INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI
A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600
PART I. STRUCTURE-FUNCTION STUDIES OF ADENYLATED KINASE WITH UNNATURAL AMINO ACIDS

PART II. STRUCTURE-FUNCTION STUDIES OF PHOSPHATIDYLINEOSITOL-SPECIFIC PHOSPHOLIPASE C: KINETIC ANALYSIS AND THE ROLES OF HIS-32 AND ASP-274

DISSERTATION

Presented in Partial Fulfillment of the Requirement for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By
Zhong Zhao, B.S.

* * * *

The Ohio State University
1996

Dissertation Committee:
Ming-Daw Tsai, Adviser
Robert S. Coleman
Dehua Pei

Approved by

Adviser

Department of Chemistry
ABSTRACT

Unnatural amino acid mutagenesis was used to probe the importance of the aromaticity of Tyr-95 and the ring size of Pro-17 in the catalysis by adenylate kinase (AK). Previous studies indicated that replacement of Tyr-95 with Phe did not cause a perturbation in the activity, whereas replacement with leucine caused a significant decrease in activity. 2,5-Dihydrophenylalanine (DiHPhe) was incorporated into AK to replace Tyr-95. The Tyr95DiHPhe mutant showed k\text{cat} and K\text{m,AMP} similar to those of WT, suggesting that the aromaticity at Tyr-95 is not critically important for the catalysis by AK. Four ring analogs of proline, pipecolic acid, homopipecolic acid, 3,4-dehydroproline and azetidine-2-carboxylic acid (Aze) were used to replace Pro-17. Among the four proline mutants, only Pro17Aze showed a large decrease in activity. The rigidity of the four-membered ring of Aze could restrict the movement of the P-loop and impair the conformational changes obligatory for the catalysis by AK.

A new continuous assay of phosphatidylinositol-specific phospholipase C (PI-PLC) was set up with a thio substrate analog (2R)-1,2-dipalmitoxypropanethiophospho-1-D-myoinositol (DPsPI). With diheptanoyl-sn-glycerol-3-phosphocholine as the detergent, V\text{max} of 53.5 μmol·min⁻¹·mg⁻¹ and K\text{m,app} of 0.18 mM were obtained. The activity represents 8.5 fold increase over previously
reported thio assay with hexadecylthiophosphoryl-1-myoinositol, while $K_{m,app}$ is relatively small. To probe the structure-function relationship of PI-PLC, mutagenesis studies combined with NMR, CD and kinetic analysis were carried out on two active site residues: His-32 and Asp-274. H32A, H32N, H32Q and D274A showed large decrease in specific activity, while D274N and D274E retain 0.5% and 23.5% activity respectively. Asp-274 mutants showed large decrease in conformational stability. D274A and D274N have a bell-shape curve in pH-rate profile, while WT PI-PLC has a broad pH range for optimal activity. H32A mutant showed a similar feature of pH-rate profile. Based on the result, His-32 is expected in a protonated form for PI-PLC to function normally for the first step reaction. PI-PLC is suggested to carry the catalysis through a ribonuclease-like mechanism, with ribonuclease T1 more likely. His-32 could act as a general acid for the catalysis, Asp-274 could act as a general base. In another possibility, His-32 acts as a general base.
ACKNOWLEDGMENTS

I am grateful to my advisor, Professor Ming-Daw Tsai, for the support through the years of my graduate study at The Ohio State University. I have benefited greatly from his encouragement, patience and perspectives of science.

With respect to the collaborative nature of AK-unnatural amino acid mutagenesis project, I thank Dr. Zhengtao Shi for various technical assistance and construction of Pro17Amber DNA, Dr. Xiaohong Liu for construction of Tyr95Amber DNA and for the kinetic analysis of Tyr95DiHPhe mutant, Dr. Baohua Huang for purification of T4 ligase, for construction of pUK vector and for preparation of tRNA_{CUA}(CA), Ru-Tai (Ted) Jiang for preparation of large amounts of pdCpA precursor.

Regarding PI-PLC project, I thank Dr. Karol S. Bruzik at University of Illinois-Chicago for providing the substrate analog DPspI, Suzette R. Riddle for kindly providing initial various technical assistance and for providing pHN1403 PI-PLC plasmid and other materials, Robert J. Hondal for sharing some of his unpublished results and for subcloning PI-PLC into M13mp19, Hua Liao for purification of the mutants, especially
D274A, D274N and D274E, Alexander V. Kravchuk for obtaining NMR spectra of the mutants and for providing help on NMR related problems.

I thank Dr. Brian Wemeburg, Jinwoo Ahn and Xuejun Zhong for the helpful discussion and assistance. I thank Xuejun Zhong, Karen Ericson and Vadim Kraynov for running the DNA dye-deoxy sequencing. I thank all the group members for various help. I thank Dr. In-Ja Byeon for her assistance on computer modeling and Dr. Gordon Renkes assistance on CD experiments.

Most importantly, I thank my wife Qing for her love, patience and support through the up and downs of my experiments. I thank my mother and my sister for their love and support. I thank my father for introducing me chemistry, for encouraging me to come to US for my advanced study, and it is to him that I dedicate this dissertation. I really wish him live long enough to see me finishing my Ph.D. education.
VITA

May 7, 1967 ................................................................. Born - Linfen, Shanxi
P. R. China

1987 .............................................................................. B. S., Peking University
Beijing, P. R. China

1987-1990 ........................................................................ Research Associate
Peking University
Beijing, P. R. China

1990-present ................................................................. Graduate Teaching and
Research Associate
The Ohio State University
Columbus, Ohio

PUBLICATIONS

"Mechanism of Adenylate Kinase. Probing the Importance of the Aromaticity in
Tyrosine-95 and the Ring Size in Proline-17 with Unnatural Amino Acids" J. Am. Chem.
Soc. 118, 3535-3536

FIELDS OF STUDY

Major Field: Chemistry
TABLE OF CONTENTS

ABSTRACT ................................................................. ii
ACKNOWLEDGEMENTS ........................................ iv
VITA ................................................................. vi
LIST OF TABLES .................................................. x
LIST OF FIGURES ............................................... xi
LIST OF ABBREVIATIONS ................................... xv

PART I. STRUCTURE-FUNCTION STUDIES OF ADENYLATED KINASE WITH UNNATURAL AMINO ACIDS ........................................ 1

CHAPTER 1. INTRODUCTION .................................. 2
  General Background of AK .................................. 2
  Background of Unnatural Amino Acid Mutagenesis
    Structure-Function Studies of Proteins with Unnatural Amino Acids ...... 6
    General Method of Site-Directed Unnatural Amino Acid Mutagenesis ...... 8
    Structure-Function Studies of AK with Unnatural Amino Acids .......... 21

CHAPTER 2. MATERIALS AND METHODS FOR THE UNNATURAL AMINO ACIDS MUTAGENESIS ........................................ 24

  Preparation of Plasmid DNA .................................. 24
  Synthesis of Aminoacyl tRNA ................................... 28
  In Vitro Protein Synthesis
    Materials ......................................................... 28
    Ribonuclease-Free Techniques .................................. 29
    SDS-PAGE ....................................................... 29
    In Vitro Expression of HAK .................................... 31
    In Vitro Suppression of Amber Mutants of HAK ......................... 38
CHAPTER 3. SYNTHESIS OF AMINOACYL TRANSFER RNA ................. 46

Introduction ......................................................... 46
Experimental Section
Materials ................................................................. 47
Synthesis of Dinucleotide pdCpA .................................. 48
Synthesis of Homopipeolic Acid and Dihydrophenylalanine .......... 52
Protection of Unnatural Amino Acids ............................ 58
Syntesis of Aminoacylated pdCpA ................................ 62
Results and Discussion ............................................... 64

CHAPTER 4. RING SIZE OF PRO-17 AND AROMATICITY OF TYR-95 .... 67

Introduction .......................................................... 67
Results ................................................................. 76
Discussion ............................................................ 83

PART II. STRUCTURE-FUNCTION STUDIES OF PHOSPHATIDYLINOSITOL-
SPECIFIC PHOSPHOLIPASE C: KINETIC ANALYSIS AND THE ROLES
OF HIS-32 AND ASP-274 .............................................. 92

CHAPTER 5. INTRODUCTION ............................................. 93

General Background of PI-PLC ...................................... 93
Properties of PI-PLC Enzymes ...................................... 95
Chemical Mechanism of Bacterial PI-PLC Reaction .................. 107
Site-Directed Mutagenesis .......................................... 110

CHAPTER 6. MATERIALS AND METHODS ............................... 114

Materials .............................................................. 114
Mutant Construction ................................................ 116
Purification of *B. thuringiensis* PI-PLC ......................... 127
Assay of *B. thuringiensis* PI-PLC ................................ 131
pH-Activity Profile ................................................... 132
Proton NMR Structural Characterization ......................... 132
Guanidine Hydrochloride-Induced Denaturation .................... 133
Sample Preparation and Ellipticity Measurement
Denaturation Curve Analysis
Calculation of Conformational Stability
Molecular Graphics ................................................. 136
CHAPTER 7. CONTINUOUS ASSAY OF PI-PLC WITH DP3PI ......................... 137
  Background ....................................................................................................... 137
  Materials and Methods .................................................................................... 145
  Results and Discussion .................................................................................... 147

CHAPTER 8. THE ROLES OF HIS-32 AND ASP-274 IN PI-PLC CATALYSIS .................................................................................. 155
  Introduction ........................................................................................................ 155
  Results ................................................................................................................ 158
  Discussion .......................................................................................................... 177

LIST OF REFERENCES .......................................................................................... 188

APPENDIX A. NMR SPECTRA OF NEWLY SYNTHESIZED ORGANIC COMPOUNDS ................................................................. 198

APPENDIX B. 1D PROTON NMR SPECTRA OF D274E AND WT PI-PLC ..... 212
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Summary of steady-state studies for WT and Pro-17 mutants</td>
<td>70</td>
</tr>
<tr>
<td>2</td>
<td>Summary of kinetic data on mutants at Tyr-95 in hAK1 and Phe-86 in Ake</td>
<td>74</td>
</tr>
<tr>
<td>3</td>
<td>Summary of suppression efficiencies and kinetic data</td>
<td>82</td>
</tr>
<tr>
<td>4</td>
<td>Properties of phosphoinositide-specific phospholipase C enzymes</td>
<td>96</td>
</tr>
<tr>
<td>5</td>
<td>Specific activities of WT and mutant PI-PLC $^3$H-PI</td>
<td>159</td>
</tr>
<tr>
<td>6</td>
<td>Steady-state kinetic parameters of WT and mutant PI-PLC with DPsPI</td>
<td>160</td>
</tr>
<tr>
<td>7</td>
<td>Free energies of unfolding induced by guanidine hydrochloride</td>
<td>176</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure

1  Primary sequences of three different types of AK .................................... 4
2  Scheme of the random Bi Bi kinetic mechanism of AK  ......................... 5
3  Strategy for the general biosynthetic method to site-specifically incorporate unnatural amino into proteins ................................................ 9
4  Examples of unnatural amino acids that have been successfully incorporated into proteins .......................................................... 10
5  Generation of yeast tRNA^{Phe}_{CUA}(-CA) by runoff transcription .......... 15
6  tRNA^{Phe}_{CUA}(-CA) prepared by anticodon-loop replacement and runoff transcription methods ....................................................... 16
7  Strategy for the chemical aminoacylation of tRNA^{Phe}_{CUA} ................. 19
8  The expression vector pUK ...................................................................... 26
9  Scheme of the enzyme-coupled assay used for AK kinetics .................... 44
10 Synthesis of Protected pdCpA .............................................................. 49
11 Deprotection to Release pdCpA ............................................................. 51
12 Synthesis of L-homopipecolic acid ........................................................ 53
13 Synthesis of 2,5-dihydrophenylalanine .................................................... 56
14 Synthesis of aminoacyl tRNA ................................................................. 59
15 Stereoview of the structure of *E. coli* AK complexed with AMP and AMPPNP .............................................................. 68
<table>
<thead>
<tr>
<th>Page</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Structure of amino acids substituted for Pro-17 in adenylate kinase</td>
</tr>
<tr>
<td>17</td>
<td>Structures of tyrosine, phenylalanine, and 2,5-dihydrophenylalanine</td>
</tr>
<tr>
<td>18</td>
<td>Autoradiogram of <em>in vitro</em> piperolic acid suppression in the presence of [^{35}S]-L-methionine</td>
</tr>
<tr>
<td>19</td>
<td>Autoradiogram of <em>in vitro</em> suppression labeled with [^{35}S]-L-methionine</td>
</tr>
<tr>
<td>20</td>
<td>Silver stained SDS-PAGE gel showing determination of the concentration of Pro17HPip elute from the purification with His Bind resin</td>
</tr>
<tr>
<td>21</td>
<td>Plots of (1/v ) vs (1/[ATP]) and (1/v ) vs (1/[AMP]) from saturation kinetic experiments carried out on Pro17HPip purified from <em>in vitro</em> suppression reaction with His.Bind resin</td>
</tr>
<tr>
<td>22</td>
<td>Stereoview of P-loop of AK1p</td>
</tr>
<tr>
<td>23</td>
<td>Stereoview of the structure of <em>E. coli</em> AK complexed with AP5A with Phe-86 and AP5A shown</td>
</tr>
<tr>
<td>24</td>
<td>Interactions between residue 95 and AMP's adenosine</td>
</tr>
<tr>
<td>25</td>
<td>Reactions catalyzed by PI-PLC</td>
</tr>
<tr>
<td>26</td>
<td>The schematic diagram of the network of IP3/Ca^{2+} interactions in the active site of mammalian PI-PLCδ</td>
</tr>
<tr>
<td>27</td>
<td>The crystal structure of <em>B. cereus</em> PI-PLC with bound myo-inositol</td>
</tr>
<tr>
<td>28</td>
<td>Contacts between the side chains of <em>B. cereus</em> PI-PLC residues bound myo-inositol</td>
</tr>
<tr>
<td>29</td>
<td>Sequence comparison of PI-PLCs from several bacterial sources</td>
</tr>
<tr>
<td>30</td>
<td>General structure of GPI anchors</td>
</tr>
<tr>
<td>31</td>
<td>Proposed mechanism of catalysis by <em>B. cereus</em> PI-PLC</td>
</tr>
<tr>
<td>Page</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>32</td>
<td>Expression vector pHN1403 with PI-PLC gene inserted 115</td>
</tr>
<tr>
<td>33</td>
<td>Molecular structures of two substrate analogs 2-naphthyl-<em>myo</em>-inositol-1-phosphate and 4-nitrophenyl-<em>myo</em>-inositol-1-phosphate 138</td>
</tr>
<tr>
<td>34</td>
<td>Scheme for spectrophotometric determination of phospholipase A2 activity using thio ester analogs of phospholipids 140</td>
</tr>
<tr>
<td>35</td>
<td>Effect of pH on the extinction coefficients of 4-thiopyridine at 324 nm and 2-nitro-5-thiobenzoic acid at 412 nm 141</td>
</tr>
<tr>
<td>36</td>
<td>Structures of Cn-thio-PI and cleavage of Cn-thio-PI by PI-PLC enzymes 143</td>
</tr>
<tr>
<td>37</td>
<td>Structure of DPsPI and the cleavage of DPsPI by PI-PLC enzyme 144</td>
</tr>
<tr>
<td>38</td>
<td>Initial reaction rate as a function of the ratio ([\text{DHPC}]/[\text{DPsPI}]) 149</td>
</tr>
<tr>
<td>39</td>
<td>The linear relationship of initial reaction rate with the amount of WT PI-PLC enzyme used in the assay 150</td>
</tr>
<tr>
<td>40</td>
<td>Initial reaction rate as a function of substrate concentration under the condition of ([\text{DPsPI}]/[\text{DHPC}] = 1 : 4) 151</td>
</tr>
<tr>
<td>41</td>
<td>Initial reaction rate as a function of substrate concentration under the condition ([\text{DHPC}] = 8.07 \text{ mM}) 152</td>
</tr>
<tr>
<td>42</td>
<td>The two step reaction catalyzed by bacterial PI-PLC 156</td>
</tr>
<tr>
<td>43</td>
<td>Steady-state kinetic analysis of D274N and D274E 161</td>
</tr>
<tr>
<td>44</td>
<td>pH activity profiles of WT PI-PLC and D274N 163</td>
</tr>
<tr>
<td>45</td>
<td>The 1D proton NMR spectra of mutants and WT PI-PLC 166</td>
</tr>
<tr>
<td>46</td>
<td>NOESY spectrum of WT PI-PLC 168</td>
</tr>
<tr>
<td>47</td>
<td>NOESY spectrum of H32A mutant PI-PLC 169</td>
</tr>
<tr>
<td>48</td>
<td>NOESY spectrum of H32N mutant PI-PLC 170</td>
</tr>
<tr>
<td>Page</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>49</td>
<td>NOESY spectrum of H32Q mutant PI-PLC</td>
</tr>
<tr>
<td>50</td>
<td>NOESY spectrum of D274A mutant PI-PLC</td>
</tr>
<tr>
<td>51</td>
<td>NOESY spectrum of D274N mutant PI-PLC</td>
</tr>
<tr>
<td>52</td>
<td>NOESY spectrum of D274E mutant PI-PLC</td>
</tr>
<tr>
<td>53</td>
<td>The CD spectra of mutant and WT PI-PLC</td>
</tr>
<tr>
<td>54</td>
<td>The stereoview of \textit{B. cereus} PI-PLC complexed with myo-inositol. His-32, Asp-274, His-82, and Asp-33 are shown with their side chains</td>
</tr>
<tr>
<td>55</td>
<td>RNase T1-like mechanisms of \textit{B. thuringiensis} PI-PLC</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>one-dimensional</td>
</tr>
<tr>
<td>2D</td>
<td>two-dimensional</td>
</tr>
<tr>
<td>A</td>
<td>alanine</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>AK</td>
<td>adenylate kinase</td>
</tr>
<tr>
<td>AKe</td>
<td><em>E. coli</em> adenylate kinase</td>
</tr>
<tr>
<td>AKy</td>
<td>yeast adenylate kinase</td>
</tr>
<tr>
<td>AKl</td>
<td>vertebrate cytosolic adenylate kinase</td>
</tr>
<tr>
<td>AKlc</td>
<td>chicken cytosolic adenylate kinase</td>
</tr>
<tr>
<td>AKlh</td>
<td>human cytosolic adenylate kinase</td>
</tr>
<tr>
<td>AKlp</td>
<td>porcine cytosolic adenylate kinase</td>
</tr>
<tr>
<td>AK2</td>
<td>mitochondrial intermembrane space adenylate kinase</td>
</tr>
<tr>
<td>AK3</td>
<td>GTP:AMP phosphotransferase from mitochondrial matrix</td>
</tr>
<tr>
<td>Am</td>
<td>amber</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine 5'-monophosphate</td>
</tr>
<tr>
<td>amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>AMPPNP</td>
<td>adenylyl-imidodiphosphate</td>
</tr>
<tr>
<td>AP₅A</td>
<td>Pⁱ,P⁵-bis(5'-adenosyl)pentaphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C₁₆S-PI</td>
<td>hexadecylthiophosphoryl-1-my-ino-sitol</td>
</tr>
<tr>
<td>CAPS</td>
<td>3-(cyclohexylamino)-propanesulfonic acid</td>
</tr>
<tr>
<td>CD</td>
<td>circular dichroism</td>
</tr>
<tr>
<td>cPI-PLC</td>
<td>cytosolic phosphatidylinositol-specific phospholipase C</td>
</tr>
<tr>
<td>D</td>
<td>aspartate</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>dATP</td>
<td>2'-deoxyadenosine 5'-triphosphate</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DHPC</td>
<td>diheptanoyl-sn-glycerol-3-phosphocholine</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dNTP</td>
<td>2'-deoxynucleoside 5'-triphosphate</td>
</tr>
<tr>
<td>DPPI</td>
<td>dipalmitoylpropylphospho-my-ino-sitol</td>
</tr>
<tr>
<td>DPsPI</td>
<td>(2R)-1,2- dipalmitoylpropylthiophospho-1-D-my-ino-sitol</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-dithiobis-(2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>DTP</td>
<td>4,4'-dithiobispyridine</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
</tbody>
</table>

xvi
EGTA: ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
F: phenylalanine
FAD: flavin adenine dinucleotide
G: glycine
Gdn-HCl: guanidine hydrochloride
GPI: glycosylphosphatidylinositol
GPI-PLC: glycosylphosphatidylinositol-specific phospholipase C
GTP: guanosine 5'-triphosphate
H: histidine
HEPES: N-(2-hydroxyethyl)piperazine-Ν'-2-ethanesulfonic acid
IcP: inositol 1,2-cyclic phosphate
IP₃: myo-inositol 1,4,5-triphosphate
HPLC: high performance liquid chromatography
IPTG: isopropyl-β-D-thiogalactopyranoside
kDa: kilodalton
L: leucine
LDH: lactate dehydrogenase
MCS: multiple cloning site(s)
MES: 2-(N-morpholino)ethanesulfonic acid
MOPS: 3-(N-morpholino)propanesulfonic acid
MW: molecular weight
N: asparagine

xvii
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD⁺</td>
<td>β-nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>β-nicotinamide adenine dinucleotide, reduced form</td>
</tr>
<tr>
<td>NADP</td>
<td>β-nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NOE</td>
<td>nuclear Overhauser effect</td>
</tr>
<tr>
<td>NOESY</td>
<td>nuclear Overhauser enhanced spectroscopy</td>
</tr>
<tr>
<td>NPIP</td>
<td>myo-inositol 1-(4-nitrophenyl)phosphate</td>
</tr>
<tr>
<td>NTP</td>
<td>nucleoside 5'-triphosphate</td>
</tr>
<tr>
<td>P</td>
<td>proline</td>
</tr>
<tr>
<td>pCpA</td>
<td>5'-phospho-cytidylyl(3',5')adenosine</td>
</tr>
<tr>
<td>pdCpA</td>
<td>5'-phospho-deoxycytidylyl(3',5')adenosine</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PEP</td>
<td>phosphoenolpyruvate</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PIP</td>
<td>phosphatidylinositol phosphate</td>
</tr>
<tr>
<td>PIP₂</td>
<td>phosphatidylinositol diphosphate</td>
</tr>
<tr>
<td>Pip</td>
<td>pipecolic acid</td>
</tr>
<tr>
<td>PI-PLC</td>
<td>phosphatidylinositol-specific phospholipase C</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>sPI-PLC</td>
<td>secreted phosphatidylinositol-specific phospholipase C</td>
</tr>
<tr>
<td>STII</td>
<td><em>E. coli</em> heat stable enterotoxin signal sequence</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethlenediamine</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
</tr>
<tr>
<td>Tris</td>
<td>2-amino-2-(hydroxymethyl)-1,3-propanediol</td>
</tr>
<tr>
<td>TSP</td>
<td>sodium 3-(trimethylsilyl)propionate-2,2,3,3-d₄</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>v/v</td>
<td>volume to volume ratio</td>
</tr>
<tr>
<td>w/v</td>
<td>weight to volume ratio</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>Y</td>
<td>tyrosine</td>
</tr>
</tbody>
</table>
PART I.

STRUCTURE-FUNCTION STUDIES OF ADENYLATE KINASE WITH UNNATURAL AMINO ACIDS
CHAPTER 1
INTRODUCTION

1.1 General Background of AK

Adenylate kinase (EC 2.7.4.3) catalyzes the conversion between adenine nucleotides ATP, AMP and ADP: \( \text{MgATP} + \text{AMP} \rightleftharpoons \text{MgADP} + \text{ADP} \). It plays an important role in biological systems by maintaining adenine homeostasis in tissues where energy turnover is significant (Noda, 1973). AK, a monomer, is the smallest member known in the kinase family (21-26 kDa). The small size of AK provides ease for cloning and structural analysis, and makes AK a model enzyme for mechanism study of the kinase family.

There are five types of AK: type 1 AK (from muscle, AK1), AKy (from yeast), AKe (from *E. coli*), AK2 (mitochondrial intermembrane space and matrix), and AK3 (using GTP as the phosphate donor). The work presented here was performed with chicken muscle AK1 (AK1c). Sequence homology within AK1 family (among chicken, human, porcine and rabbit) is more than 89% (Schulz, 1987). However, AK1 shares a much lower sequence homology with the rest of AK family, 24-28% (Schulz, 1987). AK1 does not contain an "insertion segment" of approximately 30 amino acids.
highly conserved among other AK. This difference has led to the classification of AK as small and large variants, and has made sequence alignment difficult. Several alignments have been made (Schulz, 1986, 1987; Haase et al., 1989). Based upon the spatial location of the insert in the crystal structure of AKy+MgAP5A complex (Egner et al., 1987), the second alignment of Schulz (1987) placed the “insertion segment” between residue 132 and 133 of AK1. Figure 1 shows this alignment for AK1, AKe, and AKy. The work throughout this dissertation uses this alignment.

AK transfers a phosphoryl group via an in-line displacement mechanism demonstrated by the inversion of a chiral γ-P analogue of ATP (Richard & Frey, 1978). The reaction proceeds by a random Bi Bi mechanism (Rhoads & Lowenstein, 1968) as illustrated in Figure 2. AK achieves a rate enhancement of greater than $10^{12}$ over the uncatalyzed reaction (Sanders et al., 1989) with the requirement of a divalent metal cation, preferably Mg$^{2+}$ (Noda, 1973). The chemical conversion of the ternary complex is partially rate-limiting (Tian et al., 1990). Both internal and external equilibrium constants are close to unity (Nageswara Rao et al., 1978; Tian et al., 1990). Substrate specificity varies within different types of AK and between the two binding sites. The specificity of the AMP binding site is strict (Su & Russell, 1967; Secrist et al., 1972), while the ATP site could bind and convert a variety of nucleotide analogues (Noda, 1958).

One structural motif of AK is of great interest: the glycine rich loop, represented by the consensus sequence of $^{15}$GXPGXGKGT$^{23}$. This motif, prevalent among nucleotide binding proteins such as ras proteins and elongation factors, is generally

3
Figure 1. Primary sequences of three different types of AK. The alignments and family numbering shown in parentheses are according to Schulz (1987). The bracket directly above the AKlp sequence indicates the main difference in alignment between Haase et al. (1989) and Schulz (1987). It should be noted that AKlc has one additional methionine at the N-terminal (Kishi et al., 1986) which is absent in the enzyme overproduced in E. coli (Tanizawa et al., 1987). This figure is reproduced from Tsai & Yan (1991).
Figure 2. Scheme of the random Bi Bi kinetic mechanism of AK.
efined as GXXXXGK[TS] (Saraste et al., 1990). This loop is also called “phosphate binding loop” (or “P-loop”), since this loop forms a giant cation hole that binds the negatively charged phosphate groups in ATP or GTP (Dreusicke & Schulz, 1986; Pai et al., 1990). In AK, the P-loop interacts with the phosphate 2 of substrate analogs in AK$_e$+AP$_3$A and AK$_e$+AMP+AMPNP complexes by backbone amide hydrogen bonds to the phosphate (Müller & Schulz, 1988, 1992; Berry et al., 1994). Studies of P-loop in AK will be further discussed in Chapter 4 of this dissertation.

1.2 Background of Unnatural Amino Acid Mutagenesis

1.2.1 Structure-Function Studies of Proteins with Unnatural Amino Acids

Site-directed mutagenesis is a powerful tool for structure-function studies of proteins; it allows manipulation of gene sequence and results in site-specific mutants. However, this approach only substitutes a specific amino acid (or several amino acids) in a protein with any other nineteen natural amino acids (Zoller & Smith, 1983). Many times, one would like to tailor the steric or electronic properties of an amino acid in a protein in order to pursue specific structure-function studies. For studies of small molecules, one could synthesize analogues with virtually any structural changes. Substituting with unnatural amino acids of desired structure, beyond the common 20 amino acids, would greatly enhance our ability in protein engineering and in the studies of protein structure-function, especially those issues which can not be easily, directly
addressed by the traditional site-directed mutagenesis. There are several methods which
can be used to insert unnatural amino acids into proteins.

Solid-phase peptide synthesis has allowed the synthesis of proteins containing
novel amino acids (Bayer, 1991; Rose, 1994; O'Donnell et al., 1996). The peptide chain
length is limited by the difficulty of separating the product from accumulated impurities
resulting from incomplete coupling and deprotection steps. Segment synthesis-
condensation has to be used to synthesize longer peptides or small proteins. During this
process, peptides are synthesized separately, purified and characterized prior to solution-
phase coupling. This chemical method is limited for synthesis of proteins with molecular
weight no more than 12 kDa.

Protein semisynthesis has been used to insert unnatural amino acids into larger
proteins (Wasserman et al., 1980; Offord, 1987). In this method, a naturally derived
protein is cleaved near the target site into two or more fragments. The full-length protein
is produced by ligating a synthetic peptide containing the desired unnatural amino acids
to one of the protein fragments. This strategy requires cleaving and rejoining the
fragments, and is complicated by the difficulties in cleaving the protein specifically at the
peptide ligation site and in coupling the protein and peptide fragments selectively.

Chemical modification also has been used to introduce unnatural amino acids into
proteins by chemically modifying the protein directly (Kasier et al., 1985; Pollack et al.,
1988). The unnatural side chains include cofactors, spin labels and oligonucleotides. This
method which has been widely used by biochemist, was the only means available for site-
specific incorporation of unnatural amino acids into proteins not very long ago.
However, this method has requirements which greatly limit the applications: (1) the chemical condition must be tolerated by the protein molecule; (2) the target residue must be on the protein surface and must have reactive side chains such as lysine, cysteine, tyrosine etc. This method also has selectivity difficulties when several residue can be modified at the same time.

There are biosynthetic methods employing chemically modified aminoacyl-tRNAs to introduce unnatural amino acids into proteins synthesized in vitro (Krieg et al., 1986; Brunner, 1993). The limitations associated with this method are: (1) both the modified and natural amino acids are incorporated at the site of interest (due to difficulties in removing endogeneous aminoacyl-tRNA from the in vitro extract); (2) substitutions are at multiple sites; (3) unnatural amino acids could only be derivatives of natural amino acids.

There is a method recently developed by Schultz for general site-specific incorporation of unnatural amino acids into proteins. It eliminates most of the limitations of the previous methods and allows for generation of new proteins with a variety of unnatural amino acids site-specifically incorporated.

1.2.2 General Method of Site-Directed Unnatural Amino Acids Mutagenesis

This method first developed in 1989 by Schultz and co-workers (Noren et al., 1989) is a more general method. This approach combines molecular biology techniques with organic synthesis. Figure 3 shows the strategy for this method. The codon for the amino acid of interest is changed to the amber nonsense codon TAG
Figure 3. Strategy for the general biosynthetic method to site-specifically incorporate unnatural amino acids into proteins. Reproduced from Noren et al. (1989).
Figure 4. Examples of unnatural amino acids that have been successfully incorporated into proteins. Reproduced from Cornish et al. (1995).
through conventional oligonucleotide-directed mutagenesis. A suppressor tRNA is constructed to recognize the amber nonsense codon. This suppressor tRNA is then chemically aminoacylated with the desired unnatural amino acid. The aminoacylated suppressor tRNA is then added to an in vitro extract capable of supporting protein biosynthesis. A mutant protein with the unnatural amino acid site-specifically incorporated at the desired site is then generated.

This methodology has been applied to different proteins to incorporate a variety of unnatural amino acids. It has been used for mechanism investigation of β-lactamase, ras-21, staphylococcal nuclease and structure-function studies of T4 lysozyme (Ellman et al., 1992; Mendel et al., 1992; Chung et al., 1993; Judice et al., 1993). Approximately eighty unnatural amino acids have been incorporated into about thirty sites of a variety of proteins using this methodology (Figure 4). The development of this general methodology is not incidental, as it is based on numerous previous studies on the chemical and biological aspects of protein biosynthetic systems. The method itself requires the generation of the requisite suppressor tRNA, a general method for acylating the tRNA with unnatural amino acids, and an in vitro protein synthesis system to obtain enough protein for meaningful analysis. Those studies as well as preparation of several important components required in the approach are addressed in the following.

Recognition of Aminoacylated tRNA by Translation Machinery

Two of the observations that this general methodology is based upon are: (i) Anticodon-codon recognition is independent of the amino acid at the acceptor stem of the
tRNA; (ii) The transcription apparatus shows a broad substrate specificity. These two observations ensure that the exogenous tRNA aminoacylated with a variety of unnatural amino acids can be accommodated by the translational machinery in a site-specific fashion through codon-anticodon recognition, an essential requirement for the strategy shown in Figure 3 to work. The first observation has been demonstrated by a series of experiments in which misacylated tRNAs were shown to direct incorporation of attached noncognate amino acids into polypeptide chains in response to the normal codons for each tRNA. In one of these experiments, Chapeville et al. (1962) generated Ala-tRNA^{Cys} by enzymatically reducing \textit{E. coli} Cys-tRNA^{Cys}. The resulting misacylated tRNA was observed to insert alanine in response to the cysteine codon UGU. That EF-Tu and the ribosome can accommodate 20 natural amino acids with the side chain differences ranging from Gly to Trp and Arg suggests that the translational apparatus has a broad range of side chain specificities (Chladek et al., 1985). As indicated previously (Figure 4), more than 70 unnatural amino acids have been incorporated into a variety of proteins since the development of this general biosynthetic method for site-directed unnatural amino acid mutagenesis.

Amber Nonsense Codon as the Signal for Suppressor tRNA

There are three nonsense or stop codons among the sixty-four genetic codons: amber (UAG), orche (UAA), and opal (UGA). The normal function of these nonsense codons is to signal the termination of protein elongation. If a natural mutation generates a nonsense codon within the gene, premature termination of protein synthesis occurs, which
may be lethal to the cell. Cells overcome this problem by producing mutant tRNA molecules called suppressor tRNA molecules. Suppressor tRNAs can suppress the effect of nonsense mutation, because they have a mutated anticodon which can recognize the nonsense codon and insert one of the twenty amino acids into that position. This mechanism can be applied to the incorporation of unnatural amino acids. In order to signal for unnatural amino acid insertion, the genetic codon must not be recognized by tRNAs normally present in the biosynthetic system. Since only one nonsense codon is required for termination of protein synthesis (not recognized by normal tRNA) (Brenner et al., 1965), one of the nonsense codons could be used for the signal for the unnatural amino acid incorporation: creating a nonsense codon at the position of interest, constructing a suppressor tRNA which can be acylated with unnatural amino acids and which can recognize that nonsense codon in the gene. This suppressor tRNA helps the cell readthrough the termination codon, and insert the attached unnatural amino acid to the protein at the desired position.

There are several reasons accounting for the choice of amber codon over opal or ochre codon. First, the genes of interest are least likely to end with the amber codon. A tabulation of the termination codon usage in prokaryotic genes of known sequences lists 561 genes ending with the ochre codon, 263 genes ending with the opal codon and 99 genes ending with the amber codon (Aota et al., 1988). In the less possible cases when genes end with the amber codon, the amber suppressor can still be used to direct the incorporation of unnatural amino acids after the termination codon is mutated from amber to either the opal or ochre codon. Other reasons include the high efficiency of known
amber suppressors and the low sensitivity of amber suppression to the mRNA context (Miller & Albertini, 1983; Bossi, 1983). The higher suppression efficiency, the more mutant protein produced with the less amount of wild type protein produced.

Amber Suppressor tRNA

As discussed earlier, an amber suppressor needs to be constructed to be recognized by the protein biosynthetic machinery sufficiently to suppress a stop codon with high efficiency. However, the suppressor tRNA should not be a substrate for any of the endogenous aminoacyl-tRNA synthetases. Otherwise, deacylation of the noncognate amino acids and/or aminoacylation with one of the natural amino acids would happen to the tRNA. This ensures high suppression efficiency and avoids incorporation of a natural amino acid as well as the unnatural amino acid in response to the nonsense codon.

Schultz initially used a truncated amber suppressor tRNA made from commercially available yeast tRNA^{Phe} by the "anticodon-loop replacement" method developed by Bruce and Uhlenbeck (1982). The truncated form, lacking the 3' terminal pCpA at the acceptor stem, is designed to facilitate the aminoacylation procedure (this will be discussed later). The anticodon-loop region was replaced with $3^\text{45}S'\text{'-CUAA}-3^\text{37}$. Suppressors from yeast tRNA^{Phe} have high suppression efficiency and are likely to be poor substrates for *E. coli* aminoacyl-tRNA synthetases (Bruce et al., 1982; Kwok & Wong, 1980). Anticodon-loop replacement method was used for the early work. This method is labor-intensive and of low yield because of the numerous chemical and enzymatic steps involved. A runoff transcription method was used (Noren et al., 1990).
Figure 5. Generation of yeast tRNA\textsubscript{\textsuperscript{\textsc{phe}}\textsubscript{\textsc{cuA}}} (-CA) by runoff transcription. Reproduced from Noren (1990).
Figure 6. tRNA$^{Phe}_{CUA}$(-CA) prepared by: (a) anticodon-loop replacement procedure; (b) runoff transcription method. $\psi$ is pseudouridine and $Y$ is wyosine. Base modifications are abbreviated as $m$, $s$, $o$, $I$, $h$, and $n$ represent methyl, thio, oxy, isopentenyl, dihydro, and amino groups, respectively. Superscripts and subscripts denote the position and degree of substitutions, respectively. Reproduced from Noren (1990).
As shown in Figure 5, a PUC18 derived vector, pYPhe2, was constructed with the amber suppressor tRNA gene placed immediately downstream of a T7 promoter. Cleavage of the pYPhe2 vector with the FokI restriction enzyme resulted in the template from which the truncated amber tRNA\textsubscript{CUA(-CA)} could be generated when supplemented with T7 RNA polymerase and other necessary components of the transcription reaction. The differences between the suppressors obtained from the two methods are the presence of 5'-terminus triphosphate and absence of base modifications with the suppressor from runoff transcription (Figure 6). But both suppressors incorporate unnatural amino acids efficiently and exhibit little readthrough at optimal Mg\textsuperscript{2+} concentration.

Aminoacylation of the Suppressor tRNA

A general method for aminoacylation of the suppressor had to be developed with the constructed amber suppressor. The ideal way would be an enzymatic method with aminoacyl-tRNA synthetases. This enzymatic method would allow \textit{in vitro} production of unnatural amino acid labeled proteins. Due to the high specificity of these enzymes, the enzymatic approach would require generating a variety of aminoacyl-tRNA synthetases, each being specific for a desired unnatural amino acid and for the corresponding suppressor. The is still well beyond our current reach. Direct chemical aminoacylation would be a consideration, as it does not have the specificity problem encountered in the enzymatic approach. The chemical method would involve coupling of an activated aminoacyl ester with the 2'(3') diol of the acceptor stem of the suppressor tRNA. This method is not practical because it is complicated by the presence of the large number of
reactive sites (e.g., exocyclic amines and 2'-hydroxyls) in the tRNA molecule. This problem can be solved by using a chemical/enzymatic synthesis.

A modification of a two-step method initially developed by Hecht et al. (1984) was used: the dinucleotide pCpA is chemically aminoacylated with a N-protected amino acid, followed by enzymatically ligating the aminoacylated dinucleotide to a truncated suppressor tRNA (tRNA_{CUA}^{CA}, missing the terminal dinucleotide pCpA at the 3'-acceptor stem). As discussed earlier, the truncated suppressor tRNA can be generated directly by run-off transcription of a Fok I linearized plasmid encoding tRNA gene. The low yields of desired product's are associated with side reactions including diacylation and acylation of the exocyclic amino groups of the bases. The fact that the aminoacyl tRNA linkage itself is relatively unstable in the pH 7-8 required for efficient T4 RNA ligase function also contributes to the low yields of the desired product. It was later found that cyanomethyl ester of an amino acid reacts selectively with the 2,3-hydroxyl groups of the ribose to give the monoacylated product without the need to protect the dinucleotide prior to aminoacylation (Robertson et al., 1991). Schultz further modified the method. Shown in Figure 7 is the current synthesis strategy for aminoacyl tRNA. The dinucleotide pCpA was replaced with pdCpA, the substitution of cytidine in the dinucleotide with deoxycytidine significantly simplifying the synthesis and eliminating another 2'-OH group without affecting biological activity. The α-amino group and reactive side chains are protected as the nitroveratryloxy (NVOC) carbamate, ester, or ether. Aminoacyl pdCpA is prepared by coupling of cyanomethyl active ester of N-NVOC amino acids with pdCpA. N-NVOC aminoacyl tRNA is obtained by ligating the
Figure 7. Strategy for the chemical aminoacylation of tRNA\textsuperscript{Phe}\textsubscript{CUA}. Reproduced from Ellman et al. (1991).
aminoacylated pdCpA with the truncated tRNA. Photochemical removal of the NVOC protecting group affords the aminoacyl tRNA in high yield. The strategy proceeds in high yield and is applicable to a variety of unnatural amino acids. More detailed description and discussion about synthesis of aminoacyl tRNA will be covered in Chapter 3 of this dissertation.

In Vitro Protein Synthesis

Suppressor tRNA chemically aminacylated with unnatural amino acids needs to be introduced into the translational apparatus to direct the site-specific incorporation of the desired unnatural amino acids into proteins. Since no general methodology exists for introducing large quantities of the aminoacylated suppressor tRNA into intact, dividing cells, protein synthesis has to be carried out in vitro. The current in vitro system is an *E. coli* transcription/translation protein synthesis system.

The coupled system was originally developed by Zubay (1973), and was later modified by Collins (1979) and Pratt (1984). Schultz further modified the system by lowering the pH from 8.2 to 7.4 to better stabilize the base-labile acyl linkage of the added aminoacylated suppressor (Ellman et al., 1991). A coupled system can avoid decreased expression of a prokaryotic gene of interest since transcription and translation are closely coupled in prokaryotes. A coupled system can also avoid the problems associated with mRNA synthesis and purification since mRNA are very susceptible to nuclease degradation.
The main component of this expression system is an *E. coli* S-30 cell extract, which contains all the high molecular weight factors necessary for transcription and translation (RNA polymerase, rho factor, tRNAs, aminoacyl-tRNA synthetases, ribosomes, initiation factors, elongation factors, and termination factors). A gentle method of lysis (French press) was used to prepare the S-30 extract to avoid degradation of synthesized protein by membrane-bound proteases released during cell lysis. To reconstitute protein synthesis *in vitro*, the following components are added to the S-30 extract: nucleotide triphosphates, bacterial tRNAs, amino acids, enzymatic cofactors, salts, phosphoenolpyruvate, pyrophosphatase and the plasmid DNA encoding the gene of interest.

1.2.3 *Structure-Function Studies of AK with Unnatural Amino Acids*

Although AK is a well studied enzyme, there are some important aspects of the AK mechanism that are still unresolved. This is partially due to the limitation of the natural amino acid mutagenesis. With all the advantages offered by this newly developed unnatural amino acid site-directed mutagenesis, we could extend the structure-function studies of AK to the problems left behind by the conventional mutagenesis method. The target of the first half of this dissertation will be the “P-loop” residue Pro-17 and AMP binding site residue Tyr-95.

As briefly discussed earlier in this chapter, the “P-loop” in AK is shown as: $^{15}$GGPGSGKGT$^{23}$. Pro-17 is absolutely conserved in the AK family. There have been amino acid mutagenesis studies on Pro-17 (Reinstein et al., 1988; Tagaya et al., 1989;
Reinstein et al., 1990; Tian et al., 1990). These changes cannot retain the unique characteristic of proline-ring structure. The actual role that Pro-17 plays is still unclear. Unnatural amino acid mutagenesis could allow the variation of the structure while retaining the ring structure of Pro-17, and provide direct information on the role of Pro-17.

Another interesting residue is Tyr-95. Tyr-95 is in close proximity to the adenosine moiety of AMP, and could play an important role in the substrate binding. Replacement of Tyr-95 with phenylalanine resulted little change in the catalytic properties (Kim et al., 1989), which suggested that the OH moiety of Tyr-95 residue is not required at this position. Tyr-95 in AKlc corresponds to phenylalanine in AKe, and the key point could be the aromaticity of Tyr-95. Applying the unnatural amino acid mutagenesis method to AK, we could replace Tyr-95 with dihydrophenylalanine, which retains most of the structural aspects of phenylalanine but removes the aromaticity at this position. This could provide direct evidence of the requirement at this position, and give an answer to the following question: Is the aromaticity at Tyr-95 required for the AK normal function?

A major limitation of *in vitro* protein synthesis remains the relatively small amount of protein that can be obtained (Cornish et al., 1995). The typical yield for this *in vitro* protein synthesis is only µg quantities. Routine structural analysis of mutants cannot be conducted with reasonable cost, but with the sensitive kinetic analysis of AK, steady-state kinetic studies would be the major tool to characterize the resulting mutants. Meaningful interpretation will be offered with the carefully designed substitution.
Although every step of the general procedure for this methodology has been reported by Schultz, its application to AK is still a challenge. It could be shown by the following facts: this method involves molecular biology, organic synthesis and protein chemistry; AK has its own specific expression and purification problems when in vitro protein synthesis is carried out; the method is technically demanding and time consuming due to the complicated procedures for preparing components; there are strict technical requirements of experiments involving RNA.
CHAPTER 2

MATERIALS AND METHODS FOR THE UNNATURAL AMINO ACID
MUTAGENESIS OF ADENYLATE KINASE

2.1 Preparation of Plasmid DNA

2.1.1 Materials

The AK gene used in this work was the short version with 3'-untranslated region 7 bases long. The AK gene was modified by Zhengtao Shi to encode AKH, which is AK with six-histidine residues attached to its C-terminus (Shi, 1994). The pUK vector was constructed by Baohua Huang (Huang et al., 1994). E. coli strain JM 105 was purchased from Pharmacia. Ultracentrifugation was carried out on a Beckman L8-70M Ultracentrifuge. Pro-17 and Tyr-95 amber mutants were constructed by Zhengtao Shi and Xiaohong Liu respectively. All other reagents were purchased from Sigma.

2.1.2 Methods

In order to facilitate and simplify the purification of in vitro synthesized AK, AKH (modified AK with six-histidine residues attached to its C-terminus) was studied instead. AKH has the similar catalytic properties as AK (Shi, 1994). In vitro synthesized
AKH could be purified simply by using one column with His Bind Ni\(^{2+}\) chelating resin purchased from Novagen. The expression vector pUK (Figure 8) is only 2.4 kb in size but has all the elements for efficient expression: the strong tac promoter, the expanded MCS, and the rrrB transcription terminator. It has a high copy number of 500-700, and can produce high quality supercoiled plasmid DNA (Huang et al., 1994). The purity of the template DNA is one of the most critical factors affecting the yield of protein synthesized \textit{in vitro}. The desired purity requires not only that the purified DNA is free of any protein or RNA contamination, but also that it should be in the closed circular or supercoiled form. The cesium chloride gradient purification method can provide high quality plasmid DNA. This method can separate plasmid DNA from chromosomal DNA, proteins and RNAs; it can also separate closed circular or supercoiled DNA from nicked ones. A simple alkali method was used for cell lysis.

Plasmid DNAs were prepared on large scale via the following procedure:

Twenty mL LB/amp were inoculated with a single colony picked from a fresh LB/amp plate and incubated with shaking for 10-12 hours at 37 °C. The above culture was then added aseptically to 1 L TB/amp media and incubated with vigorous shaking for 20-24 hours at 37 °C. The cells were then collected by centrifugation at 4,000 rpm for 15 minutes at 4 °C in a JA-10 rotor. The cell pellet were resuspended in 30 mL solution I (50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl, pH 8.0) and left at room temperature for 20 minutes. 60 mL of freshly made solution II (1% SDS and 0.2 N NaOH) were then added to the above suspension and mixed gently but thoroughly by inverting the tube 5-6 times. The tube was allowed to sit on ice for 10 minutes before the
Figure 8. The expression vector pUK. Reproduced from Huang et al., 1994.
addition of 45 mL of solution III (5 M acetate and 3 M potassium). The contents were again mixed thoroughly by gentle inversion. The tube was then left on ice for 20 minutes and the debris was removed by centrifugation at 4,000 rpm for 15 minutes at 4 °C in a JA-10 rotor. The supernatant was then filtered through four layers of cheesecloth into a new centrifuge tube. Crude plasmid DNA was precipitated from the supernatant by the addition of 0.6 volume of isopropanol, followed by the incubation at room temperature for 20 minutes. The precipitate was collected by centrifugation at 7,500 rpm for 15 minutes at 4 °C, washed with 70% EtOH, air dried briefly, and dissolved in 7 mL TE (pH 8.0).

The solution was then transferred to a 15 mL graduated disposable tube and more TE buffer was added to the final volume of 10 mL. Ten grams of CsCl and 0.6 mL of an ethidium bromide solution (EB, 10mg/mL) were then added to the tube and the contents were mixed well. Precipitates produced during this step were removed by centrifugation at 8,000 rpm for 5 minutes at 20 °C in a JA-20 rotor. The supernatant was then transferred to a Beckman Polyallomer Quick-Seal centrifuge tube (16 x 76 mm) and the tube was sealed with a Beckman Quick-Seal Tube Sealer. The tube was then centrifuged at 45,000 rpm for 48 hours in a Beckman Ti50 rotor at 20 °C. Consistent with normal procedure, two bands were observed under a UV lamp in a darkroom. The lower band was pulled out from the tube with a syringe equipped with an 18 gauge needle. The ethidium bromide associated with the DNAs was extracted with 10 mL of 1-butanol saturated with CsCl and water four times. After the solution was diluted with TE buffer to obtain the final volume of 6 mL, plasmid DNAs were precipitated with the addition of
12 mL of ethanol, followed by the incubation at -20 °C for 1 hour. The precipitated DNAs were collected via centrifugation at 15,000 rpm for 10 minutes and washed with 10 mL of 70% ethanol. The pellet was then dried in a Savant SC100 Speed Vac for 10 minutes and dissolved in an appropriate amount of RNase-free TE (pH 7.2). The plasmid was quantitated by measuring the absorbance of a 100-fold diluted sample at 260 nm and 280 nm using a Uvikon spectrophotometer. The OD\textsubscript{260} reading was converted to the DNA concentration according to the relationship of 1 OD\textsubscript{260} = 40 \mu g/mL plasmid DNA. In accordance with normal protocol, the ratio of OD\textsubscript{260}/OD\textsubscript{280} fell into the range of 1.6-1.9. The typical yield of pUK derived plasmid DNAs was 1 mg/L. In several cases, a second CsCl gradient purification was applied to ensure the high quality of the DNA.

2.2 Synthesis of Aminoacyl tRNA

In order to provide a more complete description of this process, synthesis of aminoacyl tRNA including synthesis of unnatural amino acids, pdCpA dinucleotides, aminoacyl pdCpA will be addressed in Chapter 3.

2.3 In Vitro Protein Synthesis

2.3.1 Materials

\textit{E. coli} strain D10 was provided by P. G. Schultz at the University of California, Berkeley. Ultra-pure acrylamide and bisacrylamide were purchased from Boehringer Mannheim, [\textsuperscript{35}S]-Met (15 \mu Ci/\mu L) was purchased from Amersham. Diethyl pyrocarbonate and TEMED were purchased from Sigma.
2.3.2 Ribonuclease-Free Techniques

All of the buffers and solutions used in the RNA related experiments were prepared with diethyl pyrocarbonate (DEPC)-treated double distilled water. The solution was stirred for at least 12 hours and autoclaved at 20 psi for 20 minutes to hydrolyze any remaining DEPC. All of the apparatus used in the RNA related experiments were either purchased presterilized or treated with DEPC as described below. They were soaked with 0.1% DEPC in double distilled water for at least 2 hours, rinsed several times with DEPC-treated water, and autoclaved. Disposable gloves were worn during the preparation of solutions and changed frequently in order to avoid RNase from the skin.

2.3.3 SDS-Polyacrylamide Gel Electrophoresis

Isotope labeled proteins obtained from the in vitro protein synthesis were separated by SDS-PAGE with the conventional apparatus (Bethesda Research Laboratories). The procedure was essentially the same as described by Sambrook, et al. (1989). The polyacrylamide gel with size of 0.75 mm x 15 cm x 17 cm consisted of the resolving gel and the stacking gel. The resolving gel contained 15% polyacrylamide (29:1 acrylamide:bisacrylamide), 0.375 M Tris-HCl, pH 8.8, and 0.1% (w/v) SDS.

Immediately prior to pouring the resolving gel, 1/100 volume of 10% (w/v) ammonium persulfate and 1/2,500 volume of TEMED were added to initiate the polymerization. A layer of water saturated with 1-butanol was then added on top of the resolving gel to prevent the diffusion of oxygen into the gel which could inhibit the polymerization. This butanol layer was then washed off thoroughly before pouring the stacking gel. The
stacking gel contained 5% polyacrylamide, 0.126 M Tris-HCl, pH 6.8, and 0.1% (w/v) SDS. Its polymerization was initiated with the addition of 1/100 volume of 10% ammonium persulfate and 1/1,000 volume of TEMED. The electrophoresis running buffer (25 mM Tris, 250 mM glycine, pH 8.3, and 0.1% SDS) was made freshly. Protein samples were prepared in the SDS-gel loading buffer, which was diluted from 5 x stock solution (60 mM Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM mercaptoethanol, 0.1% bromophenol blue). The resulting mixture was heated at 100 °C for 5 minutes, cooled on ice, then centrifuged. The supernatant was loaded onto the gel. The electrophoresis was carried out at 30 mA for 2-4 hours. The resulting gel was soaked in the fixing solution (40% ethanol and 10% acetic acid) for 30 minutes and in the fluorographic reagent Amplify TM (Amersham) for 30 minutes. It was then dried with a Bio-Rad 583 dryer for an hour. The dried gel was scanned through a β-scanner (from Betagen) to obtain the quantitative intensities of the AKH band. Then the dried gel was exposed for 12-24 hours to Kodak X-OMAT RP film (5 x 7 in) at -70 °C.

For SDS-PAGE gel of cold proteins, the gel was silver-stained as the follows (ref et al., 1988):

**FIXING:** deionized water 250 mL for 5 minutes; 12.5 mL ethanol, 12.5 mL acetic acid, 225 mL distilled water for 3 hours; deionized water 250 mL for 5 minutes; 10% glutaraldehyde 250 mL for 30 minutes. **WASHING:** deionized water 3 x 10 minutes, 4 x 30 minutes, 250 mL each time. **STAINING:** ammoniacal silver nitrate solution 250 mL (a solution of 2 g of AgNO₃ in 10 mL of distilled water was added slowly to a solution of 3.3 mL NH₃-H₂O, 0.5 mL 10 N NaOH and 53 mL distilled water. More distilled water
was added to obtain the final volume to 250 mL) for 10 minutes, then deionized water 3 x
5 minutes, 250 mL each time. DEVELOPING: 0.025 g of citric acid and 0.25 mL of
formaldehyde/250 mL distilled water, 18 °C, 3 minutes; glycerol 17.5 mL, ethanol 17.5
mL and distilled water 207.5 mL.

2.3.4 In Vitro Expression of AKH

Preparation of Solution.

All solutions used in the in vitro protein synthesis except growth media were
prepared in sterile DEPC-treated double distilled water and each of the solutions was
autoclaved again unless indicated. pH of the solutions was adjusted whenever a pH value
was given. A ORION Research digital pH meter 611 was used.

2.2 M Tris-acetate, pH 7.4, 100 mL. Acetic acid was used to adjust the pH. Stored as
10 mL aliquots at -20 °C.

0.1 M DTT, 100 mL. Sterilized by filtration using a Nalgene disposable membrane filter
unit (0.2 μm). Stored at -20 °C.

0.55 M DTT, 1 mL. Not autoclaved. Stored in aliquots at -70 °C.

0.1 M magnesium acetate, 10 mL. Stored in aliquots at -70 °C.
1.4 M magnesium acetate, 500 mL. Stored at 4 °C.

6.0 M potassium acetate, 500 mL. Filtered through a Nalgene disposable membrane filter unit (0.2 mm). Stored at 4 °C.

KCA mix (2.8 M potassium acetate, 1.4 m ammonium acetate, 0.38 calcium acetate), 10 mL. Stored in aliquots at -70 °C.

40% (w/v) polyethylene glycerol 8000, 10 mL. Stored in aliquots at -70 °C.

38 mM ATP (Na salt), 20 mL. Adjusted pH to 7 with NaOH stock solution. Not autoclaved. Stored in aliquots at -70 °C.

GCU mix (88 mM CTP, 88 mM UTP, Na salts), 1 mL. Adjusted pH to 7 with stock NaOH solution. Not autoclaved. Stored in aliquots at -70 °C.

0.42 M phospho(enol)pyruvate (K salt), 10 mL. Adjusted pH to 7 with 1 N NaOH. Not autoclaved. Stored in aliquots at -70 °C.

E. coli transfer RNA (Sigma, Type XXI), 17.4 mg/mL, 1 mL. Not autoclaved. Stored in aliquots at -70 °C.
Folinic acid (Sigma, Ca salt), 2.7 mg/mL, 1 mL. Not autoclaved. Stored in aliquots at -70 °C.

Vitamin mix (2.1 mg/mL pyridoxine-HCl, 2.1 mg/mL NADP, 2.1 mg/mL FAD, 0.86 mg/mL p-aminobenzoic acid), 5 mL. Not autoclaved. Stored at -70 °C.

Amino acid suspension (55 mM each). Each amino acid was weighed the appropriate amount from the amino acid kit (Sigma) and suspended in 10 mL water. Not autoclaved. Stored in aliquots at -70 °C.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Amount (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ala</td>
<td>50</td>
</tr>
<tr>
<td>Glu</td>
<td>82</td>
</tr>
<tr>
<td>Leu</td>
<td>72</td>
</tr>
<tr>
<td>Ser</td>
<td>58</td>
</tr>
<tr>
<td>Arg</td>
<td>116</td>
</tr>
<tr>
<td>Gln</td>
<td>80</td>
</tr>
<tr>
<td>Lys</td>
<td>100</td>
</tr>
<tr>
<td>Thr</td>
<td>66</td>
</tr>
<tr>
<td>Asn</td>
<td>72</td>
</tr>
<tr>
<td>Gly</td>
<td>42</td>
</tr>
<tr>
<td>Met</td>
<td>82</td>
</tr>
<tr>
<td>Trp</td>
<td>112</td>
</tr>
<tr>
<td>Asp</td>
<td>74</td>
</tr>
<tr>
<td>His</td>
<td>106</td>
</tr>
<tr>
<td>Phe</td>
<td>68</td>
</tr>
<tr>
<td>Tyr</td>
<td>100</td>
</tr>
<tr>
<td>Cys</td>
<td>66</td>
</tr>
<tr>
<td>Ile</td>
<td>72</td>
</tr>
<tr>
<td>Pro</td>
<td>64</td>
</tr>
<tr>
<td>Val</td>
<td>64</td>
</tr>
</tbody>
</table>

Preparation of Low molecular Weight Mixture (LM).

The stock solutions were mixed in the indicated order. All of the stock solutions were kept on ice during the procedure. The LM mixture was stored at -70 °C and should be stable for several months. Prior to use, it was thawed on ice and was returned to -70 °C immediately after use.
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2 M Tris-acetate, pH 7.4</td>
<td>40 μL</td>
</tr>
<tr>
<td>0.55 M DTT</td>
<td>5 μL</td>
</tr>
<tr>
<td>38 mM ATP</td>
<td>50 μL</td>
</tr>
<tr>
<td>GCU mix (88 mM each)</td>
<td>15 μL</td>
</tr>
<tr>
<td>0.42 M phospho(enol)pyruvate</td>
<td>100 μL</td>
</tr>
<tr>
<td>Amino acid suspension*</td>
<td>10 μL</td>
</tr>
<tr>
<td>40% PEG 8000</td>
<td>75 μL</td>
</tr>
<tr>
<td>Folinic acid</td>
<td>20 μL</td>
</tr>
<tr>
<td>Vitamin mix*</td>
<td>20 μL</td>
</tr>
<tr>
<td>E. coli transfer RNA</td>
<td>15 μL</td>
</tr>
<tr>
<td>KCA mix</td>
<td>40 μL</td>
</tr>
</tbody>
</table>

*These stocks were vortexed thoroughly before being added to the mixture.

Preparation of the S-30 Cell Extract.

S-30 extracts were prepared from *E. coli* strain D10 (*rna*-10, *reA*1, *spoT1*, *metB1*) which lacks ribonuclease I activity and contains very low endogeneous suppressor activity. The glycerol stock of D10 was streaked on a M9 Minimal/Met plate (12.8 g Na$_2$HPO$_4$·7H$_2$O, 3 g KH$_2$PO$_4$, 0.5 g NaCl, 2 mM MgSO$_4$, 0.4% glucose, 0.1 mM CaCl$_2$, and 0.1 g methionine/L) prior to use.

*Preparation of the Apparatus.* All of the apparatus used in the preparation of S-30 should be RNase-free. The day before the S-30 preparation, the following apparatus were soaked in 0.1% DEPC in double distilled water for at least one hour, wrapped by aluminum foil, and then autoclaved at 20 psi for 0.5 hour: disassembled French pressure
cell (Fred S. Carver Inc.), 20 polypropylene JA-20 centrifuge tubes with caps, a 125 mL Erlenmeyer flask, 2 spatulas, and several magnetic stirring bars and clips for dialysis tubing. Three pieces of dialysis tubing (10,000 MW cutoff, 20 cm in length) were washed extensively with DEPC-treated water and autoclaved while being soaked in DEPC-treated water. All of the treated materials were stored at 4 °C for next day use.

**Cell Growth and Harvest.** Three mL of LB media were inoculated with a single colony of D10 from the M9 Minimal/Met plate and incubated with shaking at 37 °C for 7 hours. The above culture was then added to 500 mL of LB media and grew under the same condition overnight. One liter of LB media in a 2L flask (total 10 flasks) was inoculated with 5 mL of the overnight culture and incubated with aeration at 37 °C until late log phase (OD$_{450}$ = 1.2, 3 hours). Cells were collected by centrifugation at 6,000 rpm at 4 °C in a JA-10 rotor using DEPC-treated centrifuge tubes. Cell pellets were then resuspended in 0.3 L of an ice-cold, freshly made S-30 buffer (4.5 mL of 2.2 M Tris-acetate, pH 7.4, 10 mL of 0.1 M DTT, 10 mL of 1.4 M (AcO)$_2$Mg, 10 mL of 6.0 M AcOK/L of DEPC-treated water) with 2-mercaptoethanol added to 0.5 mL/L. The suspension was combined in one preweighed DEPC-treated centrifuge tube and spun down at 5,000 rpm at 4 °C for 5 minutes. The pellet was washed twice more under the same conditions (total 1 L of S-30 buffer). The cell pellet was stored at -70 °C.

**Preparation of S-30 Extract.** All of the following operation were carried out at 4 °C unless otherwise indicated. On the day of the S-30 preparation, the D10 cell pellet was thawed at 4 °C for about 2 hours and suspended in 16 mL S-30 buffer (containing 0.05 mL of mercaptoethanol/L buffer) per gram of cell pellet. Foaming was avoided by
gentle swirling or stirring rather than vigorous shaking. The suspension was transferred
to the preweighed JA-20 centrifuge tubes and centrifuged at 10,000 rpm for 30 minutes at
4 °C. Cells were weighed, transferred to a homogenizer with spatulas, and resuspended
in 4 mL of S-30 buffer (without mercaptoethanol) per gram of cells. The homogeneous
suspension was then loaded to the chilled French press cell with a disposable syringe
equipped with a 221/2 gauge needle (volume recorded), lysed under the internal pressure
of 8400 psi, and collected in a centrifuge tube containing 100 µL of 0.1 M DTT per 10
mL of loaded sample. In contrast to normal procedure, no attempt was made to
“improve” the lysis by passing the suspension twice through the French press. The lysate
was centrifuged immediately at 16,000 rpm at 4 °C for 30 minutes in 1 JA-20 rotor, and
the supernatant was transferred with a serological disposable pipette to a new centrifuge
tube, leaving behind any viscous material associated with the pellet. The transferred
supernatant was then recentrifuged under the same conditions for another 30 minutes. At
this stage, 10 mL of preincubation mix were prepared: 1.3 mL 2.2 M Tris-acetate, pH
7.4; 67 µL 1.4 M (AcO)2Mg; 3.5 mL 38 mM ATP; 2.0 mL 0.42 M
phospho(enol)pyruvate; 80 µL of 0.55 M DTT; 8 µL of amino acid suspension (vortexed
well before pipetting); 187.5 units of pyruvate kinase; and DEPC-treated water to the
total volume of 10 mL. At the end of the centrifugation, approximately 80% of the
supernatant was transferred into a DEPC-treated 125 mL Erlenmayer flask and mixed
with 0.3 mL of preincubation mix per mL of transferred supernatant. The mixture was
incubated at 37 °C with gentle shaking (200 rpm) for 80 minutes, transferred into dialysis
tubing, and dialyzed against three changes of 2 L S-30 buffer (no mercaptoethanol added)
for the total of 3 hours. The dialyzed solution was centrifuged at 16,000 rpm for 15 minutes before being dispensed into 100 µL aliquots in cold room as quickly as possible. These aliquots of S-30 extract were stored at -70 °C and should remain active for several years. The typical yield of the S-30 extract produced from this procedure was 20-30 mL.

In Vitro Protein Synthesis.

The components of the in vitro protein synthesis included plasmid DNA, magnesium ion, LM and S-30 extract. The magnesium ion concentration was optimized for each batch of DNA and S-30 extracts preparation. The optimum concentration of magnesium ion in the in vitro reaction mixture fell into the range of 5-14 mM. This was determined by carrying out four parallel reactions with varying magnesium ion concentrations (adding various amounts of Mg$^{2+}$ from a 100 mM (AcO)₂Mg stock solution to each in vitro reaction). The concentration of calcium ion, provided from LM to the final concentration of 9.7 mM in the in vitro reaction mixture, was optimized by the addition of chelant EGTA from a 100 mM stock solution. For the in vitro experiments carried out in this work, the optimal calcium concentration was 9.7 mM.

Except for the S-30 extract, stock solutions of the in vitro reaction components were thawed on ice prior to use. Routinely, four different volumes of 0.1 M magnesium acetate (1, 2, 3, 4 µL) were mixed with 2 µL of 0.1 M EGTA, 3 µg of plasmid and DEPC-treated water to the final volume of 14 µL in microtubes at room temperature. Seven and half µL of thawed and well mixed LM were added to each tube. The mixture was left on ice while tubes of the S-30 aliquot were removed from the freezer and quickly thawed in
hand until the last bit of ice disappeared. Eight and half μL of the thawed S-30 were immediately added into each tube and mixed well by pipetting. The contents of the tube were spun down in a microcentrifuge tube for 30 seconds and were then incubated at 37 °C for 1 hour with shaking (200 rpm). After the incubation, the tube was placed on ice for 5 minutes and vortexed briefly. Any insolubles were collected by centrifugation for 1 minute. A portion of the supernatant was then mixed with SDS-loading buffer for SDS-polyacrylamide gel analysis. The rest of the reaction mixture was stored at -20 °C for future use.

The expression level of AK was examined by the amount of AK radiolabeled with \[^{35}\text{S}\]-Met, which was detected by a SDS-PAGE gel. For this purpose, 1 μL of DEPC-treated water in the above reaction mixture was replaced with 1 μL of \[^{35}\text{S}\]-Met (15 μCi/μL). After the last centrifugation step, 12 μL of the supernatant were mixed with 3 μL of 5× SDS gel loading buffer, heated at 95 °C for 3 minutes, and loaded on a SDS-PAGE gel to run the electrophoresis.

2.3.5 In Vitro Suppression of Amber Mutants of AKH

In the in vitro suppression experiments, the plasmid DNA contained the amber codon at the position of interest within the AK gene. Three μL of DEPC-treated water in the 30 μL reaction mixture described above were replaced by aminoacyl-tRNA\textsubscript{CUA} (10 μg). The aminoacyl-tRNA was used immediately after photodeprotection to remove the NVOC protecting group (to be discussed in detail in Chapter 3). Aminoacyl-tRNA\textsubscript{CUA} and S-30 extract were added to the opposite side of the inner wall of a microtube.
tube was then spun 1-2 seconds in a microcentrifuge so that the two components were quickly mixed with the rest of the components of the reaction mixture already added at the bottom of the tube. Adding aminoacyl-tRNA\textsubscript{CUA} in this way minimized its contact with LM or S-30 prior to the initiation of the suppression reaction, which could accelerate the deacylation of aminoacyl-tRNA\textsubscript{CUA} and resulted in low suppression efficiency. The rest of the steps were carried out in the same way as described for \textit{in vitro} expression. Since tRNAs can chelate magnesium ions, the optimal concentration of magnesium in \textit{in vitro} suppression reaction was higher than the corresponding \textit{in vitro} expression reaction.

Small scale protein synthesis of 30 \(\mu\)L was routinely carried out for optimization of \([\text{Mg}^{2+}]\). Once the optimum \([\text{Mg}^{2+}]\) was determined, large scale protein synthesis was carried out to obtain enough \textit{in vitro} synthesized AKH for kinetic characterization. Typically, a 300 \(\mu\)L reaction mixture was used for \textit{in vitro} expression of AKH and a 1,000 \(\mu\)L reaction mixture was used for \textit{in vitro} suppression of AKH. The suppression efficiency was measured by comparing the amount of \(\text{[}^{35}\text{S]}\)-Met incorporated into mutant AKH synthesized from 30 \(\mu\)L \textit{in vitro} suppression reaction to that of WT AKH generated from 30 \(\mu\)L \textit{in vitro} expression reaction. The two 30 \(\mu\)L reactions were carried out at the same time as the 1 mL reaction using exactly the same materials.

A typical 300 \(\mu\)L \textit{in vitro} expression mixture contained the following components:

- DEPC-treated water 85 \(\mu\)L
- 0.1 M EGTA 20 \(\mu\)L
- 0.1 M (AcO)\textsubscript{2}Mg 10 \(\mu\)L
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKH-pUK plasmid (1.2 μg/μL)</td>
<td>25 μL</td>
</tr>
<tr>
<td>LM</td>
<td>75 μL</td>
</tr>
<tr>
<td>S-30 extract</td>
<td>85 μL</td>
</tr>
<tr>
<td>Total</td>
<td>300 μL</td>
</tr>
</tbody>
</table>

A typical 1000 μL *in vitro* suppression mixture contained the following components:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC-treated water</td>
<td>143 μL</td>
</tr>
<tr>
<td>0.1 M EGTA</td>
<td>67 μL</td>
</tr>
<tr>
<td>0.2 M (AcO)(_2)Mg</td>
<td>80 μL</td>
</tr>
<tr>
<td>pUK-AKH amber plasmid (1.3 μg/μL)</td>
<td>77 μL</td>
</tr>
<tr>
<td>LM</td>
<td>250 μL</td>
</tr>
<tr>
<td>S-30 extract</td>
<td>283 μL</td>
</tr>
<tr>
<td>aminoacyl-tRNA (3.6 μg/μL)</td>
<td>100 μL</td>
</tr>
<tr>
<td>Total</td>
<td>1000 μL</td>
</tr>
</tbody>
</table>

Purification of *In Vitro* Synthesized AKH.

The standard purification procedure of AK involves two chromatography steps: P11 and gel-filtration chromatography. Most of the AK mutants require different elution conditions from the WT in the P11 purification step, and some mutants do not even bind to the P11 column. As many as three columns may be needed in order to obtain the final
pure enzymes (Dahnke, et al., 1993). These complications are also expected for the purification of in vitro synthesized AK mutants. Due to the limited amount of in vitro synthesized AK passage through many columns would not be realistic. These problems were solved by attaching a six-histidine tag (His Tag) to AK's C-terminus and purifying the resulting AK by a metal-affinity chromatography. This method, by taking advantage of the strong chelation between histidines and nickel ions immobilized on metal-chelating resins, allows a single-step purification of the in vitro synthesized AKH from a crude lysate (Shi, 1994). The purification procedure was basically the same as described in “pET System Manual” from Novagen with little modification. The purification was carried out in cold room (4°C).

In vitro expressed WT AKH was purified from a 300 μL reaction mixture. This required about 360 μL resin (settled volume) for total binding of AKH. The 360 μL resin was prepared by taking 720 μL of resin slurry, washing it with 3 x 360 μL sterile distilled water, 5 x 360 μL of Charge Buffer (50 mM NiSO₄), 3 x 360 μL of Binding Buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9), and finally adding 150 μL of Binding Buffer to the resin. The in vitro protein synthesis mixture was first mixed well with the resin; after 10 minutes, the supernatant was removed. The resin was subsequently washed with Binding Buffer 5 times and Wash Buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) 5 times, each time with a 360 μL buffer and a duration of 5 minutes. After the last wash, the buffer was removed as completely as possible and 150 μL of Elution Buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) were added and mixed well with the resins. After 10 minutes, the mixture
was centrifuged and the supernatant was taken. AKH concentration in the supernatant was determined by running a full-length SDS-PAGE gel with a protein marker of various concentrations and comparing the band intensities. The supernatant was used directly and immediately for saturation kinetic analysis (discussed in the following section).

The \textit{in vitro} suppressed AKH mutant was purified by column chromatography from a 1000 µL reaction mixture. A slurry of 2.4 mL (to get resin of 1.2 mL settled volume) was applied to a 2.5 mL polypropylene column. The column was charged and equilibrated by the following washes: 4.2 mL of sterile deionized water, 6.0 mL of Charge Buffer, and 6.0 mL of Binding Buffer. The \textit{in vitro} suppression reaction mixture of 1000 µL was loaded to the pre-equilibrated His Bind column with the Binding Buffer drained just to the top of the column bed. The flow rate was controlled at 0.4 mL/min during all of the following steps. The column was washed with 6.0 mL of Binding Buffer, and 6.0 mL of Wash Buffer. The bound proteins were then eluted with 3.0 mL of Elution Buffer; the elute were collected as 0.5 mL/fraction. The enzyme concentrations were determined by analyzing the fractions on a SDS-PAGE gel. The fraction which had the most concentrated AKH mutant protein (usually the 3rd fraction) was assayed by saturation kinetic analysis immediately. Nearly all of the "junk" proteins were washed away during the washing steps. In the final elute, HAK was the major band, while there were some other bands which were of very small portions. A control experiment was performed at the same time with the \textit{in vitro} synthesis. This control experiment showed that without adding HAK and plasmid DNA, \textit{in vitro} mixture purified this way showed
no activity using the AK assay method (Shi, 1994). Those impurity bands that showed up in the final elute of \textit{in vitro} synthesized AKH also showed up in the control experiment.

**Kinetic Analysis**

The forward reaction catalyzed by AK, \( \text{MgATP} + \text{AMP} = \text{Mg ADP} + \text{ADP} \), was used in the steady-state kinetics. A pyruvate kinase/lactate dehydrogenase coupled enzyme assay (Rhoads & Lowenstein, 1968) was used to monitor the rate of ADP formation. As illustrated in Figure 9, in the presence of excess coupling enzymes, ADP is phosphorylated in the conversion of phosphoenolpyruvate to pyruvate with concomitant formation of two molecules of ATP. The resulting pyruvate is reduced to lactic acid by the spectrophotometrically detectable (340 nm) oxidation of NADH.

A full kinetic analysis involves fitting kinetic data to the following equation for a random bi bi kinetic mechanism according to Cleland (1986):

\[
V = \frac{ABV}{(K_a K_{ib} + K_b A + K_b B + AB)}
\]

where \( V \) is the reaction rate, the subscripts a and b represent the two substrates MgATP and AMP, respectively, A and B are the concentrations of the corresponding substrates, \( K_a \) and \( K_b \) are Michaelis constants, \( K_{ib} \) is a dissociation constant for the binary complex, and \( V \) is the maximal velocity.

Due to the amount of protein obtained from \textit{in vitro} synthesis, only saturation kinetic analysis was carried out for the AKH mutants. Saturation studies were performed by holding one substrate at a high level (2.0 mM) and varying the concentration of the other substrate (0.05 ~ 0.5 mM). The data were treated by fitting to
Figure 9. Scheme of the enzyme-coupled assay used for AK kinetics.
Michaelis-Menton equation:

\[ v = \frac{V[S]}{K + [S]} \]

Two Lineweaver-Burk plots of \(1/v - 1/[ATP]\) and \(1/v - 1/[AMP]\) were carried out to obtain apparent kinetic parameters, which included \(K_{m,\text{app(AMP)}}\), \(K_{m,\text{app(MgATP)}}\), and \(k_{\text{cat}}\).

All stock solutions used in the kinetic experiments were prepared in the reaction buffer (75 mM Tris-HCl and 65 mM KCl, pH 8.0) and their pH readjusted to 8.0. Three stock solutions were prepared for both ATP and AMP with approximate concentrations of 1 mM, 5 mM and 20 mM. The exact concentration of these stock solutions was measured spectrophotometrically based on \(\varepsilon_{260} = 15,400 \text{ M}^{-1}\text{cm}^{-1}\) for both ATP and AMP. These ATP and AMP stocks were stored at -70°C. All other stock solutions were freshly made. The AKH eluate was used directly for the kinetic analysis. Its concentration was estimated by running a SDS-PAGE gel with protein markers of various known concentrations and comparing band intensities after silver-staining. The composition of the final assay reaction was as follows: 75 mM Tris-HCl, 65 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1 mM PEP, 0.2 mM NADH, 50 μg each of pyruvate kinase and lactate dehydrogenase, and proper amounts of ATP, AMP and AKH. Every component except AKH was first added to a 1 mL quartz cuvette, mixed well and preincubated at 30 °C for 9 minutes. A baseline was then taken right before the addition of AKH. The initiated reaction was immediately monitored by the decrease of absorbance at 340 nm with a Kontron Uvikon 930 spectrophotometer in the time drive mode. The initial velocity (μmol/min) was calculated from the change in absorbance at 340 nm by subtraction of the base line and multiplication by a factor of 0.0804.
CHAPTER 3
SYNTHESIS OF AMINOACYL SUPPRESSOR TRANSFER RNA

3.1 Introduction

Aminoacyl tRNA is one of the most critical components for this *in vitro* suppression system. Due to substrate specificity of aminoacyl tRNA synthetases, the enzymatic misaminoacylation is not a general approach for acylating tRNAs with unnatural amino acids. Hecht has developed a solution to this problem, which depends on the T4 RNA ligase mediated coupling of 2'(3')-O-acylated pCpA derivatives to tRNAs missing the 3'-terminal cytidine and adenosine moieties (Heckler et al., 1984). The α-amino-protecting group, which stabilize the aminoacyl linkage, was not removed from the aminoacyl tRNA. tRNA of this type cannot function in peptide elongation until the amino acid protecting group is removed. Brunner demonstrated that tert-butylloxy carbonyl- (t-Boc) protected aminoacyl pCpA can be prepared and deprotected with trifluoroacetic acid prior to ligation to provide an aminoacyl tRNA with a free α-amino group that can function in peptide elongation (Baldini et al., 1988). Pyroglutamyl aminoacyl tRNAs have been prepared from pyroglutamyl aminoacyl dinucleotides, and the α-amino group can be deprotected by pyroglutamate aminopeptidase (Rosser et al.,
In the initial studies, Schultz reported that 5'-phospho-2'-deoxyribocytidylyriboadenosine (pdCpA) can be acylated with nitrosulfenyl (NPS) or carbobenzoxy- (Cbz-) protected amino acids and can be subsequently deprotected by treatment with sodium thiosulfate or hydrogenation, respectively.

All of the above methods require a number of protection and deprotection steps that result in low yields of aminoacyl tRNAs. Most of the methods also involve coupling of the hydrolytically labile unblocked aminoacyl dinucleotide to tRNA-C\textsubscript{OH}. More recently, Schultz reported a method whereby unprotected pdCpA can be selectively aminoacylated with an N-NVOC blocked amino acid and efficiently ligated to tRNA. The carboxylic group is activated by forming a cyanomethyl ester. This N-NVOC blocked aminoacyl tRNA can be deprotected photochemically in high yield under conditions where the unblocked aminoacyl tRNA is stable. The aminoacylated tRNA can be directly added to the protein biosynthesis reaction without further purification. This method was applied to this study with some modifications. In this work, pdCpA was ion-exchanged to bear 3 eq of tetrabutylammonium ion. PdCpA thus prepared affords a higher coupling yield with the protected amino acid. L-Homopipeolic acid and L-dihyrophenylalanine are not commercially available. These two amino acids were synthesized according to the methods of Seebach (1989) and Snow (1967) with little modification.

### 3.2 Experimental Section

#### 3.2.1 Materials
The fully protected dinucleotide 5'-phospho-2'-deoxycytidy1 (3',5')adenosine (pdCpA) was prepared by Ru-Tai Jiang. Figure 10 shows the steps for the synthesis of fully protected pdCpA (Robertson et al., 1989). The truncated suppressor tRNA was a yeast tRNA\textsuperscript{pre}_{CUA} (-CA) generated with runoff transcription method (Ellman et al., 1991) by Baohua Huang. High-pressure liquid chromatography (HPLC) was performed on a Waters 600 E solvent delivery system with a Waters 996 Photodiode Array detector. T4 RNA ligase was purified from an overproducing E. coli strain E/MG583 (Ellman et al., 1991) by Baohua Huang. Chloroacetonitrile was distilled prior to use. All reagents were purchased from Aldrich unless otherwise indicated; all reagents were used without further purification unless otherwise indicated. All flash column chromatography was carried out on silica gel (Merck Kieselgel 60, Art. 9385). \textsuperscript{1}H NMR spectroscopy was recorded on the Bruker AM-500 (500 MHz) Fourier transform NMR spectrometer of CCIC at The Ohio State University facility. The internal standard for \textsuperscript{1}H NMR spectra determined in CDCl\textsubscript{3} was Me\textsubscript{4}Si; for those determined in DMSO or D\textsubscript{2}O, the solvent was used as the reference at \(\delta\) 2.49, 4.63 respectively. \textsuperscript{13}C NMR spectra were recorded on the Bruker AM-500 (127 MHz) Fourier transform spectrometer; all spectra were proton decoupled and reported in units of ppm downfield from CDCl\textsubscript{3} (\(\delta\) 77.0), or DMSO-d\textsubscript{6} (\(\delta\) 39.5).

3.2.2 Synthesis of Dinucleotide pdCpA

The fully protected pdCpA is stable, and it can be stored in a -70 °C freezer for a long time. pdCpA was prepared by deprotection of the fully protected pdCpA when
Figure 10. Synthesis of Protected pdCpA. Reproduced from Robertson et al., 1989.
pdCpA was needed. Figure 11 shows the deprotection reaction. Crude product of D was used directly in the deprotection step.

Fully protected pdCpA D (0.81 g) was dissolved in dioxane (5 mL) and methanol (40 mL) and added to fresh concentrated ammonium hydroxide (45 mL) in a sealed pressure bottle equipped with a magnetic stir bar. The reaction mixture was sealed and heated at 55 °C for 36 hours. The reaction mixture was concentrated under reduced pressure, then redissolved in 6.0 mL of 25 mM aqueous ammonium acetate (pH 5). Reverse phase HPLC was used to purify the product by being run through a Waters 25 x 20 cartridge, HR C18, 6 μm, 60 Å. The flow rate was 5.0 mL/min. Wave length was 260 nm. The gradient was 100:0 (25 mM aqueous AcONH₄:CH₃CN) to 75:25 (25 mM aqueous AcONH₄:CH₃CN) over 50 minutes. The product eluted at tᵣ ≈ 18 minute was collected, and the solution was lyophilized. The resulting solid was then desalted by being run through HPLC using the same gradient but with water replacing 25 mM AcONH₄ (pH 5). Lyophilization of the desalted fractions afforded the product as a white solid 0.20 g (52% from crude protected pdCpA). It was store at -70 °C. ¹H NMR (300 MHz, D₂O) δ 1.85 (m, 1H), 2.34 (m, 1H), 3.92 (s, 2H), 4.04 (s, 2H), 4.20 (s, 1H), 4.27 (s, 1H), 4.45 (t, 1 H, J = 4.5 Hz), 4.60 - 4.75 (m, 2 H), 6.01 (m, 2 H), 7.81 (d, 1 H, J = 7.8 Hz), 8.15 (s, 1H), 8.40 (s, 1H); ³¹P NMR (D₂O) δ -0.70, -1.90 ppm; Mass spectrum, m/e 637.14 (MH⁺).

To ion-exchange the pdCpA, a 1 x 10 cm column was filled with Dowex-50W hydrogen form resin; the column was washed with 25% EtOH (50 mL), 0.1 N NaOH (50 mL), 0.1 N HCl (50 mL), double distilled water (100 mL), and then 0.1 N Bu₄NOH. The
Figure 11. Deprotection to Release pdCpA. Reproduced from Robertson et al., 1989.
color changed to pink, indicating the bearing of Bu₄N⁺; 26.6 mg of lyophilized pdCpA was dissolved in 1.0 mL of double distilled water; the solution was loaded to the column and eluted with double distilled water; the UV active fraction (using a monitor) was collected, and it was immediately lyophilized. ¹H NMR indicated the pdCpA contained 3 eq of Bu₄N⁺. The peak area at δ 8.32 (which stands for 1 eq of pdCpA) and peak area at δ 2.97 (which stands for 8 eq of nBu₄N⁺) were compared. See Appendix for the detailed ¹H NMR spectrum. The ion-exchanged pdCpA was stored at -70 °C.

3.2.3 Synthesis of Homopipecolic Acid and Dihydrophenylalanine

Synthesis of L-Homopipecolic Acid

The chiral auxiliary of (S)-tert-butyl 2-(tert-butyl)-3-methyl-4-oxo-1-imidazolidinecarboxylate (Boc-BMI) derivative was used for the synthesis of L-homopipecolic acid (Seebach et al., 1989). Figure 12 shows the scheme for the synthesis of L-homopipecolic acid.

Alkylation. To a 250 mL round bottom flask was added 34 mL of dry THF, and 1.54 mL of diisopropyl amine (5.5 mmol); the solution was then cooled at -78 °C (dry ice-acetone bath). 7.34 mL of nBuLi (1.6 M in hexane) was added by syringe dropwise; 5 minutes later, a solution of (S)-Boc-BMI (2.56 g, 10 mmol) in 26 mL of dry THF was added by syringe (not dropwise). The stirring was continued for 40 minutes. 1.98 mL of 1-bromo-5-chloropentane (15 mmol) was added to the solution. After the addition, the reaction mixture was allowed to warm to room temperature. 24 hours later, 10 mL of saturated aqueous was poured into the reaction mixture, then stirred for a while. A white
Figure 12. Synthesis of L-homopipeolic acid. Reproduced from Seebach et al., 1989.
solid showed up on the bottom. The reaction mixture was poured into a 250 mL separation funnel containing 40 mL of Et₂O and 60 mL of H₂O, shaken well and separated the two layers. The aqueous layer was extracted with Et₂O (2 x 40 mL). The combined organic layer was dried (MgSO₄). Removal of solvents in vacuo afforded the product as a yellow liquid (3.82 g, 100%).

**N-Boc Deprotection.** The crude product from the last step was dissolved in 20 mL of dry CH₂Cl₂. The solution was cooled down to 0 °C, and TFA of 5.0 mL was added dropwise under Ar. After the addition, the mixture was warmed to r.t. and then was heated in an oil bath of 35 - 40 °C for 2 hours. The mixture was then put at r.t. and stirred for another 2 hours. Saturated aq. NaHCO₃ was added while stirring until neutral pH was reached. The mixture was poured into a 250 mL separation funnel containing 40 mL of H₂O, and extracted with Et₂O (3 x 40 mL). The combined Et₂O layer was dried over Na₂SO₄. Removal of solvents afforded crude product as a dark red oil (2.62 g). The crude product was purified with flash chromatography over 120 g of silica gel. A mixture of Et₂O and hexane was used to elute. The product fractions were combined. Removal of solvents in vacuo afforded the product as a yellow oil (1.70 g, 65.1% two steps).

**Cyclization.** To a solution of the product of the last step in 29 mL of anhydrous CH₃CN was added anhydrous Na₂CO₃ (0.34 g, 0.5 eq) and anhydrous NaI (0.97 g, 1.0 eq). The heterogeneous mixture was heated in an oil bath to reflux (~ 95 °C). A dry guard tube was applied. After 12 hours, the reaction mixture was poured into 39 mL of H₂O, then extracted with Et₂O 2 x 26 mL. The organic phase was combined, and dried (Na₂SO₄). Removal of solvents in vacuo afforded the crude product as a yellow solid
(1.29 g), which was purified with flash chromatography over 25 g of silica gel. It was eluted with 1:1 of Et$_2$O: Hexane. The product fractions were collected. Removal of solvents afforded a white solid (1.17 g, 80%).

**Preparation of the Free homopipecolic Acid.** The product from the last step was dissolved in 0.75 M HCl with the addition of 2 mL of acetic acid in a Wheaton bottle. A Dowex 50-WX8 cation-exchange resin (19 mL of dry resin) was added (prewashed with EtOH, double distilled water, then 0.75 M HCl). After 6.8 mL of toluene were added, the bottle was capped (screw cap) and was heated at ~ 105 °C. It was stirred for 72 hours. The flask was then cooled down. The mixture was transferred to a column, and the resin was washed with double distilled water. The amino acid was eluted with a 10% NH$_3$ solution. Nihyrin-positive fractions were combined. Most NH$_3$ was removed in vacuo. High vacuum was applied to remove traces of NH$_3$. Freeze-drying of the solution afforded L-homopipecolic acid as a white solid (0.567 g, 76%). $^1$H NMR (300 MHz, D$_2$O) $\delta$ 1.45 -1.60 (m, 4 H), 1.65 - 1.85 (m, 3 H), 2.02 - 2.15 (m, 1 H), 3.01 - 3.10 (m, 1H), 3.10 - 3.22 (m, 1 H), 3.68 (dd, $J_1 = 9.06$ Hz, $J_2 = 3.72$ Hz). $[\alpha]_D = -29$ (c = 1.03 in H$_2$O).

**Synthesis of L-Dihydrophenylalanine**

L-2,5-dihydro phenylalanine (DiHPhe) was prepared from L-phenylalanine by a Birch reduction using a large molar excess of Na and methanol in NH$_3$ at -40 °C (Snow et al., 1968). Figure 13 shows the scheme for synthesis of 2,5-dihydrophenylalanine. The product was found to contain less than 1% of unreacted phenylalanine based upon $^1$H
Figure 13. Synthesis of 2,5-dihydrophenylalanine. Reproduced from Snow et al., 1968.
NMR analysis. Due to the instability of DihPhe, the product was converted to copper salt and liberated as needed.

**Birch reduction and Formation of Copper Salt.** In a 500 mL three-neck round bottom flask fitted with a gas inlet tube and protected by a drying tube of powder KOH, 1.7 g of L-phenylalanine were placed in 41 mL of dry MeOH (Aldrich sure seal). The flask was immersed in a dry ice-cellosolve bath, and 333 mL of liquid ammonia were collected. The temperature of the bath was maintained around -40 °C while Na (6.0 g, 0.26 mol) was added to the clear solution in portions with magnetic stirring over a period of 1.0 hour. The blue color was allowed to fade before each successive addition of sodium. When addition of sodium was complete, NH₄Cl (14 g, 0.26 mol) was added carefully in portions, and the ammonia was allowed to evaporate overnight. Methanol and residual ammonia were removed in vacuo for 2 hours. The solid residue was dissolved in 120 mL of water. The solution was adjusted to pH 5 with concentrated HCl and clarified by filtration. To the solution was added a hot solution of 2.0 g of Cu(OAc)₂·H₂O in 16.7 mL of boiling water. It was chilled overnight, and the crystalline copper chelate was collected by filtration, and washed with 170 mL of cold water, 50 mL of cold EtOH, and 10 mL of cold ether. Removal of solvents in vacuo afforded a blue solid 1.76 g (86%). Stored at 4 °C.

**Liberation of Dihydro Phenylalanine from Copper Chelate.** The copper chelate (1.5 g) was powdered finely and dissolved in concentrated ammonia (45 mL). Moist Chelex resin (Bio-Rad) in NH₄⁺ cycle (100 mL) was added, and the mixture was stirred magnetically for 10 minutes. The resin was filtered off, and the filtrate was first
put in vacuo to remove NH₃, then put to pump for 36 hours to obtain a dry solid. 10 mL of water were used to resuspend the solid, then use a pump was used to take the solid to dryness. It was then resuspend in 10 mL of water, and the pH was adjusted to 5.5 (from ~ 7.5) with several drops of 6 N HCl. The mixture was then applied on a pump to dry. Recrystallization with 27 mL of 80% EtOH afforded the product as a white solid 0.33 g (26%). ¹H NMR (500 MHz, D₂O) δ 2.28 - 2.33 (m, 2 H), 2.46 - 2.56 (m, 4 H), 3.65 (m, 1 H, α-H), 5.53 (s, 1 H), 5.63 (s, 2 H).

3.2.4 Protection of Unnatural Amino Acids

Figure 14 shows the protection of amino acids and formation of aminoacyl tRNA.

**General Procedure for N-NVOC Protection of Amino Acids.** To a solution of 0.77 mmol (1 eq) of amino acids, 8.16 mg (0.77 mmol, 1 eq) of Na₂CO₃ in 2.0 mL of H₂O was added dropwise to a solution of 212 mg (0.77 mmol, 1 eq) of 6-nitroveratryloxycarbonyl chloride in 10.0 mL of dioxane over 5 minutes. The reaction mixture was continuously stirred at room temperature for 1.5 hours. After the reaction was complete (monitored by TLC using 98:2 of EtOAc:AcOH), 18 mL of aqueous NaOH (1.5 mL of 2N solution was diluted to 18 mL) solution were added, and CH₂Cl₂ (3 x 10 mL) was used to extract unreacted NVOC-Cl. After separation, the pH of the aqueous phase was brought to about 4 by approximately 4.0 mL of 1 M NaHSO₄. The mixture was extracted by EtOAc (3 x 15 mL). The combined EtOAc layer was dried (Na₂SO₄). After filtration, removal of solvents in vacuo afforded the crude product as a yellow foam, which was used directly in the next step without further purification.
Figure 14. Synthesis of aminoacyl tRNA. NVOC = 6-nitroveratryloxycarbonyl. (a) NVOC-Cl (1.0 eq), NaCO₃ (1 eq), H₂O/dioxane; (b) CICH₂CN (2.0 eq), Et₃N (3.0 eq), DMF, rt, 24 hr; c) 3.0 eq n-Bu₄N⁺, dry DMSO; (d) T4 RNA ligase; (e) hv, 350 nm.
General Procedure for Formation of Cyanomethyl ester from N-NVOC-Amino Acids. To a solution of crude N-NVOC-Amino acid (1eq) in 0.20 mL of dry DMF was added dry Et₃N (2 eq) and freshly distilled chloroacetonitrile (3 eq). The reaction solution was stirred at room temperature for 24 hours. After the reaction was complete (monitored by TLC using 98:2 of EtOAc:AcOH), 20 mL of 1 M NaHSO₄ and 30 mL of CH₂Cl₂ were added to dilute the mixture. After separation, the aqueous phase was extracted using CH₂Cl₂ (3 x 10 mL). All the organic phases were combined and then dried (Na₂SO₄). Removal of solvents in vacuo afforded a yellow foam as a crude product; this was purified by flash chromatography over 7.0 g of silica gel to give the N-NVOC-amino acid cyanomethyl ester as a yellow foam, which was fully characterized.

N-NVOC-L-Azetidine-2-Carboxylic Acid Cyanomethyl Ester (ZZ1050A2).

¹HNMR (500 MHz, DMSO-d₆, 353K) δ 2.28 (m, 1H, β-H), 2.65 (m, 1H, β-H), 3.87 (s, 3H, OCH₃), 3.94 (s, 3H, OCH₃), 3.98 (m, 2H, γ-H), 4.90 (m, 1H, α-H), 5.03 (s, 2H, CH₂CN), 5.34, 5.38 (AB, J = 14.5 Hz, 2H, CH₂BzI), 7.13 (s, 1H, ArH), 7.67 (s, 1H, ArH); ¹³C NMR (126 MHz, DMSO-d₆, 353K) δ 19.9 (t), 47.2 (t), 49.2 (t), 56.0 (q), 59.3 (d), 62.7 (t), 108.4 (d), 110.7 (d), 114.9 (s), 126.4 (s), 139.3 (9s), 147.8 (s), 153.2 (s), 154.2 (s), 169.3 (s); HRMS calcd for C₁₇H₁₇N₃O₇ 379.1016 found 379.1012. Anal calcd for C₁₇H₁₇N₃O₇: C, 50.66; H, 4.52; N, 11.08. Found: C, 50.74; H, 4.56; N, 10.79.

N-NVOC-L-3,4-Dehydro Proline Cyanomethyl Ester (ZZ1033B). ¹HNMR (500 MHz, DMSO-d₆, 353K) δ 3.88 (s, 3H, OCH₃), 394 (s, 3H, OCH₃), 4.28 (broad, 2H, δ-H), 5.00 (s, 2H, CH₂CN), 5.21 (m, 1H, α-H), 5.42 (s, 2H, CH₂BzI), 5.87 (dd, J = 2.1, 6.1 Hz, 1H, β-H), 6.17 (m, 1H, γ-H), 7.15 (broad, 1H, ArH), 7.68 (s, 1H, ArH); ¹³C NMR
(126 MHz, DMSO-d$_6$, 353K) $\delta$ 49.2 (t), 52.9, 53.5 (twin peaks, t, t), 56.0 (q), 63.2 (t), 65.3, 65.6 (twin peaks, d, d), 108.4 (d), 111.0 (d), 114.8 (s), 123.4 (d), 126.3 (s), 129.7 (d), 139.4 (s), 147.8 (s), 152.8 (twin peaks, s), 153.2 9s), 168.2 (twin peaks, s); HRMS calcd for C$_{17}$H$_{17}$N$_3$O$_8$ 391.1016 found 391.1018. Anal calcd for C$_{17}$H$_{17}$N$_3$O$_8$ : C, 52.18; H, 4.38; N, 10.74. Found: C, 52.20; H, 4.50; N, 10.45.

N-NVOC-L-Pipelic Acid Cyanomethyl ester (ZZ1062A2). $^1$HNMR (500 MHz, DMSO-d$_6$, 353K) $\delta$ 1.21 - 1.29 (m, 1H, $\gamma$-H), 1.37 - 1.46 (m, 1H, $\gamma$-H), 1.66 -1.78 (m, 3 H, 2 $\delta$-H, 1 $\beta$-H), 2.11 (d, J = 14.0 Hz, 1H, $\beta$-H), 3.00 (broad, 1H, $\varepsilon$-H), 3.88 (s, 3H, OCH$_3$), 3.92 (s, 3H, OCH$_3$), 3.96 (m, 1H, $\varepsilon$-H), 4.92 (m, 1H, $\alpha$-H), 5.02 (s, 2H, CH$_2$CN), 5.41 (s, 2H, CH$_2$Bzl), 7.11 (s, 1H, ArH), 7.67 (s, 1H, ArH); $^{13}$C NMR (126 MHz, DMSO-d$_6$, 353K) $\delta$ 19.6 (t), 23.6 (t), 25.8 (t), 41.2 9t), 49.2 (t), 53.8 (d), 56.0 (q), 63.3 (t), 108.5 (d), 111.1 (d), 115.0 (s), 126.3 (s), 139.6 (s), 147.9 9s), 153.1 (s), 154.5 (s), 169.9 (s); HRMS calcd fpr C$_{18}$H$_{21}$N$_3$O$_8$ 407.1329 found 407.1322. Anal calcd for C$_{18}$H$_{21}$N$_3$O$_8$: C, 53.07; H 5.20; N, 10.31. Found: C, 52.97; H, 5.26; N, 10.10.

N-Methyl-N-NVOC-L-Glycine Cyanomethyl Ester (ZZ1057A2). $^1$HNMR (500 MHz, DMSO-d$_6$, 353K) $\delta$ 2.97 (s, 3H, NCH$_3$), 3.87 (s, 3H, OCH$_3$), 3.92 (s, 3 H, OCH$_3$), 4.21 (s, 2H, $\alpha$-CH$_2$), 4.99 (s, 2H, CH$_2$CN), 5.39 (s, 2H, CH$_2$Bzl), 7.11 (s, 1H, ArH), 7.67 (s, 1H, ArH); $^{13}$C NMR (126 MHz, DMSO-d$_6$, 353K) $\delta$ 35.1 (q), 49.0 (t), 49.6 (t), 56.0 (q), 63.4 (t), 108.4 (d), 110.9 (d), 115.0 (s), 126.4 (s), 139.5 (s), 147.9 (s), 153.2 (s), 168.3 (s); HRMS calcd for C$_{15}$H$_{17}$N$_3$O$_8$ 367.1016 found 367.1015. Anal calcd for C$_{15}$H$_{17}$N$_3$O$_8$: C, 49.05; H, 4.66; N, 11.4. Found: C, 49.90; H, 4.96; N, 10.84.
N-NVOC-L-Homopipeolic Acid Cyanomethyl Ester (ZZ1065A2). $^1$HNMR (500 MHz, DMSO-d$_6$, 353K) δ 1.33 - 1.47 (m, 3H), 1.70 - 1.83 (m, 4H), 2.23 - 2.50 (m, 1H), 3.15 (m, 1H, $\alpha$-H), 3.81 (d, J = 14.4 Hz, 1H, $\xi$-H), 3.88 (s, 3H, OCH$_3$), 3.96 (s, 3H, OCH$_3$), 4.64 (broad, 1H, $\alpha$-H), 4.97 (s, 2H, CH$_2$CN), 5.37 - 5.46 (m, 2H, CH$_2$Bzl), 7.08, 7.13 (2 broad s, 1H, ArH), 7.68 (s, 1H, ArH); $^{13}$C NMR (126 MHz, DMSO-d$_6$, 353K) δ 24.7, 24.9 (twin peaks, t, t), 27.7 (t), 28.5, 28.7 (twin peaks, t, t), 29.1, 29.4 (twin peaks, t, t), 43.5, 43.7 (twin peaks, t, t), 48.9 (t), 56.0 (q), 58.2 (d), 63.4 (t), 108.4 (d), 110.9, 111.1 (twin peaks, t, t), 115.0 (s), 126.4 (s), 139.5 (s), 147.8 (s), 153.1 (s), 154.3, 155.0 (twin peaks, s, s), 107.5, 170.8 (twin peaks, s, s); HRMS calcd for C$_{19}$H$_{23}$N$_3$O$_8$ 421.1486 found 421.1486. Anal.calcld for C$_{19}$H$_{23}$N$_3$O$_8$: C, 54.15; H, 5.50; N, 9.97. Found: C, 53.96; H, 5.51; N, 9.72.

N-NVOC-L-Dihydrophenylalanine Cyanomethyl Ester (ZZ3018C). $^1$HNMR (500 MHz, CDCl$_3$) δ 2.35 - 2.46 (m, 1H), 2.52 - 2.62 (m, 3H), 2.67 - 2.75 (m, 2H), 3.94 (s, 3H), 3.99 (s, 3H), 4.45 - 4.55 (m, 1H, $\alpha$-H), 4.70, 4.84 (AB, J = 15.7 Hz, OCH$_2$CN), 5.21 - 5.30 (d, J = 7.3 Hz, N-H), 5.46, 5.59 (AB, J = 15.3 Hz, 2H, benzyl), 5.57 (s, 1H), 5.69 (s, 2H), 6.97 (s, 1H), 7.70 (s, 1H). $^{13}$C NMR (125 MHz, CDCl$_3$) δ 26.7 (t), 28.4 (t), 39.8 (t), 48.9 (t), 51.8 (d), 56.4 (q), 56.5 (q), 64.0 (t), 108.2 (d), 109.9 (d), 113.7 (s), 123.4 (d), 123.9 (d), 124.2 (d), 127.7 (d), 129.1 (s), 139.7 (s), 148.2 (s), 153.7 (s), 155.3 (s), 171.1 (s). HRMS calcd for C$_{21}$H$_{23}$N$_3$O$_8$: 445.1486 found 445.1463. Anal. Calcd. for C$_{21}$H$_{23}$N$_3$O$_8$: C, 56.63; H, 5.20; N, 9.43.

3.2.5 Synthesis of Aminoacylated tRNA
General Procedure for Coupling N-NVOC-Amino Acid Cyanomethyl Ester

with pdCpA. PdCpA bearing n-Bu₄N⁺ (0.048 mmol, cation exchange from 26.6 mg of pdCpA bearing 3 H, 1.0 eq) and active ester (5.0 eq) were put in a one dram vial containing a micro stir bar. The mixture was then applied in a high vacuum overnight to achieve complete dryness. 0.400 mL of dry DMSO was then added under Ar after the vacuum was release. The solution was stirred at room temperature for 3.0 hours. The reaction mixture was diluted by adding 4.0 mL of 50 mM aqueous AcONH₄ and 1.0 mL of CH₃CN. Reverse phase HPLC was used to separate the product by being run through a Waters 25 x 20 cartridge, HR C18, 6 μm, 60 Å. The flow rate was 5.0 mL/min. The wavelength was 260 nm. The gradient was 90:10 (50 mM aqueous AcONH₄:CH₃CN) to 10:90 (50 mM aqueous AcONH₄:CH₃CN) over 80 minutes. The appropriate fraction was collected and freeze-dried to obtain a solid which was then dissolved in 3.0 mL of solution of 10 mM aq. AcOH and CH₃CN (9:1). HPLC was used to desalt the solid by using the same gradient table but with the replacement of 50 mM aq. AcONH₄ by 10 mM aq. AcOH. The fraction corresponding to pdCpA was collected. Assuming that the extinction coefficient at 260 nm for the NVOC-protected aminoacyl pdCpA was additive: ε₂₆₀ = 25140 cm⁻¹M⁻¹ (ε₂₆₀ = 23000 cm⁻¹M⁻¹ for pdCpA and ε₂₆₀ = 2140 cm⁻¹M⁻¹ for each 6-nitroveratryl group), the concentration of the acylated pdCpA was determined by measuring UV absorbance at 260 nm. The solution was then dispensed into Eppendorf tubes with 40 μg/tube. Lyophilization of the solution afforded pdCpA as a white solid (30%), which was stored immediately in a -70 °C freezer.
Ligation of Aminoacylated pdCpA to tRNA\textsubscript{CUA}(-CA). Lyophilized aminoacylated pdCpA (40 μg) was dissolved in 160 μL of ligation solution [55 mM HEPES (Na), pH 7.5, 0.25 mM ATP, 15 mM MgCl\textsubscript{2}, dimethyl sulfoxide (DMSO) 10% (v/v), 40 μg truncated tRNA\textsubscript{CUA}(-CA), and 400 units of T4 RNA ligase]. The reaction mixture was incubated at 37 °C for 8 minutes followed by successive extractions with equal volumes of phenol/chloroform and chloroform. The acyl tRNA was then precipitated by adding 0.1 volume of 2.5 M sodium acetate, pH 4.5, and 3 volumes of ethanol, and then incubated at -70 °C for 15 minutes. The precipitate were collected by microcentrifugation for 15 minutes at 4 °C. The pellet was washed by 1 mL of 70% ethanol and recentrifuged for 5 minutes at 4 °C. The supernatant was carefully removed and the pellet was dried with Speed Vac. for 1 minute. The resulting acyl tRNA was either used immediately in the photodeprotection reaction or stored at -70 °C.

Photodeprotection of Aminoacyl-tRNA. The aminoacyl-tRNA pellet obtained from the ligation step was dissolved in 12.5 mL of 1 mM potassium acetate, pH 4.5. To completely dissolve the pellet, the solution was vortexed for 10 seconds, and left at room temperature for 10 minutes. It was vortexed again, and transferred to a transparent Eppendorf tube (Robins Scientific). The contents were irradiated (350 nm) for 15 minutes employing a 450 W Immersion lamp lamp (ACE Glass, 7825-34) with a Pyrex glass filter. After irradiation, the solution was either directly added to the in vitro suppression reaction mixture or immediately stored on dry ice to minimize hydrolysis.

3.3 Results and Discussion
The strategy for aminoacylation of pdCpA developed by Schultz was proven in this study to produce a higher yield. Previous protocols suffered several drawbacks that led to low aminoacylation yield. These methods either resulted in diacylation of pCpA or required protection of pCpA prior to aminoacylation. The protecting groups were removed in relatively low overall yields. Moreover, most of the protocols involved coupling of free aminoacyl pCpA to tRNA-C_{OH} under conditions where the unprotected aminoacyl linkage was hydrolytically liable. Schultz's protocol uses pdCpA and N-NVOC amino acid cyanomethyl ester. Aminoacyl pdCpA can be prepared in one step. No protecting groups are needed for pdCpA. In order to increase the solubility of pdCpA in DMSO, tetrabutylammonium salt was formed. Schultz found that when pdCpA containing 2.2 eq of the tetrabutylammonium countion was employed, a convenient reaction time of 2.5 hours was obtained. In this dissertation research, pdCpA containing 3.0 eq of the tetrabutylammonium countion was employed, and the coupling yield was also high after 3 hours of reaction at room temperature. Several unnatural amino acids were employed in this work; these included L-homopipecolic acid and L-2,5-dihydrophenylalanine. This research thus adds more amino acids to the variety of amino acids that can be used for aminoacylation of pdCpA with the cyanomethyl esters.

The α-amino group was protected with nitroveratryloxy (NVOC) group, which could be removed by photolysis. Nitroveratryloxy carbamates and esters and nitroveratryl ethers have been used to protect amino, hydroxyl, carboxyl, and phosphoryl moieties (Amit et al., 1974). Deprotection can be carried out with a 350 nm light, which is known not to damage RNA (Bartholomew et al., 1975). Because the photolysis reaction leads to
nitroso and aldehyde byproducts, deprotection can be carried out under slightly acidic conditions in the case of N-blocked amines to prevent secondary reaction of the free amine with reaction photoproducts. Since, in the procedure a long wavelength of 350 nm is employed, carbon-carbon double bonds of the unnatural amino acids (for example dihydrophenylalanine and dehydroproline) will not be affected.

The protected aminoacyl pdCpA can be directly ligated to tRNA-COH employing T4 RNA ligase to provide protected aminoacyl tRNA. Importantly, N protection of the α-amino group greatly enhances the stability of the labile aminoacyl bond to the ligation buffer condition. The photochemical removal of NVOC occurs in near quantitative yield. Either the 2′- or 3′-hydroxyl group of adenosine could be acylated. This is not a problem since the interconversion between the two isomers is rapid in aqueous buffer, pH 7.3, 37 °C (t$_{1/2}$ = 1-11 s$^{-1}$) (Schuber et al., 1974). The aminoacylated tRNA can be added directly to the in vitro protein biosynthesis reaction. In this work, it has been able shown that this strategy could be applied to several newly utilized unnatural amino acids.
CHAPTER 4
RING SIZE OF PRO-17 AND AROMATICITY OF TYR-95

4.1 Introduction

4.1.1 Ring Size of Pro-17

Phosphate binding loop (P-loop) is a common motif in ATP- and GTP-binding proteins. These adenine and guanine nucleotide-binding proteins include adenylate kinases, *ras* proteins, elongation factors, ATP-synthase β-subunits, myosin heavy chains, thymidine kinases, and phosphoglycerate kinases (Saraste et al., 1990). The primary structure of P-loop typically consists of a glycine-rich sequence followed by a conserved lysine and a serine or threonine. The consensus sequence for the P-loop motif is normally quoted in the literature as GXXXXGK[TS].

The P-loop of adenylate kinase is located close to the N-terminus, where it forms a loop between a β-strand and an α-helix. Figure 15 shows stereoview of the structure of *E. coli* AK complexed with AMP and AMPPNP (Berry et al., 1994). Four glycine residues, a proline, a lysine and a threonine are conserved in the nine-residue sequence. Its pattern is $^15GXPGXGKGT^{23}$. There are two features that distinguish adenylate kinase from the other P-loop-containing families: a conserved proline residue; a glycine residue
Figure 15. Stereoview of the structure of *E. coli*. AK complexed with AMP and AMPPNP (Berry et al., 1994). Pro-9 and Phe-86 correspond to Pro-17 and Tyr-95, respectively, in muscle AK.
following the invariant lysine. The conserved proline residue is particularly interesting. In ras p-21, another P-loop containing protein, the P-loop has the sequence of \textsuperscript{10}GAGGGVKS\textsuperscript{17}. It has been demonstrated that any mutation by natural amino acids at Gly-12 of ras p-21 besides Pro led to decreased GTPase activity of this protein (Seeburg et al., 1984; Barbacid, 1987). From the comparison of the two P-loop patterns of adenylate kinase and ras protein, Gly-12 in ras p-21 corresponds to Pro-17 in adenylate kinase.

Pro-17 is absolutely conserved in the AK family (Egner et al., 1987; Dreusicke et al., 1988; Saraste et al., 1990). Site-directed mutagenesis studies have been reported at the Pro-17 position (Tagaya, 1989; Reinstein et al., 1988, 1990), and the results are summarized in Table 1. All substitutions cause concomitant increase of both Michaelis constants, $K_{m,MgATP}$ and $K_{m,AMP}$, but not $k_{cat}$. According to the current substrate binding model of AK revealed by crystal structure, NMR studies and mutagenesis, Pro-17 is located at the ATP site, and is quite far from the AMP site. The perturbations in substrate binding parameters can not simply be attributed to direct interaction between Pro-17 and both substrates. The explanation provided by Reinstein et al. (1988) related these observation to the induced-fit mechanism of AK. According to the kinetic consequences described by Fersht (1985) on an enzyme conforming to the induced-fit mechanism, AK exists in two conformations, $E_{\text{inact}}$ that does not bind substrates, and $E_{\text{act}}$ that does. Mutations at Pro-17 are suggested cause to changes in this equilibrium between these two conformations, which favoring $E_{\text{inact}}$. This would result in concomitant increase of $K_m$ of both substrates but no change of $k_{cat}$.
<table>
<thead>
<tr>
<th>parameters</th>
<th>unit</th>
<th>WT</th>
<th>P17G (P9G)(^b)</th>
<th>P17V</th>
<th>P17L (P9L)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(V_{\text{max}})</td>
<td>units/mg</td>
<td>1900</td>
<td>1400</td>
<td>1300</td>
<td></td>
</tr>
<tr>
<td>(K_{m,\text{app,MgATP}})</td>
<td>mM</td>
<td>0.19</td>
<td>1.4 (7)</td>
<td>8.0 (42)</td>
<td></td>
</tr>
<tr>
<td>(K_{m,\text{app,AMP}})</td>
<td>mM</td>
<td>0.19</td>
<td>1.4 (7)</td>
<td>4.5 (24)</td>
<td></td>
</tr>
<tr>
<td>(k_{\text{cat,app}})</td>
<td>s(^{-1})</td>
<td>303</td>
<td>389</td>
<td>548</td>
<td></td>
</tr>
<tr>
<td>(K_{m,\text{app,MgATP}})</td>
<td>mM</td>
<td>0.071</td>
<td>0.775 (11)</td>
<td>1.18 (17)</td>
<td></td>
</tr>
<tr>
<td>(K_{m,\text{app,AMP}})</td>
<td>mM</td>
<td>0.026</td>
<td>0.350 (13)</td>
<td>0.442 (17)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Summary of steady-state studies for WT and Pro-17 mutants\(^a\).

\(^a\)Numbers in parentheses indicate the rations between the mutant and WT. \(^b\)Mutant names are based on the numbering of AKe. \(^c\)The kinetic data for AK1c are from Tagaya et al. (1989). \(^d\)The kinetic data for WT and P9G of AKe are from Reinstein et al. (1990). The kinetic data for P9L of AKe are from Reinstein et al. (1988).
One of the mutants listed in Table 1, P9L of AKc, was also studied by X-ray crystallography (Müller and Schultz, 1993). Neither global conformational change nor local structural perturbation at AMP binding site was observed in the crystal structure of P9L·MgAP₅A solved at 3.4 Å resolution. However, Ile-120, a residue that may be involved in the induced-fit movement, was drawn toward the P-loop, where the β-methylene group of Pro-17 was displaced. The structural study thus suggests a direct or indirect involvement of Pro-17 in the induced-fit mechanism of AK. However, the actual role Pro-17 plays is still not clear. This is due to the limitation of the natural amino acid mutagenesis and the unique structure of Pro among the 20 natural amino acids. The key feature of proline’s structure is the ring, which will be incorporated into the protein backbone. None of the rest 19 natural amino acids can have or mimic this structure. Therefore those mutations could provide limited information as that it is likely to be involved in the induced-fit movement of AK and is very sensitive to amino acid substitutions. The current development in unnatural amino acid mutagenesis offers a new tool to study this residue by its geometrical and electronic requirement at Pro-17 for the normal function of AK. Several proline analogs were designed for replacement for Pro-17 (Figure 16). These analogs include pipecolic acid (Pip), homopipecolic acid (HPip), azetidine 2-carboxylic acid (Aze), N-methyl glycine (MeGly), and 3,4-dehydroproline (Dhp). The first four analogs were designed to probe the ring size effect at Pro-17, and Dhp was designed to probe Dhp-17’s electronic effect.
Figure 16. Structure of amino acids substituted for Pro-17 in adenylate kinase. Pro (proline); Dhp (3,4-dehydroproline); Pip (pipecolic acid); HPip (homopipecolic acid); Aze (azetidine 2-carboxylic acid); MeGly (N-methyl glycine).
4.1.2 Aromaticity of Tyr-95

On the basis of structural analysis by X-ray (Berry et al., 1994; Müller et al., 1992; Dreusicke et al., 1988) and NMR (Tsai & Yan 1991), Tyr-95 is located in proximity to the adenosine moiety of AMP and is also in close contact with Phe-12 and Phe-105 (distances between centroids are 6.1 and 5.9 Å, respectively). The aromaticity of Tyr-95 in adenylate kinase is absolutely conserved in the AK family. While Tyr-95 is conserved in muscle AKs, it corresponds to Phe in yeast and *E. coli* AK. Mutagenesis studies have been done on this position in both muscle AK (Kim et al., 1989) and *E. coli* AK (Liang et al., 1991). The results are summarized in Table 2. Actually, there is little change in enzymatic activity and binding properties in Y95F compared with WT hAK1. The hydroxy group of Tyr-95 is not required. Although no NMR or X-ray data is available for this mutant, it is reasonable that there is no major conformational perturbation change in the mutation. Y95A showed a large decrease in activity (Tian 1989). The mutation F86L resulted in an inactive enzyme that bound poorly to the gel blue A affinity column during purification. F86W had a 44-fold increase in $K_{m,\text{app,AMP}}$ and a 3 ~ 6 fold increase in $K_{m,\text{app,MgATP}}$. Since Phe-86 of AKe is a highly conserved aromatic residue in adenylate kinase, Liang et al (1991) suggested that an aromatic residue at this position be essential for an active enzyme. However, no further explanation or study was pursued to investigate how this tyrosine (or phenylalanine) residue functions in the catalysis of AK, and whether the aromaticity at this position is truly required.
<table>
<thead>
<tr>
<th></th>
<th>hAK1&lt;sup&gt;a&lt;/sup&gt;</th>
<th>AKe&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{m,app,MgATP}$ (μM)</td>
<td>$K_{m,app,AMP}$ (μM)</td>
<td>$k_{cat,app}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</td>
</tr>
<tr>
<td></td>
<td>(AMP = 0.2 mM)</td>
<td>(AMP = 10 mM)</td>
<td>(AMP = 0.2 mM)</td>
</tr>
<tr>
<td>WT</td>
<td>313.0 (± 52.0)</td>
<td>306.0 (± 31.4)</td>
<td>742 (± 47.3)</td>
</tr>
<tr>
<td>Y95F</td>
<td>567 (± 72.0)</td>
<td>743.7 (± 152)</td>
<td>1415 (± 20.6)</td>
</tr>
</tbody>
</table>

|        | $K_{m,app,MgATP}$ (μM) | $V_{max}$ (units/mg) |
|        | (AMP = 0.2 mM) | (AMP = 10 mM) |
| WT     | 90 ± 14 | 400 ± 18 |
| F86W   | 287 ± 32 | 1430 ± 69 |
| F86L   | 0 | 0 |

|        | $K_{m,app,AMP}$ (μM) | $V_{max}$ (units/mg) |
|        | ATP = 5 mM | ATP = 5 mM |
| WT     | 48 ± 10 | 1316 ± 160 |
| F86W   | 2100 ± 220 | 1461 ± 36 |
| F86L   | 0 | 0 |

Table 2. Summary of Kinetic Data on Mutants at Tyr-95 in hAK1 and Phe-86 in AKe.

<sup>a</sup>Kim et al., 1989.  <sup>b</sup>Liang et al., 1991.
Figure 17. Structures of tyrosine, phenylalanine, and 2,5-dihydrophenylalanine
In order to directly probe the importance of aromaticity at Tyr-95, we designed an unnatural amino acid 2,5-dihydrophenylalanine (DiHPhe). DiHPhe had been used as an analog of phenylalanine, and it can provide π-electrons without aromaticity, and its side chain structure is close to planar (Hermes et al., 1985; Snow et al., 1968; Hanson et al., 1979). Figure 17 shows the structures of both tyrosine and 2,5-dihydrophenylalanine.

4.2 Results

The experiments were described in Chapter 2. The *in vitro* suppression reaction conditions were optimized by small scale experiments. Typically a 30 μL *in vitro* suppression reaction was carried out. The amount of *in vitro* synthesized AKH were examined by the amount of AK radiolabeled with [35S]-Met. For this purpose, 1 μL of DEPC-treated water in the 30 μL cold reaction mixture was replaced with 1 μL of [35S]-Met (15 μCi/μL). Four different Mg2+ concentrations were used: 8.0, 10.0, 12.0, and 14.0 mM. Suppression usually needs more Mg2+ than expression, and this may due to the addition of aminoacyl tRNA. tRNA with the negative charge could chelate Mg2+. For expression, [Mg2+] from 1.7 ~ 6.7 mM were used. Figure 18 shows the small scale suppression with pipecolic acid charged tRNA. As shown by the autoradiogram, a [Mg2+] of 12.0 mM gave the highest suppression for those components preparations. Optima [Mg2+] varies with different batch of S-30, plasmid, LM, and different stock solutions of 0.1M EGTA and 0.1 M Mg(OAc)2. Before each large scale protein synthesis, the small experiments were carried out to optimize the conditions. Figure 19 shows the small scale suppressions with Aze and HPip charged tRNAs. The suppression
Figure 18. Autoradiogram of in vitro pipecolic acid suppression in the presence of $[^{35}\text{S}]$-L-methionine. Lane 1: expression of WT AKH; Lanes 2 - 5: suppressions with pipecolyt suppressor tRNA, with $[\text{Mg}^{2+}] = 6.0, 8.0, 10.0, \text{and } 12.0 \text{mM}$, respectively. The samples were centrifuged, and the supernatants were analyzed by 0.1% SDS-15%PAGE. Bla: $\beta$-lactamase.
Figure 19. Autoradiogram of \textit{in vitro} suppression labeled with $[^{35}\text{S}]-\text{L-methionine}$. Lane 1 - 3 showing expression of WT AKH; Lane 4 - 7 showing suppression with Aze charged suppressor tRNA; Lane 8 - 11 showing suppression with HPip charged suppressor tRNA. [Mg$^{2+}$] for all the lanes 1 - 11 are: 1.7 mM, 3.3 mM, 6.7 mM, 8.0 mM, 10.0 mM, 12.0 mM, 14.0 mM, 8.0 mM, 10.0 mM, 12.0 mM and 14.0 mM respectively.
efficiencies were determined by comparing the intensities of the AKH mutant bands in the optimized [Mg$^{2+}$] with WT expression AKH band. A β-scanner was used to count the radioactivity of the AKH bands. The second column in Table 3 lists the determined suppression efficiencies. The four ring analogs of Pro have a suppression efficiency of 12 ~ 20. Visual estimation of the Pro17Dhp mutant band indicated suppression efficiency with Dhp fell within the range of 12 ~ 20. In addition to the four ring analogs, substitution of Pro-17 with N-methylglycine (MeGly) was also attempted, but the suppression efficiency was too low to detect, and no further large scale preparation of this suppression reaction was carried out.

In order to obtain enough mutant enzymes for kinetic characterization, the in vitro suppression with each unnatural amino acid was carried out in 1.0 mL scale after the conditions were optimized with a 30 μL small reaction. The ligation reaction was scaled-up correspondingly to a total volume of 1.44 mL (divided into 4 microtubes). The ligation mixtures were incubated for 10 minutes, purified, and photodeprotected for 16 minutes. No [$^{35}$S]-Met was used for the large scale protein synthesis. All the materials used for the large scale suppression were the same ones used for the small scale suppression.

After the in vitro synthesis reaction, the reaction mixture was applied immediately for purification with His'Bind resins. The mutant enzyme concentration of the elute was analyzed by running a SDS-PAGE gel with a standard protein marker of various amounts. Visual comparison of the intensity of mutant AKH band with the standard marker bands of various amounts determines the mutant enzyme concentration.
Figure 20. Silver stained SDS-PAGE gel showing determination of the concentration of Pro17Hpip elute from the purification with HisBind resin. The concentration of Pro17Hpip in the third elute was 0.25µg/mL. Lane 1 ~ 5: protein marker of 1, 2, 3, 4, 5 ng respectively; Lane 6: second elute 32 µL; Lane 7: third elute 32 µL; Lane 8 ~ 14: protein marker of 6, 7, 8, 9, 10, 20, 30 ng respectively.
Figure 21. Plots of $1/v$ vs $1/[\text{ATP}]$ and $1/v$ vs $1/[\text{AMP}]$ from saturation kinetic experiments carried out on Pro17Hpip purified from in vitro suppression reaction with His Bind resin.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Suppression Efficiency (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>$K_{m,AMP}$ (mM)</th>
<th>$K_{m,MgATP}$ (mM)</th>
<th>$k_{cat}$ (s&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT AKH</td>
<td>100</td>
<td>0.11</td>
<td>0.019</td>
<td>420</td>
</tr>
<tr>
<td>Pro17HPip</td>
<td>12</td>
<td>0.059</td>
<td>0.039</td>
<td>200</td>
</tr>
<tr>
<td>Pro17Pip</td>
<td>20</td>
<td>0.060</td>
<td>0.037</td>
<td>500</td>
</tr>
<tr>
<td>Pro17Dhp</td>
<td>nd</td>
<td>0.30</td>
<td>0.14</td>
<td>410</td>
</tr>
<tr>
<td>Pro17Aze</td>
<td>17</td>
<td>nd</td>
<td>nd</td>
<td>&lt;4.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tyr95DiHPhe</td>
<td>nd</td>
<td>0.54</td>
<td>nd</td>
<td>520</td>
</tr>
</tbody>
</table>

Table 3. Summary of Suppression Efficiencies and Kinetic Data<sup>a</sup>

<sup>a</sup>The kinetic data should be considered as apparent values since they were obtained by holding one substrate constant while varying the other, instead of varying both substrates.

The AMP concentration was held at 2.0 mM while [ATP] was varied, and [ATP] was held at 2.0 mM while [AMP] was varied; [Mg<sup>2+</sup>] was kept at 4.8 mM. The abbreviation nd means not determined. Pro17Pip's data was obtained from Shi (1994); Kinetic analysis on Pro17Aze was carried out by Xiaohong Liu. <sup>b</sup>The suppression efficiency was measured by comparing the radioactivity of the AK mutant band with that of in vitro expressed WT AKH. <sup>c</sup>The activity of this mutant was too low to detect and was estimated to be <1% of the $k_{cat}$ of WT AKH.
Figure 20 shows the SDS-PAGE of Pro17HPip elute and protein markers of various amounts. The concentration of Pro17HPip in the elute was determined as 0.25 μg/mL. Since there were other minor bands presence in addition to the AKH major band, a negative control experiment was carried out at the same time. This control uses all the rest components, but does not contain plasmid DNA. *In vitro* mixture synthesized and purified this way gave no activity using the AK assay method, while the other minor bands were presence. Therefore, presence of the minor bands do not interfere the activity assay of HAK.

The eluate was used immediately for saturation kinetic analysis. Figure 21 shows Lineweaver-Burk plots for Pro17HPip kinetic analysis. Table 3 shows the summary of suppression efficiencies and kinetic data. Replacement of Pro-17 with Dhp, Pip, and HPip resulted in little changes in kinetic parameters, except a 7-fold increase in the $K_{m,MgATP}$ of Pro17Dhp. The only mutant displaying a large decrease in activity is Pro17Aze, whose activity is beyond the limit of detection (<1% of WT's activity). The Tyr95DiHPhe mutant behaves very similarly to WT. The $k_{cat}$ is virtually unchanged while the $K_{m,AMP}$ shows a small, 5-fold increase.

4.3 Discussion

4.3.1 Ring Size of Pro-17

As discussed in 4.1, one feature that distinguish adenylate kinase from other P-loop-containing families is that it has a conserved proline residue in the P-loop pattern GXPGXGKGT. It is a conserved glycine residue in *ras* protein; replacement of this
glycine residue with amino acids other than proline leads to great loss of activity. However, in adenylate kinase, replacement of this proline residue with glycine, valine or leucine resulted in little change in $k_{cat}$, but with in $K_{m,AMP}$ and $K_{m,MgATP}$. Considering proline's unique feature of a ring structure, either glycine, valine or leucine is quite different from proline. With the unnatural amino acids site-directed mutagenesis, several real proline analogs were designed to probe the ring size at Pro-17: Pip, HPip, Dhp, and Aze.

While Pip, HPip and Dhp mutants retained the WT activity and both $K_m$, Aze mutant has a greatly decreased activity. In the absence of structural analysis, one cannot rule out the possibility that the functional perturbation of Pro17Aze was caused by global structural changes. However, a possible interpretation of the result is that the reduced activity of Pro17Aze is caused by the restricted conformational freedom of the four-membered ring of Aze. It could restrict the movement of the P-loop and impair the conformational changes obligatory for the catalysis by adenylate kinase. Torsional angle is an important parameter for conformational analysis. From one aspect, the torsion angle of the possible conformation of a ring suggest the ring's freedom and the ring possible conformation it could accommodate. The four-membered ring of Aze, having a torsion angle of 25 degree, is less flexible than the five-membered ring (Bucourt 1974; Cesari et al., 1975). The four-membered ring restricts the movement of the P-loop, possibly not allowing the conformation to accommodate one which is suitable for ATP binding, thus causing an inactive enzyme. Tsai et al (1990) showed in a tetrapeptides, azetidine is
capable of restricting the flexibility of the N-terminal group, whereas proline is less restricting.

Pipecolic acid's six-membered ring and homopipecolic acid's seven-membered ring have a greater flexibility than the five-membered ring of proline (Bucourt, 1974). They could accommodate a conformation suitable for ATP binding. Unchanged kinetic parameters were observed for both Pro17Pip and Pro17HPip mutants. Pro17Dhp shows the same $k_{cat}$ as well as $K_{m,AMP}$ of WT, while the $K_{m,MgATP}$ is increased 7-fold. This suggests that electronic factors do not play an important role at Pro17 for catalysis. The slightly perturbed $K_{m,MgATP}$ agreed with the crystal structure of AK, as Pro-17 is located at the ATP site. It appears that Pro17Dhp mutant's global conformation is not perturbed. In summary, unnatural amino acid mutagenesis was used to show that the P-loop of AK can tolerate replacement of Pro-17 with more flexible analogs, but not the more rigid four-membered ring analog Aze.

4.3.2 Aromaticity of Tyr-95

On the basis of structural analysis by X-ray and NMR, Tyr-95 is located in proximity to the adenosine moiety of AMP. Tyr-95 in AK1 corresponds to Phe in AKe. As mentioned in 4.1, changing it to Phe resulted in little effect in AK1, changing to Leucine resulted in an inactive enzyme, and changing to alanine made it even worse. So, Tyr-95’s role was suggested lie in its aromaticity (Liang et al., 1991). However, when Tyr-95 was replaced with non-aromatic analogs of Phe, 2,5-dihydrophenylalanine, the mutant Tyr95DiHPhe behaves very similarly to WT. The results suggests that the
Figure 22. Stereoview of P-loop of AK1p.
Figure 23. Stereoview of the structure of *E. coli* AK complexed with AP₅A, only Phe-86 and AP₅A are shown.
aromaticity of Tyr-95 is not critically important for AK-AMP interactions. While replacements with natural nonaromatic amino acids caused large decrease in $k_{cat}$, the more isosteric DiHPhe is a reasonably good mimic of tyrosine or phenylalanine at position 95.

In WT adenylate kinase, Tyr-95 could have amino-aromatic interaction with AMP, a weakly polar interaction. As shown in Figure 24, the amino group of adenosine moiety is in close contact with the aromatic ring of Tyr-95, an interaction between them could occur. One of the weakly polar interactions in protein is amino-aromatic interaction (Burley and Petsko, 1988). While oxygen-aromatic, sulfur-aromatic and aromatic-aromatic interactions derive from the polar attraction of the $\delta^+$ hydrogen of an aromatic ring to a negatively polarized atom or $\delta^-$ $\pi$-electron cloud. Amino-aromatic interaction derives from $\delta^+$ polarized hydrogen on an amino group attraction of $\delta^-$ $\pi$-electron cloud of an aromatic ring (dipole-quadrupole interaction). Such an interaction would lead to a preference for amino groups to be found in axial orientations above and below the plane of the ring, rather than close to the equatorial distributed $\delta^+$ ring hydrogen atoms. Amino-aromatic interactions in proteins were first described by Burley and Petsko (1986). Geometric analyses of a crystallographic data base of 33 protein structures documented that side-chain amino and aromatic groups in proteins have preferred separation distance of 3.0 to 6 Å between the nitrogen atom and the centroid of the nearby aromatic ring. Perutz et al. (1986) described a series of X-ray crystallographic studies of drugs and peptides bound to deoxy-Hb A. An amino-aromatic interaction between the $\delta^+$ N$\delta$-H group of asparagine-108$\beta$ with the $\delta^-$ $\pi$-electron cloud of one of the
phenyl rings of benzafibrate was characterized in that work. In addition, a pair of aminooaromatic interactions in basic pancreatic trypsin inhibitor has been characterized by neutron and X-ray crystallography and proton magnetic resonance (Wlodawer et al., 1984; Tüchsen and Woodward, 1987). The $\delta^-$ $\pi$-electron cloud of the aromatic ring of tyrosine-35 is sandwiched between the main chain amino group of glycine-37 and the side chain amino group of asparagine-44.

The above evidence suggest that amino-aromatic interactions could happen in the WT adenylate kinase between amino group of adenosine moiety of AMP and Tyr-95. The distance between the nitrogen atom of amino group in AMP adenosine moiety and Tyr-95's benzene ring centroid is 4.95 Å, and the amino group is in a orientation close to axial to the benzene ring of the side chain of Tyr-95 (Müller et al., 1992; Berry et al., 1994). This interaction derives from the polar attraction of the $\delta^+$ hydrogens of amino group of AMP's adenosine to the $\delta^-$ $\pi$-electron cloud of the aromatic ring of Tyr-95's side chain.

2,5-Dihydrophenylalanine (DiHPhe) is a much better analog of tyrosine (or phenylalanine) than other nonaromatic amino acids. DiHPhe can provide $\pi$-electrons without introducing aromaticity, and its side chain is very close to a planar ring (Snow et al., 1968; Hanson et al., 1979; Hermes et al., 1985). With the geometric structure of the side chain almost unchanged, DiHPhe could be used to probe the aromaticity of Tyr-95. Tyr-95DiHPhe showing no different catalytic parameters suggests the non-essential role of the aromaticity. Tyr95DiHPhe has two carbon-carbon double bonds in the side chain of DiHPhe-95, the $\delta^-$ $\pi$-electrons of these two double bonds could interact with $\delta^+$ polarized
Figure 24. Interactions between residue 95 and AMP's adenosine
hydrogen on the amino group of AMP's adenosine moiety (Figure 22). The kinetic result of DiHPhe mutant obtained suggests that amino-aromatic interaction could be replaced with amino-carbon-carbon double bond interaction. The δπ-electrons at Tyr-95 could be essential for the normal function of adenylate kinase. Tyr-95 is also close to Phe-12 and Phe-105; however the aromatic-aromatic interaction between Tyr-95 and these two aromatic residues are not expected to play important structural roles of adenylate kinase since neither Phe-12 nor Phe-105 is conserved in yeast and E. coli adenylate kinase.

In summary, unnatural amino acid mutagenesis was applied on adenylate kinase to show that the aromaticity of Tyr-95 (or Phe-95) is not critically important for the function of AK and that the P-loop of AK can tolerate replacement of Pro-17 with more flexible analogs, but not the more rigid four-membered ring analog Aze. The research presented here also contributes to this area by adding more unnatural amino acids to the library. Prior to this work, only one of the analogs used in the research, pipecolic acid has been incorporated into other proteins. Aze, which was suggested unable being incorporated into proteins due to the small size, was successfully incorporated into adenylate kinase.
PART II
STRUCTURE-FUNCTION STUDIES OF PHOSPHATIDYLINOSITOL
-SPECIFIC PHOSPHOLIPASE C: KINETIC ANALYSIS
AND THE ROLES OF HIS-32 AND ASP-274
CHAPTER 5
INTRODUCTION

5.1 General Background of PI-PLC

Phospholipase C (PLC) includes two major types: phosphatidylinositol-specific phospholipase C (PI-PLC) enzymes and nonspecific PLC. PI-PLCs with a high specificity for PI and phosphorylated PI show no functional or structural similarity to the nonspecific PLC. Nonspecific PLC prefers phosphatidylcholine and also accepts a variety of phospholipids. No sequence homology is found between PI-PLCs and nonspecific (or PC-specific) PLC (Kuppe et al., 1989). Receptor-mediated turnover of inositol phospholipids and inositol phosphates has attracted vigorous research activity, due to its importance in cellular signal transduction (Bruzik & Tsai, 1994; Roberts, 1996). PI-PLCs are the key enzyme triggering the cascades of PI-mediated signal transduction (Rhee & Choi, 1992).

PI-PLCs cleave the phosphodiester moiety of PI, its phosphorylated, or glycosylated derivatives to produce the corresponding water-soluble myo-inositol 1,2-cyclic phosphates or myo-inositol 1-phosphates and the lipid-soluble diacylglycerol
Figure 25. Reactions catalyzed by PI-PLC. Bacterial PI-PLC (a), mammalian PI-PLC (b & c), GPI-PLC (d). a. $R^1 = R^2 = R^3 = H$; b. $R^1 = R^2 = H, R^3 = PO_3^-$; c. $R^1 = H, R^2 = R^3 = PO_3^-$; d. $R^1$ = glycosaminoglycan, $R^2 = R^3 = H$. Reproduced from (Bruzik & Tsai, 1994).
(DAG). Figure 25 shows the reactions catalyzed by PI-PLCs. Several products of the enzymatic cleavage of inositol phospholipids, including myo-inositol 1,4,5-trisphosphate (IP$_3$) and DAG are second messengers in the signal transduction cascades. IP$_3$, being proposed as calcium-mobilizing second messenger, elevates the intracellular calcium level delivering the original extracellular message carried by a hormone, neurotransmitter or growth factor inside the cell (Berridge, 1993). DAG activates protein kinase C (PKC) (Dekker et al., 1995), and rapid production of this lipid by PI-PLC conversion of the phosphorylated PI is a primary step in signaling. Glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC), a subclass of PI-PLC, cleaves the spacer arm of GPI-anchored proteins to release extracellular enzymatic activities of these proteins. GPI-PLC has been proposed to play key roles in viral and parasite infections (Thomas et al., 1990; Carrington et al., 1991).

PI-PLC enzymes occur both in secreted (sPI-PLC) and cytosolic (cPI-PLC) forms in a wide variety of tissues and organisms. sPI-PLC are excreted by a variety of bacteria, including Bacillus thuringiensis and Bacillus cereus, and by the animal and human pathogens Staphylococcus aureus and Listeria monocytogenes. (Kuppe et al., 1989; Leimeister-Wächter et al., 1991; Mengaud et al., 1919; Daugherty and Low, 1993). The parasite Trypanosoma brucei was found be able to secrete a GPI-PLC (Hereld et al., 1988). cPI-PLC exist as several isoenzymes in mammalian cells.

5.2 Properties of PI-PLC Enzymes

Table 4 lists the summary of characteristics of PI-PLC enzymes.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Molecular weight (kDa)</th>
<th>Substrate specificity</th>
<th>Cofactor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI-PLC</td>
<td>35</td>
<td>PI</td>
<td>none</td>
</tr>
<tr>
<td>Mammalian</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PI-PLC-(\beta)</td>
<td>134-154</td>
<td>PI; prefer</td>
<td>(\text{Ca}^{2+})</td>
</tr>
<tr>
<td>-(\gamma)</td>
<td>145</td>
<td>PIP and PIP(_2)</td>
<td></td>
</tr>
<tr>
<td>-(\delta)</td>
<td>85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPI-PLC</td>
<td>37-52</td>
<td>GPI</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Properties of phosphoinositide-specific phospholipase C enzymes\(^a\).

\(^a\) Reproduced from Bruzik and Tsai, 1994.
Mammalian PI-PLC. Mammalian PI-PLCs have a larger size compared with bacterial PI-PLC. There are at least five isozymes of mammalian PI-PLC (Rhee et al., 1991), with the molecular weights ranging from 60 to 150 kDa. However, the isozymes share two common regions: the conserved domain X (about 60% homologous among different isozymes), and the conserved domain Y (about 40% homologous). There are about 170 residues in X domain; and there are about 260 residues in Y domain (Majerus, 1992). Most mammalian PI-PLCs require calcium ion for activity (Rhee and Choi, 1992). Mammalian PI-PLCs cleave PI and its phosphorylated forms PI-4-P and PI-4,5-P$_2$ to produce a mixture of inositol 1,2-cyclic and acyclic phosphates. The preference of PI-PLCs for phosphorylated or nonphosphorylated substrates depends on the level of calcium ion and the protein type. PIP$_2$ is a preferred substrate at low (physiological) concentrations of calcium ion while PI becomes a preferred substrate at millimolar calcium concentrations (Ryu et al., 1987). Some types of PI-PLC such as those from guinea pig and melanoma cell line are active in the absence of calcium and are only mildly activated by calcium. Melanoma PI-PLC prefers the nonphosphorylated PI, and guinea pig PI-PLC cleaves various PI derivatives with similar efficiency (Perrella et al., 1991; Cruz-Rivera et al., 1990). There are three main classes of mammalian PI-PLCs: PI-PLC-β, PI-PLC-γ and PI-PLC-δ (Rhee et al., 1989). The δ-isozymes are smaller (85 kDa) than β- and γ-isozymes (150 kDa). The 2.4 Å crystal structure of a mammalian PI-PLC-δ with inositol-1,4,5-trisphosphate and Ca$^{2+}$ reveals the X-region and Y-region as the catalytic domains (Essen et al., 1996). Figure 26 shows the schematic diagram of the network of IP$_3$/Ca$^{2+}$ interactions in the active site. The residues of X and Y domains
Figure 26. The schematic diagram of the network of IP$_3$/Ca$^{2+}$ interactions in the active site of mammalian PI-PLC-δ (Essen et al., 1996).
which have contacts with the inhibitor are very similar to the active site residues of bacterial PI-PLC revealed by the 2.6 Å crystal structure of *Bacillus cereus* PI-PLC with myo-inositol (Heinz et al., 1995).

**Bacterial PI-PLC.** Bacterial PI-PLCs are the smaller size with a molecular weight of about 35 kDa. Two representatives of this type are from *Bacillus cereus* and *Bacillus thuringiensis*. *Bacillus cereus* PI-PLC shows limited similarity to the X domain in the mammalian PI-PLC that is associated with the catalytic activity. Around the same region, the sequence is also similar to bacterial GPI-PLC enzymes, suggesting catalytic similarities for all the diverse PI-PLCs. PI-PLC enzymes from *B. cereus* and *B. thuringiensis* cleave nonphosphorylated and glycosylated forms of phosphatidylinositol in a Ca²⁺-independent manner in two steps (Volwerk et al., 1990; Bruzik et al., 1992): (1) an intramolecular phosphotransfer reaction to form IcP; and (2) hydrolysis of the IcP to produce IP. The second step has a much lower $V_{\text{max}}$ and much higher $K_m$. Mammalian PI-PLCs produce both cyclic and acyclic inositol phosphates simultaneously. Despite the smaller size, bacterial PI-PLC is a much more efficient catalyst than mammalian PI-PLCs (67 times faster for the first step) (Takenawa et al., 1991; Griffith et al., 1991).

Another difference between bacterial PI-PLC and mammalian PI-PLC is that phosphorylated PIs are not substrates for bacterial PI-PLC (Volwerk et al., 1989; Griffith et al., 1991). Bacterial PI-PLC is widely used as a tool to release GPI-anchored proteins from the membrane (Ikezawa, 1991; Bruzik and Tsai, 1994). With an intermediate of the corresponding 1,2-cyclic phosphate, palmitoylation at the 2-hydroxy group of the inositol makes GPI-anchors resistant to PI-PLC cleavage (Ferhuson, 1992).
Figure 27. Structure of *B. cereus* PI-PLC with bound myo-inositol showing the active site pocket (Heinz et al., 1995).
Figure 28. Contacts between the side chains of *B. cereus* PI-PLC residues and bound myo-inositol. Reproduced from Heinz et al., 1995.
bacterial PI-PLC, possible similar catalytic mechanism, and close relation with GPI-PLC, bacterial PI-PLC serves as a good target for studying of the PI-PLCs.

Recently the crystal structure of the PI-PLC from *Bacillus cereus* (EC 3.1.4.10) with bound myo-inositol was determined at 2.6 Å resolution (Heinz et al., 1995). The crystal structure of free enzyme does not differ much from the myo-inositol PI-PLC's structure which was also solved at 2.6 Å in the same report. PI-PLC from *Bacillus cereus* contains 298 amino acid residues (Kuppe et al., 1989). There is no cysteine, and no disulfide bond in PI-PLC from *Bacillus cereus*. Based on the crystal structure, this enzyme consists of a single globular domain of approximately 40x40x50 Å³ in dimension. It exhibits extensive β-sheet structure (eight β-sheets) forming an imperfect (βα)₈-barrel. The β-barrel is not strictly closed, due to the absence of main chain hydrogen bonding interactions between β-strands V and VI. The amino and carboxyl terminus of the protein are spatially close. Figure 27 shows the crystal structure of *Bacillus cereus* PI-PLC with bound myo-inositol. The active site is located in a deep pocket: a wide and solvent-accessible cleft at the C-terminal end of the β-barrel. Figure 28 illustrates the contacts of myo-inositol with various protein side chains. His-32 and His-82 are positioned in the active site similarly to RNase. It was suggested His-32 and His-82 act as general base and acid in a mechanism similar to that of the ribonuclease. Asp-274, due to the close contact with His-32, was suggested form a catalytic triad along with His-32 and 2-OH group of the inositol (Heinz et al., 1995). Due to the weak binding of myo-inositol with *Bacillus cereus* PI-PLC, the crystal structure may not reveal the actual structure when PI-PLC bound with PI. However, with no structural information available before, the report
Figure 29. Sequence comparison of PI-PLCs from several bacterial sources. Bt: *Bacillus thuringiensis*, Bc: *Bacillus cereus*, Lm: *Listeria monocytogenes*, Sa: *Staphylococcus aureus*. Reproduced from Daugherty and Low, 1993. Isolated identical residues and regions of similarity are indicated by underlining. Only sequence numbers of Bt PI-PLC are shown.
of the crystal structure of the enzyme with bound myo-inositol provides a great amount of information.

PI-PLC from *Bacillus thuringiensis* is another natural variant of PI-PLC, it differs from *Bacillus cereus* PI-PLC in only eight amino acid residues (Kuppe et al., 1989). Figure 29 shows the sequence comparison of PI-PLCs from several bacterial sources (Daugherty and Low, 1993). The eight different residues between *Bacillus thuringiensis* and *Bacillus cereus* PI-PLCs are Asn (Ser)-20, Gly (Asp)-126, Asn (Lys)-135, Ser (Pro)-170, Val (Ala)-192, Asn (Ser)-203, Asp (Tyr)-259, and Thr (Ala)-266. The amino acid residues in parenthesis are those of PI-PLC from *B. cereus* (Griffith et al., 1991). All of the eight amino acid residues are located on the surface of the enzyme and not directly involved in either substrate binding or catalysis (Heinz et al., 1995).

The PI-PLC variant studied in this dissertation is PI-PLC from *B. thuringiensis*. Since the structures of PI-PLC from *B. cereus* and *B. thuringiensis* are essentially identical (Heniz et al., 1995), the model of *B. cereus* PI-PLC crystal structure will be used for *B. thuringiensis* PI-PLC studies in this dissertation. *B. thuringiensis* was the richest source among phosphatidylinositol-specific phospholipase C-producing bacteria (Taguchi et al., 1980).

Although a clear physiological function has yet to be established for bacterial PI-PLCs including from *B. cereus*, *B. thuringiensis* and *Staphylococcus aureus*, there are a lot of aspects of importance and significance to study bacterial PI-PLC. (1) The size of bacterial PI-PLC is the smallest among the PI-PLCs. The relative small size provides ease in study the enzymes, in various enzyme characterizations, and in
Figure 30. General structure of GPI anchors. The glucosamine (GlcN) is bonded to 6-OH of the myo-inositol ring of PI. The point of cleavage by bacterial PI-PLCs, including GPI-PLCs is marked by an arrow (Reproduced from Heinz et al., 1996).
elucidation of mechanisms. (2) The studies of bacterial PI-PLCs may contribute our understanding of the role of the eucaryotic PI-PLCs, which are a key player in the signal transduction pathways. (3) Bacterial PI-PLCs have been reported to show insulinlike effects when incubated with insulin-sensitive membrane preparations or cells, such as hepatocyte membranes (Saltiel and Cuatrecasas, 1986), myocytes (Saltiel et al., 1986), and hepatoma cells (Mato et al., 1987). (4) Bacterial PI-PLCs are useful tools to study GPI anchored proteins, due to their ability to cleave the GPI anchors to release these proteins from the surface of their resident membrane (Ferguson and Williams, 1988; Low and Saltiel, 1988).

GPI-PLC. The 37 - 40 kDa GPI-PLC from Trypanosoma brucei (Bruzik and Tsai, 1994) is involved in the removal of the variant surface glycoprotein during the life cycle of the parasite. GPI-PLC cleaves the space arm of GPI-anchored proteins. Figure 29 shows the general structure of GPI anchors (Heinz et al., 1996). The preferred substrates for GPI-PLCs are GPI and GPI-protein conjugates such as membrane form of Variant Surface Glycoprotein (mfVSG) or their corresponding lyso-derivatives (Mensa-Wilmot and Englund, 1992; Fox et al., 1986). As discussed in last section, bacterial PI-PLCs can also cleave the space arm of the GPI anchor. A crystal structure of B. cereus PI-PLC with bound glucosaminyl(α1→6)-D-myo-inositol has been just reported (Heinz et al., 1996).

5.3 Chemical Mechanism of Bacterial PI-PLC Reaction

Interfacial Activation. PI-PLC are enzymes which utilize membrane-bound substrates in their natural environment. Most PI-PLCs prefer micellar substrates dispersed with
detergents such as deoxycholate and Triton X-100 (Ikezawa and Taguchi, 1981). Bacterial PI-PLC display little activity with pure PI as a substrate (Low, 1981; Godfine and Knob, 1992), but are activated when PI is presented to the enzyme as mixed micelles with other phospholipids (Stieger and Brodbeck, 1991; Kume et al., 1991). PI-PLC from *B. thuringiensis* is activated by short-chain PC by a rate factor of $10^{-10}$ (Rosenberry, unpublished result). However, some of the variant bacterial PI-PLCs exhibit different dependence on phospholipids and detergents for activity. For example, *Staphylococcus* enzyme is readily deactivated by either noninoic or inoic detergents (Low, 1981).

*General Base and Acid Catalysis.* In the reaction catalyzed by *B. cereus* PI-PLC, the acyclic inositol 1-phosphate is a secondary product derived from inositol 1,2-cyclic phosphate, and the stereochemical results suggested a sequential nature of the reaction (Volwerk et al., 1990; Bruzik et al., 1992). The rate of formation of IP was estimated to be slower than that of IcP by a factor of $10^3$, on the basis of $^{31}$P NMR analysis. $^{31}$P NMR studies found that isotope-labeled (chiral at phosphorus) substrate DPPI was converted to IcP by *B. cereus* PI-PLC with inversion of configuration at phosphorus (Lin et al., 1990); conversion to IP was shown to have overall retention of configuration at phosphorus (Bruzik et al., 1992). The sequential mechanism of bacterial PI-PLC was suggested similar to the mechanism of ribonuclease A. Pancreatic ribonuclease, RNase A (Walsh, 1979; Hammes, 1982), catalyze the hydrolysis of the phosphodiester bonds of ribonucleic acids in a two-step mechanism. In the first step, clevage of the RNA chain by the RNase occurs via an intramolecular phosphotransfer reaction involving the 2'-hydroxy of the ribose ring with formation of a pyrimidine 2',3'-cyclic phosphate intermediate. In the
Figure 31. Proposed mechanism of catalysis by PI-PLC from *B. cereus*. Reproduced from Heinz et al., 1995).
second step, the cyclic intermediate is hydrolyzed to the 3'-phosphate final product. The rate constants for the RNase-catalyzed phosphotransfer reaction is up to 3 orders of magnitude higher than the rate constants for the hydrolysis of the corresponding cyclic phosphates, depending on the nature of the leaving alcohol (Witzel, 1963).

From the recent crystal structure of *B. cereus* PI-PLC with bound myo-inositol, two potential catalytic histidines were identified (Heinz et al., 1995). A mechanism of general base and acid catalysis with the two histidines was proposed based on the crystal structure. Figure 31 shows the proposed catalytic mechanism of the action of PI-PLC from *B. cereus*. Asp-274 has a hydrogen bonding with His-32, and a possible catalytic diad forming between these two residues is very likely. Asp-274 and His-32 along with the 2-OH group of inositol ring of PI could form a catalytic triad analogous to the well known catalytic triad in serine proteases. In this study, site-directed mutagenesis studies along with functional and structural analysis are used to investigate the roles of these two residues in the catalysis of PI-PLC, these studies have lead to new insight look of the mechanism of bacterial PI-PLC.

### 5.4 Site-Directed Mutagenesis

Mutagenesis studies with bacterial PI-PLC have been very limited so far. With the chemical mechanism revealed by stereochemistry and the active site residues revealed by the crystal structure, mutagenesis studies are needed to further investigate the mechanism. The beauty of site-directed mutagenesis approach is that it has the ability to link the structural information directly to the functional information. A mutant enzyme
can be analyzed with quantitative kinetic and structural methods to evaluate the contribution that a particular side-chain makes toward enzyme catalysis, structure, conformational stability. This approach has been widely used in the field of protein chemistry to elucidate the structure-function relationship of a protein. However, kinetic results of mutants could be over interpreted if possible structural perturbations in the mutants are not carefully examined.

In this study, the mutagenesis is combined with rigorous structural analysis. The mutants are subjected to functional characterizations including kinetic analysis and pH-activity profile analysis (if active). Structural analysis including 1D, 2D NOESY NMR, circular dichroism (CD), and conformational stability studies with Gdn-HCL-induced denaturation monitored by CD spectroscopy. The properties of mutants are compared with those of WT to see the possible conformational perturbation.

Functional Analysis of PI-PLC Mutants

There are many methods which could be used for PI-PLC activity analysis (Bruzik and Tsai, 1994). Those methods include assays employing radiolabeled substrates; spectrophotometric assays; coupled enzymatic assay; fluorometric assays; monolayer assay; release of GPI-anchored proteins; $^{31}$P NMR; mass spectrometry. Among the methods, the one utilizing $[^3$H]-labeled PI as the substrate is by far the most commonly used, though it is laborious and discontinuous (Rittenhouse, 1982). A modified method is used for the specific activity analysis of the WT and mutant enzymes (Griffith et al., 1991). A trace amount of [myo-inositol-2-$^3$H]-PI is mixed with cold PI to be the
substrate, the conversion of hot PI to IcP and IP after 10 minutes reaction is monitored by scintillation counting of the water phase which contains IcP and IP but not PI. PI concentration is 2 mM, a detergent of sodium deoxycholate is used with a final concentration of 3.9 mM. This assay is only a measurement of the first step reaction.

$^{31}$P NMR analysis is another often used method. It does not require separation of reaction products, and thus offers ease and quick result. This method is also a way to measure the second half reaction. However, it needs a significant amount of enzymes, especially a mutant enzyme with low activity. This method was widely used in stereochemistry studies with isotope-labeled DPPI (Bruzik et al., 1992).

Thio assay, one of the spectrophotometric methods, offers an easy way to conduct the continuous assay of PI-PLC. After PI-PLC catalysis, the substrate analog is cleaved to relieve a free thiol which could be coupled with DTNB or DTP. The coupled product could then be monitored by UV-Vis spectrometer. However, the substrate analogs used so far in the thiol assay are quite different from the DPPI. In this work, a new thiol substrate: DPsPI is used to set-up a new thiol assay. DPsPI is synthesized by Dr. Bruzik at the University of Illinois at Chicago. This thiol analog differs from DPPI in the DAG 3-O only, and the 3-O is replaced by sulfur. Chapter 7 will discuss detailed set-up of this steady-state kinetic analysis. This assay is performed also on mutants with measurable activity.

Due to the possible general base and acid catalysis mechanism, the pH-activity profile is also performed with WT PI-PLC and interesting mutants. The pH profile is obtained by analyzing the specific activities in different pHs. The specific activities
analysis is carried out with cold PI and [myo-inositol-2-\textsuperscript{3}H]-PI, and a short chain PC: DC\textsubscript{7}PC is used as the detergent.

Structural Analysis of PI-PLC Mutants.

NMR spectroscopy is chosen as the major tool to obtain structural information. Compared to X-ray crystallography, NMR is easier to carry out, and it reveals the solution structure of a protein which is closer to its structure under physiological condition. One-dimensional proton NMR experiments were first carried out on the mutant. Comparison between the 1D spectra of mutants and that of WT PI-PLC offers a qualitative assessment of the extent of conformational change in the mutant. A further analysis is obtained from two-dimensional NMR experiments which offers much better resolution of intra- and interresidue cross peaks. Since the NMR spectra peaks have not been assigned yet, both 1D and 2D NOESY spectra are used for qualitative comparison between WT and mutant PI-PLC to monitor the conformational perturbation.

Another structural analysis is conformational stability studies. The conformational stability were measured by Gdn-HCl-induced denaturation monitored by circular dichroism spectroscopy. This could provide more structural information which may not be revealed by NMR analysis, as in the case of D274E mutant.

In this work, mutagenesis studies combined with functional and structural analysis on two of the active site residues His-32 and Asp-274 reveals the structural and functional roles of the two residues. At the same time, these studies also provide new information on the mechanism of bacterial PI-PLC. The previously proposed mechanism involving
His-32 and His-82 as the general base/acid does not agree with some of the results in the studies, several other mechanisms are suggested. Chapter 8 will address this issue in detail. In this work, a new continuous assay with a substrate analog DPPI is also set-up. This steady-state analysis provides an easy way to carry the full kinetic analysis.
6.1 Materials

The *E. coli* expression system for *Bacillus thuringiensis* PI-PLC was kindly provided by Dr. Volwerk (Koke et al., 1991). The expression vector pHN1403 (Figure 32) was used, and it has a lac-tac-tac triple promoter. PI-PLC gene with the STII signal sequence was obtained from plasmid pMY31 (generously provided by Dr. Volwerk). Site-directed mutants were constructed according the method of Kunkel (1985). Dye-deoxy terminator cycle sequencing kits were purchased from Applied Biosystems. Oligonucleotides were obtained from Integrated DNA Technologies, Inc., and were used directly for mutagenesis. The mutagenic primers were as follows: H32A, TCCAGGAACAGCTGATAGTGGGA; H32N, CCAGGAACAAACGATAGT; H32Q, AGGAACAGGATAGTGGG; D274A, TGGGTAATTCAAGCTTACATAAATGAAAAG; D274N, GGGTAATTCAAAACTACATAAATG; D274E, GTAATTCAAGAATACATAAATG. The underlined bases represent the mutation site(s).

All DNA-modifying enzymes were purchased from New England Biolabs. Gene-Clean kits were purchased from Bio-101, Qiaquick Gel Extraction Kits were purchased from Qiagen. WT PI-PLC M13 mp19 phage stock was provided by Robert Hondal. *E.
Figure 32. Expression vector pHN1403 with PI-PLC gene inserted. Reproduced from Koke et al. (1991)
coli strains CJ236 and JM101 were used for mutagenesis and were purchased from Stratagene. *E. coli* strain MM294 was obtained from New England Biolabs. DEAE resin (DE52) was purchased from Whatman, Sephadex G-100 and Phenyl Sepharose CL-4B resins were purchased from Pharmacia. PI and DHPC were purchased from Avanti Polar Lipids. Sodium deoxycholate was purchased from Sigma. Ultrapure guanidine hydrochloride was obtained from ICN Biochemicals. Perdeuterated Tris, DCl, 99.9% D D$_2$O, “100%” D D$_2$O, and TMSP-d$_4$ were obtained from Isotech. All other chemicals were of reagent grade.

### 6.2 Mutant Construction

*Plaque Purification of Phage Stock WT PI-PLC in M13mp19.* Due to unidentified reasons, WT PI-PLC-M13mp19 phage stock was found to have deletion problems. Therefore, it was necessary to purify the phage stock to get rid of the deleted M13mp19 before the phage stock was used to prepare the pure single-stranded uracil M13 DNA. The protocol used for plaque purification of phage stocks was adapted from a procedure described in the Amersham mutagenesis manual. Prepare an overnight culture of JM101 cells in LB (Bacto-tryptone, 10 g; yeast extract, 5 g; and NaCl 10 g/L). Some molten H top agar were prepared and kept at 45-50 °C. A 1 in 10$^6$ dilution of phage stock was made as the follows: 1 µL of the phage stock was added to 99 µL LB (1 in 10$^5$), then 1 µL of this dilution was added to 99 µL LB (1 in 10$^4$) and the dilution was repeated once more (1 in 10$^6$). A 5 µL of the diluted phage were added to 0.2 mL of the JM101 overnight culture in LB. The mixture was added to a tube containing 3 mL of molten top agar,
followed by quick addition of 50 μL of 2% X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside; w/v in dimethylformamide in a glass vial) and 20 μL of 100 mM IPTG (isopropyl-β-D-thio-galactopyranoside in water). The contents in the tube were mixed by vortexing, and were immediately poured onto LB plates. After 10 minutes of setting in room temperature, the plate was inverted and incubated at 37 °C overnight. A white plaque was picked by inserting a sterile Pasteur pipet through the plaque and blowing the entire plug into 1 mL of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). This would transfer 10⁷ or mor phage. The phage will be stable at 4 °C for several months. The new phage stock and M13mp19 RF PI-PLC DNA were prepared by adding 60 μL of the plaque suspension in TE and 20 μL of JM101 overnight culture in LB to 2.0 mL of LB, and incubating at 37 °C for 4.0 hours. The culture was centrifuged twice in a microcentrifuge. The supernatant was saved as the new phage stock, and the pellet was used for M13 RF DNA preparation. The RF DNA was digested with XbaI and SphI to check the insert. When the phage is plated out, if there is no blue plaque and the M13 RF DNA has PI-PLC insert, the phage stock is considered pure and ready for preparation of template uracil M13mp19 PI-PLC. If there is still blue plagues, this purification is repeated until there is no blue plagues. This is normally repeated two or three times.

Purification of Single-Stranded Uracil M13mp19 PI-PLC DNA. The protocol used for single-stranded uracil DNA template purification was adapted from a procedure described in the Bio-Rad mutagenesis manual. CJ236 was streaked out onto a LB plate containing chloramphenicol. The plate was incubated at 37 °C for 24 hours. An isolated colony was picked and placed in 5 mL of LB containing 25 μg/mL chloramphenicol. An
overnight culture of CJ236 was obtained by incubating the mixture at 37 °C overnight. 120 mL of 2xYT (Bacto-tryptone, 16 g; yeast extract, 10 g; and NaCl, 5 g/L) medium in a 500 mL flask containing chloramphenicol were inoculated with 2.4 mL of the overnight culture of CJ236. The culture was incubated with shaking at 37 °C. When the cell's OD₆₀₀ of 0.3 was obtained, which took from 1 to 4 hours, 120 μL of purified phage stock of PI-PLC M13mp19 were added to the cell. The incubation was continued with shaking at 37 °C for 6 hours. The culture was transferred into three JA-20 sterile centrifuge tubes (40 mL/each). The tubes were centrifuged at 17,000 x g (12,000 rpm in the JA-20 rotor) for 15 minutes at 4 °C. 3x36 mL of the supernatant were transferred into three fresh JA-20 tubes respectively. The tubes were centrifuged at 12,000 rpm for 15 minutes at 4 °C. 3x32 mL of the second supernatant were transfer to three fresh JA-20 tubes respectively. To each of the three fresh tubes was added 1/4 vol (8 mL/each) of a solution containing 3.5 M ammonium acetate and 20% PEG 8000. After thoroughly mixed, the tubes were hold on ice for 50 minutes. The tubes were then centrifuged at 12,000 rpm for 15 minutes at 4 °C. The supernatant was poured off carefully, and the tubes were drained thoroughly. Surplus fluid was wiped off. 3x100 μL of high salt buffer (300 mM NaCl, 100 mM Tris, pH 8.0, 1 mM EDTA) were added to each of the tubes to resuspend the DNA pellet. The suspension from the three tubes were combined and transferred into a fresh microcentrifuge tube. The tube was hold on ice for 30 minutes. Insoluble material was removed by centrifuging for 2 minutes at 4 °C. The supernatant was transferred to a fresh 2 mL tube, and stored at 4 °C. DNA from this preparation (phage stock) was extracted within a week.
The entire phage stock (~ 500 µL) was extracted 2x with an equal volume of neutralized phenol, 1 x with phenol/chloroform (1:1:1/48 phenol:chloroform:isoamyl alcohol), and 3 x with chloroform/isoamyl alcohol. The chloroform extractions were continued until there was no visible interface, then once more. The first three extractions were vortexed vigorously for 30 seconds. Back-extracting each step were performed to increase the yield: 100 µL of TE were added to the first phenol extraction tube, after vortexing, the resultant aqueous phase was added to the next phenol extraction tube, vortexed, and so on. The aqueous phase was pooled together (~ 0.61 mL), and 1/10 volume (61 µL) of 7.8 M ammonium acetate and 2.5 volume (1.53 mL) of ethanol were added to the aqueous phase. The 2 mL tube containing the above mixture was kept at -80 °C for 45 minutes. The tube was then centrifuged in full speed for 15 minutes at 4 °C, and the supernatant was carefully removed. The pellet was gently washed with 90% ethanol, and then resuspended in 400 µL of TE. The suspension was subjected to one more phenol-extraction. Then, to 0.38 mL of the supernatant were added 38 µL of 7.5 M NH₄OAc and 0.95 mL of EtOH. The tube was put in -80 °C for ~ one hour, and then centrifuged for 15 minutes at 4 °C. The pellet was washed with 90% EtOH, and was resuspended in 50 µL of TE. Any residue that may cling to the side of the tube was avoided dissolving. The concentration of DNA (0.4 - 1.0 µg/µL) was determined by the absorbance at 260 nm (1 OD₂₆₀ ≈ 40 µg/µL) and the template purity was determined by the OD₂₆₀/OD₂₈₀ ratio and running a 1% agarose gel.

**Purification of Single-Stranded M13 DNA.** QIAprep Spin M13 Kits (QIAGEN) were used to purify the single-stranded M13 DNA. The procedure was described in the
QIAprep Spin M13 manual. A single viral plaque was picked into 1 mL of TE in a microtube. To a sterile test tube containing 5 mL of LB was added 50 μL of JM101 overnight culture in LB and 150 μL of the plaque suspension. The tube was then incubated with shaking at 37 °C for 4.5 hours. The bacterial cells were spun down at 5000 rpm for 15 minutes at room temperature. The supernatant containing M13 bacteriophage was transferred to a fresh micro tube. 1/100 volume of M13 Precipitation Buffer was added to the supernatant. The tube was vortexed, and then incubated at room temperature for at least 2 minutes. A QIAprep spin column was placed in a 2 mL microcentrifuge tube, and 0.7 mL of the sample was applied to the QIApre spin column. The tube along with spin column was centrifuged for 15 seconds at 8000 rpm, and the flow-through from collection tube was discarded. The loading, centrifuging, and discarding steps were repeated until all of the samples had been loaded onto the spin column. The spin column was washed by adding 0.7 mL of M13 Lysis & Binding Buffer, and then centrifuged for 15 seconds at 8000 rpm. Another 0.7 mL of M13 Lysis & Binding Buffer was added, and the column was incubated for 1 minutes at room temperature to lyse bacteriophages completely. The tube with the column was centrifuged for 15 seconds at 8000 rpm. 0.7 mL of PE Wash Buffer was added, and the column was centrifuged for 15 seconds at 8000 rpm. The PE Wash Buffer in the collection tube was discarded, and the column was centrifuged for an additional 15 seconds at 8000 rpm to remove residual PE Buffer. The spin column was then placed in a clean 1.5 mL microcentrifuge tube. 40 μL of 1 mM Tris (pH 8.5) was added to the column, which was then incubated for 1 hour. The DNA was eluted by centrifuge for 30
seconds at 8000 rpm. Thus obtained single-stranded DNA was used directly for sequencing.

**Purification of Double-Stranded M13 RF and Plasmid DNA.** Small preparations of double stranded DNA were carried out according to the alkaline lysis method described by Maniatis et al. (1989). However, for larger preparations of double-stranded PI-PLC M13 mp19 RF DNA and pHN PI-PLC plasmid, QIAGEN Plasmid Midi Kits were used. The protocol was adapted from QIAGEN Plasmid Handbook (1995). A single colony of *E. coli* strain XL1-Blue harboring the pHN1403-PI-PLC plasmid was inoculated into 5 mL LB with 0.1 mg/mL ampicillin and grown for 9 hours (late log phase, OD_{600} = 0.65) at 37 °C. 100 mL of LB with ampicillin was inoculated with the 5 mL above late log phase cell, and grown for 2.5 hour exactly. Stock solution of chloramphenicol was added to get a final concentration of 0.17 mg/mL, and the cells were grown 12 hours more. For viral DNA purification, a single plaque was picked. 2 mL of LB was inoculated with 50 μL JM101 overnight culture and 100 μL of the plaque suspension, the cells were grown for 4.5 hours, after centrifuge the supernatant was saved as the phage stock. 100 mL of LB was inoculated with 1.0 mL of JM101 overnight culture and 0.2 mL of the phage stock and grown for 5 hours at 37 °C. At this stage of purification, both DNA preparation were treated identically. The cells were harvested by centrifuge at 5000 rpm (JA-10 rotor) for 15 minutes at 4 °C. The supernatant was discarded (retained for M13 DNA if SS DNA was needed). For viral DNA, the cell pellet was resuspended with 35 mL of ice-cold STE (100 mM NaCl; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA) and recentrifuged. The bacterial pellet was resuspended in 4 mL of Buffer
PI (RNase A was added). 4 mL of Buffer P2 were added, and the contents were mixed gently, and incubated at room temperature for 5 minutes. 4 mL of chilled Buffer P3 were added, and the contents were mixed immediately but gently, and incubated on ice for 15 minutes. After centrifuge at 11,000 rpm for 30 minutes at 4 °C, the supernatant was transferred to another centrifuge tube, and was recentrifuged at 14,000 rpm for 15 minutes at 4 °C. The supernatant was transferred to a new tube. The QIAGEN-tip 100 was equilibrated by applying 4 mL of Buffer QBT, and the column was allowed to empty by gravity flow. The supernatant was applied to the column, and was allowed to enter the resin by gravity flow. The column was then washed with 2x10 mL of Buffer QC. The DNA was eluted with Buffer QF: 0.5 mL, 0.8 mL, and 0.8 mL three elutions. The second and the third elution contained the most DNA, and were combined. The DNA was precipitated with 0.7 volume of isopropanol, the sample was centrifuged immediately at full speed for 30 minutes at 4 °C, and the supernatant was carefully removed. DNA was washed with 0.4 volum of 70% EtOH, air-dried for 5 minutes, and redissolved in 20 μL of TE. The concentration was determined by the absorbance at 260 nm and the purity was determined by 260/280 nm absorbance ratio. The DNA was also run on a 1% agarose gel stained with ethidium bromide.

Preparation of Competent Cells. A single colony of desired cell strain was picked from a fresh plate, inoculated into 3 mL of LB and incubated at 37 °C with shaking overnight. 210 mL of SOB media (Component I: Bacto-tryptone, 4 g; yeast exact, 1 g; NaCl, 0.12 g; and KCl, 0.10 g/0.2 L. Component II: 0.2 M MgSO$_4$ and 0.2 M MgCl$_2$, 10 mL. 0.2 L of Component I and 10 mL of Component II were mixed right before use)
were inoculated with 1 mL of the overnight culture. The contents were incubated at 37 °C with gentle shaking until the cell density reached an absorbance of 0.3 - 0.4 at 550 nm (2 - 3 hours). The cell culture was then transferred to a sterilized centrifuge bottle and stored on ice for 15 minutes. The cells were then pelleted by centrifugation for 5 minutes in a Beckman JA-10 rotor at 5000 rpm at 4 °C. The supernatant was carefully decanted, and the cells were resuspended in 35 mL of ice-cold transformation buffer I (30 mM KOAc, 100 mM KCl, 10 mM CaCl₂·H₂O, 50 mM MnCl₂·4H₂O, and 12% v/v glycerol/0.2 L, pH 5.8; sterilized by filtration with a 0.2 μm Gelman Acrodisc) by gentle swirling on ice. The suspension was stored on ice for an additional 10 minutes. Following a second centrifugation under identical conditions, the supernatant was removed and the cells were suspended in 8 mL of ice-cold transformation buffer II (10 mM MOPS, 10 mM KCl, 75 mM CaCl₂·H₂O, and 12% v/v glycerol/0.2 L, pH 5.8; sterilized by filtration). 300 μL aliquots were transferred into 1.5 mL microcentrifuge tubes, and immediately frozen in liquid nitrogen. The competent cells were stored at -70 °C and remained viable for several months after preparation.

Transformation and Transfection of DNA. 5 μL of ligarion mixture or 50 ng of DNA was added to 0.3 mL competent cells thawed on ice for 15 minutes. The contents were mixed thoroughly by rolling the tube on ice for 10 seconds and were then incubated on ice for 30 minutes. The tube was then placed in 42 °C water bath for exactly 90 seconds followed by storage on ice for 2 minutes (heat shock). 1 mL of LB media was added to the tube and the mixture was incubated at 37 °C for 30 minutes. 10 μL of the culture and 10 μL of the concentrated transformation mixture (the mixture was
centrifuged 1 minute, and was decanted to leave 0.1 mL supernatant, and mixed well) were plated out onto two LB/ampicillin plates (agar, 15 g/L LB media, with ampicillin 0.1 g/L added aseptically when the liquid had cooled to 50 °C). The plates were then left at room temperature for 5 minutes and incubated at 37 °C for 8 ~ 12 hours. To transfet competent cells with viral DNA, 300 μL of lawn cells (overnight JM101 cells) were added to the competent cells after the “heat shock” step. 50 μL of the mixture were then added to 3 mL of H top agar (tryptone, 10 g; NaCl, 8 g; and agar, 8 g/L) kept at 50 °C using a heat block. The contents were immediately mixed and poured onto a pre-warmed LB plate. The plate was kept at room temperature for 10 minutes, and then incubated overnight at 37 °C.

Site-Directed Mutagenesis. Mutants were constructed based upon the method developed by Kunkel (1985). The procedure was essentially the same as described in the manual from the Bio-Rad mutagenesis kit. Mutant oligonucleotide was first phosphorylated with T4 polynucleotide kinase and dATP in T4 polynucleotide kinase buffer (NEB). The phosphorylated mutant oligonucleotide was then annealed with the SS uracil M13 DNA template in T4 DNA polymerase buffer. The complementary DNA strand was synthesized by extension and ligation with T4 DNA polymerase and T4 DNA ligase in the presence of dGTP, dCTP, TTP, dATP, DTT, and BSA in T4 DNA polymerase buffer. The reaction was stopped by adding small amount of 100 mM EDTA and freezing. The reaction products were analyzed by running a 1% agarose gel. The reaction products were used to transfet JM101 competent cell. SS DNAs were prepared
from selected recombinant plaques. The mutant was screened by SS DNA sequencing of the mutation site.

**DNA Sequencing.** Single-stranded DNA sequencing was carried out to screen the mutant after mutagenesis experiment, and double-stranded DNA full sequencing was carried out to confirm the PI-PLC mutant gene after subcloning of the PI-PLC mutant gene into the expression vector pHN1403. The sequencings were obtained using “Taq DyeDeoxy Terminator Cycle Sequencing Kit” from Applied Biosystems. The procedure was adapted from the sequencing manual. The following reagents were mixed in a microcentrifuge tube: 9.5 μL of Reaction Premix (5x TACS Buffer, dNTP mix, DyeDeoxy™ A Terminator, DyeDeoxy™ T Terminator, DyeDeoxy™ G Terminator, DyeDeoxy™ C Terminator, AmpliTag® DNA Polymerase); 5.0 μL of DNA template (0.5 ~ 1 μg); 0.8 pmol of ss DNA (3.2 pmol for ds DNA) of Primer; distilled water. Final reaction volume was 20 μL. The reaction mixture was overlaid with one drop of mineral oil (~ 40 μL). The tube was placed in a thermal cycler (Perkin Elmer Cetus Model 480) preheated to 90 °C. Immediately, the thermal cycling was running as follows: rapid thermal ramp to 96 °C; 96 °C for 30 seconds; rapid thermal ramp to 50 °C; 50 °C for 15 seconds; rapid thermal ramp to 60 °C; 60 °C for 4 minutes. After 25 cycles, the thermal cycler was held to 4 °C. Thus obtained reaction mixture was subjected to Phenol/chloroform extraction. At the end of the thermal cycling, 80 μL of H₂O and 100 μL of chloroform were added to the reaction mixture. The terminators were extracted with 100 μL of phenol:H₂O:chloroform (68:18:14) reagent. The lower organic phase was discarded after the sample was vortexed and centrifuged. The aqueous layer was
reextracted with a second 100 µL aliquot of phenol:H₂O:chloroform. The sample was vortexed and centrifuged for 1 minute; then the aqueous upper layer was transferred to a clean tube. The extension products were precipitated by adding 15 µL of 2 M sodium acetate, pH 4.5, and 300 µL of 100% EtOH. The mixture was centrifuged for 15 minutes at room temperature, and the pellet was washed with 70% EtOH, and dried. 4 µL of a mixture of 5 µl deionized formamide and 1 µL 50 mM EDTA, pH 8.0 were added to each tube containing the dried residue. The tube was agitated vigorously to dissolve the residue. The sample was heated at 90 °C for 2 minutes, and immediately put on ice. The sample was loaded on the gel on an Applied Biosystems 373A DNA Sequencer. One reaction could read about 300 bases.

Subcloning. Double-stranded M13 recombinant DNA was subcloned into the polylinker region of the expression vector pHN1403. The procedure was described as follows. 5 µg of M13 RF double-stranded DNA were digested with 20 units of restriction enzyme XbaI and 10 units of SphI (2 ~ 3 hours at 37 °C in a total volume of 20 µL); 1.1 µg of pHN1403-WT PI-PLC were digested with 40 units of XbaI and 20 units of SphI (2 ~ 3 hours at 37 °C in a total volume of 40 µL). The mutant AK gene fragment and the linear pHN1403 vector fragment were separated from other DNA fragments by running 1% agarose gel. The vector was extracted using a Gene Clean kit, and the mutant AK gene fragment was purified using a "QIA quick" Gel Extraction kit. The purified insert and 1/5 of the purified vector (molar ratio of 3:1) were ligated together by 40 units of T4
DNA ligase with 1 mM dATP in 11.2 μL of ligation buffer at 16 °C for 16 hours. 5 μL of the ligation mixture were transformed into XL1 Blue competent cells.

To screen for recombinant DNA, several colonies were picked, and DNA were purified by inoculating 100 mL of LB culture containing ampicillin with each of the picked colonies. Presence of mutant PI-PLC gene insert was confirmed by all the following experiments: plasmid DNA was digested with XbaI and SphI, PI-PLC gene insert was visualized under UV lamp on 1% agarose gel stained for 2 minutes with ethidium bromide (5 μg/mL); sequencing of the full PI-PLC gene were carried out on the plasmid DNA; the DNA was also checked for expression, this was accomplished by inoculating 100 μL overnight culture into 4 mL LB/amp and growing at 37 °C for 4~5 hours before adding IPTG to the final concentration of 1 mM. The incubation continued for another 6 hours at 30 °C, and 100 μL of the cell culture were removed to run the protein SDS-PAGE gel to check for AK expression.

6.2 Purification of B. thuringiensis PI-PLC

Cell Growth and Harvest. 20 mL of LB/amp media were inoculated with a single colony picked from a LB/amp plate with freshly transformed MM294/pHN1403 PI-PLC (mutant or WT), and were incubated with vigorous shaking at 37 °C overnight. 5 x 200 mL of LB/amp were inoculated with 5 x 2 mL of the above overnight culture, and were incubated for 12 hours under the same condition. 10 flasks of 1 L LB/amp media were then inoculated with 10 x 90 mL of the above culture, and were incubated with vigorous shaking at 34 °C until OD₆₀₀ reaches 1.0 (1 ~ 2 hours). 10 x 1.1 mL of 1 M IPTG were
added to the flasks, and the induced culture was incubated at 30 °C (Asp-274 mutants were grown at 20 °C for 20 hours) overnight. Cells were harvested via centrifugation with JA-10 rotor at 6000 rpm for 15 minutes at 4 °C. The pellets were resuspended in cold wash buffer (20 mM Tris, pH 7.5, 50 mM NaCl) 50 mL/each tube, and the suspended cell were combined then recentrifuged. The cell pellet was stored at -20 °C. The typical yield of cell pellet from the 10 L culture was 30 ~ 40 g.

Preparation and Equilibration of DEAE Resin (DE52). 400 g of pre-swollen DE52 resin were suspended in 1.5 L of 1 M Tris, pH 8.5, and the supernatant was decanted. The resin was resuspended in another 1.5 L of the buffer. The final slurry was then packed into a column (d = 2.5 cm) and equilibrated with the buffer overnight. A final bed volume of 200 mL was obtained. The column was further equilibrated with at least 5 times bed volume of 20 mM Tris-HCl buffer, pH 8.5. The pH of the eluate was checked before cell lysis was carried out.

Cell Lysis and DEAE Ion-Exchange Chromatography. Cell pellets were thawed and suspended in 20 mM Tris-HCl, pH 8.5 (65 mL/8 g pellet) to homogeneity with mild stirring at 4 °C. The cells were lysed on ice via sonication using 300-Watt high intensity ultrasonic processor from Sonics & Materials, Co. 6 x 40 seconds pulses were applied with 60% duty cycle and 9 output control. The cells were cooled down for 2 minutes between two sonifications. The lysate was spun at 10,000 rpm for 1 hour in a JA-10 rotor at 4 °C, and the supernatant was then loaded onto the pre-equilibrated DE52 column (flow rate 1 mL/min). Once the loading was complete, the column was washed with 1 L of 20 mM Tris-HCl, pH 8.5 with a flow rate of 1 mL/min. The eluate was collected in
case the enzyme did not bind to the column. When the absorbance of the eluate at 280 nm dropped below 0.05, elution was started with a linear gradient of 0 - 200 mM NaCl in the Tris-HCl buffer, and in some cases, more high salt buffer were used until the absorbance of the eluate at 280 nm was below 0.1. The flow rate was 1 mL/minute. 10 to 15 mL fractions were collected and monitored by absorbance at 280 nm. Peak fractions were examined by SDS-PAGE on a Pharmacia PhastSystem using 12.5% acrylamide gel and silver staining methods. PI-PLC fractions with reasonable purity were pooled. The proteins were then precipitated with ammonium sulfate (708 g/L solution) added slowly and with mild stirring in cold room. The solution was further stirred for at least 2 hours. The precipitate was spun down at 19,000 rpm for 1 hour in JA-20 rotors at 4 °C. The supernatant was removed completely and the pellet was stored at 4 °C for further purification with the gel-filtration column. The column could be regenerated via washing with 1 M NaOH.

*Gel-Filtration Chromatography.* Sephadex G-100 resin was used in the gel-filtration chromatography of PI-PLC. 40 g of dry resin were swelled with 1.5 L of the Tris buffer for 24 hours. The slurry was then packed into a well-cleaned column with special care taken to avoid bubble formation in the column. The G-100 column was equilibrated overnight with the 20 mM Tris buffer. The bed volume was 500 mL. The pellet from the ammonium sulfate precipitation was then dissolved in 7 mL of the Tris buffer. The solution was then loaded onto the gel-filtration column which had its liquid surface right above the surface of the resin (the resin in the column was never allowed to dry). When the surface of the loaded sample dropped right above the surface of the resin,
gel-filtration buffer was carefully added to elute the proteins. 5 mL fractions were collected and monitored by the absorbance at 280 nm. Peak fractions were examined by SDS-PAGE using 12.5% acrylamide gel and silver staining method. PI-PLC fractions were pooled, and solid NaCl was added to get a final concentration of 1.5 M NaCl.

Hydrophobic Interaction Chromatography, Dialysis, and Lyophilization. Phenyl Sepharose CL-4B resin was used for the Hydrophobic Interaction Chromatography. 40 mL of the gel slurry were applied onto a column of 1.5 cm diameter, and the column was equilibrated first with Tris buffer, then with 40 mL of 1.5 M NaCl in the Tris buffer. The protein in the high salt buffer obtained from last step was loaded onto the equilibrated phenyl sepharose column. The proteins were eluted with a gradient of 1.5 M - 0 M NaCl in Tris buffer. 5 mL fractions were collected and monitored by absorbance at 280 nm. The fractions purity was analyzed by running a SDS-PAGE gel. Those fractions with high purity PI-PLC were pooled and precipitated with ammonium sulfate. The precipitate were then collected by centrifugation and dissolved in 5 mL of Tris buffer. The solution was then transferred into knotted dialysis tubing (with molecular weight cutoff of 6000 - 8000 and washed extensively with distilled water) and dialyzed first against 8 L of 1 mM Hepes buffer for 8 hours, then against 8 L of 0.1 mM Hepes buffer for another 8 hours. The enzyme solution was then transferred to a round bottom flask with the volume 10 times of that of the solution. The flask was then dipped into liquid nitrogen. The frozen solution was then lyophilized for 8 - 12 hours and stored at -20 °C. For Asp-274 mutants, the pooled fractions were reloaded onto a small phenyl sepharose column with NaCl added. The mutant enzyme was concentrated by eluting with 20 mM Tris buffer directly,
and some of the fractions were dialyzed and lyophilized for NMR usage, the rest fractions were combined, frozen and stored at -20 °C. The typical yield of PI-PLC enzymes was about 40 mg from 10 L cell culture.

6.3 Assay of B. thuringiensis PI-PLC

The specific activities of PI-PLC enzymes were measured according to a modification (Volwerk, et al., 1994) of the method described by Griffith et al. (1991). 100 mg of cold PI were dissolved in 2.75 mL of CHCl₃, and the solution is aliquoted into 11 ependorf tubes (250 μL/each tube). The solution in each tube was speed-vac dried with no heat. The aliquoted tubes were stored in -80 °C freezer. Only one tube was used at a time. To one of the tubes containing the aliquoted cold PI was added 12.5 μL (1.25 x 10⁶ cpm) of L-α-[myo-inositol-2-³H]-phosphatidylinositol (³H-PI). To the mixture was added 1.0 mL of 0.8% sodium deoxycholate and 1 mL of water, and the final concentration of PI was 5 mM. The suspension was vortexed, and then sonicated using a Branson model 1210 water bath sonicator for 5-10 minutes to ensure micelle formation. For the reaction, 40 μL of the substrate solution were added to a microtube containing 40 μL of 0.1 M sodium borate buffer. 20 μL of diluted enzyme solution were added to the tube, and the tube was immediately capped, vortexed, and then incubated at 37 °C for exactly 10 minutes. After exact 10 minutes, the reaction was stopped by adding 0.5 mL of CHCl₃:MeOH:HCl (66:33:1). The mixture was vortexed, and briefly centrifuged to separate the phases. 50 μL of the aqueous phase was counted on a Beckman LSC3801 Scintillation counter. The specific activity was calculated in terms of μmol min⁻¹ mg⁻¹ by
multiplying the aqueous counts by four, dividing the cpm/μmol (determined by counting 5 μL of the unreacted substrate), the reaction time (10 minutes), and the amount of PI-PLC added.

In addition to the specific activities measurement with ³H-PI, a continuous assay with DPPI was set-up, and used to assay the active enzymes. The detailed set-up of this steady-state kinetic analysis will be discussed in Chapter 7.

6.4 pH-Activity Profile

The pH-Activity profile was obtained by measuring the corresponding specific activities under different pH in a range of from 2 to 10. A new buffer of 50 mM gly-gly, 50 mM Tris-HCl, 50 mM CAPS was used, and 40 mM of DHPC replaced sodium deoxycholate as the detergent.

6.5 Proton NMR Structural Characterization

Sample Preparation. The enzyme (approximately 4 mg) was transferred into a microtube and dissolved in D₂O. The pH was adjusted carefully with DCl and NaOD to 6.8. The sample was then centrifuged to precipitate denatured enzyme and the supernatant was transferred to a fresh microtube. The opening of the tube was covered in parafilm, pricked several times with a syringe needle, and the contents were immediately frozen by partial immersion in liquid nitrogen. The sample was lyophilized to dryness. The lyophilized enzyme was dissolved in 500 μL of 99.96% D₂O and the pH was
adjusted as required (enzyme was about 0.2 mM). After a final centrifugation step, the supernatant was carefully transferred to a NMR tube.

**One-Dimensional Proton NMR.** All NMR experiments were performed on a Bruker DMX-600 spectrometer at 37 °C. Chemical shifts were referenced to internal TMSP-d₄ and the residual water peak was suppressed by 3-9-19 pulse sequence with gradients (Piotto et al., 1992; Sklenar et al., 1993). The spectral width was 12 ppm and 16 K data points were acquired in the time domain with quadrature detection mode. A total of 256 transients were collected for each spectrum. Prior to Fourier transformation, the time domain data were zero-filled to 32 K points. They were then processed with resolution enhancement by a Gaussian function (GB = 0.1, LB = -3).

**Two-Dimensional Proton NMR.** Standard pulse sequences and phase cycling were used for the 2D NOESY experiments (Bodenhausen et al., 1984). All spectra were obtained in the phase-sensitive mode (Marion & Wüthrich, 1983) with mixing time 100 ms. Generally, a 2 K x (400-512) time domain matrix was recorded. The two-dimensional data matrix was multiplied by a shifted sine bell function (SSB₁ = 8) in t₁ and by a Gaussian function (LB = -3, GB = 0.1) in t₂. The data matrix was then zero-filled to 2K x 1K matrix prior to Fourier transformation.

6.6 Guanidine Hydrochloride-Induced Denaturation

**Sample Preparation and Ellipticity Measurement.** Guanidine hydrochloride was dissolved in CD buffer (10 mM borate, 0.1 mM EDTA pH 7.5) to a final concentration of about 7 M. The absolute concentration of the denaturant was calculated from its
refractive index (Nozaki, 1972), where $\Delta N$ is the difference between the refractive index of the denaturant solution and CD buffer at the sodium D line:

$$[\text{Gdn-HCl}] (\text{M}) = 57.147 (\Delta N) + 38.68 (\Delta N)^2 - 91.60 (\Delta N)^3$$  \hspace{1cm} (1)$$

A 2 - 3 mg/mL stock solution of enzyme was prepared by dissolving the enzyme in a buffer consisting of 10 mM borate, and 0.1 mM EDTA pH 7.5. Enzyme precipitate was removed by centrifugation with a benchtop centrifuge. The reversible denaturation of PI-PLC was followed by monitoring the change in ellipticity at 215 nm with a JASCO J-500C polarimeter in conjunction with DP-500/AT system software. For each point, five spectra were signal-averaged and obtained in the far UV region 250-200 nm. The scan speed, sensitivity, time constant, band width, and step resolution were 50 nm/minute, 50 mdeg/FS, 2.0, 2 nm, and 0.04 nm respectively. Gdn-HCl solutions of concentration from 0.25 - 5 M were prepared by diluting the concentrated stock solution, and the exact concentration were determined by measuring refractive index of each solution. Components of Gdn-HCl in buffer and enzyme were mixed in a quart cuvette (pathlength 10 mm), and allowed to equilibrate at 19 °C for 5 minutes. Typically 15 - 30 points were obtained in the range of 0 - 3 M Gdn-HCl. A more comprehensive review of many of these experimental details has been described by Pace (1986).

The denaturation data was analyzed assuming a two-state mechanism shown in equation 2, there are folded state (native, N) and various unfolded states (denatured, D) in equilibrium:
Based upon the denaturation curve, ellipticity values ($y$) for the native ($y_N$) and denatured ($y_D$) states of a protein can be determined by extrapolation from the linear portions of the curve. At any point along the transition curve,

$$y = f_N y_N + f_D y_D$$  \hspace{1cm} (3)$$

where $f_N$ and $f_D$ represent the fraction of enzyme in the native and denatured state, respectively. Since $f_N + f_D = 1$, the equation (3) could be rewritten as:

$$f_D = (y - y_N) / (y_D - y_N)$$  \hspace{1cm} (4)$$

and

$$f_N = (y_D - y) / (y_D - y_N)$$  \hspace{1cm} (5)$$

A free energy of unfolding ($\Delta G_d$) can be calculated as the following:

$$\Delta G_d = -RT \ln K_D = -RT \ln (f_D / f_N) = -RT \ln[(y - y_N) / (y_D - y)]$$  \hspace{1cm} (6)$$

**Conformational Stability.** One of the simplest methods to estimate $\Delta G_d^{H_2O}$ (the free energy change at zero concentration of denaturant) is linear extrapolation (Pace, 1975). It is assumed that the linear dependence of $\Delta G_d$ on denaturant concentration in the
transition region of the curve extends to zero concentration of denaturant. This model was first proposed by Greene and Pace (1974) and the data fits the equation:

\[ \Delta G_d = \Delta G_d^{\text{H}_2\text{O}} - m \text{[denaturant]} \]  

(7)

where \( m \) measures the dependence of \( \Delta G_d \) on denaturant concentration. To obtain \( \Delta G_d^{\text{H}_2\text{O}} \) and \( m \), \( \Delta G_d \) was plotted against [Gdn-HCl] in this experiment.

6.7 Molecular Graphics

Crystal structure of PI-PLC deposited in the Brookhaven Protein databank was examined using either Sybyl or Insight II on a Silicon Graphics work station.
CHAPTER 7
CONTINUOUS ASSAY OF PI-PLC WITH DPsiPI

7.1 Background

Continuous analysis of an enzyme provides an easy and straightforward way to obtain steady-state kinetic properties of the enzyme. The most frequently used kinetic analysis method for PI-PLC is the discontinuous assay with $^3$H-PI. The assay with $^3$H-PI not only is laborious and discontinuous, it also is radioactive, thus health and environmental hazardous. Kinetic analysis of PI-PLC have, until early 90’s, been hampered by the lack of a continuous assay. The first reported continuous assay (Shashidhar et al., 1991a) was a fluorometric method using synthetic 2-naphthyl-myo-inositol-1-phosphate. The measurement is based upon the batochromic shift of the emission maximum of product 2-naphthol as compared to the substrate phosphodiester. However, the specific activity of PI-PLC for this substrate was extremely low (0.003% of the rate with PI). Another assay was also developed using a chromogenic substrate, 4-nitrophenyl-myo-inositol-1-phosphate (Shashidar et al., 1991b). Formation of IcP was accompanied by the release of p-nitrophenol which was quantified by UV spectrophotometry. The specific activity with this substrate (77 μmol min$^{-1}$ mg$^{-1}$ at 1 mM) was higher than that of the fluorometric assay. However, the substrate binds
Figure 33. Molecular structures of two substrate analogs. A. 2-naphthyl-myoinositol-1-phosphate. B. 4-nitrophenyl-myoinositol-1-phosphate.
only weakly to bacterial PI-PLC ($K_m > 15$ mM), and it is water soluble and can not be incorporated into interfaces. Figure 33 shows the structures of these two substrates.

Thio-based phospholipase assay has been well studied, and was used for phospholipase $A_1$ and $A_2$ and other phospholipases (Yu and Dennis, 1991). The thiol assay is a continuous, spectrophotometric assay which is very convenient. It directly detects one of the products liberated upon cleavage, and it is also suitable for full kinetic studies. Figure 34 shows the scheme for the spectrophotometric determination of phospholipase A2 activity using thio ester analogs of phospholipids. After enzymatic cleavage, one of the products, a thiol, is allowed to react with a thiol-sensitive reagent included in the assay. The formation of the resulting chromophore is measured continuously by monitoring the increase in absorption associated with its production.

Two thiol coupling reagents, 4, 4'-dithiobispyridine (DTP) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), are routinely used to detect the free thiol products. DTP has limited solubility in water, and DTNB is a little more soluble in water. As shown in Figure 34, the chromophore liberated by the reaction of DTP with a free thiol affords 4-thiopyridine, which absorbs at 324 nm; the chromophore liberated by the reaction of DTNB with a free thiol affords 5-thio-2-nitrobenzoic acid which absorbs at 412 nm. The extinction coefficients for both chromophores vary dramatically with pH (Yu and Dennis, 1991). Figure 35 shows the pH effects on the extinction coefficient ($\varepsilon$) of 4-thiopyridine (derived from DTP) at 324 nm and 2-nitro-5-thiobenzoic acid (derived from DTNB) at 412 nm. As shown in the figure, the extinction coefficient of 4-thiopyridine decreases rapidly at pH values above 7 but is constant below pH 7. On the other hand, 2-
Figure 34. Scheme for the spectrophotometric determination of phospholipase A2 activity using thio ester analogs of phospholipids (Yu and Dennis, 1991).
Figure 35. Effect of pH on the extinction coefficient ($e$) of 4-thiopyridine at 324 nm (○) (derived from DTP) and 2-nitro-5-thiobenzoic acid (derived from DTNB) at 412 nm (□). The extinction coefficient was determined in the presence of 100 mM KCl, 10 mM CaCl$_2$, and 25 mM of one of the following buffers (at 30 °C): glycine, Tris, acetate, HEPES (Yu and Dennis, 1991).
nitro-5-thiobenzoic acid has a constant extinction coefficient above pH 7 but a decreasing extinction coefficient as the pH drops below 7. Therefore, the buffer used in the assay must have sufficient buffering capacity to maintain the pH at the appropriate level. The thiol assay method can detect rates as low as 1 nmol/min and as high as 400 nmol/min with 5% error across the entire range (Yu and Dennis, 1991). However, derivations in buffer pH can dramatically affect the reproducibility of the assay. It is better to use DTNB in basic solution ($\varepsilon = 12,800 \text{ M}^{-1}\text{cm}^{-1}$), and DTP in acidic solution ($\varepsilon = 19,800 \text{ M}^{-1}\text{cm}^{-1}$). The limitation has been the availability of the thio substrate analogs.

The thiol assay was first applied to PI-PLC using a series racemic substrates of $C_n$-thio-PI (Hendrickson, et al., 1992). Figure 36 shows the structure of the substrate analogs and the cleavage by PI-PLC. Three substrate analogs were studied in the buffer of 50 mM MES, pH 7, with or without detergent of HDPC (hexadecylphosphocholine). The DTP was used as the thiol coupling reagent. $C_{10}$-thio-PI gave a hyperbolic curve with activity versus concentration between 0 and 2 mM, and addition of HDPC did not have effects on $V_{\text{max}}$ and $K_s$ (the bulk concentration of thio-PI which gives the half $V_{\text{max}}$). $C_{12}$-thio-PI had a similar story. Pure $C_8$-thio-PI did not give a hyperbolic curve in activity against concentration plot, but a hyperbolic curve was obtained with the addition of detergent HDPC. The $V_{\text{max}}$ of PI-PLC with pure $C_{16}$-thio-PI micelles was 6.25 $\mu$mol$^{-1}$ mg$^{-1}$, and half $V_{\text{max}}$ was obtained at 0.022 mM of substrate. This assay offers continuity and ensures relatively good interfacial binding of the substrate to the enzyme. However, the $V_{\text{max}}$ is still low (1% of that with natural PI). Compared with natural substrate PI, those substrate analogs only have one long hydrophobic alkyl chain, and they do not have
Figure 36. Structure of $C_n$-thio-PI and cleavage of $C_n$-thio-PI by PI-PLC enzyme.
Figure 37. Structure of DPsPI and the cleavage of DPsPI by PI-PLC enzyme.
the glycerol moiety. Those analogs were racemic compounds. The best thio-substrate analog would be DPsPI, which differs from PI in only the glycerol 3-oxygen (replaced 3-sulfur). This substrate analog DPsPI was synthesized by our collaborator Dr. Karol Bruzik at University of Illinois at Chicago in an enantiomerically pure form (2R, same as that in natural PI). In this work, the thiol assay of PI-PLC with this DPsPI substrate analog (2R)-1,2-dipalmitoylpropylthiophospho-1-D-myo-inositol was set-up. Figure 37 shows the structure of DPsPI and the cleavage of DPsPI by PI-PLC enzyme.

7.2 Materials and Methods

DTNB, DTP and MOPS [3-(N-morpholino) propanesulfonic acid] were purchased from Aldrich Chemical Co. DHPC was purchased from Avanti Polar Lipids, Inc. Enantiomerically pure DPsPI was synthesized by Dr. K. Bruzik (University of Illinois at Chicago). *B. thuringiensis* PI-PLC was over-expressed in *E. coli* and purified as described in Chapter 6. The protein concentration of PI-PLC was determined using an extinction coefficient of 1.83 mL mg⁻¹ cm⁻¹ at 280 nm (Hendrickson et al., 1992; Volwerk et al., 1994). A water bath type sonicator Branson 1210 was used to completely disperse the substrate lipid. The microcuvettes were purchased from Starna Cells, Inc. A Kontron Uvikon 930 spectrophotometer with a Samsung computer was used.

The procedure used in this assay was a modification of the one described in literature (Yu and Dennis, 1991; Hendrickson et al., 1992). A stock of concentrated DPsPI solution containing 2.20 mM of DPsPI and 8.80 mM of DHPC in 50 mM MOPS buffer pH 7.5 was prepared for several sets of assays by the following procedure: a
measured amount of DPsPI was dissolved in chloroform with small amount of methanol; the solution was dried under a stream of argon and then in vacuo for 10 minutes; 50 mM DHPC in MOPS buffer and MOPS buffer were added to achieve a final concentration of 2.20 mM DPsPI and 8.80 mM DHPC in 50 mM MOPS; the mixture was vortexed for several minutes, then sonicated (bath-type sonicator) for 5 minutes at room temperature to completely solubilize the substrate analog.

The stock solution was diluted with 50 mM MOPS (pH 7.5) buffer into different concentrations (0 to 2.0 mM) prior to assaying. The diluted substrate solution (225 μL) was placed in a microcuvette (2 x 10 mm), 5 μL of 50 mM DTNB in ethanol were added. The mixture was stirred well with a gel-loading tip on a Pipetteman (Ranin Instrument, Inc.), and care was taken to limit bubble formation. The microcuvette was equilibrated for 5 minutes at 25 °C in the UV-Vis spectrophotometer with a water bath circulating the cuvette holder. A background slope was recorded for 2 minutes at 412 nm with a sampling rate of 150 with the spectrophotometer in the time drive mode. 5 μL of PI-PLC of appropriate concentration (10 ng/μL of WT) in 20 mM Tris, 2 mM EDTA, pH 7.5 were added to the cuvette, and the sample was mixed quickly and the absorbance was recorded for 2 minutes or more. The extinction coefficient (ε) of 12,800 M⁻¹ cm⁻¹ for 5-nitro-2-nitro-benzoic acid was used to calculate the enzyme activity:

\[
\nu_0 = \frac{\Delta A \text{ min}^{-1}}{1.0 \text{ cm} \times 12.8 \text{ mM}^{-1} \text{ cm}^{-1}} \times 0.235 \text{ mL} \times \frac{1}{W_{\text{TE}} \text{ mg}}
\]

(8)
$v_0$ is initial reaction rate (mmol min$^{-1}$ mg$^{-1}$), $\Delta A$ is the absorbance change rate at 412 nm (min$^{-1}$), $W_{TE}$ is the weight of the enzyme used in one assay (mg). $v_0$ as a function of substrate concentration showed a hyperbolic curve, and was treated by curve-fitting the results to the Michaelis-Menten equation using SigmaPlot (Jandel Corporation):

$$v_0 = \frac{V_{max} [S]}{K_m + [S]}$$  \hspace{1cm} (9)

$V_{max}$ is the maximal reaction rate, $[S]$ is the substrate bulk concentration, $K_m$ is the substrate concentration which gives half $V_{max}$ reaction rate.

### 7.3 Results and Discussion

The assay was first carried out with pure substrate suspension, and turnover of DPsPI by PI-PLC was undetectable with the 50 ng of WT PI-PLC added to the total 235 $\mu$L of assay mixture. This may be due to the inability of PI-PLC to act on the vesicles of DPsPI. The high absorbance of the substrate suspension could also interfere with UV-Vis monitoring of the reaction. In order to better use this thiol assay with UV-Vis, a micelle phase must be formed. The aggregated state of micelle is a clear solution, and will not interfere with the UV-Vis monitoring. Sodium deoxycholate and Triton X-100 are the two traditional detergents, and were used initially to solubilize DPsPI forming mixed micelles. However, these two detergent still could not form a clear solution when 12 times amount of the detergents were mixed with DPsPI suspension. DHPC, the short chain C7 PC, has been used recently to substitute for sodium deoxycholate and Triton X-
100 as the new generation of solubilizing reagent (Kessi et al., 1994; Roberts, 1996). It always forms micelles when dissolved in water. It has been shown that the PI-PLC from *B. thuringiensis* is activated by short-chain PC by a rate factor of $10^{3}$-$10^{4}$ (Rosenberry, to be published). When DHPC was used in this study to solubilize DPsPI, a clear solution was formed with 2.20 mM DPsPI and 8.80 mM DHPC. The solution of 1.0 mM DPsPI with 4.0 mM DHPC absorbed only 0.031 when added to the microcuvette. The initial reaction rate at 1.0 mM substrate was 48.3 $\mu$mol min$^{-1}$ mg$^{-1}$.

DTP was also tested as the thiol-detecting reagent. When DTP was used in the same condition, the reaction rate was essentially the same (59.3 $\mu$mol min$^{-1}$ mg$^{-1}$). In the continuous assay of PI-PLC with C$_{8}$-thio-PI (Hendrickson et al., 1992), a ratio of 1:4 (C$_{8}$-thio-PI / DHPC) mixed micelles gave the best activity. A series of ratios of detergent to substrate were tested for the activity with DTP as the thiol reagent. Figure 38 shows the activities in different amount of detergent DHPC. The activities were not too much different when the ratio (DHPC/DPsPI) were 4 and 5. When the ratio was small, the reaction rate was decreasing; when more DHPC was used an inhibition occurred. So, a ratio of 1:4 (DPsPI/DHPC) was chosen to form the mixed micelle. The pH of 7.5 of the buffer was chosen in order to compare the results with results of $^{3}$H-PI assay. Since 2-nitro-5-thiobenzoic acid (derived from DTNB) has a more constant extinction coefficient ($\varepsilon$) compared with 4-thiopyridine (derived from DTP) in the pH of 7.5, DTNB was chosen over DTP for detecting thiol in the assay.

Enzyme activity was linear with respect to the amount of enzyme used (up to at least 100 ng WT) in these assays and the time course were initially linear with no lag for
Figure 38. Initial reaction rate as a function of the ratio [DHPC]/[DPsPI]. The assay was carried out with 1.0 mM DPsPI, 4.0 mM DHPC, and 1 mM DTP in the buffer of 50 mM MOPS, pH 7.5 at 25 °C.
Figure 39. The linear relationship of initial reaction rate ($v_0$) to the amount of WT PI-PLC enzyme used in the assay. 1.0 mM DPsPI, 4.0 mM DHPC, and 1 mM DTNB in 50 mM MOPS buffer, pH 7.5 at 25 °C.
Figure 40. Initial reaction rate as a function of substrate concentration. [DPsPI]/[DHPC] = 1:4. 1 mM DTNB in 50 mM MOPS, pH 7.5 at 25 °C.
Figure 41. Initial reaction rate as a function of substrate concentration. [DHPC] = 8.07 mM. 1 mM DTNB in 50 mM MOPS buffer, pH 7.5 at 25 °C.
both DTNB and DTP. Figure 39 shows the linear relationship of initial reaction rate with the amount of enzyme used in the assays.

The full kinetic studies of PI-PLC with DPsPI were carried out in the following two conditions respectively: a constant molar ratio of DPsPI/DHPC (1:4) was used in the various concentrations of substrate DPsPI; a constant concentration of DHPC (8.07 mM) was used in the various substrate concentrations (up to 2.0 mM). Figure 40 and Figure 41 show the initial reaction rate $v_0$ as a function of substrate concentration in the two experiments respectively. Both experiments afforded a hyperbolic curve with $v_0$ against [S]. After curve-fitting of the data to Michaelis-Menten equation, $V_{\text{max}}$ of 53.3 μmol min$^{-1}$ mg$^{-1}$ and $K_{\text{m,app}}$ of 0.184 mM were obtained for the assay with a constant ratio of DPsPI/DHPC (1:4). For the assay with a constant concentration of DHPC of 8.07 mM, $V_{\text{max}}$ was estimated as 129 μmol min$^{-1}$ mg$^{-1}$, and $K_{\text{m,app}}$ was estimated as 3.68 mM. Although the $V_{\text{max}}$ was higher with a constant DHPC concentration, the saturation could not be approached even with [DPsPI] of 2.0 mM. The $V_{\text{max}}$ obtained with a constant ratio of DPsPI/DHPC (1:4) was comparable with $V_{\text{max}}$ obtained with a constant [DHPC], but the saturation was easily observed at 2.0 mM. Considering the amount of substrate needed to run each assay and the limited amount of DPsPI, the method with a constant ratio of DPsPI/DHPC was chosen for the assay. And the assay was utilized to analyze mutants.

Compared with previous continuous assay of PI-PLC, especially the one using C$_{16}$-thio-PI as the substrate, the assay presented in this work has a much higher $V_{\text{max}}$ of 53.5 μmol min$^{-1}$ mg$^{-1}$, a 8.2 fold increase over $V_{\text{max}}$ with C$_{16}$-thio-PI. The second acyl
chain along with the DAG moiety in the substrate does contribute to the activity of PI-PLC toward the substrate. The maximal activity of PI-PLC with DPsPI is still low compared with the activity with natural PI (53.5 compared with 1600 μmol min⁻¹ mg⁻¹). The difference could be due to the replacement of oxygen with sulfur. Thioester analogs of phosphatidylmethanol are hydrolyzed by phospholipase A₂ at about 7-fold lower rate as compared to oxy analogs (Jain et al., 1992). And the differences in electronegativity, hydrophobicity and interfacial packing of substrate in the interface. The alcohol product may be more readily released into water than thiol product. Due to the interfacial binding, the $K_m$ obtained should be considered as an apparent parameter. Even though, the $K_{m,app}$ of 0.18 mM is relatively small, and it suggests that DPsPI bind well with the PI-PLC enzyme.

This assay uses the most natural thiophosphate analog of PI, and afforded a reasonable $V_{max}$ of 53.5 μmol min⁻¹ mg⁻¹. It is continuous, non-radioactive, and easy to carry out. It can be used to assay WT PI-PLC as well as mutant enzymes. It would be interesting to see how short chain thio-analog, such as DC₈₅PI, performs in the assay. Studies with mammalian PI-PLC would be able to show whether it also could be used in those enzymes. The disadvantages of this assay include the sensitivity, although high enough for most purposes, is still low compared with $^3$H-PI assay and the availability of the substrate DPsPI is limited due to the long synthesis.
CHAPTER 8
THE ROLES OF HIS-32 AND ASP-274 IN PI-PLC CATALYSIS

8.1 Introduction

As discussed in Chapter 5, the stereochemistry studies (mainly via the method of $^{31}$P NMR) of the reaction catalyzed by bacterial PI-PLC showed a two-step reaction: the single displacement forming IcP proceeds with inversion of the configuration at phosphorus and the second reaction forming IP is accompanied by another inversion (Lin et al., 1990; Volwerk et al., 1990; Bruzik et al., 1992; Lewis et al., 1993). The two reactions are shown in Figure 42. The sequential mechanism proposed bears a resemblance to the well-known mechanism of ribonuclease A (Richard & Wyckoff, 1971), which is a mechanism of general base and acid catalysis. In ribonuclease A, two active site histidines are believed to be the general acid and base.

Structural information was not available until recently when a crystal structure of B. cereus PI-PLC with bound myo-inositol was reported at a resolution of 2.6 Å (Heinz et al., 1995). Based upon the crystal structure obtained, two histidines His-32 and His-82 were projected as the general base and acid: for the first half reaction, His-32 acted as the base and His-82 acted as the acid; for the second half reaction, His-32 and His-82's roles were reversed. His-32 forms hydrogen bonds with myo-inositol and Asp-274: His-32's
Figure 42. The two-step reaction catalyzed by bacterial PI-PLC (Volwerk et al., 1990).
$\varepsilon_2\text{-N}$ forms a hydrogen bond with 2-OH group of the bound myo-inositol (distance between the nitrogen and the oxygen atoms is 2.9 Å); His-32's $\delta_1\text{-N}$ along with its hydrogen forms a hydrogen bond with the side chain carboxylic group of Asp-274 (distance between the nitrogen and the oxygen atoms is 2.6 Å). It was suggested that Asp-274, His-32 and the 2-OH group of inositol form a "catalytic triad". Compared with the catalytic triad in serine protease, the nucleophilic hydroxyl group of this proposed triad is from the substrate itself instead of from the enzyme (Ser). And the Asp-274 and His-32 pair could be called catalytic diad as in the case of phospholipase $A_2$ (PLA$_2$) (Kumar et al., 1994). However, the bound inhibitor myo-inositol is a weak inhibitor of PI-PLC with IC$_{50}$ of 8 mM (Shashidhar et al., 1990), and the crystal structure of the PI-PLC bound with myo-inositol does not differ from that of the free enzyme. Normally, a enzyme is likely to undergo induced conformational change once bound to a substrate. The conformation of PI-PLC with bound myo-inositol may not reveal the actual conformation when PI-PLC binds with PI. So, it is very interesting to study the structural and functional roles of His-32 and Asp-274. This can provide more information of whether the His-32 and Asp-274 pair acts as a catalytic diad, and lead to new aspects of the catalytic mechanism of PI-PLC, which is still unclear.

In this study, six mutants were constructed in these two positions: His-32 was replaced with Ala, Asn, and Gln; Asp-274 was replaced with Ala, Asn, and Glu. The alanine mutants could provide initial information of the side chain functionality of these residues. For His-32, amide nitrogen atoms of Asn and Gln were reported to be able to mimic the $\delta_1$-nitrogen and $\varepsilon_2$-nitrogen respectively of His (Leatherbarrow and Fersht, 157
1987). For Asp-274, Asn replacement removed the negative charge with retaining the rest side chain properties; Glu mutant is a conservative replacement with one extra methylene on the side chain. All the six mutants were purified into homogeneity. The mutant enzymes were subjected to kinetic analysis, structural analysis with 1D and 2D NMR, conformational stability analysis. Some of the mutants also were tested for pH-activity profile. Analysis of these results allowed the X-ray model evaluated, and new aspects of the PI-PLC mechanism revealed.

8.2 Results

Expression and Purification of His-32 and Asp-274 Mutants. The three His-32 mutants H32A, H32N, and H32Q behaved similarly to WT PI-PLC in the expression and purification process: cells were grown at 30 °C; the cell lysate were subjected to DEAE anion-exchange column (DE52), G-100 gel-filtration column, and Phenyl Sepharose CL-4B hydrophobic column. However, difficulties were encountered in the purification of the Asp-274 mutants. Extremely low yields were obtained with D274A and D274E mutants. This was mainly due to the very poor binding of the mutant enzymes to the anion-exchange column. It was very possible that the problem arose from the insolubility of the expressed mutant form rather than the mutant enzyme binding problems. Considering the possibilities, we tried to grow the cell under a lower temperature (20-25 °C). The newly grown cell bind to the DE52 column very similarly to WT PI-PLC and His-32 mutants. D274A and D274E were then purified to homogeneity following the other two columns: gel-filtration and hydrophobic columns. D274N were grown at 25 °C, and was purified
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific Activities (μmol min(^{-1}) mg(^{-1}))</th>
<th>Relative activities (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1350</td>
<td>100</td>
</tr>
<tr>
<td>H32A</td>
<td>0.030</td>
<td>0.0022</td>
</tr>
<tr>
<td>H32N</td>
<td>0.014</td>
<td>0.0010</td>
</tr>
<tr>
<td>H32Q</td>
<td>0.012</td>
<td>0.00089</td>
</tr>
<tr>
<td>D274A</td>
<td>0.0099</td>
<td>0.00073</td>
</tr>
<tr>
<td>D274N</td>
<td>21.0</td>
<td>1.56</td>
</tr>
<tr>
<td>D274E</td>
<td>168</td>
<td>12.4</td>
</tr>
</tbody>
</table>

Table 5. Specific Activities of WT and Mutant PI-PLC with \(^3\)H-PI\(^a\).

\(^a\)The specific activities were measured at 37 °C, with 2.0 mM of DPPI, 0.4% sodium deoxycholate in 50 mM borate buffer, pH 7.5.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$V_{\text{max}}$ (μmol min$^{-1}$ mg$^{-1}$)</th>
<th>$K_{\text{m,app}}$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>53.5</td>
<td>0.18</td>
</tr>
<tr>
<td>H32A</td>
<td>&lt;0.0026</td>
<td></td>
</tr>
<tr>
<td>H32N</td>
<td>&lt;0.0028</td>
<td></td>
</tr>
<tr>
<td>H32Q</td>
<td>&lt;0.0031</td>
<td></td>
</tr>
<tr>
<td>D274A</td>
<td>&lt;0.0028</td>
<td></td>
</tr>
<tr>
<td>D274N</td>
<td>0.29</td>
<td>0.040</td>
</tr>
<tr>
<td>D274E</td>
<td>13.2</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Table 6. Steady-State Kinetic Parameters of WT and Mutant PI-PLC with DPsPI.

*The steady-state kinetic analysis were carried out at 25 °C, with 0 ~ 2.0 mM of DPsPI, [DHPC]/[DPsPI] = 4.0, in 50 mM Mops buffer, pH 7.5.
Figure 43. Steady-state kinetic analysis of D274N and D274E mutants. A. D274N; B. D274E. The experiments were carried out at 25 °C, with 0 - 2.0 mM of DPsPI, [DHPC]/DPsPI = 4.0, in 50 mM Mops buffer, pH 7.5. The initial reaction rate ($v_0$) were obtained according to the procedure described in Chapter 7. $v_0$ were plotted against the bulk concentration of DPsPI. The data were processed by fitting into Michaelis-Menten equation. Thus obtained $V_{max}$ and $K_{m,app}$ parameter were listed on Table 6.
to homogeneity. SDS-PAGE gel analysis also showed the size of the mutant enzymes were the same as that of WT PI-PLC.

*Kinetic Analysis of Mutants.* The results of the specific activities analysis carried out on the pure mutant enzymes are shown in Table 5. The three His-32 mutants H32A, H32N, and H32Q had large decreased activities, which decrease $10^4 - 10^5$ fold from WT. D274A also had a large decrease in the specific activity ($10^5$ fold) too. Replacement of Asp-274 with Asn only resulted modest decrease in the specific activity, which decrease 64 fold. D274E mutant had the highest activity among the mutants constructed in this study, it had a specific activity of 168 $\mu$mol min$^{-1}$ mg$^{-1}$, which was only 8 fold less than that of WT PI-PLC.

The newly developed continuous assay with DPsPI method was also utilized to analyze these mutants. The results are shown in Table 6. The low activities of H32A, H32N, H32Q and D274A were undetectable in the continuous assay even with about 50 $\mu$g of mutant enzymes were added to each assay. Full steady-state kinetic analysis were carried out on D274N and D274E, which had higher activities. Figure 43 shows the initial reaction rate plot against the bulk concentration of the substrate DPsPI. The relative activities obtained agree with results obtained from hot assay with $^3$H-PI.

*PH-Activity Profile.* pH-activity profile were obtained by measuring the specific activities in a wide range of pH. The pH-activity profile of WT PI-PLC showed a broad range of pH for optimum specific activity, from 5.0 to 8.5. This actually agreed with the broad pH optimum range reported in the literature (Griffith et al., 1991; Steiger and Brodbeck, 1991; Hendrickson et al., 1992). The experiments were also carried out with
Figure 44. pH-activity profiles of WT PI-PLC, D274N, H32A, and D274A mutants. The experiments were carried out at 37 °C, with 2.0 mM PI and \(^3\)H-PI in a buffer of 50 mM gly-gly, 50 mM Tris-HCl and 50 mM CAPS. 40 mM of DHPC was used to form mixed micelles with the substrate. pH of the buffers were checked before each set of assay. Relative activities as percentage of maximum activity were plotted against pH.
Figure 44.
D274N, D274A, and H32A mutants. Figure 44 showed the pH profiles. Compared with the pH profile of WT PI-PLC which had a broad range, the pH profile of D274N was a classical bell-shape curve. The ascending limb was slightly shifted to low pH (~ 0.3 pH), and the descending limb was shifted greatly to low pH (~ 1.5 unit). The maximum activity of D274N was obtained at pH 5.5. D274A’s pH profile was shifted to low pH greatly with ascending limb shifted 1.0 pH unit and descending limb shifted 3.5 pH units. H32A’s pH profile showed little difference from WT: ascending limb was shifted to low pH slightly (0.5 pH unit); descending limb was actually not changed.

Proton NMR Properties of Mutants. Since the proton NMR spectra of WT PI-PLC had not been assigned yet, both the 1D NMR and 2D NOESY spectra were obtained to monitor the global conformational change of the mutants by comparing the spectra of mutants with those of WT. The well resolved spectra allowed the comparison possible. The 1D proton NMR spectra of the six mutants as well as the WT PI-PLC are shown in Figure 45. The peak shape, width, chemical shift were compared. It can be seen that five out of the six mutants H32A, H32N, H32Q, D274A, and D274N showed a very similar 1D proton spectra to that of WT. D274E, although had a similar characteristics in the aromatic region, did not show the main chain amide proton peaks in the corresponding region (8 ~ 10). The 2D NOESY spectra of the mutants and WT were shown in Figure 46 through 52. These 2D NOESY spectra of mutants are very similar to those of WT. Most of the cross peaks showed up in the mutant enzymes. Although there are a few chemical shift change in some of the peaks, they are very minor. These qualitative comparisons indicated that all the mutants except D274E retained the global
Figure 45. The 1D proton NMR spectra of mutants and WT PI-PLC (to be continued).
Figure 45 (continued).

D274E

D274N

D274A

WT

167
Figure 46. NOESY spectrum of WT PI-PLC (Riddle et al., 1996)
Figure 47. NOESY spectrum of H32A mutant PI-PLC.
Figure 48. NOESY spectrum of H32N mutant PI-PLC.
Figure 49. NOESY spectrum of H32Q mutant PI-PLC.
Figure 50. NOESY spectrum of D274A mutant PI-PLC.
Figure 51. NOESY spectrum of D274N mutant PI-PLC.
Figure 52. NOESY spectrum of D274E mutant PI-PLC.
Figure 53. The CD spectra of mutants and WT PI-PLC.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$\Delta G_{d}^{H_2O}$ (kcal/mol)</th>
<th>$m$ [kcal/(mol M)]</th>
<th>$D_{1/2}$ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT$^b$</td>
<td>7.0</td>
<td>3.6</td>
<td>2.0</td>
</tr>
<tr>
<td>H32A</td>
<td>7.2</td>
<td>3.9</td>
<td>1.8</td>
</tr>
<tr>
<td>H32N</td>
<td>7.1</td>
<td>4.0</td>
<td>1.8</td>
</tr>
<tr>
<td>H32Q</td>
<td>6.7</td>
<td>4.4</td>
<td>1.5</td>
</tr>
<tr>
<td>D274A</td>
<td>2.0</td>
<td>1.8</td>
<td>1.1</td>
</tr>
<tr>
<td>D274N</td>
<td>3.1</td>
<td>2.6</td>
<td>1.2</td>
</tr>
<tr>
<td>D274E</td>
<td>1.5</td>
<td>0.89</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Table 7. Free energies of unfolding induced by guanidine hydrochloride$^a$.

$^a$Measured at 19 °C, pH 7.5, 10 mM borate, 0.1 mM EDTA. The error limit of $\Delta G_{d}^{H_2O}$ is estimated to be ± 0.5 kcal/mol. $^b$Obtained from Hondal, R (unpublished result).
conformation, and the conformation perturbation resulted from mutation are very minor and local. D274E, due to the missing main chain amide proton peaks, is expected to have a more flexible conformation, which allows the rapid proton exchange with H\textsubscript{2}O in the solvent.

*Conformational Stability of Mutant Enzymes.* The CD spectra of the mutants are shown in Figure 53, and they are essentially the same as that of WT PI-PLC. The free energy of unfolding was determined for all the mutants by monitoring the change in ellipticity of CD spectra with varying concentrations of Gdn-HCl. The data are summarized in Table 7. \(\Delta G_d^{H\textsubscript{2}O}\) of His-32 mutants are similar to the WT values, which suggests that His-32 is not important for the conformational stability of PI-PLC. \(\Delta G_d^{H\textsubscript{2}O}\) of Asp-274 mutants showed a substantial perturbation from that of WT. D274A, D274N, and D274E had a decrease in \(\Delta G_d^{H\textsubscript{2}O}\) of 5.0, 3.9, and 5.5 kcal/mol respectively. These results suggests that Asp-274 plays an essential role in the conformational stability of PI-PLC.

**8.3 Discussion**

*Structural Perturbation of the Mutations.* The structural characterization is important for the interpretation of the kinetic data and the structure-function relationship. If the conformation of a mutant is unperturbed, the change in kinetic parameters can be used to analyze the functional roles of the mutated residue. One can not attribute the change in kinetic constants solely to the functional role of the mutated residue if there is a large perturbation in conformation is observed. Circular dichroism (CD) was used in this
study to preliminarily check the conformational perturbation of the mutants. CD spectrum of a enzyme mainly provides information of secondary structures of the enzyme. The CD spectra of the six mutants all showed similar feature as the CD spectrum of WT PI-PLC. The CD spectra suggest that the secondary structures of all the six mutants are intact. Proton NMR studies provide a complimentary method for the structural analysis. Compared with CD spectra, NMR spectra is more sensitive, and it reveals information of the global conformation. With the relative large size of the PI-PLC (35 kDa), we have been able to get a well resolved 1D and 2D NOESY spectra of the WT PI-PLC (Riddle et al., 1996). The NMR studies showed little conformational perturbation in the mutants of H32A, H32N, H32Q, D274A, and D274N. The disappearance of amide proton peaks in D274E suggests that this mutant is much more flexible than WT, and it may be a “molten globule” structure, a conformational state with great flexibility while still preserving secondary structures (Baum et al., 1989). It is very interesting that this relaxed form of D274E retains a high activity (12.5% of WT). The conformation stability studies (monitored by CD) showed that D274E is much less stable than WT, and the “m” value is also greatly perturbed. Other two Asp-274 mutants also showed greatly decreased conformational stability though not to the extent of D274E. This may be the reason why difficulties were found in purifying the Asp-274 mutants. It appears that Asp-274 mutants were obtained in a wrong form (may be insoluble form) when the cell were grown at 30 °C. The results suggest that Asp-274 plays an critical role in the PI-PLC structure. In summary, the conformational analysis ensure that the kinetic
Figure 54. The stereoview of *B. cereus* PI-PLC complexed with *myo*-inositol. His-32, Asp-274, His-82, Asp-33, and Asp-198 are shown with their side chains. The crystal structure was obtained at a resolution of 2.6 Å (Heinz et al., 1995).
analysis of the mutants (except D274E) could be interpreted without the interference from the global structural perturbation.

**Functional Role of His-32 and Asp-274.** The pK$_a$ of His-32 was reported as 7.6 based upon NMR titration experiments (Liu et al., 1996). The pK$_a$ value is close to the pK$_a$ of histidine side chain imidazole 6.00 (Rawn, 1989), and His-32's determined pK$_a$ value of 7.6 is reasonable. The pH-activity profile of WT showed a broad range of pH for optimal PI-PLC activity. Two apparent pK$_a$ values of 4.5 and 8.5 were obtained from the pH-activity profile. The pH profile of WT PI-PLC and the pK$_a$ values suggest that His-32's side chain imidazole is in protonated form in order for the enzyme to function normally in the first step reaction. And His-32 is somehow associated with the descending limb of the pH profile. This is supported by the pH profile of D274N mutant. His-32 and Asp-274 are two residues in the active site (Heinz et al., 1995). Figure 55 shows the stereoview of *B. cereus* PI-PLC complexed with myo-inositol, illustrating the orientation of His-32 and Asp-274 as well as other active site residues including His-82, Asp-33, and Asp-198. Asp-274 and His-32 forms a strong hydrogen bond indicated by the short distance between these tow residues in structures of both free enzyme and enzyme with bound myo-inositol (2.6 Å). And this hydrogen bond is not expected to be disrupted during the conformational change upon binding of PI. Replacement of Asp-274 with Asn removed the negative charge in the position of 274. The change is expected to destabilize the positive charge of the protonated imidazole of His-32, and to lower the pK$_a$ of His-32. The pH-rate profile of D274N had a greatly shifted descending limb, which is in agreement of His-32 being protonated and associated with the descending
limb. The shift to lower pH agrees with the theoretical predication of the His-32’s pKₐ lowering effect by mutation of Asp-274 → Asn-274. As His-32 is protonated when PI-PLC could function normally, His-32 could be very likely acting as a general acid for the first step reaction. This general acid of His-32 could interact with other residues or interact with the substrate to enable the enzyme to catalyze the cleavage of PI. However, His-32 could not act as a general base to abstract the 2-OH proton, as in the mechanism proposed by Heinz et al. (1995). The Asp-274 and His-32 pair, although has strong hydrogen bonding, they do not form a “catalytic triad” with 2-OH group of inositol ring.

All the three mutations at His-32 result in great drop in enzyme activity, which means that His-32 is essential for PI-PLC normal function. H32N’s Asn-32 residue could have interaction with Asp-274, but this interaction may not be as effective as the interaction in WT between imidazole and Asp-274’s carboxylic group, and Asn-32 does not have another nitrogen atom, which could be critical. H32Q’s Gln-32 amide may not have interaction with Asp-274. The interaction is critical, which is also indicated by large decrease in activity of D274A. Although Asp-274 and His-32 could not form a “catalytic diad”, the interaction between Asp-274 and His-32 could still be needed to help the catalysis in some ways. Asp-274 could position His-32 in a proper orientation and proper tautomer, so His-32 could interact effectively with other residues or substrates, for example, His-32 may be required in a proper plane to have effective interaction with other functional groups. On the other hand, His-32 could help Asp-274 to have interaction with other groups too, for example, histidine has been proposed to help
aspartate to act as a general base in the mechanism of ribonuclease T₁ (Steyaert et al., 1990).

**Ribonuclease A-Like Mechanism with Two Asp-His Diads.** Bruzik et al. (1992) postulated that the catalytic mechanism of PI-PLC resembles that of ribonuclease A. A main difference between the mechanisms of the two enzymes at the time was that PI-PLC releases its cyclic intermediate while RNase A does not. This difference is no longer valid now as Thompson et al. (1994) have demonstrated by $^{31}$P NMR that RNase A and several other ribonuclease also release the cyclic intermediate.

The crystallographic work by Heinz et al. (1995) further supported the resemblance between PI-PLC and RNase A. Ribonuclease A has a pair of histidines His-12 and His-119 (Eftink and Biltonen, 1983) acting as general base/acid in the suggested mechanism (Kartha et al., 1967; Nishikawa et al., 1987; Thompson and Raines, 1994; Nogëes et al., 1995). Based on the X-ray structure of Bacillus cereus PI-PLC bound with a weak inhibitor myo-inositol, two histidines: His-32 and His-82 were suggested acting as the general base and acid for the catalysis of PI-PLC. According to the proposed mechanism, for the first half reaction, His-32 abstracts the 2-OH proton, His-82 donates a proton to the DAG moiety to make it an easy leaving group. For the second half reaction, the roles of His-32 and His-82 are reversed: His-82 serves as a general base to activate the water molecule; His-32 serves as a general acid to facilitate the ring to open.

A possible difference between PI-PLC and RNase A is that His-32 and His-82 of PI-PLC are hydrogen-bonded to Asp-274 and Asp-33, respectively, possibly forming two Asp ••• His catalytic diads. According to the crystal structure, Asp-274 is within 2.6 Å to
the $\delta_1$-nitrogen of His-32, and Asp-33 is within 3.2Å to the $\delta_1$-nitrogen of His-82. The activity of D274N mutant is consistent with the Asp-His catalytic diads in other enzymes. D274N mutant has a $V_{\text{max}}$ decrease of only 180 fold compared with that of WT. The result is comparable to D99N in PLA2 and D102N in trypsins. D274E mutant showed only four-fold decrease in $V_{\text{max}}$. D33N (Hondal, R. unpublished result) retained the global conformation of WT PI-PLC, and it has about 3% activity of WT.

In this mechanism of PI-PLC catalysis, both histidines are protonated appropriately for the second step reaction after the first step reaction (transphosphorylation); both histidines are returned to their initial protonation states for another cycle of reaction after completion of the first and second step reactions. As suggested by Thompson et al. (1994) for ribonuclease A, an iso mechanism (medwedew, 1937) may also occur in this mechanism: protonation states of unbound enzyme are interconverted by a pathway that does not involve substrates.

**Do His-32/His-82 Function as General Base/Acid?** The pH-activity profile of WT PI-PLC is broad and significantly different from the classic bell-shape curve. In addition, the $pK_a$ of neither the ascending limb (4.5) nor the descending limb (8.8) falls in the usual $pK_a$ range of histidines in enzymes (ca 5 - 7.5). Although it is not impossible that the two histidines have unusually high or low $pK_a$ due to the local structures, the data suggest a possibility that pH-rate profile is not controlled by the two histidines, and/or that the two histidines do not function as general acid/general base. NMR titration experiments of free *B. cereus* PI-PLC gave $pK_a$ values of 7.6 and 6.9 for His-32 and His-82 respectively (Liu et al., 1996). Unlike PI-PLC, RNase A exhibits a normal bell shape.
Figure 55. RNase T1-like mechanism of *B. thuriginesis* PI-PLC.
pH-rate profile (Findlay et al., 1961; Richards and Wyckoff, 1971; Eftink and Biltonen, 1983) and normal histidine pKa of 5.8 (His-12) and 6.2 (His-119) (Markley, 1975; Patel et al., 1975; Eftink and Biltonen, 1983). RNase A-like mechanism cannot explain the pH profile of WT, D274N, as well as the determined pKₐ of His-32 and His-82. WT PI-PLC has optimal activity in the pH range of 5.0-8.5. His-32 should be protonated for PI-PLC to function normally for the first step. D274 mutation is expected to affect His-32's pKa, but D274N's pH profile's descending limb is shifted greatly to low pH.

Ribonuclease Tl-Like Mechanism of PI-PLC. Another ribonuclease, RNase Tl, exhibits a broad range of pH-rate profile (Steyarert et al., 1990) similar to PI-PLC. The mostly accepted mechanism of RNase Tl is that Glu-58 and His-92 act as a general base and a general acid, respectively (Heinemann & Saeaga 1982; Steyaert et al., 1990; Heydenreich et al., 1993; Ishikawa et al., 1996), and His-40 interacts with Glu-58 to optimize the pKₐ of Glu-58. If this mechanism is applied to PI-PLC, the roles of His-32 and Asp-274 would be reversed, with Asp-274 functioning as a general base and His-32 enhancing the basicity of Asp-274 and perhaps also helps to orient its conformation. Mutants of D274N, H32A have similar relative activities as those of corresponding mutants for RNase Tl (E58Q and H40A). The charged His-82 may act as the general acid to activate the leaving group DAG. It may also deshield the second negative charge. Asp-33 may be involved in a step which is not rate-limiting but catalytically important, such as orienting the position of His-82, interacting with the DAG leaving group. For the second step the roles of Asp-274 and His-82 would be reversed.
The RNase T1-like mechanism is also supported by the crystal structure of a mammalian PI-PLC 81-isomechanism (from rat) complexed with InP$_3$ (Essen et al., 1996). Although mammalian PI-PLC differs from the bacterial PI-PLC, the reported active site residues are quite similar. In the mammalian PI-PLC structure, His-356 is in a position similar to His-82 in \textit{B. cereus} PI-PLC. However, His-311, which corresponds to His-32 in \textit{B. cereus} PI-PLC, is too far to have hydrogen bonding with 2-OH group. It was suggested that Glu-341 or Glu-390 be the alternates. There are several carboxylate residues in the active site of \textit{B. cereus} PI-PLC: Asp-274, Asp-33, Asp-67, Asp-198, Asp-180, and Glu-117. Mutagenesis studies on Asp-67 concluded that Asp-67 is not essential for catalysis (Hondal et al., 1996). Glu and Asp-180 are far from this region, but mutagenesis studies are needed to see their roles; Asp-198 is possibly reachable for the 2-OH group, and the mutagenesis studies are underway. Although these residues and other unspecified Asp or Glu residues are possible, the better candidates for the general base for the first step are Asp-274 and Asp-33.

\textit{Other Possible Mechanism of PI-PLC Catalysis}. It is assumed that the weak inhibitor myo-inositol binds to the active site in the same manner as the myo-inositol moiety of PI. If this is not the case, the other mechanism involving different roles of the four residues, His-32, His-82, Asp-33 and Asp-274, are also possible. For example, His-82 could act as general base and His-32 as general acid for the first step reaction, reversing the roles of the two residues in RNase A-like mechanism.

\textit{Conclusion}. All the mutants except D274E have little global structural perturbation. His-32 and Asp-274 play essential roles in the \textit{B. cereus} PI-PLC catalysis.
His-32 are in protonated form for PI-PLC to catalyze the first step reaction. D274N has modest decrease in activity, D274E retains one quarter of WT PI-PLC's activity. D274E has a more flexible global conformation, compared with WT and D274N. Asp-274 plays an important role structurally, and it contributes greatly to the conformational stability. Several possible mechanisms including ribonuclease A-like mechanism and ribonuclease T1-like mechanism are discussed.

Future Studies. The complicated situation associated with PI-PLC catalysis arises from two steps reaction of different rates, interfacial catalysis. More studies are needed to probe the PI-PLC mechanism. For His-32, mutagenesis with other mutants such as His-32 → Lys may provide more information about the role of this residue. NMR peak assignments and pH-titration may determine which Asp or Glu residue plays the general base role. X-ray structure of PI-PLC complexed with a strong inhibitor could offer a structural feature closer to the real one when PI-PLC is bound with PI. X-ray studies of D274N is currently underway in corporation with another research group, and that structural result could allow us to further evaluate Asp-274's role. The second half reaction has not been studied much, and research on that reaction could provide us more information about why the second reaction is much slower than the first half reaction. And finally, the interfacial catalysis is also an interesting aspect of the PI-PLC catalysis.
LIST OF REFERENCES


Bruzik, K. S. unpublished results.


Hondal, R. unpublished results.


Noda, L. (1973) *Enzymes (3rd Ed.*)* 8, 279-305.


Patel, D., Canuel, L. and Bovey, F. (1975) *Biopolymers* 14, 987-997.


195


APPENDIX A.

NMR SPECTRA OF NEWLY SYNTHESIZED ORGANIC COMPOUNDS
pdCpA containing 3.0 eq of n-Bu₄N⁺.
ZZ1050A2
in DMSO-d$_6$, 353K, 500MHz
ZZ1050A2
in DMSO-d$_6$, 353K, 126MHz
ZZ1033B
in DMSO-d6, 353K, 126MHz
N O, CN
ZZ1057A2
In DMSO-d$_6$, 353K, 500MHz
ZZ1057A2
In DMSO-d₆, 353K, 126MHz
In DMSO-d$_6$, 353K, 126MHz
ZZ3018C
in CDCl₃, 500 MHz
ZZ3018C
In CDC\textsubscript{3}, 126 MHz
APPENDIX B.

1D PROTON NMR SPECTRA OF D274E AND WT PI-PLC