems in the interpretation of the structure obtained. A major goal of any X-ray experiment is the determine the orientation of amino acid side chains in the active site of a protein. Inhibitor molecules may interfere with the correct orientation of these side chains in the active site and thus give a poor representation of these residues in the catalytically active form of the enzyme.

The probe proposed to be used as a heavy atom derivative in the isomorphous phase determination of an X-ray crystallography experiment to solve the structure of NADP\(^+\)-dependent enzymes is O-Se-NADP\(^+\) (see figure 5). Glutamate dehydrogenase is of interest due to its unique ability to exploit either NAD\(^+\) or NADP\(^+\) with equal affinity.\(^{(32)}\) NAD(P)\(^+\) is used as a cofactor in the oxidative deamination of glutamate and functions by transferring a hydride radical to the nicotinamide moiety resulting in the 1,4-dihydronicotinamide ring system as shown in figure 6.\(^{(33)}\) The adenine ribose portion of NAD(P)\(^+\) is thought to be important in the interactions involved in the binding of the cofactor to the enzyme.\(^{(33)}\) Changing the nicotinamide moiety of NAD(P)\(^+\) should not change the interactions needed for the specificity of an enzyme for one cofactor over another but depending on the type of change may affect the ability of the cofactor to be reduced in the oxidation-reduction reaction. The thio-derivative of NAD(P)\(^+\) (see
Figure 5: The proposed heavy atom affinity probe to be used to solve the crystal structure of NADP+ dependent enzymes. O-Se-NADP⁺. The nicotinamide ring is replaced with a five membered ring containing selenium. Both the nicotinamide ring and the new ring systems are aromatic.
Figure 6: Functioning of NAD(P)$^+$ as a cofactor. NAD(P)$^+$ accepts one hydride ions resulting in the reduction of the nicotinamide ring system to the 1,4-dihydronicotinamide ring system.\(^{33}\)
figure 7) has been shown to be catalytically active because the modified nicotinamide ring is still able to be reduced.

Using the binary NAD⁺-glutamate dehydrogenase complex as the parent protein in an X-ray experiment, thio-NAD(P)⁺ would probably not be a good probe due to the fact that a carbon to sulfur replacement in the past has been found not to cause enough change in electron density. Seleno-NAD⁺ has also been made and has been found to inhibit glutamate dehydrogenase competitively with NAD⁺ thus indicating that it binds to the enzyme at the same binding site as NAD⁺. If the binary NAD⁺-glutamate dehydrogenase complex is used as the parent protein for phase determination, then the carbon to selenium replacement would probably provide enough difference in electron density to resolve the phases. However, Se-NAD⁺ would not be a good probe for the structure of glutamate dehydrogenase for two reasons. First, glutamate dehydrogenase has two binding sites for NAD⁺, a regulatory site and the catalytic site. Therefore two or more different derivatives are possible with some sites not being fully saturated making the determination of phases more difficult. The second reason is that the probe needs to be covalently attached to the enzyme. Periodate oxidation of NAD(P)⁺ (figure 8a) will convert the diol to a dial which then could form a Schiff’s base with any lysine residue which may be in the site (figure 8b). Reduction with sodium borohydride (figure 8c) would covalently attach the probe. NAD⁺ has two different diols one on the nicotinamide ribose and one on the adenine ribose both of which could undergo periodate oxidation causing two possible products. NADP⁺ only has the diol on the nicotinamide ribose thus providing only one possible product.
Figure 7: Thionicotinamide analog of NAD(P)$^+$.  
$R=\text{OH}; \text{Thio-NAD}^+ \quad R=\text{OPO}_4^{2-}; \text{Thio-NADP}^+$
Figure 8: Attachment of Heavy Atom Probe

a) Periodate treatment of a cis diol results in the hydroxyl groups being oxidized to aldehyde groups.

b) The resulting aldehydes can form a Schiff's base with any nearby amine groups.

c) Sodium borohydride reduction reduces the double nitrogen to carbon bond in the Schiff's base to a single nitrogen to carbon bond.
In the X-ray experiment to solve the crystal structure of glutamate dehydrogenase, the reduced binary O-NADP⁺-glutamate dehydrogenase complex will be the parent complex and the reduced binary O-Se-NADP⁺-glutamate dehydrogenase complex will be the heavy atom isomorphous derivative used to solve the phase problem inherent in the diffraction pattern. While O-Se-NADP⁺ is most likely an inhibitor of glutamate dehydrogenase, there should be very little difference in the conformation of glutamate dehydrogenase when bound to either O-NADP⁺ or O-Se-NADP⁺ in view of the fact the difference is in the catalytic portion of the cofactor and not the portion responsible for the interactions needed to bind to the enzyme. These are the interactions that would most likely be responsible for triggering any conformational changes. O-Se-NADP⁺, at least on paper, seems to be a reasonable probe for the structure of NADP⁺-dependent enzymes. The protocol for synthesizing O-Se-NADP⁺ is shown in figure 9.
Figure 9: The strategy for preparing O-Se-NADP⁺.
EXPERIMENTAL

Reactivity of Lys-126

Materials

Bovine liver L-glutamate dehydrogenase (EC 1.4.1.3) was obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.) as a 67 mg/ml solution in 50% glycerol containing sodium phosphate buffer, pH 7.3. Before use the enzyme was diluted to a stock solution of 2 mg/ml in 0.1 M phosphate buffer, pH 7. The enzyme concentration used for the inactivation was estimated using an extinction coefficient of 0.93 cm\(^{-1}\)*(mg/ml)\(^{-1}\) for a 1 mg/ml solution at 280 nm.\(^{(33)}\) All buffer salts with the exception of sodium dihydrogen phosphate, substrates, cofactors and modulators were purchased from Sigma Chemical Company and made up fresh just before use in 0.1 M phosphate buffer, pH 7. The phosphate buffer was made with sodium diphosphate, monohydrate in the crystalline form purchased from J.T. Baker Chemical Company (Phillipsburg, N.J., U.S.A.). The standard assay mixture used for rate measurements consisted of 20 mM glutamate and 250 M NAD\(^+\) in 0.1 M phosphate buffer, pH 7. The cuvettes used in rate measurements were supplied by Sarstedt Company (Pennsauken, N.J., U.S.A.).

Methods

All enzyme activity measurements were performed using the SLM-AMINCO 3000 Array spectrophotometer (SLM-AMINCO3000). Because the cuvettes used absorb at 340 nm but not at 360 nm, the OD was monitored at 360 nm to monitor for NADH production as opposed to 340 nm. The slope of the absorbance at 360 nm vs time graph was determined using the rate analysis program.
in the applications package of the SLM-AMINCO3000. The absorbance of a 1 mg/ml NADH solution was measured both at 340 nm and 360 nm. Since the absorbances at both wavelengths were within three percent of one another, the rate of enzyme activity was found by dividing the slope of the absorbance vs time line by the extinction coefficient of NADH at 340 nm, 6.22 cm⁻¹. The rates of inactivation and reactivation were found by fitting the rate of enzyme activity vs time graph to the exponential function, \( y = A_0e^{-kt} + B \) where \( A_0 \) is the amplitude of the exponential; \( k \) is the rate of either inactivation or reactivation; \( t \) is the time and \( B \) is the residual activity, using SigmaPlot 4.0 (Jandel Scientific, CA) on a Hewlett Packard RS-16 IBM compatible PC.

\textbf{Inactivation}

The rate of inactivation was found by assaying the enzyme for activity at times of 0, 1.5, 3, 5, 10, 15, 20, 30, and 60 minutes after 1 mM pyridoxal 5' phosphate was added. The enzyme's activity was found by adding 10 uls of the enzyme mixture to 3 ml of standard assay mix.

\textbf{Reactivation}

From the inactivation mixture, a 200 ml aliquot is taken and separated from free pyridoxal 5' phosphate by chromatography on a Sephadex G-25 column. The enzyme containing fraction was determined from its absorbance at 280 nm (figure 10). Aliquots of the enzyme containing fraction were assayed using 3 mls of standard assay mix and the increase in absorbance at 360 nm was monitored on the SLM-Aminco 3000 for 25 cycles with an exposure time of 540 msec.
Determination of Fraction containing GDH from Sephadex G-25 Column

Figure 10: Graph of OD (absorbance) at 280 nm vs. fraction number from the fractions collected on a Sephadex G-25 column. The column was equilibrated with 0.1 M phosphate buffer pH 7.0. A 0.2 mL sample of 0.41 mg/mL solution of glutamate dehydrogenase in 0.1 M phosphate buffer, pH 7 was loaded onto the column. One ml fractions were collected. The OD at 280 nm of each fraction was measured.
Kinetic Calculations

When glutamate dehydrogenase is in the presence of pyridoxal 5' phosphate, glutamate dehydrogenase is inactivated as a result of the Schiff's base formed between the two (figure 1). The rate of inactivation is a function of the relative rates of $k_{\text{inact}}$ and $k_{\text{react}}$. Since the inactivation is done in the presence of excess pyridoxal 5' phosphate, the Schiff's base formation is essentially first order with respect to the concentration of pyridoxal 5' phosphate and the observed rate of the inactivation is:

$$k_{\text{observed}} = k_{\text{inact}}[\text{pyridoxal 5' phosphate}] - k_{\text{react}}$$

Since the reactivation is done without an excess of pyridoxal 5' phosphate, the rate observed for the reactivation is the rate of reactivation; $k_{\text{observed}} = k_{\text{react}}$. The rate of inactivation can be found once $k_{\text{observed}}$ and $k_{\text{react}}$ are both known by:

$$k_{\text{inact}} = (k_{\text{observed}} + k_{\text{react}})/[\text{pyridoxal 5' phosphate}]$$

However, since the rate of reactivation is small compared to the rate of inactivation the rate of inactivation is effectively:

$$k_{\text{inact}} = k_{\text{observed}}/[\text{pyridoxal 5' phosphate}]$$
Criteria For Determining a Significant Result

For each type of data, the data set with no ligand present was used for comparison. A result was classified as significant if the magnitude of the average deviations was less than the difference between the numbers being compared. For example if with no ligand present the result was 0.20 +/- 0.03 and with a ligand present the result was 0.30 +/- 0.03, then this would be regarded as a significant difference. However if the result with a ligand present was 0.25 +/- 0.03 this would not be regarded as a significant result.

Preparation of Selenium NADP+ Analog

Separation of NAD+ from NADP+ using Ion Exchange Chromatography

Materials

AG1-X8 resin was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). The sodium chloride was purchased from J.T.Baker Incorporated (Phillipsburg, N.J., U.S.A.). Both the cofactors NAD+ and NADP+ were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Disposable culture tubes were bought from VWR Scientific Incorporated (San Francisco, CA, U.S.A.). An ISCO Model 1200 fraction collector was used to collect fractions. The OD's were measured on a SLM-Aminco 3000 Array spectrophotometer. Due to the fact that the plastic cuvettes are not transparent at low wavelengths, quartz cuvettes made by Fisher Scientific (Fair Lawn, N.J., U.S.A.) were used to measure the OD at 260 nm.
Methods

Bio Rad AG1-X8 anion exchange resin was slurried with 0.1 M sodium chloride solution and packed into Bio Rad column with a radius of 5 cm to a height of 19 cm. The column was then equilibrated with 100 ml of 0.1 M NaCl solution. One milliliter containing 1.3 mM NADP⁺ and 1.5 mM NAD⁺ in 0.1 M NaCl solution was loaded onto the column. Using a gradient formed by a MRA gradient maker with 55 mL distilled water and 55 mL 1.0 M NaCl solution the cofactors were eluted from the column and 3 mL fractions were collected in disposable culture tubes using a fraction collector. The OD at 260 nm of each fraction was measured to determine the fractions containing NAD⁺ or NADP⁺ (figure 11). AG1-X8 is a anion exchange resin that is derivatized with quatenary ammonium groups. Since NADP⁺ has more negative charge due to its extra phosphate group we would expect it to bind tighter to the column than NAD⁺ (figure 12). To confirm that NAD⁺ eluted first and NADP⁺ eluted last, the above procedure was repeated using first a one mL sample containing either 1.5 mM NAD⁺ or 1.3 mM NADP⁺ (see figure 11b,c).

Determination of Salt Gradient Used to Elute NAD⁺ and NADP⁺

Materials

AG1-X8 resin was purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). The sodium chloride was purchased from J.T.Baker Incorporated
Figure 11: Graph of OD at 260 nm versus fraction number. The column was equilibrated with 0.1 M sodium chloride solution. a) 1.5 mM NAD$^+$ and 1.3 mM NADP$^+$ were dissolved in 0.1 M sodium chloride solution and loaded onto the column. The sample was washed onto the column with 5-10 mL of 0.1 M sodium chloride solution and eluted via a gradient formed with 55 mL of distilled water and 55 mL of 1.0 M sodium chloride solution. b) Same procedure was repeated as described in a but done with a solution of 1.3 mM NAD$^+$. c) Same procedure was repeated as described in (a) but with a solution of 1.5 mM NADP$^+$. 
Figure 12: Structure of a) NAD$^+$ and b) NADP$^+$ at a pH of 6.5. Charges shown using pk$_a$ of primary phosphate as 0.9 and secondary phosphate as 6.1 and assuming that at a pH of 6.4 that the secondary phosphate has an equal probability of being protonated since it is not much past its pk$_a$. 
(Phillipsburg, N.J., U.S.A). Both the cofactors NAD$^+$ and NADP$^+$ were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). Disposable culture tubes were bought from VWR Scientific Incorporated (San Francisco, CA, U.S.A.). An ISCO Model 1200 fraction collector was used to collect fractions. Using a gradient maker purchased from MRA (Clearwater, Fl, U.S.A.), the salt gradient was formed. The salt gradient was determined using an Expandable Ion Analyzer EA940 made by Orion Research (Cambridge, MA, U.S.A.) with a Corning sodium selective electrode Model #476210 (Medfield, MA, U.S.A.), Fisher saturated calomel electrode model # 13-620-57-SN8095112 (Fairlawn, N.J., U.S.A.), and an Orion ATC probe Model # 917002 (Cambridge, MA, U.S.A.). Triethanolamine, the ionic strength buffer, was purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). The OD's were measured on a SLM-Aminco 3000 Array spectrophotometer. Quartz cuvettes made by Fisher Scientific (Fair Lawn, N.J., U.S.A.) were used to measure the OD.

**Methods**

As is shown in figure 10, there is poor resolution of the NAD$^+$ and NADP$^+$ peaks. Better resolution can be achieved by either changing the relative amounts of water and salt to get a slower gradient or by determining the salt gradient and washing through the column a salt solution that has a concentration between that needed to elute NAD$^+$ and the concentration needed to elute NADP$^+$. This will cause the NAD$^+$ to be washed off of the column and the NADP$^+$ will remain bound. Using this method it is possible to separate the two cofactors by many more fractions and to even totally wash NAD$^+$ off of the column if desired.
The sodium chloride gradient used to elute NAD$^+$ and NADP$^+$ was determined using a sodium selective electrode. First the column was equilibrated with 100 mLs of 0.1 M NaCl solution. Three mL fractions of the gradient formed by a MRA gradient maker using 55 mL distilled water and 55 mL of 1.0 M NaCl solution were collected. Two ml of each fraction was diluted to 10 mL with distilled water and then mixed with 10 mL of 0.5 M triethanolamine in order to bring all the samples to the same ionic strength. Triethanolamine to be used as the ionic strength buffer, effectively brings all the samples to the same ionic strength so that the activity coefficients are equal and the concentration is proportional to the activity. The Expandable Ion Analyzer by Orion research with a sodium selective electrode, Fisher saturated calomel electrode, and an Orion ATC probe was first calibrated. The solutions used to calibrate the ion analyzer were prepared by mixing 20 mL of sodium chloride solutions with concentrations of 0.1000M, 0.0100M, 0.0010M and 0.0001M with an equal amount of 0.5 M triethanolamine resulting in final concentrations of 50.00mM, 5.000mM, 0.500mM, and 0.050 mM. The slope of the resulting calibration curve was 57.2 mV/Dec. Using the sodium selective electrode the concentration of each fraction was determined. The Expandable Ion Analyzer made by Orion Research with a Corning sodium selective electrode, Fisher saturated calomel electrode, and an Orion ATC probe was recalibrated after fraction 20 resulting in a slope of 53.4 mV/Dec and after fraction 26 resulting in a calibration curve of 53.7 mV/Dec using standards with final concentrations of 100.0mM, 50.00mM, 5.000mM, 0.5000mM and 0.0500 mM. The concentration of the fractions collected from the gradient are shown in figure 13.

From the graph of concentration versus fraction number, the data for the fractions where the cofactors eluted looked questionable so the Expandable Ion
Analyzer made by Orion Research with a Corning sodium selective electrode, Fisher saturated calomel electrode, and an Orion ATC probe was recalibrated. Final concentrations of the standard solutions used to calibrated the ion analyzer were 100mM, 50mM, 5mM, 0.5mM and 0.05 mM NaCl. The slope of the calibration curve was 54.4 mV/Dec. Concentrations of these fractions is shown in figure 13.

**New Protocol for the Separation of NAD\(^+\) and NADP\(^+\)**

**Materials**
Same materials as for previous trial.

**Methods**
From figure 9 we can see that NAD\(^+\) eluted mainly in fractions 12 and 13 which we see from figure 11 corresponds to a concentration of 0.059 M NaCl and NADP\(^+\) eluted mostly in fraction 20 which corresponds to a concentration of 0.18 M NaCl. Taking this information into account a new protocol was devised.

The AG1-X8 anion exchange column was equilibrated with 0.1 M NaCl. A 1.3 mM NADP\(^+\) and 1.5 mM NAD\(^+\) solution in 0.1 M NaCl was loaded onto the column. Fifty mL of 0.1 M NaCl was washed through the column to force the NAD\(^+\) down the column. Using a gradient formed using a MRA gradient maker with 55 mL of distilled water and 55 mL of 1.0 M NaCl solution, the cofactors were eluted from the column. Three mL fractions were collected. The OD at 260
Figure 13: Graph of sodium concentration versus fraction number.
nm of each fraction was measured to identify the fractions containing cofactors (figure 14a). The peaks were confirmed by repeating the above procedure using solutions that contained just NAD$^+$ and NADP$^+$ (see figure 14 b,c).

**Enzymatic Synthesis of NADP$^+$ from NAD$^+$ Using NAD$^+$-Kinase**

**Materials**

NAD$^+$, NAD$^+$-kinase ((EC 2.7.1.23), type 4 from chicken liver), ATP, morpholinoethanesulfonic acid and 2-mercaptoethanol were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). A Haake FK constant temperature water bath was used. The OD’s were measured on a SLM-Aminco 3000 Array spectrophotometer. Quartz cuvettes made by Fisher Scientific (Fairlawn, NJ, U.S.A.) were used to measure the OD.

Materials used for ion exchange chromatography were as described in the materials section on page 29.

**Methods**

In a culture tube 100 uls of 20 mM NAD$^+$, 200 microliter of 10 mM ATP, 5 uls of 1 mM 2-mercaptoethanol, 20ul of 25 mM MnCl$_2$ and 475 uls of 0.5 M morpholinoethane sulfonic acid buffer, pH 6.5 were added. The reaction mixture was incubated in a constant temperature bath for 30 seconds at 31ºC. Addition of 200 uls a 37.4 units of NAD$^+$-kinase per 200 uls solution initiated the reaction.$^{(36)}$ The reaction was run at 31ºC for 30 minutes and was then loaded onto the AG1-X8 ion exchange column that had been equilibrated with 0.1 M NaCl. Fifty mL of 0.1 M NaCl was washed through the column. The cofactors were eluted by a sodium chloride gradient formed by a MRA gradient.
Figure 14: Graph of OD at 260 nm versus fraction number. The column was equilibrated with 0.1 M sodium chloride solution. a) 1.5 mM NAD$^+$ and 1.3 mM NADP$^+$ were dissolved in 0.1 M sodium chloride solution and loaded onto the column. After the sample was loaded onto the column, 50 mL of 0.1 M sodium chloride solution was washed through the column and the cofactors were eluted via a gradient formed with 55 mL of distilled water and 55 mL of 1.0 M sodium chloride solution. b) Same procedure was repeated as described in a but done with a solution of 1.3 mM NAD$^+$. c) Same procedure was repeated as described in a but done with a solution of 1.5 mM NADP$^+$. 
maker using 55 mL of distilled water and 55 mL of 1.0 M NaCl. Three mL fractions were collected and the OD at 260 nm of each fraction was monitored (figure 15).

**Determination of Extra Peaks in Enzymatic Synthesis**

**Materials**

ATP and ADP were purchased from Sigma Chemical company. All chromatography materials were the same as described in the materials section on page 29.

**Methods**

From a plot of the OD at 260 nm versus fraction number (figure 15) two additional peaks were discovered. After the realization that ATP and ADP would also absorb light at 260 nm and that they are also negatively charged (figure 16 a,b), the ion exchange of ATP and ADP were performed using the above procedure. The results are shown in figure 17.

ADP and ATP were determined to account for one of the additional peaks. However nothing was done to correct for this since it both elute after the NADP+ peak. In order to check the reproducibility of the enzymatic synthesis, the enzymatic synthesis was repeated and the results are shown in figure 18. The procedure is reasonably reproducible given the difficulty of adding the enzyme to the reaction mixture.
Figure 15: Graph of OD at 260 nm versus fraction number of the enzymatic synthesis reaction mixture. In a culture tube 100 uls of 20 mM NAD$^+$, 200 uls of 10 mM ATP, 5 uls of 1 mM 2-mercaptoethanol, 20 uls of 25 mM MnCl$_2$ and 475 microliter of 0.5 M morpholinoethane sulfonic acid buffer, pH 6.5 were added. The reaction mixture was incubated in a constant temperature bath for 30 seconds at 31°C. Addition of 200 microliter of a solution containing 37.4 units of NAD$^+$-kinase initiated the reaction.$^{36}$ The reaction was run at 31°C for 30 minutes and was then loaded onto the AG1-X8 ion exchange column that had been equilibrated with 0.1 M NaCl. Fifty mL of 0.1 M NaCl was washed through the column. The cofactors were eluted by a sodium chloride gradient formed by a MRA gradient maker using 55 mL of distilled water and 55 mL of 1.0 M NaCl. Three mL fractions were collected and the OD at 260 nm of each fraction was monitored.
Figure 16: Structure of a) ADP and b) ATP at a pH of 6.5. Charges shown using pKₐ of primary phosphate as 0.9 and secondary phosphate as 6.1 and assuming that at a pH of 6.4 that the secondary phosphate has an equal probability of being protonated since it is not much past its pKₐ.
Figure 17: Graph of OD at 260 nm versus fraction number. The column was equilibrated with 0.1 M sodium chloride solution. a) 1 mM ATP was dissolved in 0.1 M sodium chloride solution and loaded onto the column. After the sample was loaded onto the column, 50 mL of 0.1 M sodium chloride solution was washed through the column and the cofactors were eluted via a gradient formed with 55 mL of distilled water and 55 mL of 1.0 M sodium chloride solution. b) Same procedure was repeated as described in a), but with a solution of 1mM ADP.
Figure 18: Graph of OD at 260 nm versus fraction number of the enzymatic synthesis reaction mixture for trial 1 and 2. Reproducibility is reasonable given difficulty in quantitatively transferring the NAD+-kinase.
RESULTS

Reactivity of Lys-126

Determination of pyridoxal 5' phosphate Concentration
to Use for Inactivation

First, the appropriate pyridoxal 5' phosphate concentration to study the reactivity of lys-126 was determined in experiments where varying concentrations of pyridoxal 5' phosphate were incubated with a fixed enzyme concentration. At the end of the incubation period, aliquots were assayed for activity using the standard assay protocol described on pages 25-26.

From the plot of the activity vs pyridoxal 5' phosphate concentration (figure 19), it was determined that a pyridoxal 5' phosphate concentration of 1 mM results in approximately 90% inactivation of glutamate dehydrogenase. Since these results were obtained with an enzyme concentration of 7.3 uM per active sites (i.e. the concentration of pyridoxal 5' phosphate is 136 fold excess over the enzyme), we have analyzed the rates of inactivation reported below in terms of a pseudo first order reaction.

Determination of the Rate of Inactivation
of GDH by pyridoxal 5' phosphate

No Modulator

The time course of inactivation of glutamate dehydrogenase by pyridoxal 5' phosphate was determined for a 0.41 mg/mL (7.3 uM per active site) glut-
Figure 19: Effects of the concentration of pyridoxyl 5' phosphate on the inactivation of glutamate dehydrogenase by pyridoxylation. A 0.41 mg/mL solution of glutamate dehydrogenase in 0.1 M phosphate buffer, pH 7 was incubated with different concentrations of pyridoxyl 5' phosphate varying from 0 to 3 mM in 0.1 M phosphate buffer, pH 7. After 1 hour the mixture was assayed for activity.
mate dehydrogenase with 1 mM pyridoxal 5' phosphate. The absorbance at 360 nm vs time data at each incremental assay point resulted in straight lines (figure 20). The rate analysis software of the SLM-AMINCO3000 was used to calculate rates from the data. The plot of the rate vs time is shown in figure 21 and the apparent rate of inactivation was found by fitting the data to an exponential using SigmaPlot 4.0. The rate of inactivation was determined from the equation 
\[ k_{\text{inact}} = \frac{k_{\text{observed}}}{[\text{pyridoxal 5'} \ \text{phosphate}]} \]. For quadruplicate determinations the average rate of inactivation was found to be 0.15 mM$^{-1}$* min$^{-1}$ with an average deviation of 0.02.

**Determination of the Rate of Inactivation of Glutamate Dehydrogenase by Pyridoxal 5' Phosphate In Presence of Various Modulators**

The effects of various modulators on the inactivation were studied using similar techniques. The results are tabulated in table 1.

During the course of the above study, the slope of the OD at 360 nm vs time graph became negative towards the end of the time course when the activity of the enzyme was low. Since this can not be a property of glutamate dehydrogenase and pyridoxal 5' phosphate absorbs at 360 nm (see figure 22 for spectrum), the effect of the standard assay conditions on the absorbance of pyridoxal 5' phosphate was examined.

A blank was made up consisting only of 1 mM pyridoxal 5' phosphate and 0.1 M phosphate buffer, pH 7. Ten microliters of the blank was assayed into cuvettes containing 3 mls of standard assay mix consisting of 20 mM glutamate and 250 uM NAD$^+$ at times of 0, 1.5, 3, 5, 10, 15, 20, 30 and 60 minutes.

46
Figure 20: Absorbance at 360 nm versus time lines obtained from the applications package of the SLM-AMINCO 3000 Array at the various incremental assay points of the inactivation of 7.3 uM per active site glutamate dehydrogenase by 1 mM pyridoxal 5’ phosphate buffer, pH 7.
Figure 21: The time course of inactivation of 7.3 uM per active site glutamate dehydrogenase by 1 mM pyridoxyl 5' phosphate in 0.1 M phosphate buffer, pH 7 with no ligands present. The rate of enzyme activity was determined by dividing the slope of the OD at 360 nm versus time data by 6.22 cm$^{-1}$ mM$^{-1}$, the extinction coefficient of NADH. The rate of glutamate dehydrogenase activity was then plotted as a function of time and the apparent rate constant of inactivation was determined by fitting the exponential using Sigma Plot 4.0.
Table 1 Effects of various modulators and substrate analogs on the time course of inactivation. Concentration used: 0.5 mM ADP, 100 uM GTP, 5 mM glutarate, 5 mM L-2-hydroxyglutarate, 5 mM 2-oxoglutarate, and 1 mM succinate. All solutions were made in 0.1 M phosphate buffer. All inactivations were done with 1 mM pyridoxal 5' phosphate and 7.3 uM per active site glutamate dehydrogenase. \( k = \text{observed rate constant of inactivation}; \ A_0 = \text{amplitude of the exponential}; \) and \( B = \text{residual of the exponential.} \)

<table>
<thead>
<tr>
<th>Modulator</th>
<th>K</th>
<th>Ao</th>
<th>B</th>
<th>% red act</th>
<th>avg Kobs</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0.12</td>
<td>0.04</td>
<td>0.02</td>
<td>29.82</td>
<td>0.14</td>
</tr>
<tr>
<td>none</td>
<td>0.17</td>
<td>0.03</td>
<td>0.00</td>
<td>-10.29</td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.13</td>
<td>0.03</td>
<td>0.00</td>
<td>-1.02</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>0.37</td>
<td>0.05</td>
<td>4.8E-03</td>
<td>8.76</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>0.35</td>
<td>0.01</td>
<td>-3.6E-05</td>
<td>-0.36</td>
<td>0.37</td>
</tr>
<tr>
<td>ADP</td>
<td>0.40</td>
<td>0.02</td>
<td>-2.9E-04</td>
<td>-1.47</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>0.26</td>
<td>0.02</td>
<td>-2.5E-03</td>
<td>-14.29</td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>0.29</td>
<td>0.03</td>
<td>-3.9E-03</td>
<td>-14.94</td>
<td>0.30</td>
</tr>
<tr>
<td>GTP</td>
<td>0.34</td>
<td>0.02</td>
<td>-3.5E-03</td>
<td>-21.21</td>
<td></td>
</tr>
<tr>
<td>Glutarate</td>
<td>0.21</td>
<td>0.03</td>
<td>3.4E-03</td>
<td>10.18</td>
<td></td>
</tr>
<tr>
<td>Glutarate</td>
<td>0.25</td>
<td>0.01</td>
<td>1.3E-04</td>
<td>1.28</td>
<td></td>
</tr>
<tr>
<td>Glutarate</td>
<td>0.26</td>
<td>0.03</td>
<td>0.00</td>
<td>0.00</td>
<td>0.22</td>
</tr>
<tr>
<td>Glutarate</td>
<td>0.17</td>
<td>0.02</td>
<td>0.0E+00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>L-H-Gluta</td>
<td>0.29</td>
<td>0.02</td>
<td>-3.4E-03</td>
<td>-20.48</td>
<td></td>
</tr>
<tr>
<td>L-H-Gluta</td>
<td>0.25</td>
<td>0.01</td>
<td>0.00</td>
<td>-20.48</td>
<td>0.32</td>
</tr>
<tr>
<td>L-H-Gluta</td>
<td>0.36</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>L-H-Gluta</td>
<td>0.38</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>2-Oxogl</td>
<td>0.19</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>2-Oxogl</td>
<td>0.27</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.19</td>
</tr>
<tr>
<td>2-Oxogl</td>
<td>0.12</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.18</td>
<td>0.02</td>
<td>4.3E-03</td>
<td>17.70</td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.16</td>
<td>0.02</td>
<td>-4.3E-03</td>
<td>-27.39</td>
<td>0.16</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.14</td>
<td>0.01</td>
<td>-2.1E-03</td>
<td>-26.58</td>
<td></td>
</tr>
</tbody>
</table>
Figure 22: Spectrum from 300 to 500 nm of a 1 mM pyridoxyl 5' phosphate solution in 0.1M phosphate buffer pH 7 taken on the SLM-AMINCO 3000 Array spectrophotometer.
The absorbance at 360 nm versus time lines obtained are shown in figure 23. The slopes of these lines are listed in table 2. A negative slope for the OD at 360 n vs time graph was obtained suggesting that pyridoxal 5' phosphate is responsible for the "negative rate of enzyme activity" observed. This decrease was determined to be constant over the time course of modification. In order to correct for this, the average rate of decrease in pyridoxal 5' phosphate absorbance was subtracted from each point on the rate vs time graph from the inactivation. Table 3 summarizes the results after the data has been corrected for the decrease in absorbance due to pyridoxal 5' phosphate (figure 23). Since a constant was subtracted from the data, the rates remain essentially the same, however the percent residual activity changed significantly to positive values which are more credible.

In order to characterize the source of this decrease in absorbance in the presence of pyridoxal 5' phosphate, additional experiments were performed. Pyridoxal 5' phosphate is known to be sensitive to light and also is known to form a Schiff's base with free amino groups. The decrease in absorbance could be a result of either or both. Under the conditions used for this study it is possible for either to occur. The assay mix containing pyridoxal 5' phosphate is exposed to light at 360 nm which is near the absorption maxima of pyridoxal 5' phosphate. In addition, the assay mix contains two non-enzymatic sources of amine groups, the amine group on glutamate and the amine group on the adenine ring of NAD+, both of which may form a Schiff's base with excess pyridoxal 5' phosphate. Only Schiff's base formation between pyridoxal 5' phosphate and lys-126 of glutamate dehydrogenase results in the loss of enzymatic activity. The enzymatic activity was measured by monitoring at 360 nm for the production of NADH, at various times during the inactivation. Towards the end of the time course of inactivation most of the enzyme molecules are modified by pyridoxal 5' phosphate and are inactive.
Figure 23: Absorbance at 360 nm versus time lines obtained from the applications package of the SLM-AMINCO 300 Array of the pyridoxal 5' phosphate blank assayed into 3mL of standard assay mix containing 250 uM NAD⁺ and 20 mM glutamate at the various incremental time points. Note the decrease in absorbance is constant over time.
Table 2  Time Course of "Inactivation" of Blank

10 ul aliquot of a 1 mM solution of pyridoxal 5' phospahte was assayed at times of 0, 1.5, 3, 5, 10, 15, 20, 30, and 60 minutes. The OD at 360 nm was monitored and the rate of change in the absorbance was determined using the rate analysis program on the SLM-AMINCO 3000 to determine the slope of the OD at 360 nm vs time lines.

<table>
<thead>
<tr>
<th>time(min)</th>
<th>rate(abs/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-0.00487</td>
</tr>
<tr>
<td>1.5</td>
<td>-0.00579</td>
</tr>
<tr>
<td>3</td>
<td>-0.00569</td>
</tr>
<tr>
<td>5</td>
<td>-0.00564</td>
</tr>
<tr>
<td>10</td>
<td>-0.00308</td>
</tr>
<tr>
<td>15</td>
<td>-0.00534</td>
</tr>
<tr>
<td>20</td>
<td>-0.006</td>
</tr>
<tr>
<td>30</td>
<td>-0.00524</td>
</tr>
<tr>
<td>60</td>
<td>-0.00452</td>
</tr>
<tr>
<td>average:</td>
<td>-0.00513</td>
</tr>
</tbody>
</table>
Table 3  Effects of various modulators and substrate analogs on the time course of inactivation. This data has been corrected for the decrease in absorbance due to pyridoxal 5' phosphate. Concentration used: 0.5 mM ADP, 100 uM GTP, 5 mM glutarate, 5 mM L-2-hydroxyglutarate, 5 mM 2-oxoglutarate, and 1 mM succinate. All solutions were made in 0.1 M phosphate buffer. All inactivations were done with 1 mM pyridoxal 5' phosphate and 7.3 uM per active site of glutamate dehydrogenase.

\( k \) = observed rate constant of inactivation; \( A_0 \) = amplitude of the exponential; and \( B \) = residual of the exponential.

<table>
<thead>
<tr>
<th>Modulator</th>
<th>( K )</th>
<th>( A_0 )</th>
<th>( B )</th>
<th>% red act</th>
<th>( \text{avg Kobs} )</th>
<th>( \text{avg dev} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0.1244</td>
<td>0.0048</td>
<td>0.0007</td>
<td>13.25</td>
<td>0.1496</td>
<td>0.0168</td>
</tr>
<tr>
<td>ADP</td>
<td>0.2126</td>
<td>0.0027</td>
<td>1.6E-04</td>
<td>5.69</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AMP</td>
<td>0.3249</td>
<td>0.0042</td>
<td>8.0E-03</td>
<td>1.86</td>
<td>0.3303</td>
<td>0.0821</td>
</tr>
<tr>
<td>GTP</td>
<td>0.4535</td>
<td>0.0039</td>
<td>6.4E-04</td>
<td>14.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.2147</td>
<td>0.0017</td>
<td>7.4E-04</td>
<td>29.96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.1730</td>
<td>0.0039</td>
<td>1.2E-03</td>
<td>23.36</td>
<td>0.2166</td>
<td>0.0303</td>
</tr>
<tr>
<td>Glutamate</td>
<td>0.2621</td>
<td>0.0043</td>
<td>0.0007</td>
<td>13.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-H-Glutal</td>
<td>0.2927</td>
<td>0.0028</td>
<td>2.7E-04</td>
<td>8.74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-H-Glutal</td>
<td>0.1944</td>
<td>0.0020</td>
<td>3.5E-04</td>
<td>14.83</td>
<td>0.2768</td>
<td>0.1242</td>
</tr>
<tr>
<td>L-H-Glutal</td>
<td>0.1729</td>
<td>0.0027</td>
<td>0.0001</td>
<td>3.28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-H-Glutal</td>
<td>0.3571</td>
<td>0.0028</td>
<td>0.0011</td>
<td>28.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-H-Glutal</td>
<td>0.3668</td>
<td>0.0033</td>
<td>0.0012</td>
<td>26.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>0.1938</td>
<td>0.0027</td>
<td>0.0010</td>
<td>27.73</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>0.3281</td>
<td>0.0019</td>
<td>0.0012</td>
<td>38.24</td>
<td>0.2303</td>
<td>0.0652</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>0.1692</td>
<td>0.0021</td>
<td>0.0013</td>
<td>38.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.1789</td>
<td>0.0026</td>
<td>1.3E-04</td>
<td>4.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.1575</td>
<td>0.0032</td>
<td>1.4E-04</td>
<td>4.14</td>
<td>0.1595</td>
<td>0.0130</td>
</tr>
<tr>
<td>Succinate</td>
<td>0.1421</td>
<td>0.0022</td>
<td>4.9E-04</td>
<td>18.15</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Thus at the end of the time course very little increase in the OD at 360 nm due to the production of NADH will be observed. However the excess pyridoxal 5' phosphate is still available to be either photo-oxidized and/or form a Schiff's base with glutamate and/or NAD⁺. Both options would result in a decrease in the absorbance at 360 nm. In order to determine the cause of the decrease in absorbance, the pyridoxal 5' phosphate blank was assayed in a cuvette containing just 250 uM NAD⁺ in 0.1 M phosphate buffer at pH 7 at times of 0, 1.5, 3, 5, 10, 15, 20, 30, and 60 minutes. A decrease in absorbance was observed and this rate of decrease was again constant over time. However the decrease in the absence of glutamate in the assay mix was only -0.00399 min⁻¹ compared to -0.00513 with glutamate present. This suggests that Schiff's base formation between pyridoxal 5' phosphate and glutamate contributes to the decrease in absorbance. The remainder of the decrease could be a result of the photo-oxidation of pyridoxal 5' phosphate.

*Time Course of Reactivation of Pyridoxylated Glutamate Dehydrogenase with No Modulator Present*

Since the inactivation of glutamate dehydrogenase by Schiff's base formation with pyridoxal 5' phosphate is a reversible process, the rate of reactivation was determined. After the completion of the inactivation, an aliquot of the reaction mixture was run through a Sephadex G-25 column equilibrated with 0.1 M phosphate in order to remove excess pyridoxal 5' phosphate. Removal
of excess pyridoxal 5' phosphate allows for the following reaction to occur.

\[
\text{k}_{\text{react}} \quad \rightarrow \quad \text{GDH-PLP} \quad \quad \text{GDH} + \text{PLP} \\
\text{\textless} \quad \quad \quad \quad \text{\textless} \quad \text{\textless} \quad \text{k}_{\text{inact}}
\]

Since no excess pyridoxal 5' phosphate is present the dissociation is essentially irreversible and the enzyme should regain activity. The rate of this reactivation was determined by assaying for enzyme activity over a two hour time period and fitting the resulting rate vs time data (figure 24) to a single exponential function. The rate of reactivation with no modulator present was found to be 0.02 min\(^{-1}\) with a average deviation of 0.01.

**Time Course of Reactivation of Pyridoxylated Glutamate Dehydrogenase In Presence of Various Modulators**

The effects of the various modulators on the reactivation of pyridoxylated glutamate dehydrogenase were determined using procedures similar to the ones above.

The rates of reactivation are summarized in Table 4.

In our study we observed no effect on the reactivation of pyridoxylated glutamate dehydrogenase by the various modulators.
Figure 24: Time course of reactivation after being modified with 1 mM pyridoxal 5' phosphate for 1 hour. Pyridoxylated glutamate dehydrogenase was assayed for activity at times of 0, 5, 10, 15, 20, 30, 60 and 120 minutes. The slope of the OD at 360 nm versus time data at each incremental assay point was divided by the extinction coefficient of NADH and the resulting rate of enzyme activity versus time graph is shown for the reactivation done in the presence of 20 mM 2-oxoglutarate.
Table 4  Effects of the various modulators on the time course of reactivation. A Sephadex G-25 column was equilibrated with one of the following: 2.5 mM ADP, 300 uM GTP, 20 mM glutarate, 20 mM L-2-hydroxyglutarate, 10 mM 2-oxoglutarate, and 5 mM succinate. All solutions were made in 0.1 M phosphatse buffer. Reactivation was monitored after 7.3 uM per active site of glutamate dehydrogenase was incubated with 1 mM pyridoxal 5' phosphatse for 1 hour. k=rate constant of reactivation; A_o=amplitude of the exponential; and B=the residual of the exponential.

| Modulator | K     | Ao    | B     | avgK(rect) | avg.dev.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>0.019</td>
<td>-0.019</td>
<td>0.017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.022</td>
<td>-0.014</td>
<td>0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.022</td>
<td>-0.011</td>
<td>0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>0.029</td>
<td>-0.015</td>
<td>0.013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>0.047</td>
<td>-0.010</td>
<td>0.009</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>0.043</td>
<td>-0.002</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>0.036</td>
<td>-0.001</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>0.028</td>
<td>-0.011</td>
<td>0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTP</td>
<td>0.029</td>
<td>-0.005</td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-H-Gluta</td>
<td>0.039</td>
<td>-0.007</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-H-Gluta</td>
<td>0.024</td>
<td>-0.008</td>
<td>0.011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L-H-Gluta</td>
<td>0.026</td>
<td>-0.009</td>
<td>0.010</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutarate</td>
<td>0.022</td>
<td>-0.007</td>
<td>0.062</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glutarate</td>
<td>0.030</td>
<td>-0.014</td>
<td>0.014</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Oxogl</td>
<td>0.025</td>
<td>-0.002</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Oxogl</td>
<td>0.028</td>
<td>-0.002</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.023</td>
<td>-0.012</td>
<td>0.006</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.019</td>
<td>-0.013</td>
<td>0.007</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate</td>
<td>0.013</td>
<td>-0.002</td>
<td>0.012</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Preparation of Selenium NADP⁺ Analog**

*Separation of NAD⁺ from NADP⁺ using Ion Exchange Chromatography*

Using the procedure described on page 29 (see figure 11) it is obvious that resolution was not achieved. To achieve better resolution, the salt concentrations needed to elute the cofactors were determined. The salt gradient used to elute NAD⁺ and NADP⁺ was determined (figure 13) using the procedure described on pages 33-35. From figure 13 we can see that NAD⁺ eluted at a concentration of 0.059 M NaCl and NADP⁺ eluted at a concentration of 0.18 M NaCl.

A new protocol was devised using this information. Resolution is greatly improved using this protocol as can be seen from the OD at 260 nm versus fraction number graph (figure 14).

*Enzymatic Synthesis of NADP⁺ from NAD⁺ using NAD⁺-Kinase*

Using the procedure described on pages 37-39, NADP⁺ was formed by enzymatically phosphorylating NAD⁺ using NAD⁺-kinase. Two trials were performed and the trials were reasonable reproducible considering the difficulty encountered in quantitatively transferring the enzyme solution.
DISCUSSION

Reactivity of Lys-126

Effects on the Rate Constant of Modification

The rate constant of inactivation of 0.41 mg/ml (7.3 uM per active site) glutamate dehydrogenase by 1 mM pyridoxal 5' phosphate in 0.1 M phosphate buffer, pH 7 was determined to be 0.15 +/- 0.02 sec\(^{-1}\). No effect was observed on the rate constant of inactivation of glutamate dehydrogenase by pyridoxal 5' phosphate when the inactivation was done in the presence of the substrate analogs (see figure 25) L-2-hydroxyglutarate (\(k_{\text{inact}}=0.28 +/- 0.12 \text{ sec}^{-1}\)) or succinate, a four carbon dicarboxylic acid (\(k_{\text{inact}}=0.16 +/- 0.01 \text{ sec}^{-2}\)). In the presence of the product of the oxidative deamination reaction, 2-oxoglutarate, the rate constant of inactivation was discovered to be 0.23 +/- 0.07. Within experimental error the rate constants with 2-oxoglutarate and no modulator are indistinguishable. A slight increase in the rate constant of inactivation of glutamate dehydrogenase by pyridoxylation is observed when the enzyme is modified in the presence of glutarate, a five carbon, catalytically inactive substrate analog of glutamate. With glutarate present a rate constant of inactivation of 0.22 +/- 0.03 is obtained. Considering the magnitude of the errors of this determination and that with no modulator present, the rate constant for inactivation in the presence of glutarate is, statistically, significantly faster than that with no modulator present. With the exception of glutarate, the substrate analogs have little if any affect on the rate constant of inactivation of glutamate dehydrogenase by pyridoxal 5' phosphate.
Figure 25: Structures of substrate analogs: a) L-glutamate; b) 2-oxoglutarate; c) glutarate; d) 2-D-hydroxyglutarate; e) 2-L-hydroxyglutarate and f) succinate.
The effects of the purine nucleotides ADP and GTP (see figure 26) were also investigated. When the modification is done in the presence of ADP the rate constant of inactivation is greatly increased to 0.33 +/- 0.08 sec^-2. GTP brings about a similar increase in the rate constant of inactivation to 0.29 +/- 0.03 sec^-1. Both purine nucleotides facilitate inactivation of glutamate dehydrogenase by pyridoxal 5' phosphate. This observed increase in the rate constant of inactivation of the enzyme could be the result of the binding of the purine nucleotide resulting in an induced conformational change in glutamate dehydrogenase leading to a greater accessibility of lys-126 toward modification by pyridoxal 5' phosphate (figure 27a). Another possibility is that the pK of lys-126 could change due a conformational alteration, resulting in the lys-126 being in either the protonated or deprotonated form, enhancing the reactivity of this lysine residue (figure 27b).

Preliminary results show no pH dependence on the reactivity of lys-126 toward pyridoxylation. Studies done by Schellenberg and Metzler revealed that Schiff's base formation with free lysine was also independent of pH. Both our preliminary results and previous work done by others strongly suggest that the increase in the rate of inactivation by pyridoxal 5' phosphate is not due to a change in pK of lys-126, but the result of greater accessibility due to an induced conformational change.

Since modification of glutamate dehydrogenase by pyridoxal 5' phosphate is a result of reversible Schiff's base formation, the effects of the various modulators and analogs on the reactivation was examined as well. With no ligands present, the rate of reactivation was determined to be 0.021 +/- 0.013. In the presence of modulators ADP and GTP, the rate of reactivation was found to be 0.039 +/- 0.010 and 0.028 +/- 0.001 respectively. The purine nucleotides thus have no significant effects on the rate of reactivation of pyridoxylated glutamate.
Figure 26: Structure of allosteric regulator: a) GTP and b) ADP
Figure 27: Conformational effects of the binding of the purine nucleotides.

a) Binding of the purine nucleotide to glutamate dehydrogenase results in a conformational change resulting in greater accessability of lys-126.

b) Binding of the purine nucleotide to glutamate dehydrogenase results in a conformational change where lys-126 is in a more reactive form due to a change in its pK as a result of a local environment effect.
Various substrate analogs were also examined for their effects on the reactivation of glutamate dehydrogenase. The rate constant of reactivation in the presence of succinate was determined to be 0.018 +/- 0.004. When L-2-hydroxyglutarate, glutarate or 2-oxoglutarate were independently present in the reactivation mixture, the rate constants of reactivation were determined to be 0.026 +/- 0.004, 0.030 +/- 0.006, and 0.027 +/- 0.002 respectively. From our studies neither the purine nucleotides nor the substrate analogs exhibited any effect on the rate constant of reactivation of pyridoxylated glutamate dehydrogenase suggesting that either the modified enzyme can no longer bind these ligands or, if the modified enzyme can bind these ligands, any conformational changes resulting from the binding of these ligands do not affect the ability of the Schiff's base to dissociate.

**Effects of the Ligands on the Extent of Modification**

The effects of the various ligands on the percent residual activity after modification of glutamate dehydrogenase by pyridoxyl 5' phosphate was also investigated. With no modulator present in the inactivation mixture, the percent residual activity(% R.A.) was determined to be 9% +/- 3%. When the purine nucleotides, ADP and GTP were present the % R.A. were discovered to be 7% +/- 5% and 7% +/- 2% respectively. ADP and GTP have no effect on the extent of modification of glutamate dehydrogenase by pyridoxal 5' phosphate. This observation is consistent with work published by Chen and Engel.(37,38)

The consequences of substrate analogs on the percent residual activity were also determined. With L-2-hydroxyglutarate present, the % R.A. was found to be 16 +/- 9. Due to the magnitude of the average deviation, no conclusions can be
drawn. The presence of glutarate results in a % R.A. of 22% +/− 5%, indicating a large increase in the residual activity at the end of the modification of glutamate dehydrogenase by pyridoxal 5' phosphate. 2-oxoglutarate, the product of the oxidative deamination reaction of glutamate dehydrogenase, leads to a percent residual activity of 35% +/− 5% which is even larger than that of glutarate. The four carbon dicarboxylic acid analog, succinate, has no effect on the percent residual activity as indicated by the result of 9% +/− 6% residual activity. With the exception of succinate and possibly L-2-hydroxyglutarate, the substrate analogs result in a higher percent residual activity remaining after modification with pyridoxyl 5' phosphate for one hour.

**Conclusions about ADP and GTP**

When bound in the absence of other ligands both purine nucleotides, ADP and GTP, increase the rate constant of inactivation of glutamate dehydrogenase by pyridoxal 5' phosphate. The observed increase in the rate constant is comparable for both GTP and ADP. This suggests that both ADP and GTP have similar conformational effects on the enzyme at least in the vicinity of lys-126. GTP has historically been regarded as a potent inhibitor and under most conditions it is. ADP has both inhibitory and activatory effects on the glutamate dehydrogenase depending on the conditions. Recently, however, GTP has been suggested to show effects similar to ADP when present at low concentrations of cofactor (conditions in which GTP will bind to only one of the two or more potential binding sites\(^{40}\)). The results of the current work demonstrate that ADP and GTP have similar effects when either ADP or GTP is bound to only one of their two or more potential binding sites, providing the first supporting evidence for this suggestion.
The effects of ADP and GTP on the residual activity was previously investigated by Chen and Engel.\(^{(38,39)}\) Our results are in agreement with their finding that the purine nucleotides have no effect on the residual activity. Chen and Engel also demonstrated that in the presence of the reduced cofactor NAD(P)H, GTP lessened the extent of the inactivation however no rate constants were reported. In the presence of NAD(P)H, GTP is known to have at least two binding sites while in the absence of NAD(P)H, GTP has only a single binding site available.\(^{(39)}\) The protection of modification of lys-126 when both NAD(P)H and GTP are present could be a result of a conformational change triggered by the ternary complex preventing lys-126 from being modified by pyridoxal 5' phosphate. Another possibility is that either the NAD(P)H or GTP binding sites are close to the substrate binding site resulting in lys-126 being blocked.

**Conclusions about Five Carbon Dicarboxylic Acid Substrate Analogs**

The presence of various substrate analogs in the inactivation mixture had little effect on the rate constants of inactivation. However, with the exception of succinate and L-2-hydroxyglutarate, the various analogs resulted in an increase in the residual activity suggesting that lys-126 is blocked from being modified by pyridoxal 5' phosphate. lys-126 is important because it is thought to be involved in the binding of substrate by forming a salt bridge with its 5' carboxyl group. Thus when substrate analogs containing a 5' carboxyl group are bound to glutamate dehydrogenase, lys-126 is inaccessible for modification by pyridoxal 5' phosphate (figure 28).

If pyridoxylation were performed at saturating substrate concentrations, no modification by pyridoxal 5' phosphate at lys-126 would occur. Due to the high
Figure 28: Model of the active site of Glutamate Dehydrogenase with Glutamate bound. This model is based on previous work (46), and is constructed using Alchemy software.
ionic strength of saturating concentrations, the modification had to be performed at sub-saturating concentrations of substrate analogs. At subsaturating conditions the enzyme molecules that have no substrate bound to them are modified by pyridoxal 5' phosphate. Thus one would expect the rate constant of inactivation in the presence of substrate analogs to have the same rate constant of inactivation as with no ligand present which is what was observed. However the residual activity would be expected to increase due to the concentration of enzyme molecules with bound substrate analogs because they were protected from modification.

**Conclusions about Succinate**

Succinate was determined to have no effect on the rate constant of inactivation indicating succinate does not affect the reactivity of lys-126. The residual activity of glutamate dehydrogenase in the presence of succinate also remained the same as with no ligands suggesting that the binding of succinate does not block lys-126 from being modified. Kinetic studies(41) have indicated that the four carbon dicarboxylic acid analog binds in a quite different orientation between two positive charges (figure 29), one shared with the 1' carboxyl group of the substrate and its analogs and the other somewhere in the cofactor binding site (figure 30). The present study clearly supports this by demonstrating that lys-126 is still available for modification even when succinate is bound.

**Summary of Results.**

The effects of the purine nucleotides on the reactivity of lys-126 are a result of a conformational change making lys-126 more accessible for modification. The
Figure 29: Active site of Glutamate Dehydrogenase with Succinate bound.
Figure 29: Active site of Glutamate Dehydrogenase with both Glutamate and Cofactor Pyrophosphate bridge shown.
substrate analogs had no effect on the rate of reaction of lys-126, however, two major conclusions that can be drawn from the residual activity results. 1) lys-126 must not interact with the 1' carboxyl group of dicarboxylic acid substrates as has been previously suggested and 2) succinate must bind differently than five carbon dicarboxylic acid substrates.

**Preparation of Selenium NADP+ Analog**

The ability to separate NAD+ from NADP+ is essential if an enzymatic synthesis is going to be used to make Se-NADP+ from Se-NAD+. Using a Bio Rad AG1-X8 anion exchange column, a procedure was developed for separating NAD+ and NADP+ with good resolution. With the ability to separate NADP+ from NAD+, an enzymatic synthesis is a feasible method to prepare Se-NADP+ from Se-NAD+. 
REFERENCES

7. Smith, T., and Bell, J.E., (1985) Arch. Biochem. Biophys. 239, 63-73
30. Personal communication with E.B.
41. Bell, J.E. to be submitted to *Biochemistry*