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CHEMICAL AND PHYSICAL STUDIES OF SWINE KIDNEY MICROSOMAL AMINOPEPTIDASE AND CHARACTERIZATION OF AN IMMOBILIZED DERIVATIVE WITH APPLICATIONS IN PROTEIN CHEMISTRY

DISSERTATION

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

By

John Parry Andrews, B. S.

The Ohio State University

1974

Reading Committee:

Dr. Garfield P. Royer
Dr. Edward J. Behrman
Dr. Roy A. Scott III
Dr. Gary E. Means
Dr. Perry A. Frey

Approved By

G.P. Royer
Adviser
Department of Biochemistry
This dissertation is humbly dedicated to the people, places, and institutions who supported, guided, helped, or in some way influenced my graduate school career:

To Molly: Memories of things past and expectations of things to come are perhaps the greatest of gifts. Your understanding, patience, and love are deeply appreciated and will be returned a hundred times over.

"Ah, Love could thou and I with Fate conspire To grasp this sorry Scheme of Things entire, Would not we shatter it to bits--and then Re-mould it nearer to the Heart's Desire?"

To my parents, John and Betty, for their love and guidance, and most of all for their understanding and support for the kid who just had to have a chemistry set in the fourth grade.

To my adviser, Dr. Garfield Royer, who taught me more in four years than in all my previous years of academic endeavors. I am eternally grateful for your guidance, patience, understanding, and, most of all, your friendship.

To all the ladies in the Drake Union cafeteria for sustaining me with their unequalled culinary delights and for providing their own inimitable logic in that, "a half piece of cheese is a whole piece, and a whole piece is a piece-and-a-half."
To the administrative bureaucracy of The Ohio State University and the Department of Biochemistry without whose help and guidance my research would have been completed six months earlier.

To John, Tina, Carol, Kathy, and all the girls at Molly McGuire's who served the finest tequila in Columbus.

"Then to this earthen Bowl did I adjourn
My Lip the secret Well of Life to learn:
And Lip to Lip it murmur'd---"While you live,
Drink---for once dead you never shall return."

To Earl's Cafe, Talita's, the Nic-Nac Club, and the Drake pool room where I spent quite a few hours trying to climb out of the pits of experimental failure and the valleys of non-motivation.

To John, Ron, Dodson, Kathy, Dan, Tim, Mike, Pat, Tony, Gil, Karen, Billy, John, Linda, Kevin, Al, Les, Brad, Cindi, and Louie:

"For I have known them all already, known them all---
Have known the evenings, mornings, afternoons,
I have measured out my life with coffee spoons,
I know the voices dying with a dying fall
Beneath the music from a farther room."

To Rosa, Larry, Kathy, Dan, Janis, Steve, Musetta, Bill, Gail, Warren, Mary, Gerri, Steve, Marty, Dave, Carol, Mike, Bob, Gary, Joan, Steve, Doug, Susan, Danny, Bill, Pat, Tom, Marilyn, Ron, Suzanne, Phil, Bob, Dolly, Jim, Ester, Barry, and Pam: Your friendship is and will remain very precious to me. I can truly say: "I'll get by with a little help from my friends."

Shantih Shantih Shantih
VITA

January 29, 1949 . . . . . . . Born - Cambridge, Ohio

1966 . . . . . . . . . . . . . Graduated - North Olmsted High School, North Olmsted, Ohio

1968 . . . . . . . . . . . . . National Science Foundation Undergraduate Research Fellow

1968-1970 . . . . . . . Ohio State University Honors Scholarship Recipient

1970 . . . . . . . . . . . . . B.S. (chemistry), The Ohio State University, Columbus, Ohio

1970-1974 . . . . . . . Graduate Teaching and Research Associate, Department of Biochemistry, The Ohio State University

PUBLICATIONS


"Immobilized Leucine Aminopeptidase", Polymer Preprints 13, 848 (1972).


"Chemical Studies on the Active Site Composition of the Microsomal Aminopeptidase of Swine Kidney", *J. Biol. Chem.*, submitted for publication.

FIELDS OF STUDY

Major Field: Biochemistry

Studies in Enzymology. Professor Garfield P. Royer

Studies in Thermodynamics. Professors Roy A. Scott and Garfield P. Royer

Studies in Physical Biochemistry. Professor Kirk C. Aune

Studies in Enzyme Reaction Mechanisms. Professor Robert M. Mayer

Studies in Chemical Kinetics. Professor Jack G. Calvert

Studies in Physical Organic Chemistry. Professor Jack Hine

Studies in Organic Reactions and Mechanisms. Professors Paul G. Gassman and John S. Swenton

CHEMICAL AND PHYSICAL STUDIES OF SWINE KIDNEY MICROSONAL AMINOPEPTIDASE AND CHARACTERIZATION OF AN IMMOBILIZED DERIVATIVE WITH APPLICATIONS IN PROTEIN CHEMISTRY

By

John Parry Andrews, Ph.D.

The Ohio State University, 1974

Professor Garfield P. Royer, Adviser

Aminopeptidase M, an aminopeptidase isolated from the microsomal fraction of swine kidney homogenates, has been covalently bound to an arylamine derivative of porous glass through a diazo linkage. The bound form of the enzyme retains 100% of its activity at saturating levels of substrate (Leu-p-NO₂-anilide). For the hydrolysis at pH 7.5 and 25°C, $k_{cat}$ values for bound and free aminopeptidase M are $23 \pm 2$ sec⁻¹ and $21 \pm 0.4$ sec⁻¹, respectively. Although the Michaelis constant increases on binding, the pH and temperature dependencies of the bound enzyme remains unchanged.

The immobilized protease is potentially valuable in the field of primary structure determination of proteins and peptides. After treatment for removal of contaminating endopeptidases, the insoluble derivative of aminopeptidase M was used successfully for the sequence determination of the first four N-terminal amino acid residues in the aminoethylated A chain of insulin. In the area of total amino acid determi-
nation, the bound enzyme catalyzes the hydrolysis of the aminoethylated A and B chains of insulin nearly to completion (>87% recovery of free amino acids in all cases). Immobilized pronase was used in concert with bound aminopeptidase M for the hydrolysis of β-lactoglobulin. The recovery of free amino acids was 93%. These bound proteolytic enzymes should be quite useful in amino acid composition determinations when acid-labile residues such as tryptophan, glutamine, or asparagine are present.

Aminopeptidase M has been reacted with an assortment of protein modification reagents. Histidine and tyrosine appear to be at the active site and necessary for catalytic activity. Photochemical oxidation of the enzyme with rose bengal inactivates it rapidly. The inactivation is pseudo-first order which is consistent with the oxidation of one residue or one type of residue. The pH dependence of the inactivation implicates the imidazole group of histidine. Approximately 60 carboxyl groups per molecule of enzyme were modified without significant loss of activity. Tryptophan residues do not appear to be necessary for catalytic activity since 20 residues can be alkylated with no loss of enzymatic activity. Similarly 80% of the lysine residues can be methylated while the enzyme retains full activity.

Diazotized sulfanilic acid, tetranitromethane, and N-acetylimidazole inactivate the enzyme. Inactivation may be retarded by the presence of a competitive inhibitor or...
a specific substrate. Inactivation by N-acetylimidazole may be reversed with hydroxylamine in a manner suggestive of deacetylation of O-acetyltyrosine. Furthermore, results of studies of solvent deuterium isotope effects are consistent with a mechanism which includes histidine as a general base and tyrosine as a general acid.

The hydrolysis of Leu-p-NO₂-anilide by aminopeptidase M is inhibited by aromatic and aliphatic hydrocarbons, amines, and amino acids. In contrast, the hydrolysis of Gly-p-NO₂-anilide is inhibited neither by aromatic nor aliphatic hydrocarbons. An argument is proposed for the existence of a hydrophobic binding site on the enzyme. However, experiments indicate that the binding of inhibitors to aminopeptidase M does not occur by an "extraction" mechanism.

Preliminary studies of some physical properties of the enzyme have been undertaken. A study of the binding of L-leucine, a competitive inhibitor, by equilibrium dialysis gave ambiguous results for the number of active sites. The subunit structure as determined by SDS-gel electrophoresis using several protein dissociating techniques is highly unusual. In all cases, a three banded pattern was obtained with related molecular weights of 130,000-140,000, 90,000, and 60,000. The size of the catalytically active species, determined at enzyme concentrations normally used in an assay, is the same as determined by conventional techniques.
TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................. ii
VITA ........................................................................ iv
ABSTRACT .................................................................. vi
LIST OF FIGURES AND TABLES .................................. xi
LIST OF ABBREVIATIONS AND SYMBOLS ................. xiv

INTRODUCTION ............................................................. 1

Immobilized Enzymes
Primary Structure Determination of Peptides
and Proteins-Use of Immobilized Enzymes
Aminopeptidase M
Chemical Modification
Purpose

EXPERIMENTAL ........................................................... 13

Materials
Diazotization of Arylamino Glass
Coupling of Enzyme to Arylamino Glass
Enzyme Assay
Determination of the Amount of Enzyme Bound
Determination of Kinetic Parameters
pH Dependence
Temperature Dependence
Aminoethylation of Insulin and Separation of Chains
Peptide Sequencing
Total Hydrolysis of Proteins
Commercial Aminopeptidase M Purification
Inhibition Studies
p-Nitrophenyltrimethyl Acetate Hydrolysis
Tetranitromethane Modification
Diazonium Salt Modification
N-acetylimidazole Modification
Photochemical Oxidation
Tryptophan Modification
Carboxyl Group Modification
Lysine Modification

ix
Deuterium Isotope Effect Studies
SDS-Acrylamide Gel Electrophoresis
Polyacrylamide Gel Electrophoresis
Equilibrium Dialysis
Reacting Species Ultracentrifugation

RESULTS

Characterization of Immobilized Aminopeptidase-
Comparison to Soluble Enzyme
Sequencing and Total Hydrolysis of Proteins
and Peptides
Inhibition Studies-Evidence Against an Extraction
Mechanism
Chemical Modification Studies-Essential Active
Site Residues
Isotope Effect Studies
Equilibrium Dialysis
Gel Electrophoresis
Reacting Species Sedimentation

DISCUSSION

Conclusions

BIBLIOGRAPHY
### LIST OF FIGURES AND TABLES

<table>
<thead>
<tr>
<th>NUMBER</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Experimental set-up for substrate or competitive inhibitor protection experiments</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>2.</td>
<td>A representative time course of aminopeptidase M coupling to diazotized arylamino glass at 0°</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>3.</td>
<td>Representative Lineweaver-Burk plots for the hydrolysis of Leu-p-NO₂-anilide by immobilized and soluble aminopeptidase M</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>4.</td>
<td>Table of raw data used to construct Lineweaver-Burk plots for soluble and immobilized aminopeptidase M</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>5.</td>
<td>Comparison of kinetic parameters for the hydrolysis of Leu-p-NO₂-anilide by the soluble and insoluble forms of aminopeptidase M</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>6.</td>
<td>Theoretical and experimental hyperbolic relationship between v/ν and [S₀]</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>7.</td>
<td>pH profiles for the hydrolysis of Leu-p-NO₂-anilide catalyzed by the soluble and insoluble forms of aminopeptidase M</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>8.</td>
<td>Arrhenius plots illustrating the temperature dependence of Leu-p-NO₂-anilide hydrolysis catalyzed by soluble and insoluble aminopeptidase M</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>9.</td>
<td>Sequential release of amino acids from the A-chain of insulin-AE catalyzed by glass-bound aminopeptidase M</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>10.</td>
<td>Analytical data for the total hydrolysis of aminoethylated insulin A chain catalyzed by glass-bound aminopeptidase M</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
<tr>
<td>11.</td>
<td>Analytical data for the total hydrolysis of aminoethylated insulin B chain catalyzed by glass-bound aminopeptidase M</td>
</tr>
<tr>
<td></td>
<td>PAGE</td>
</tr>
</tbody>
</table>
12. Recovery of free amino acids from glass-bound pronase and aminopeptidase M hydrolyzed β-lactoglobulin  
13. Table of the effect of inhibitors on the aminopeptidase M catalyzed hydrolysis of Leu-p-NO₂-anilide and Gly-p-NO₂-anilide. 
14. A plot of log (v̅/v - 1) vs. log [I] for Leu-p-NO₂-anilide hydrolysis inhibited by L-leucine. 
15. A plot of the free energy of enzyme-inhibitor complex formation vs. molecular surface area of inhibitor for APM. 
16. Time course of inactivation of aminopeptidase M and aminopeptidase M + L-leucine by diazotized sulfanilic acid. 
17. Time course of inactivation of soluble and insoluble aminopeptidase M by tetranitromethane in the presence and absence of L-leucine and Leu-p-NO₂-anilide. 
18. Inactivation of aminopeptidase M by TNM as a function of the number of tyrosine residues modified per mole of enzyme. 
19. Inactivation of insoluble aminopeptidase M by N-acetylimidazole and reactivation by hydroxylamine. 
20. Photochemical oxidation of aminopeptidase M with rose bengal as the photosensitive dye. 
21. Plots of In (% activity) as a function of time at different pH values and pH profile of the relative rates of inactivation. 
22. Inactivation of aminopeptidase M by photo-oxidation as a function of the number of histidine residues modified. 
23. A table of the chemical modification of aminopeptidase M by various reagents.
<table>
<thead>
<tr>
<th>NUMBER</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>24.</td>
<td>Time course of lysine modification as followed by the TNBS assay for primary amino groups (340 nm)</td>
</tr>
<tr>
<td>25.</td>
<td>Lineweaver-Burk plots for the hydrolysis of Leu-p-(\text{NO}_2)-anilide catalyzed by aminopeptidase M in (\text{D}_2\text{O}) and (\text{H}_2\text{O}) buffer</td>
</tr>
<tr>
<td>26.</td>
<td>(\text{pH (pD)}) profile of the solvent deuterium isotope effect on (k_{\text{cat}})</td>
</tr>
<tr>
<td>27.</td>
<td>(\text{pH (pD)}) profile of the solvent deuterium isotope effect on (K_{M(\text{app})})</td>
</tr>
<tr>
<td>28.</td>
<td>Representation of the binding of L-leucine by aminopeptidase M ((r/A) vs. (r))</td>
</tr>
<tr>
<td>29.</td>
<td>Gel chromatographic purification of commercial aminopeptidase M on a column of Sephadex G-100</td>
</tr>
<tr>
<td>30.</td>
<td>Homogeneity of aminopeptidase M as determined on polyacrylamide gel electrophoresis (7.5% gels)</td>
</tr>
<tr>
<td>31.</td>
<td>Determination of the subunit structure of aminopeptidase M by SDS-polyacrylamide gel electrophoresis under different dissociating conditions</td>
</tr>
<tr>
<td>32.</td>
<td>A plot of the relative mobilities of the subunits of aminopeptidase M as a function of molecular weight</td>
</tr>
<tr>
<td>33.</td>
<td>Successive scanner traces of the sedimentation of the reacting species of aminopeptidase M on a single frame of reference</td>
</tr>
<tr>
<td>34.</td>
<td>A plot of the log of the radius as a function of the mean time for the sedimentation of the reacting species of APM</td>
</tr>
<tr>
<td>35.</td>
<td>Proposed mechanism of action for aminopeptidase M catalysis</td>
</tr>
</tbody>
</table>
## LIST OF ABBREVIATIONS AND SYMBOLS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>aminopeptidase M (APM)</td>
<td>The aminopeptidase from the microsomal fraction of swine kidney homogenates</td>
</tr>
<tr>
<td>A</td>
<td>Ligand concentration (L-leucine)</td>
</tr>
<tr>
<td>DDM</td>
<td>Diphenyldiazomethane</td>
</tr>
<tr>
<td>DFP</td>
<td>Diisopropylphosphorofluoridate</td>
</tr>
<tr>
<td>$E_a$</td>
<td>Energy of activation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Disodium dihydrogen ethylenediaminetetraacetate</td>
</tr>
<tr>
<td>ES</td>
<td>Enzyme-substrate complex</td>
</tr>
<tr>
<td>$\Delta G^\circ$</td>
<td>Standard free energy change</td>
</tr>
<tr>
<td>Gly-p-NO$_2$-anilide</td>
<td>Glycine p-nitroanilide</td>
</tr>
<tr>
<td>Gu·HCl</td>
<td>Guanidine hydrochloride</td>
</tr>
<tr>
<td>I</td>
<td>Enzyme inhibitor</td>
</tr>
<tr>
<td>k</td>
<td>Rate constant</td>
</tr>
<tr>
<td>$k_{cat}$</td>
<td>Catalytic rate constant</td>
</tr>
<tr>
<td>$K_M$(app)</td>
<td>Apparent Michaelis constant</td>
</tr>
<tr>
<td>$K_I$</td>
<td>Association constant for the reaction: $E + I \rightleftharpoons EI$</td>
</tr>
<tr>
<td>Leu-p-NO$_2$-anilide</td>
<td>Leucine p-nitroanilide</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>r</td>
<td>Moles ligand bound per mole enzyme</td>
</tr>
<tr>
<td>Symbol</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>$r_a$</td>
<td>Molar ratio of inhibitor to enzyme in the enzyme-inhibitor complex</td>
</tr>
<tr>
<td>$R$</td>
<td>Universal gas constant (1.987 cal deg$^{-1}$ mole$^{-1}$)</td>
</tr>
<tr>
<td>$S_0$</td>
<td>Initial substrate concentration</td>
</tr>
<tr>
<td>$s_{20,w}$</td>
<td>Sedimentation coefficient relative to water at 20°</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>$T$</td>
<td>Absolute temperature</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TNBS</td>
<td>Trinitrobenzene sulfonyl acid</td>
</tr>
<tr>
<td>TNM</td>
<td>Tetranitromethane</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris hydroxymethyl aminomethane HCl buffer</td>
</tr>
<tr>
<td>$v$</td>
<td>Initial reaction velocity in moles/liter/min</td>
</tr>
<tr>
<td>$V_{\text{max}}$</td>
<td>Maximal reaction velocity</td>
</tr>
</tbody>
</table>
"Think you, that a drop of water, which to the vulgar eye is but a drop of water, loses anything in the eye of the chemist who knows that its elements are held together by a force which, if suddenly liberated, would produce a flash of lightning? . . . . Think you that the rounded rock marked with parallel scratches calls up as much poetry in an ignorant mind as in the mind of a geologist, who knows that over this rock a glacier slid a million years ago?

The truth is, that those who have never entered upon scientific pursuits know not a tithe of the poetry by which they are surrounded."

Herbert Spencer

And would it have been worth it, after all,
Among the porcelain, among some talk of you and me,
Would it have been worth while,
To have bitten off the matter with a smile,
To have squeezed the universe into a ball
To roll it towards some overwhelming question . . .
Do I dare
Disturb the universe?
In a minute there is time
For decisions and revisions which a minute will reverse.

from "The Love Song of J. Alfred Prufrock"
T. S. Eliot

Why, all the Saints and Sages who discuss'd
Of the two worlds so learnedly, are thrust
Like foolish Prophets forth, their Words to Scorn
Are scatter'd, and their Mouths are stopt with Dust.

Myself when young did eagerly frequent
Doctor and Saint, and heard great Argument
About it and about: but evermore
Came out by the same Door as in I went.

With them the Seed of Wisdom did I sow,
And with my own hand labour'd it to grow:
And this was all the Harvest that I reap'd--
"I came like Water, and like Wind I go."

from "The Rubaiyat of Omar Khayyam"
Omar Khayyam, translated by E. J. Fitzgerald
Enzymes are members of the class of biologically important compounds known as proteins. All of these naturally occurring molecules are composed of amino acids linked together through amide linkages, more properly termed peptide bonds. Because of this unique primary sequence of amino acids, proteins and enzymes possess a definite secondary structure, as a result of short range interactions between amino acid residues, and a tertiary structure, which comprises the three dimensional conformation of the intact molecule. Besides these structures, many enzymes possessing more than one subunit are arranged according to a particular quaternary structure, depending on subunit interactions and composition.

Along with these structural aspects, enzymes are extremely efficient catalysts of chemical reactions occurring in living systems. Indeed, the vast majority of biological reactions are catalyzed by enzymes with efficiencies far surpassing most, if not all, synthetic or non-biological catalysts. Associated with this remarkable catalytic activity, there exists a discrete region on the enzyme molecule where the chemical reaction being catalyzed takes place. This area, generally known as the active site or center, contains the amino acid residues necessary for
enzymatic (catalytic) activity. This activity is brought about by the enzyme in a very specific manner. Accordingly, enzyme efficiency and specificity towards substrates, the molecules on which enzymes exert catalytic activity, have been termed by Koshland (1) the "twin miracles" of enzymatic catalysis.

**Immobilized Enzymes.** The attachment of a protein or enzyme to a solid, water-insoluble support is of great practical and theoretical interest. If at least partial enzymatic activity remains upon immobilization, the insoluble enzyme derivative can catalyze a chemical reaction and be quickly and efficiently removed from reactants and products by simple filtration. Alternatively, the insoluble enzyme can be packed into a column or reactor. Because most enzymes immobilized on solid supports exhibit enhanced operational stability, they can be used repeatedly. Such reuse and stability have made their industrial and practical applications more economically feasible.

The theoretical aspects of enzymatic reactions on solid supports cannot be minimized. Since most enzymes act in vivo while embedded in membranes or at least constrained by their surroundings, enzymes immobilized on an insoluble matrix can be used as model systems for the study of their in vivo properties. Likewise, the mode of action of enzymes can be investigated by studying the microenvironmental effects introduced by the presence of the insoluble support. Several excellent review articles dealing with immobilized
enzyme derivatives have appeared in the literature (2-7).

There are several methods used for the immobilization of enzymes and proteins onto water-insoluble supports. These include: (a) physical adsorption to ion-exchange resins or other inert supports such as charcoal or cellulose; (b) physical entrapment (inclusion) of the enzyme inside the lattice of a gel, e.g. polyacrylamide; (c) direct covalent binding to a support involving linkages to amino acid residues on the enzyme not necessary for catalytic activity; (d) covalent cross-linking of the enzyme itself using bifunctional cross-linking reagents, such as $p,p'\text{-difluoro-m,m'-dinitrodiphenyl sulfone}$ (8). Of these methods, physical adsorption is an unreliable technique due to the ease of enzyme dissociation from the support. Likewise, enzyme leakage from the matrices of enzyme-gel inclusion derivatives has been reported (9). Along with this problem, large substrates cannot be used with enzymes included within a gel because of the relatively small size of the pores of the gel matrix. However, with these precautions in mind, reliable enzyme-gel inclusion complexes have been prepared with good success (10-15).

The most widely used approach to enzyme immobilization has been the covalent attachment of enzymes to solid supports (2, 6, 16-27). The first definitive review dealing exclusively with supports (organic, inorganic, organic-inorganic) for covalent enzyme insolubilization has been prepared by Royer,
Andrews, and Uy (7). Organic polymeric supports, especially agarose derivatives, have been widely used with great success (22, 24, 28-34). In recent years, inorganic supports have received a great deal of attention, especially due to the pioneering research on porous glass-enzyme derivatives by Howard Weetall (17, 25, 35-38). Inorganic supports have several distinct advantages over organic supports. These include: (a) greater resistance to microbial attack; (b) nonsusceptibility to physical deformation by pH changes or solvent composition; (c) greater stability towards pressure deformation. This last characteristic makes inorganic supported enzyme derivatives particularly suitable for use in columns or flow-type reactors. Furthermore, it has been demonstrated that several enzyme-porous glass derivatives have greater operational and storage stability than the corresponding enzymes bound to several organic supports (39). Consequently, the enzyme immobilization described in this dissertation has been carried out using porous glass as a support in all cases.

Several methods exist for the attachment of enzymes to porous glass, all of which involve an initial silanization reaction wherein a siloxane is reacted with the porous glass:

(Glass)\text{O-Si-O}^- + \text{H}_2\text{N-CH}_3\text{Si-(OC}_2\text{H}_5)_3 \rightarrow
\begin{align*}
\text{OC}_2\text{H}_5 \\
\text{H}_2\text{N-CH}_3\text{Si-O-Si-O-(Glass)} \\
\text{OC}_2\text{H}_5
\end{align*}
The primary amino group of the silanized glass can be linked to protein carboxyl groups with a carbodiimide. A more widely used approach, and the method of choice in this dissertation, involves coupling the silanized glass to ε-amino groups and phenolic rings of enzymes through diazo linkages. The silanized glass is first reacted with p-nitrobenzoyl chloride:

\[
\text{(Glass) -O-Si-O-Si-(CH}_3)_3\text{-NH}_2 + \text{Cl-C-NO}_2 \rightarrow \\
\text{(Glass) -O-Si-O-Si-(CH}_3)_3\text{-N-H-C-NO}_2 \text{Cl}^-
\]

The nitro group is now reduced to give an aryl amine, which is subsequently diazotized to yield a diazonium salt:

\[
\text{(Glass) -O-Si-O-Si-(CH}_3)_3\text{-NH-C-NO}_2 \overset{(1) \text{ reduction}}{\longrightarrow} \\
\text{(Glass) -O-Si-O-Si-(CH}_3)_3\text{-NH-C-N}_2^+ \text{Cl}^-
\]

This aryl diazonium salt can then be reacted with protein ε-amino groups (lysine residues) and phenolic moieties (tyrosine residues):

\[
\text{(Glass) -NH-C-N}_2^+ \text{Cl}^- \overset{\text{Enz-NH}_2 \text{ or Enz-} \phi-\text{OH}}{\longrightarrow} \text{(Glass) -NH-C-NN-Enz}
\]
Even though soluble diazonium salts will react with imidazole (40), guanidinium (41), and indole (42) moieties, as well as with primary amino and phenolic groups, it has been shown that trypsin bound to diazotized porous glass at pH 8.0 involves linkages between only the latter two groups (43).

**Primary Structure Determination of Peptides and Proteins-Use of Immobilized Enzymes.** The primary sequence of amino acids in proteins and enzymes directly dictates the three dimensional structure, the catalytic activity, and the subunit interactions (quaternary structure) of the macromolecule. Accordingly, the elucidation of amino acid composition and sequence of proteins and peptides is of paramount importance to the protein chemist. Such an analysis requires that the protein or peptide in question first be hydrolyzed to its constituent amino acids, followed by quantitative analysis of the resultant hydrolysate. The research of Stein and Moore on automated amino acid analysis has progressed to the point where hydrolysate analysis is simple, relatively swift, and quantitatively reliable (44-46). In addition, only a few milligrams of protein are necessary for determination of its amino acid composition.

The conventional hydrolysis of proteins and peptides is carried out in strong acid or strong base. Normal acid hydrolysis is accomplished by heating in sealed, evacuated tubes at 110°C in constant boiling, 6N HCl, for periods ranging from 24-72 hours (47). Likewise, alkaline hydro-
lysis is achieved by incubating proteins in 4N sodium hydroxide at 100° for 8-12 hours. Both of these methods, however, have several relatively serious limitations. During acid hydrolysis, tryptophan, glutamine, asparagine, and phospho- or sulfo-esters are completely destroyed, and small losses of serine and threonine are usually encountered. Also, relatively long periods of hydrolysis are needed for the complete liberation of leucine, isoleucine, and valine (48). The limitations of alkaline hydrolysis are even more drastic. Cysteine, cystine, serine, threonine, and arginine are completely destroyed, and other amino acids may be partially decomposed due to deamination. Furthermore, racemization of all the amino acids occurs.

Recently, enzymatic hydrolysis of proteins has been used to prepare hydrolysates for amino acid analysis (49-51). Obviously, the use of enzymes to hydrolyze peptide bonds alleviates the problems associated with acid or alkaline hydrolysis. However, several inadequacies are apparent with the use of soluble proteolytic enzymes. First, it is often difficult to separate the proteolytic enzymes from the amino acids after hydrolysis has occurred. Second and more important, contamination of the hydrolysate often results due to proteolysis of the enzymes themselves. The use of insolubilized proteolytic enzymes for the digestion of proteins would seem to overcome these problems. The enzymes can be easily removed from the hydrolysate
by filtration, and the danger of autolysis is essentially eliminated with solid-supported enzymes. In addition, it has been demonstrated that many immobilized enzymes retain most of their activity at higher temperatures or in solvents (e.g. urea solutions) which would denature most soluble proteins (52). Obviously, both of these conditions would facilitate protein hydrolysis while leaving the insoluble proteolytic enzymes relatively unchanged.

Along with the total amino acid composition of proteins, the sequential analysis of the primary structure is of great interest. Soluble enzymes have been used in this regard with some success (53-57). However, the use of soluble enzymes for sequential analysis has the same limitations as mentioned above for the total hydrolysis of proteins. Again, insolubilized enzymes would alleviate these problems. The time course of appearance of free amino acids could be easily obtained with no contamination from the proteolytic enzymes used in the study.

Aminopeptidase M. Of the many catalytic functions of enzymes, the hydrolysis and cleavage of the peptide bonds in other proteins or peptides has received great attention (58). The general class of enzymes known as peptidases can be divided further into enzymes hydrolyzing internal peptide bonds (endopeptidase) and enzymes cleaving terminal peptide linkages (exopeptidases). In contrast to carboxypeptidases, which catalyze peptide bond hydrolysis from the carboxyl
terminii of proteins, the aminopeptidases sequentially release amino acids from the N-terminal end of a peptide chain.

Aminopeptidases have been found in a wide variety of organs and organisms (59-68). Recently, Pfleiderer and Celliens have shown that the majority of aminopeptidase activity in swine kidney was destroyed by acetone treatment (69). They were subsequently able to isolate small amounts of this new enzyme, aminopeptidase M, from the microsomal fraction of swine kidney homogenates (69). Wachsmuth et al. have developed an improved method of preparation of homogeneous enzyme which entails swelling the microsomal particles with toluene followed by trypsin treatment (70). The enzyme is stable for long periods of time when kept cool and dry, and solutions of the enzyme can be kept for days in buffered solution even at room temperature with little lose of activity.

Aminopeptidase M, in contrast to most other aminopeptidases, catalyzes the release of amino acids from N-terminii at rates which differ by only a few fold (70-72). Also, proline is released, although much more slowly (70). Thus, aminopeptidase M has iminopeptidase (hydrolysis of peptides having a free α-imino group) as well as aminopeptidase activity, although there is some doubt as to whether the former is due to a contaminating enzyme (73). These properties make aminopeptidase M highly suited for the sequential analysis
and amino acid composition determination of proteins and peptides. Pfleiderer et al. (71) demonstrated that the soluble enzyme hydrolyzed glucagon and the oxidized B-chain of insulin almost completely into free amino acids. Likewise, the hydrolysis of the S-sulfonated B-chain of insulin, including the release of proline, was reported by Katsoyannis et al. (74). Along with these results, aminopeptidase M was used successfully in the sequential analysis of the essential cysteine peptide of pig heart lactate dehydrogenase (75).

Despite the wide distribution and central importance of this enzyme, little work has been done on either its mechanism of action, the amino acid residues necessary for catalytic activity, or its physical properties. Preliminary work on the active site composition of aminopeptidase M has been reported using rather non-specific chemical modification of amino acid residues (76-77). However, even with high molar excesses of modifying reagent, only as much as 70% of the native enzymatic activity could be destroyed. Furthermore, no protection of the active site by substrates or competitive inhibitors was demonstrated during amino acid modification.

Information about the physical properties of aminopeptidase M is even more sparse. Gel chromatography (78) and ultracentrifugation (79) data have indicated a molecular weight of about 280,000. However, in both of these techniques, the enzyme concentration was high enough that enzyme association was a distinct possibility. The molecular weight of
the catalytically active species was not reported. Data on
the subunit structure of the enzyme is also in a state of con­
fusion. Wachsmuth first reported that the enzyme was com­
posed of 10 subunits of two different types (80). Since then,
Wacker et al. (79) have cast serious doubt on this number
from preliminary results obtained from disc gel electropho­
resis following SDS treatment. No evidence for a species
of molecular weight less than 60,000 was found.

Chemical Modification. The first step in deducing even
the most simple enzymatic mechanism of action is the eluci­
dation of the amino acid residues at the active site necessary
for catalytic activity. Of the many approaches to this prob­
lem, the use of chemical reagents to specifically modify the
side chains of amino acid residues in an enzyme has become
a powerful diagnostic tool. The many reagents for chemical
modification and their specificity towards different amino
acids have been reviewed and compiled by several authors
(81-87). The correlation of a loss of enzymatic activity
with destruction (modification) of a particular amino acid
residue, as deduced by modification reagent specificity and
quantitative data, provides direct evidence for the necessity
of that residue in the catalytic mechanism of the enzyme.
Further evidence for the presence of essential residues at
the active site can then be provided by substrate or inhibitor
protection experiments. These are based on the fact that a
substrate or competitive inhibitor will protect the active
site, and any amino acid residues therein, from modification. Accordingly, the rate of essential residue modification and, hence, loss of enzymatic activity, is drastically reduced in the presence of the protecting agent than in its absence.

**Purpose.** The objectives of these investigations were: (a) to prepare and characterize an insoluble, porous glass-bound derivative of aminopeptidase M; (b) to compare the insoluble enzyme kinetic parameters to those of the soluble enzyme form; (c) to show the value of an immobilized derivative of aminopeptidase M in the sequential analysis and determination of the total amino acid composition of proteins and peptides; (d) to attempt a comprehensive chemical modification study of aminopeptidase M in the hope of establishing the catalytically essential nature of certain amino acid residues at the active site; (e) to establish the effect of aromatic and aliphatic hydrocarbon, amine, and amino acid inhibitors on Leu-p-NO₂-anilide and Gly-p-NO₂-anilide hydrolysis; (f) to demonstrate the presence or absence of a hydrophobic binding site and whether or not an extraction mechanism occurs in the binding of inhibitors to enzyme; (g) to relate the essential amino acid residues and the effect of D₂O on Leu-p-NO₂-anilide hydrolysis by aminopeptidase M to a proposed mechanism of action for the enzyme; and finally (h) to elucidate the subunit structure of aminopeptidase M and to determine the size of the catalytically active species.
EXPERIMENTAL

Materials. Aminopeptidase M from swine kidney was purchased from Rohm and Haas and Sigma Chemical Company (leucine aminopeptidase, type IV). Arylamino glass was supplied by Corning Glass Company, Corning, New York. Insulin (Lot No. T2842) was the generous gift of Dr. Bruce Frank, Eli Lilly Co. N-ethylmorpholine, p-nitrophenyl trimethylacetate, glycine ethyl ester, N-acetylimidazole, and deuterium oxide (99.6%) were purchased from Aldrich Chemical Company. Leu-p-NO$_2$-anilide, bovine serum albumin, L-leucine, tetranitromethane, L-leucine amide, and Gly-p-NO$_2$-anilide were obtained from Sigma Chemical Company. Constant boiling 6N HCl, dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide, and TNBS were products of Pierce Chemical Company. IRC-50 resin, β-mercaptoethanol, acrylamide, methylenebisacrylamide, ammonium persulfate, SDS, TEMED, and Coomassie Brilliant Blue R-250 were from Bio-Rad Laboratories. Except for the resin, all were electrophoresis purity grade. Tris (ultra pure), DFP, and β-lactoglobulin were purchased from Schwartz Mann. Ethylenimine, rose bengal, and bromophenol blue were obtained from Matheson, Coleman, and Bell. The radioactive compounds, L-leucine-C$^{14}$ and glycine-1-C$^{14}$ ethyl ester hydrochloride were supplied by New England Nuclear. Amino acid standards were provided by Beckman Instruments. Guanidine hydrochloride was from Heico, Inc., and 1-ethyl-3-dimethylaminopropylcarbodiimide from Ott Chemical Co. All other chemicals were reagent grade.
Diazotization of Arylamino Glass. In a typical reaction, 200-300 mg of arylamino glass were suspended in 50 ml of 1N HCl with mechanical stirring provided by a Tri-R Stir-R Model 563C. The reaction flask (125 ml) was suspended in an ice-water bath. Drops of 0.5N sodium nitrite were added until an excess was indicated with starch-iodide paper (about 0.2-0.3 ml). The mixture was stirred at 0°C for 15 minutes. After this time, the diazotized glass was collected on a sintered glass funnel (medium frit) and washed with 200 ml of cold, 3% sulfamic acid followed by cold, distilled water (400 ml). The diazotized glass was kept moist and coupled to the enzyme immediately.

Coupling of Enzyme to Arylamino Glass. The previously prepared diazotized glass was added to a conical centrifuge tube (12 ml) containing a solution of aminopeptidase M (3-4 mg) in 0.1M Tris buffer (pH 7.3) which was 1mM in MnCl₂. The tube was connected to a constant torque stirrer and rotated slowly in an ice-water bath. The stirrer was stopped periodically and aliquots of the supernatant were removed and assayed for enzymatic activity. When the coupling was complete, as judged by depletion of enzymatic activity in the supernatant, the enzyme-glass derivative was filtered through a sintered glass filter (medium frit) and washed with 1000 ml of cold
0.1M Tris buffer (pH 7.3). The preparation was stored at 4°C as a moist cake.

Enzyme Assay. Leu-p-NO₂-anilide was used as the substrate for all assays of aminopeptidase activity. The hydrolysis of the substrate was followed by monitoring the appearance of p-nitroaniline at 405 nm (89) with a Cary model 15 spectrophotometer. The temperature was controlled to ± 0.2°C by a Haake Constant Temperature Thermal Circulator connected to the cell holder. A typical assay performed at saturating levels of substrate included .1 ml of Leu-p-NO₂-anilide (15 mg/ml in absolute methanol), .1 ml of enzyme solution (.5 mg/ml), and 2.8 ml of 0.1M Tris buffer (pH 7.5). Initial velocities were calculated by measuring the concentration of p-nitroaniline hydrolyzed per unit of time. An extinction coefficient of 9.9 X 10³ M⁻¹ cm⁻¹ was used (89).

In order to assay the insoluble enzyme-glass derivative, a quantity of immobilized enzyme was suspended in the above assay mixture and stirred at a constant rate with a Tri-R Ms-7 immersible stirrer in a water bath. After a given time interval (1-2 min), the mixture was rapidly filtered by vacuum filtration through a Millipore filter. The optical density of the filtrate was then measured at 405 nm. The amount of enzyme-glass derivative was normalized to a definite, predetermined value. This provided a means of holding the concentration of enzyme in the assay mixture constant.
Determination of the Amount of Enzyme Bound. At timed intervals, the enzymatic activity of the supernatant in the coupling reaction was determined by following the hydrolysis of Leu-p-NO₂-anilide as previously described. The depletion of activity in the supernatant was then used to calculate the amount of enzyme bound by subtracting the amount of enzyme present at the end of coupling from the amount present at time zero. The washings of the enzyme-glass derivative were assayed for enzymatic activity which had washed off the glass and represented loosely bound enzyme. For this aminopeptidase-arylamino glass system, all of the activity was accounted for either as tightly bound enzyme or free enzyme.

The amount of enzyme bound to the glass support was also determined by amino acid analysis as outlined by Spackman et al. (46). In a typical experiment, a sample of enzyme-glass derivative was air-dried at room temperature until a constant weight was attained. This usually took no longer than 24 hours. A sample of the dry preparation was weighed (5-10 mg) to the nearest hundredth milligram and placed in a hydrolysate tube made from a 12 ml Pyrex test tube. Constant boiling 6N HCl (3 ml) was added and the contents were frozen in an acetone-dry ice bath. The tube was then evacuated with a vacuum pump and sealed with a torch. After heating at 110° for 24 hours, the tube was broken open and the HCl was removed in a heated des-
iccator (65°) with a water aspirator. Citrate buffer (1 ml, pH 2.2) was added to dissolve the hydrolysate and 0.5 ml of this was added directly to the short column of a Beckman amino acid analyzer Model 116. The arginine peak area was used to calculate the amount of enzyme in the enzyme-glass hydrolysate by comparison with the peak height from the hydrolysate of a known amount of soluble aminopeptidase. The soluble form of the enzyme was hydrolyzed in the same manner as described above.

The two methods of determining the amount of enzyme bound were in good agreement. In most cases, the average value was close to 1% (w/w).

Determination of Kinetic Parameters. Using the enzyme assay described above, the change in optical density at 405 nm per unit of time was determined using the synchronous gear system of the Cary 15 spectrophotometer. In most cases, gears 3 and 4 were used, which gave a chart speed of 6 divisions per minute. The optical density change per time was converted into initial velocities by dividing by the extinction coefficient of 9,900 (88). In this way, initial velocities of substrate hydrolysis were computed at different substrate concentrations. Standard double reciprocal or Lineweaver-Burk plots were constructed by plotting 1/v vs. 1/S₀, where v is the initial velocity and S₀ represents initial substrate concentration. The maximal velocity (Vₘₐₓ) and the apparent Michaelis constant (Kₘ(app))
were determined from the \( x \) intercept and \( y \) intercept respectively. Because this graphical method of determining kinetic parameters is tedious and statistically rather inaccurate, the constants were subsequently determined on a computer using a method of regression analysis developed by Wilkinson (89). The method is basically a weighted least squares treatment of the data. The catalytic constant, \( k_{\text{cat}} \), was then easily determined by dividing the maximal velocity by the initial enzyme concentration \( (E_0) \). The molecular weight used for the calculation of \( E_0 \) was 280,000 for the aminopeptidase (79). The kinetic constants, \( k_{\text{cat}} \) and \( K_M(\text{app}) \), occur in the Michaelis-Menten equation:

\[
\frac{\text{d}p}{\text{d}t} = \frac{k_{\text{cat}}E_0S_0}{K_M(\text{app}) + S_0}
\]

which applies to the simple scheme:

\[
E + S \underset{k_{\text{cat}}}{\overset{\text{ES}}{\rightleftharpoons}} E + P
\]

\( E, S, \) and \( P \) are the enzyme, substrate, and product concentrations, respectively. The experimental errors in the rate determinations were within \( \pm 5\% \).

**pH Dependence.** Tris buffers (0.1M) of different pH values were prepared to the nearest 0.01 pH unit at 25.0\(^\circ\)C using a Radiometer pH meter 26. Aminopeptidase was dissolved in the buffers to give an enzyme concentration of 0.01 mg/ml. Initial velocities were calculated as before from a Cary 15 spectrophotometer by following Leu-\( p \)-NO\(_2\)-anilide hydrolysis at different initial substrate concentra-
trations. After calculating the kinetic constants, $k_{\text{cat}}$ and $K_{M(\text{app})}$, pH profiles were constructed by plotting $k_{\text{cat}}/K_{M(\text{app})}$ vs. pH. The pH ranged from 6.0 to 9.5.

The procedure for assaying immobilized aminopeptidase was essentially the same as outlined in "Enzyme Assay" except that buffers of various pH values were used. The maximal velocity ($V_{\text{max}}$) and, hence, the catalytic constant, $k_{\text{cat}}$, of the insoluble derivative were normalized to the amount of enzyme used in the assay for soluble aminopeptidase.

**Temperature Dependence.** For the determination of the temperature dependence of Leu-p-NO$_2$-anilide hydrolysis with soluble aminopeptidase, the enzyme (.27 mg) was dissolved in 3 ml of 0.1M Tris buffer (pH 7.5). The anilide substrate was dissolved in absolute methanol (30 mg/ml). The rate of substrate hydrolysis was monitored with a Cary 15 spectrophotometer at different temperatures by means of a Haake constant temperature circulator connected to the cell holder. The temperature was controlled to $\pm 0.2^\circ$. At each temperature, the assay mixture consisted of 2.8 ml buffer (0.1M Tris, pH 7.5), 0.1 ml enzyme, and 0.1 ml Leu-p-NO$_2$-anilide in methanol (30 mg/ml). Standard Arrhenius graphs were constructed by plotting $\ln v$ vs. $1/T$, where $v$ is the velocity of substrate hydrolysis and $T$ is the temperature in degrees Kelvin. The points at
high temperatures where enzyme denaturation was significant were discarded and the slope of the linear line was determined by least squares analysis. The energy of activation ($E_a$) was then determined by multiplying the slope by the universal gas constant, $R$ (1.987 cal deg$^{-1}$ mole$^{-1}$).

The energy of activation of the insoluble enzyme was determined by assaying as previously described at substrate saturation. The weight of enzyme-glass derivative was normalized to a pre-determined, constant value. The same buffer system and substrate concentration were used as before. A water bath (Precision Scientific, Model 83) was used to regulate the temperature of the reaction.

Aminoethylation of Insulin and Separation of Chains.
The disulfide bonds of insulin were reduced and aminoethylated according to the procedure of Cole (90). In a 125 ml Erlenmeyer flask was added 0.5 g insulin in 50 ml 8M urea, 2.5 ml EDTA solution (2 mg/ml), 15 ml 3M Tris buffer (pH 8.6), and 0.5 ml 2-mercaptoethanol. The mixture was allowed to stand for five hours at room temperature under a nitrogen atmosphere. After this time, three aliquots (2 ml) of ethylenimine were added at 10 minute intervals. The reaction was followed by use of the nitroprusside spot test (91). A negative nitroprusside test usually resulted within thirty minutes. The protein was then separated from salts and other reagents by gel filtration. The mixture (in two 30-35 ml portions) was passed through a column of Sephadex
G-25 (5 x 60 cm) equilibrated with 0.2N acetic acid. After identifying the fractions by measuring the absorbance at 280 nm of the eluate, the aminoethylated insulin was lyophilized and stored in a desiccator at 4°.

The procedure of Humbel et al. (92) was used for the chromatographic separation of the aminoethylated insulin A and B chains. Aminoethylated insulin (130 mg) was dissolved in 5 ml of 25% acetic acid (v/v) and applied to a column (2.5 x 10 cm) of Bio-Rad IRC-50 resin (minus 400 mesh) equilibrated with 25% acetic acid. The aminoethylated A chain was eluted with 200 ml of 25% acetic acid (v/v) and identified by its absorbance at 280 nm. Subsequently, the aminoethylated B chain was eluted with 60% acetic acid (v/v). The two separate fractions were diluted with 100 ml of water and lyophilized. The yield of aminoethylated A and B chains was 46 mg and 67 mg, respectively. Purity of the two chains was determined by amino acid analysis of a 24 hour acid hydrolysate on a Beckman Model 116 amino acid analyzer according to the method of Spackman et al. (46).

Peptide Sequencing. In order to assure that contamination by serine proteases during the sequencing experiments was negligible, the immobilized aminopeptidase was treated with DFP by the standard procedure before sequencing (53). The immobilized enzyme was highly stirred for 30 minutes in .1M Tris buffer containing a 50-fold molar excess of diisopropyl phosphorofluoridate (0.1M in isopropanol)
by means of a Tri-R constant torque stirrer. The immobilized derivative was washed copiously with .1M Tris buffer (pH 7.5).

Digestions were performed with 1 mM solutions of either aminoethylated insulin A or B chains in 0.2N N-ethylmorpholine acetate buffer (pH 7.5). Occasionally, a small amount of acetic acid was used to dissolve the B chain. A screw cap test tube (15 ml) with baffled sides was used as a reaction vessel. The protein solution (5 ml) was put in the tube and 150 mg (wet weight) of insoluble aminopeptidase was added. The tube was rotated slowly in a constant temperature bath (35°C) by means of a Tri-R constant torque stirrer. At timed intervals, aliquots were removed for analysis by both thin layer chromatography and amino acid analysis. Eastman-Kodak Chromagram cellulose plates (no. 6064 without fluorescent indicator) were spotted with 15 μl of the reaction mixture and 5 μl amino acid standards (1 mg/ml in methanol). The chromatogram was developed with a solvent system of n-butanol/acetone/diethylamine/water (10:10:2:5, v/v). Amino acids were detected after spraying with ninhydrin spray (Ninspray, Pierce Chemical Co.). Samples (0.2 ml) withdrawn at timed intervals for amino acid analysis were lyophilized, dissolved in 1 ml citrate buffer (pH 2.2), and subjected directly to amino acid analysis on a Beckman Model 116 amino acid analyzer (46).
Total Hydrolysis of Proteins. The complete hydrolysis of aminoethylated insulin A and B chains was carried out with immobilized aminopeptidase (150 mg) as previously described under sequencing, except that the reaction tube was rotated for 24 hours. Aliquots (.2 ml) were removed, lyophilized, and subjected to amino acid analysis as before. The enzymatic hydrolyzate was compared to a standard acid hydrolyzate using 6N constant boiling HCl at 110°. Serine was not determined since asparagine and glutamine fall under the peak. Aminoethylcysteine appeared on the trace from the short column as a lone peak between lysine and histidine. The ninhydrin color value for aminoethylcysteine was found to be 5% below that of lysine (90).

For the total hydrolysis of β-lactoglobulin, 5 ml of the protein (1 mM) in 0.2N N-ethylmorpholine acetate buffer (pH 7.5) was placed in a baffled reaction vessel (15 ml) similar to that used in sequencing experiments. Immobilized pronase (75 mg) prepared as described in an earlier publication from our laboratory (93) was added and the tube was rotated by a constant torque motor for 6 hours at room temperature. The bound pronase was removed by filtration and immobilized aminopeptidase (150 mg) was added to the protein solution which was now partially hydrolyzed by the pronase. The tube was rotated for 24 hours in a constant temperature (35°) water bath. After this time, the contents were filtered and an aliquot (.2 ml) was lyophilized.
and dissolved in 1 ml of citrate buffer (pH 2.2). The hydrolysate (0.5 ml) was applied to both columns of a Beckman Model 116 amino acid analyzer and compared with a standard acid hydrolysate of β-lactoglobulin.

**Commercial Aminopeptidase M Purification.** The commercial Rohm and Haas aminopeptidase M was purified further by gel chromatography. The material from one vial of enzyme (50,000 units, 10 mg) was dissolved in 2 ml of 0.1M phosphate buffer (pH 8.0) and applied directly to the top of a Sephadex G-100 column (2.5 x 30 cm) previously equilibrated with 0.1M phosphate buffer (pH 8.0). To facilitate layering of the protein solution on the column, a few drops of glycerol were added to the sample before application. The column was eluted with the same buffer and 0.5 ml fractions were collected by means of an LKB Ultrorac fraction collector, Model 7000. Fractions containing protein were identified by their absorbance at 280 nm on a Cary 15 spectrophotometer. The specific activity of the protein fractions was determined using Leu-p-NO₂-anilide as substrate.

Aminopeptidase (0.1 ml of a 1:20 dilution from the fractions) was added to 2.7 ml of 0.01M phosphate buffer (pH 8.0) and 0.1 ml of Leu-p-NO₂-anilide (10 mg/ml in absolute methanol). The rate of p-nitroaniline appearance was followed at 405 nm with a Cary 15 spectrophotometer. Protein concentration was determined either by the method of Lowry et al. (94) using bovine serum albumin as a standard, or spectrophotometrically by employing the absorbancy coefficient, $E_{280}^{1\%} = 16.9$. 
Inhibition Studies. The effect of hydrocarbon, amino acid, amine, and amide inhibitors on the hydrolysis of Leu-p-NO$_2$-anilide by aminopeptidase M was studied. The kinetic parameters, $k_{cat}$ and $K_M$(app), were determined from Lineweaver-Burk plots as previously described. In most cases, the inhibitor was dissolved in methanol and added to the buffer solution (.1M Tris, pH 7.5) with rapid stirring to effect solution. The type of inhibition for each inhibitor was determined from Lineweaver-Burk graphs. In the case of competitive inhibition, $K_I$ was determined from the relationship:

$$K_I = \frac{[I]}{K_p/K_M - 1}$$

where $[I]$ is the inhibitor concentration, $K_p$ the apparent Michaelis constant of the inhibited enzyme, and $K_M$ the apparent Michaelis constant of the uninhibited enzyme. For non-competitive inhibition, $K_I$ was determined from:

$$K_I = \frac{[I]}{V_{max}/V_p - 1}$$

where $V_{max}$ and $V_p$ are the maximal velocities of substrate hydrolysis for the uninhibited and inhibited enzyme, respectively. All these parameters were determined from Lineweaver-Burk plots using the regression analysis of Wilkinson (89). Having obtained $K_I$, $\Delta G^0$ was calculated from the
equation, \[ \Delta G^0 = -RT \ln K_I \], where \( R \) is the universal gas constant and \( T \) is the absolute temperature.

For one of the amino acid inhibitors, L-leucine, \( r_a \), the molar ratio of inhibitor to enzyme in the enzyme-inhibitor complex, was determined by the method of Johnson et al. (95). A plot of \( \log \left( \frac{v_0}{v} - 1 \right) \) versus \( \log [I] \) gave a linear slope which was equal to \( r_a \). In this relation, \( v_0 \) is the velocity of uninhibited enzyme catalyzed substrate hydrolysis, while \( v \) is the velocity corresponding to the inhibited enzyme.

\textbf{p-Nitrophenyltrimethyl Acetate Hydrolysis.} In an attempt to demonstrate burst kinetics during hydrolysis by aminopeptidase M, a poor substrate, p-nitrophenyltrimethyl acetate, was used. The substrate (0.7 mg/ml in acetonitrile) was added to 2.9 ml of Tris buffer (pH 7.5) in a 4 ml quartz cuvette. Aminopeptidase (0.5 or 1 mg) was rapidly added to the cuvette with a Precision Cell Add-A-Mixer and substrate hydrolysis was followed at 400 nm by means of a Cary 15 spectrophotometer. The time between mixing and the start of substrate hydrolysis monitoring was less than two seconds in all cases. No initial burst could be detected even at the highest enzyme concentrations.
Tetranitromethane Modification. The procedure of Sokolovsky et al. (96) for the nitration of phenolic groups was used for soluble aminopeptidase M. The reaction was carried out in 0.05M Tris buffer (pH 8.0) at room temperature. The enzyme solution was treated with an aliquot (10 μl) of tetranitromethane in absolute ethanol to give a final TNM concentration of 1 x 10^{-4}M. Aliquots were removed at timed intervals and assayed in 0.1M phosphate buffer (pH 7.8) with Leu-p-NO_2-anilide (2mM) as substrate. The hydrolysis of the substrate was followed by monitoring the appearance of p-nitroaniline at 405 nm with a Cary 15 spectrophotometer. The nitrotyrosyl residues were readily quantitated from their absorbance at 428 nm and extinction coefficient of 4.1 x 10^3 (96).

Insoluble aminopeptidase was inactivated and assayed directly in a column. Five hundred milligrams of enzyme-glass derivative (27) was placed in a column and inactivated by pumping a 10^{-4}M solution of tetranitromethane in 0.05M Tris (pH 8.0) through the column system at a constant flow rate. The enzyme was assayed at various timed intervals by pumping substrate solution (2mM in 0.05M Tris buffer, pH 8.0) through the column after washing with buffer to remove TNM. A value for 100% enzyme activity was obtained by pumping substrate through the enzyme-column apparatus at the same flow rate and measuring the A_{405} of the eluate before modifying the insoluble enzyme with TNM.
For the substrate and inhibitor protection experiments, gradient bottles were attached to the column containing the immobilized enzyme as shown in Figure 1. One bottle contained a tetranitromethane solution (10^{-4} M) in 0.05 M Tris buffer (pH 8.0) and the other contained a 5.0 \times 10^{-3} M solution of Leu-p-NO_2-anilide, a specific substrate, or L-leucine, a competitive inhibitor, in the same buffer. Thus, both substrate (or inhibitor) and inactivating reagent were pumped through the column simultaneously and maintained at constant concentrations.

**Diazonium Salt Modification.** A stock solution of sulfanilic acid was prepared by dissolving one gram of the acid in water (100 ml) to which 10 ml concentrated hydrochloric acid had been added. Just before use, one volume of sulfanilic acid was added to 2 volumes of sodium nitrite (0.5%, w/v) at 0°C. Aminopeptidase M (.5 mg) was dissolved in .1 M Tris, pH 7.5 and dizotized sulfanilic acid (.2 ml) was added. The reaction was carried out at 0°C to slow the decomposition of the diazonium salt. Aliquots were removed at timed intervals and assayed with Leu-p-NO_2-anilide. To show that leucine will decrease the rate of enzymatic inactivation, the final enzyme solution was made $10^{-5}$ M with respect to L-leucine. The same concentration of diazotized sulfanilate was used throughout the experiment.
Figure 1. Experimental set-up for substrate or inhibitor protection experiments. The two reagents are mixed and pumped through the column of immobilized aminopeptidase. Side reactions between the two reagents are minimized because of the short (< 10 sec) exposure of one to the other. Protection of the enzyme by a specific substrate is made possible because of the short exposure to enzyme and by maintaining substrate at constant levels.
PROTECTING REAGENT (INHIBITOR OR SUBSTRATE)

MODIFYING REAGENT

MIXING AREA

IMMOBILIZED ENZYME COLUMN
The decomposition of diazotized sulfanilate at 0°
cwas followed spectrophotometrically (470 nm) with a
Cary 15 spectrophotometer by coupling 0.2 ml aliquots of
reagent at timed intervals with 3 ml of a 10 mM α-naphthol
solution.

**N-acetylimidazole Modification.** Soluble aminopeptidase
(1 mg) was dissolved in 2 ml of 0.05M Veronal buffer (pH 7.5)
and 1 mg of N-acetylimidazole was added. Aliquots were
removed at timed intervals and assayed. For insoluble
aminopeptidase M, a 10^-4M solution of N-acetylimidazole
in 0.05M Veronal buffer (pH 7.5) was pumped through a
column of immobilized enzyme at a constant flow rate as
described under "Tetranitromethane Modification". Activity
was measured with Leu-p-NO₂-anilide after first washing
the column with buffer to remove inactivating reagent.
After four hours, a 0.5M solution of hydroxylamine was
pumped through the column. Activity was monitored at
timed intervals as before.

**Photochemical Oxidation.** A modified procedure of Ray
and Koshland was used (97). One ml of a solution of aminopeptidase (.5 mg) and rose bengal (0.01%) in 0.1M phosphate
buffers (pH range: 5.5-8.5) was prepared in the dark and
placed in a glass water bath at 18°. A light source (Sears
model number 8820, 650 watt, 120 volt) was placed 10 cm
from the water bath. The solution was irradiated for timed
intervals and assayed immediately. Pseudo-first order rate constants were calculated from graphs of ln (% activity) vs. time. Quantitative determination of the number of histidine residues oxidized was done by amino acid analysis on a Beckman Model 116 amino acid analyzer by comparison with an unmodified aminopeptidase hydrolysate according to the method of Spackman et al. (46).

Tryptophan Modification. Indole group modification was done with a modified Koshland's reagent according to the procedure of Horton and Tucker (98). Dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide (0.5 mg was added as a solid to 1 ml of aminopeptidase solution (1 mg/ml) in 0.03M phosphate buffer (pH 5.9). The pH was kept constant by the addition of 0.5N NaOH. The enzyme was assayed at timed intervals in 0.1M phosphate buffer (pH 7.8) with Leu-p-NO₂-anilide as before. After 120 minutes of reaction, the solution was dialyzed against water for 24 hours to remove inactivating reagent. The pH of the dialysate was brought to pH 10.5 with the addition of 2N NaOH and the absorbance was read at 405 nm by means of a Cary 15 spectrophotometer. Concentration of modified tryptophan was determined using an extinction coefficient of 18,000 (99).

Carboxyl Group Modification.

a) Glycine Ethyl Ester + Carbodiimide. Carboxyl group
modification was carried out with a water-soluble carbodi-imide and glycine ethyl ester according to the procedure outlined by Hoare and Koshland (100). A solution of aminopeptidase M (1.31 mg/ml) was adjusted to pH 4.75 with 0.05N HCl. Glycine-1-C\textsuperscript{14} ethyl ester (0.2 ml, 1.29mM) and cold glycine ethyl ester (0.2 ml, .67M) were added to the enzyme solution with stirring. The reaction was initiated by the addition of 1-ethyl-3-dimethylaminopropylcarbodiimide (5 mg). The pH of the reaction mixture was maintained at pH 4.75 by the addition of 0.05N HCl. The enzyme was assayed at timed intervals in phosphate buffer (0.1M, pH 7.8) with Leu-p-NO\textsubscript{2}-anilide as the substrate. At the end of 120 minutes, the reaction mixture was placed in a Visking dialysis bag and dialyzed extensively against distilled water. An aliquot (1.0 ml) was added to 15 ml of scintillation "cocktail" (5 l dioxane, 2.5 l tetrahydrofuran, 750 g naphthalene, 75 g 2,5-diphenyloxazole, 1.87 g p-Bis-[2-(5-phenyloxazoyl)]-benzene) in a screw-capped vial. The sample was counted in a Packard liquid scintillation counter.

The number of moles of glycine bound, and hence the number of moles of carboxyl groups modified per mole of enzyme, was calculated from the specific activity of the glycine-1-C\textsuperscript{14} ethyl ester, the ratio of cold to radioactive glycine ethyl ester, and the concentration of aminopeptidase M (determined from its absorbance at 280 nm).
b) Diphenyldiazomethane. Diphenyldiazomethane was prepared by the method of Smith and Howard (101). Benzophenone hydrazone (96%, 200 mg) and yellow HgO (400 mg) were suspended in petroleum ether (20 ml, bp 30-60°) in a 50 ml round bottom flask. Water (0.04 ml) was added, the flask was stoppered, and the mixture was stirred for 2 hours at room temperature with a magnetic stirrer. After this time, anhydrous MgSO₄ was added and the mixture was stirred for an additional 30 minutes. The solution was dark red in color after completion of reaction. After gravity filtration through Whatman No. 1 filter paper, the filtrate was evaporated under reduced pressure to yield a red oil. Absolute ethanol (50 ml) was added and the stock solution was kept at -20° until required. The concentration of ethanolic DDM solutions was determined spectrophotometrically at 528 nm using ε = 102 (101).

Aliquots (0.1 ml) of the reagent were added to a 5 ml solution of aminopeptidase (0.01 mg/ml) in 0.1M Tris buffer (pH 8.0). Activity of the enzyme was monitored at timed intervals with Leu-p-NO₂-anilide.

c) Diazoacetyl-D,L-norleucine methyl ester. Glycyl-D,L-norleucine was esterified by methanol according to the procedure of Brenner and Huber (102). A solution of thionyl chloride (.9 ml, 12mM) in absolute methanol (3.4 ml, 84mM) was cooled in an ice-NaCl bath. Glycyl-D,L-norleucine (2 g, 10.7mM) was added in small increments with stirring provided by a magnetic, so that the temperature did not rise above 0°. After mixing of the reagents, the reaction vessel was transferred to a water
bath (40°C) and the mixture was incubated for two hours with stirring. The methanol and thionyl chloride were removed en vacuo and the crude product was recrystallized twice from methanol-ether, mp 117-119°C. For diazotization, esterified dipeptide (500 mg) and NaNO₂ (228 mg) were dissolved in 3 ml of 2M sodium acetate cooled to 4°C. Glacial acetic acid (.13 ml) was added and the mixture was allowed to stand at 4°C for 2 hours. After this time, the mixture was filtered and the crystals were washed with ice-cold water.

The inactivation of aminopeptidase M by the above diazonium reagent was attempted using the method of Rajagopalan et al. (103). A 2 ml solution of aminopeptidase M (1 mg/ml) was adjusted to pH 5.5 and a small amount of 0.01M cupric acetate solution (10 μl) was added. At this time, 0.1 ml of a methanolic solution of diazoacetyl-D,L-norleucine methyl ester (.5 mg/ml) was added. Aliquots were withdrawn and assayed at timed intervals.

Lysine Modification. Reductive alkylation was carried out with NaBH₄ and formaldehyde according to the method of Means and Feeney (104). A solution of aminopeptidase in 0.2M borate buffer (pH 9.0, 1 mg/ml) was kept at 0°C. Solid NaBH₄ (0.5 mg) was added, followed by three 0.5 μl portions of 37% aqueous formaldehyde over a period of 30 minutes. The enzyme was assayed at timed intervals in the usual manner. In order to quantitate the modification, the reaction of trinitrobenzene-sulfonic acid with protein amino groups was used (105). At timed intervals, 0.2 ml of 4% NaHCO₃ solution (pH 8.5) and 0.2 ml of 0.1% TNBS in water were added to 0.1 ml of the enzyme
solution. The reaction mixture was then incubated at 50° for 30 minutes. After this, 0.1 ml of 1N HCl was added and the absorbance of the solution was read on a Varian Techtron UV-VIS spectrophotometer at 340 nm. An extinction coefficient of 14,000 was used for calculation. In order to corroborate the data gained from the TNBS reaction, the alkylation mixture was dialyzed against 0.2N N-ethylmorpholine-acetate buffer (pH 7.8). This sample was dried in a Pierce hydrolysate tube and 1 ml of 6N HCl was added. The tube was evacuated and heated at 110° for 24 hours. The acid was removed under vacumn, 0.8 ml of citrate buffer (pH 2.2) was added, and 0.5 ml of this solution was subjected to amino acid analysis. The number of lysine residues modified was calculated by comparison with an unmodified aminopeptidase hydrolysate.

Deuterium Isotope Effect Studies. The pD values of 0.1M phosphate buffers were determined by adding 0.4 units to the pH reading on a Radiometer pH meter model 26 (106). Aminopeptidase M was assayed against different concentrations of Leu-p-NO2-anilide as previously described. The kinetic constants, k_cat and K_M(app) were calculated by the method of Wilkinson (89) from standard double reciprocal plots. Plots of k_cat,H_2O/k_cat,D_2O, the isotope effect on the catalytic constant, and K_M,H_2O/K_M,D_2O, the isotope effect on the Michaelis constant, versus pH (pD) were constructed. Error bars were included for each point.
**SDS-Acrylamide Gel Electrophoresis.** The following solutions were prepared according to the published procedure of Weber et al. (107): acrylamide solution (22.2 g of acrylamide, 0.6 g of methylenebisisacrylamide, water to 100 ml), gel buffer (0.2M phosphate buffer, pH 7.2, 0.2% in SDS), ammonium persulfate (15 mg/ml in water, made fresh daily). The final concentration of acrylamide in the gels was either 7.5% or 5%. The preparation of 7.5% gels required 10.1 ml of acrylamide solution, 3.4 ml water, 15.0 ml of gel buffer, 1.5 ml of ammonium persulfate solution, and 0.045 ml of tetramethylethylenediamine (TEMED). The 5% gels contained the same amounts as the 7.5% gels except for the change to 6.75 ml acrylamide solution and 6.75 ml of water. Regardless of the acrylamide concentration, the acrylamide, water, and gel buffer were placed in a 50 ml vacuum flask and deaerated for one minute at room temperature with an aspirator. The TEMED and persulfate were then added and 2 ml of the resulting solution were immediately pipetted into glass tubes (0.5 x 10 cm). Air bubbles were eliminated by gently tapping the tubes. A small volume of distilled water was carefully layered on top of the gel solution with a drawn-out Pasteur pipette. After 20-30 minutes, the gel had polymerized and was observed by a sharp interface between the gel and water.

The standard protein markers (β-galactosidase, phosphorylase-A, serum albumin, and pepsinogen) and aminopeptidase M were prepared for electrophoresis according to the three
following methods:

a) Boiling SDS-Mercaptoethanol. The standard proteins (2 mg) were dissolved in 0.01M phosphate buffer (pH 7.0) and the aminopeptidase (.5-1 mg/ml) was used directly from the gel filtration column described above. Nine parts of 0.01M phosphate buffer (pH 7.0) containing 1% SDS and 1% 2-mercaptoethanol were placed in a screw-capped tube in a boiling water bath. After a few minutes, one part of the protein solution was added and the tube was capped. After 5 minutes of incubation at 100°C, the tube was cooled to room temperature and the contents were dialyzed against sample buffer (0.01M phosphate, pH 7.2, 0.1% in SDS, and 0.1% in 2-mercaptoethanol) overnight. To account for spurious bands due to possible hydrolysis of peptide bonds, the 100°C treatment was carried out for only one minute in a control experiment.

b) Denaturation by Guanidine Hydrochloride Followed by Alkylation. To the protein (1 mg or 1 ml of a 1 mg/ml solution) in a screw-cap tube was added 1 ml of hot (100°C) 0.1M Tris buffer (pH 8.5) which was 8M in guanidine hydrochloride. The tube was immediately transferred to a boiling water bath and 15μl of 2-mercaptoethanol was added. After 5 minutes of incubation at 100°C, the tube was moved to a 37°C water bath and incubated for an additional 2 hours. Iodoacetate acid (260 mg) was dissolved in 1 ml of 1N NaOH, and 0.25 ml of this was added to 1 ml of protein solution. The pH of the mixture was raised to 8-9 by addition of 2N NaOH as indicated by pH paper. After 5 minutes, 3 more drops
of the iodoacetate solution were added and the pH was raised to 10-11 where the mixture was incubated for 10 minutes. After this time, 2-mercaptoethanol (0.05 ml) was added and the pH was readjusted to 7.0. Since guanidinium dodecyl sulfate is insoluble, the sample was first dialyzed against 9M urea in 0.1M Tris buffer (pH 8) and then against 0.01M phosphate buffer (pH 7.0) containing 0.1% SDS.

c) Performic Acid Oxidation. One part of 30% hydrogen peroxide was mixed with 9 parts of 90% formic acid and the mixture was allowed to stand at room temperature for 2 hours in a capped tube. The protein (0.5-1 mg) was dissolved in 1 ml of this reagent and placed in an ice-water bath for one hour. After this time, 15 ml of distilled water was added, the mixture was frozen in an acetone-dry ice bath, and lyophilized. The lyophilized protein was dissolved in 2 ml of 0.2M phosphate buffer (pH 8.0), containing 1% SDS, and incubated at 37° for 2 hours. Finally, the sample was dialyzed overnight against 0.01M phosphate buffer (pH 7.0), containing 0.1% SDS.

Gel buffer was added to the bottom chamber of a Bio-rad Model 150 gel electrophoresis cell. The gel tubes containing the polymerized gels were placed into the holes of the cell making sure no air bubbles were trapped at the bottom of the gels. The upper cell chamber was now filled with gel buffer and the air bubbles were teased out of the tubes with a Pasteur pipette. Several drops of glycerol
and a crystal of bromophenol blue were added to each protein solution and a sample was applied to the top of the gels with a long-tipped 0.1 ml pipette. The amount applied onto the gels varied from .005-.040 ml but was constant during any one run. The cell was connected to a power supply from Buchler Instruments (model 3-1008). Electrophoresis was carried out using 7 mamps per tube at constant current (about 40 volts). Temperature of the gels was maintained at 25°C by means of a Neslab Instruments circulating water bath attached to the lower chamber of the cell. After the tracking dye reached the bottom of the gels, the power supply was disconnected and the tubes were removed from the cell holder. The gels were removed from the tubes with the help of a fine gauge hypodermic syringe. Water was forced between the gel and the tube, thus facilitating gel removal.

Before staining, the gels were placed in 10% trichloroacetic acid for 10 minutes in 10 ml test tubes. The gels were rinsed in distilled water and stained at room temperature for 24 hours. The staining solution consisted of Coomassie Brilliant Blue R-250 (1.25 g), 227 ml of methanol, 46 ml of glacial acetic acid, and water to 500 ml. Insoluble material was removed by filtration through Whatman No. 1 filter paper. After staining, the gels were rinsed in distilled water and destained in a mixture of 50 ml methanol, 75 ml of glacial acetic acid, and 875 ml of water by means
of an electrophoretic destainer (Instrumentation Specialties Co., model 422). By this method, destaining time was less than 1 hour. Temperature was regulated to 20° with a Neslab Instruments bath cooler (model PBC-4) and circulating water bath. The destained gels were stored at room temperature in 7.5% acetic acid in capped vials.

Molecular weights of the protein bands were determined by first measuring the distance from the top of the gels to the center of the bands. The standard protein markers were graphed by plotting the logarithm of the molecular weight versus relative mobility (distance along the gel). The unknown aminopeptidase bands were placed on the linear line at their relative migration position. Molecular weights were obtained by extrapolation to the y-axis. Standard protein markers which gave a consistent linear standard curve were pepsinogen (MW 41,000), serum albumin (MW 68,000), and phosphorylase A (MW 103,000).

Polyacrylamide Gel Electrophoresis. Gel electrophoresis of aminopeptidase M was performed on 7.5% acrylamide gels. All solutions and gel preparation were identical to those described above except for the complete absence of SDS or mercaptoethanol in all cases. Aminopeptidase M (0.03 ml), either from the commercial Rohm and Haas source (1 mg in 2.7 ml water and 3 ml 0.1M phosphate buffer, pH 8.0) or from fractions of the gel chromatography step (dilute 1:10 in 0.1M phosphate buffer, pH 8.0), was applied to the top of the gels with a long-tipped 0.1 ml pipette. In all
cases, a drop of glycerol and a minute amount of bromophenol blue were added to the protein solution before sample application. Electrophoresis, staining, and destaining were carried out exactly as before. Gels were stored in 7.5% acetic acid in capped tubes.

**Equilibrium Dialysis.** Equilibrium binding was used to study the interaction of L-leucine, a good competitive inhibitor, with aminopeptidase M. Lucite equilibrium dialysis cells from Technilab Instruments (model 260) were used in all cases. Non-radioactive L-leucine (in 0.1M phosphate buffer, pH 7.9) was mixed with L-leucine-\(^{14}\)C (uniformly labeled, New England Nuclear, NEC 279) to give different ratios of "cold" to "hot" L-leucine. The ratio varied from as little as 40 to as much as 2000. Solutions of this mixture (containing different concentrations of inhibitor) were placed in one side of the dialysis cell and aminopeptidase M (0.23 mg/ml) in 0.1M phosphate buffer (pH 7.9) was added to the other side. The two solutions were separated by sheets of Union Carbide dialysis tubing which had been exhaustively boiled and washed in distilled water. The cells were sealed thoroughly with silicone grease and the components of the solution were allowed to reach equilibrium (24 hours). After this time, the cells were open and 0.1 ml of solution from each side of the dialysis membrane was placed in 20 ml screw-capped vials containing 15 ml of scintillation "cocktail" (5 l dioxane, 2.5 l tetrahydrofuran, 750 g naphthalene, 75 g 2,5-diphenyloxazole, 1.87 g p-Bis[2-(5-phenyloxazoyl)-
benzene). The samples were counted in a Parkard liquid scintillation counter. Using the specific activity of L-leucine-\(^{14}\)C, the ratio of "cold" to "hot" L-leucine, the efficiency of the scintillation counter, and the concentration of aminopeptidase, the amount of ligand bound to the enzyme was calculated by subtracting the concentration of L-leucine in the ligand side of the cell from the concentration of L-leucine in the enzyme-ligand side. Finally, a plot of \(r/A\) versus \(r\) was constructed, where \(r\) represented the number of moles of ligand bound per mole of enzyme and \(A\) was the ligand concentration.

**Reacting Species Ultracentrifugation.** The general procedure of Cohen et al. (108-110) was used in these experiments. All experiments were performed on a Beckman Model E analytical ultracentrifuge. An An-D rotor with double sector, 12 mm cells was used. The main compartment of the cell contained .29 ml of an assay mixture consisting of Tris buffer (0.1M, pH 7.8), NaCl (.5M), and Leu-p-NO\(_2\)-anilide (2.0mM). The salt prevents severe diffusion of the sedimenting band. Aminopeptidase M (8 \(\mu\)l) in .1M Tris buffer (pH 7.8) and .5M NaCl was placed in the cell reservoir. The enzyme concentration was either 2.4 \(\mu\)g/ml or 0.8 \(\mu\)g/ml. The ultracentrifuge was operated at a speed of 60,000 rpm with temperature controlled to \(\pm 0.1^\circ\) by an RTIC unit. During acceleration of the rotor, the layering of the enzyme over the assay mixture was observed with the Schlieren optical system and occurred
between 500 and 1000 rpm. As the enzyme band sedimented through the assay mixture, the absorbance by p-nitroaniline (405 nm) along the length of the cell was recorded with a photoelectric scanner at two minute intervals. The photoelectric scanner, which was first used for reacting enzyme sedimentation by Hoagland and Teller (111), provided a direct plot of product formed versus radius along the cell that is useful in evaluating the sedimentation properties of the macromolecule at concentrations used in the assay of the enzyme.

After the run had progressed for about 45 minutes, the distance (radius along the cell) which the maximum deflection in absorbance had moved from time = 0 was measured for each of the timed scans. A plot of the logarithm of the distance (log R) as a function of time was constructed. The slope of this line was equal to:

\[
\frac{s_{obs}}{2.303/60} = \frac{[2\pi (rpm/60)]^2}{2s_{20,w}}
\]

The \( s_{obs} \) value was converted to an \( s_{20,w} \) value by correcting for the density and viscosity of the solution relative to water at 20°C.
RESULTS

Characterization of Immobilized Aminopeptidase -- Comparison to Soluble Enzyme. A representative time course for the coupling of aminopeptidase M to diazotized arylamino glass is shown in Figure 2. The depletion of enzymatic activity in the supernatant was used to determine the amount of enzyme bound to the glass. This number was corroborated by amino acid analysis of an acid-hydrolyzed sample of enzyme-glass derivative. The value for glass-bound aminopeptidase M was near 1.0% (w/w) in all cases. Appropriate controls indicated that enzyme denaturation over this time period was not a problem. The enzyme derivative is extremely stable if stored at 4° as a moist cake, and can be used over many weeks without apparent loss of activity.

Kinetic studies of the hydrolysis of Leu-p-NO₂-anilide by soluble and insoluble aminopeptidase M showed that Michaelis-Menten kinetics are applicable for both systems, as is seen by the linearity of Lineweaver-Burk plots (Figure 3). The raw data used to calculate initial velocities and substrate concentrations, and, in turn, $k_{cat}$ and $K_M(app)$ from double reciprocal plots are given in Table 4 for a typical experiment using soluble enzyme. $K_M(app)$ and $k_{cat}$ values appear in Table 5. For both forms of the enzyme, the velocities of substrate hydrolysis at saturating levels
Figure 2. Representative time course of enzyme coupling. Diazotized arylamino glass was reacted at $0^\circ$ with 2 ml of aminopeptidase M (2 mg/ml in 0.1M Tris-HCl buffer, pH 7.3). Activity was measured spectrophotometrically with Leu-p-NO$_2$-anilide as substrate.
Figure 3. Lineweaver-Burk plots for the hydrolysis of Leu-p-NO₂-anilide at pH 7.5 and 25°C in Tris buffer (0.1M) by the bound and free forms of aminopeptidase M.
### TABLE 4

Buffer: 0.1M Tris (pH 7.5)

Substrate: Leu-p-NO₂-anilide in buffer (0.2 mg/ml). Dissolve 20 mg Leu-p-NO₂-anilide in 0.5 ml absolute methanol and bring up to 100 ml with buffer.

Enzyme: aminopeptidase M in 0.1M Tris buffer (pH 7.5), final concentration = 0.2667 mg/ml (MW = 280,000).

Temperature: 25.0°C.

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<th>Substrate (ml)</th>
<th>Buffer (ml)</th>
<th>Enzyme (ml)</th>
<th>OD/min</th>
<th>v(M/l/min)</th>
<th><a href="M/l">S</a></th>
<th>1/v(1/M·min)</th>
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TABLE 5

Comparison of kinetic parameters for the hydrolysis of Leu-p-NO2-anilide by the soluble and glass-bound forms of aminopeptidase M.

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<td>Soluble</td>
<td>Bound</td>
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<td>$k_{cat}(s^{-1})$</td>
<td>21 ± 0.4$^a$</td>
<td>23 ± 2$^a$</td>
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</tr>
<tr>
<td>$E_a(\text{kcal/mole})$</td>
<td>19.5 ± 0.8$^b$</td>
<td>17.9 ± 1.4$^b$</td>
<td></td>
</tr>
<tr>
<td>pH optimum</td>
<td>7.5</td>
<td>7.5</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ pH 7.5, 25°.

$^b$ pH 7.5.
of substrate \( V_{\text{max}} \) are identical. Conversely, \( K_{M}^{(\text{app})} \) is lower for soluble enzyme than for the insoluble form. These data show the importance of comparing the activities of soluble and insoluble enzyme forms at enzyme saturation. In the case of aminopeptidase M, the Michaelis-Menten equation describes both reactions as follows:

\[
\begin{align*}
V_{\text{insol}} &= \frac{k_{\text{cat, insol}} [E_0][S_0]}{K_{M, \text{insol}} + [S_0]} \quad (1) \\
V_{\text{sol}} &= \frac{k_{\text{cat, sol}} [E_0][S_0]}{K_{M, \text{sol}} + [S_0]} \quad (2)
\end{align*}
\]

Since \( k_{\text{cat}} \) values are identical for soluble and insoluble aminopeptidase, dividing equation 1 by equation 2 yields:

\[
\frac{V_{\text{insol}}}{V_{\text{sol}}} = \frac{K_{M, \text{sol}} + [S_0]}{K_{M, \text{insol}} + [S_0]} \quad (3)
\]

This equation can be described graphically by a plot of \( V_{\text{insol}}/V_{\text{sol}} \) vs. \([S_0]\) (Figure 6). The solid line is the theoretical representation of equation 3, while the points are from experimental results. As is expected from equation 3, the plot of \( V_{\text{insol}}/V_{\text{sol}} \) vs. \([S_0]\) approaches unity at high substrate concentration (\( [S_0] \gg K_{M} \)) and \( K_{M, \text{sol}}/K_{M, \text{insol}} \) at low substrate concentrations (\( [S_0] \gg K_{M} \)). Thus, until saturation is reached, \( V_{\text{insol}}/V_{\text{sol}} \) is a function of substrate concentration and reflects the differences in the apparent
Figure 6. Hyperbolic relationship between $v_{\text{insol}}/v_{\text{sol}}$ and $[S_0]$. The solid line is the theoretical representation of equation 3 (see Results). The points are from experimental data of Leu-p-NO$_2$-anilide hydrolysis by free and bound aminopeptidase M.
Michaelis parameter \( (K_M^{\text{app}}) \) between the two enzyme forms.

A shift in the pH optimum of an enzyme often occurs after attachment to a solid support \((20)\). The activities of both soluble and insoluble aminopeptidase M are compared as a function of pH in Figure 7. Since the compositions of \( k_{\text{cat}} \) and \( K_M^{\text{app}} \) are unknown, the specificity constant, \( k_{\text{cat}}/K_M^{\text{app}} \), is plotted as a function of pH \((112)\). Use of this constant also eliminates the complications introduced by possible multiple binding modes. Both soluble and insoluble forms of the enzyme exhibit virtually identical pH profiles and optima.

The temperature dependence of both enzyme forms is shown in Arrhenius plots (Figure 8). The initial velocities were calculated at substrate saturation \(([S_0] >> K_M)\). The experimental activation energies calculated from the slope of these plots are 19.5 ± 0.8 kcal/mole for soluble enzyme and 17.9 ± 1.4 kcal/mole for insoluble enzyme (Table 5), identical within experimental error. The non-linearity of the points at higher temperatures is a common feature of enzymic activity temperature dependence and is due to enzyme denaturation at high temperature. Subsequently, these points were not used in the calculation of the activation energies.

**Sequencing and Total Hydrolysis of Proteins and Peptides.** The results of using immobilized aminopeptidase M for the amino acid sequence determination of aminoethylated insulin
Figure 7. pH profiles for the hydrolysis of Leu-p-NO₂-anilide catalyzed by the soluble and insoluble forms of aminopeptidase M. Tris buffer (0.1M) was used for the entire pH range.
Figure 8. Arrhenius plots illustrating the temperature dependence of the rate of Leu-p-NO₂-anilide hydrolysis catalyzed by the immobilized and free forms of aminopeptidase M. Determinations were made in 0.1M Tris-HCl buffer (pH 7.5) with temperature controlled to ± 0.2°C.
A chain appears in Figure 9. The sequence of the first four amino acid residues starting from the N-terminus of insulin A chain is Gly-Ileu-Val-Glu (113). The experimentally determined rate of release of amino acids shown in Figure 9 correctly identifies the sequential order. The separation of amino acids is excellent as would be expected from the fact that the rate of release of amino acids by aminopeptidase M differs by only a factor of 6 for all residues (70).

The total hydrolysis of the aminoethylated A chain of insulin by immobilized aminopeptidase M goes to the extent of 96% (Table 10). The percentage of hydrolysis is based on the recovery of amino acids. The value for serine was not determined since asparagine and glutamine fall under the peak. Aspartic acid is not normally found in the A chain of insulin. However, this particular preparation of insulin contained a small amount of aspartic acid as a result of a desamido contaminant (Asn-21→Asp-21). The percentage of aspartic acid contamination found by hydrolysis with immobilized aminopeptidase is in excellent agreement with the suppliers estimate based on electrophoretic separation and determination. The exact determination of this amino acid contaminant dramatically illustrates the usefulness of bound proteolytic enzymes for the total hydrolysis of proteins.

Aminopeptidase M releases proline very slowly in comparison with other amino acids (70). Therefore, a substrate containing proline was used to determine the difficulty
Figure 9. Release of amino acids from the aminoethylated A chain of insulin catalyzed by glass-bound aminopeptidase M. The expected sequence is Gly-Ileu-Val-Glu-.
MOLE

GLYCINE

ISOLEUCINE

VALINE

GLUTAMIC ACID

\[ \mu \text{MOL} \]

\[ \text{TIME (min)} \]

\[ 0 \]

\[ 10 \]

\[ 20 \]

\[ 30 \]

\[ 40 \]

\[ 0.02 \]

\[ 0.1 \]
TABLE 10

The hydrolysis of the aminoethylated A-chain of insulin by aminopeptidase-M bound to porous glass. Reaction was carried out at 35° and pH 7.5.

<table>
<thead>
<tr>
<th>Residues/Hole A-chain</th>
<th>Found</th>
<th>Known</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE³-cys</td>
<td>3.7</td>
<td>4.0</td>
<td>93</td>
</tr>
<tr>
<td>Ser</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glu</td>
<td>2.0</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Gly</td>
<td>0.9</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>Ala</td>
<td>1.0</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Val</td>
<td>1.9</td>
<td>2</td>
<td>95</td>
</tr>
<tr>
<td>Ile</td>
<td>0.9</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>Leu</td>
<td>1.9</td>
<td>2</td>
<td>95</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.0</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Asp</td>
<td>0.41</td>
<td>0.4</td>
<td>103</td>
</tr>
</tbody>
</table>

Mean Recovery 96%
due to the presence of this residue. A good choice for this substrate was the B chain of insulin, whose COOH terminal end included the sequence -Thr-Pro-Lys-Ala-COOH. The results of the enzymatic hydrolysis are shown in Table 11. Even though the release of proline is quite low, the overall recovery of amino acids is still relatively high (87%). As would be expected by the incomplete release of proline, the recoveries of lysine and alanine, residues on the carboxyl side of proline, are also low.

The hydrolysis of a much bigger protein than insulin was attempted using β-lactoglobulin as the substrate. The protein was first cleaved into peptides by a pretreatment with immobilized pronase, a mixture of proteolytic enzymes secreted by Streptomyces griseus. After 6 hours, the degree of hydrolysis is about 65% as judged by amino acid analysis. Further treatment with immobilized aminopeptidase M increased the extent of hydrolysis to 93% (Table 12). As before, serine, asparagine, and glutamine were not determined because of merging into one peak. Despite the presence of a proline residues and their low release (11%), the overall recovery of amino acids was excellent (93%).

Inhibition Studies - Evidence Against an Extraction Mechanism. The hydrolysis of Leu-p-NO₂-anilide by aminopeptidase M is inhibited by free L-amino acids, aromatic hydrocarbons, aliphatic hydrocarbons, and aliphatic amines.
TABLE 11

The hydrolysis of the aminoethylated B-chain of insulin by aminopeptidase-M covalently linked to porous glass. Reaction was carried out at 35° at pH 7.5.

<table>
<thead>
<tr>
<th>Residues/Mole B-chain</th>
<th>Found</th>
<th>Known</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>0.6</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>His</td>
<td>2.0</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Arg</td>
<td>1.0</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>AE-cys</td>
<td>1.9</td>
<td>2</td>
<td>95</td>
</tr>
<tr>
<td>Thr</td>
<td>0.6</td>
<td>1</td>
<td>60</td>
</tr>
<tr>
<td>Ser</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glu</td>
<td>2.0</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>Gly</td>
<td>3.0</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Ala</td>
<td>1.3</td>
<td>2</td>
<td>65</td>
</tr>
<tr>
<td>Val</td>
<td>3.0</td>
<td>3</td>
<td>100</td>
</tr>
<tr>
<td>Leu</td>
<td>3.9</td>
<td>4</td>
<td>98</td>
</tr>
<tr>
<td>Tyr</td>
<td>2.1</td>
<td>2</td>
<td>105</td>
</tr>
<tr>
<td>Phe</td>
<td>3.1</td>
<td>3</td>
<td>103</td>
</tr>
<tr>
<td>Pro</td>
<td>0.5</td>
<td>1</td>
<td>50</td>
</tr>
</tbody>
</table>

Mean Recovery 87%
TABLE 12

Recovery of free amino acids from a digest of β-lactoglobulin.

Treatment with bound pronase was followed by treatment with bound aminopeptidase-M.

<table>
<thead>
<tr>
<th>Residues/Mole β-lactoglobulin</th>
<th>Found</th>
<th>Known</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp</td>
<td>2.1</td>
<td>2</td>
<td>105</td>
</tr>
<tr>
<td>Lys</td>
<td>14.5</td>
<td>15</td>
<td>97</td>
</tr>
<tr>
<td>His</td>
<td>1.7</td>
<td>2</td>
<td>85</td>
</tr>
<tr>
<td>Arg</td>
<td>2.7</td>
<td>3</td>
<td>90</td>
</tr>
<tr>
<td>Asp</td>
<td>11.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Thr</td>
<td>7.6</td>
<td>8</td>
<td>95</td>
</tr>
<tr>
<td>Ser</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glu</td>
<td>13.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gly</td>
<td>3.4</td>
<td>3</td>
<td>113</td>
</tr>
<tr>
<td>Ala</td>
<td>14.7</td>
<td>14</td>
<td>105</td>
</tr>
<tr>
<td>Val</td>
<td>11.2</td>
<td>10</td>
<td>112</td>
</tr>
<tr>
<td>Met</td>
<td>4.4</td>
<td>4</td>
<td>110</td>
</tr>
<tr>
<td>Ile</td>
<td>9.3</td>
<td>10</td>
<td>93</td>
</tr>
<tr>
<td>Leu</td>
<td>21.5</td>
<td>22</td>
<td>98</td>
</tr>
<tr>
<td>Tyr</td>
<td>3.9</td>
<td>4</td>
<td>98</td>
</tr>
<tr>
<td>Phe</td>
<td>3.9</td>
<td>4</td>
<td>98</td>
</tr>
<tr>
<td>Pro</td>
<td>0.9</td>
<td>8</td>
<td>11</td>
</tr>
</tbody>
</table>

Mean Recovery 93%
Table 13 lists the results of inhibition experiments using both Leu-p-NO\(_2\)-anilide and Gly-p-NO\(_2\)-anilide as substrate. Inhibition type was determined from Lineweaver-Burke plots. \(K_I\) and \(\Delta G^0\) are, respectively, the inhibition constant and the free energy of the enzyme-inhibitor complex formation. It was of interest to determine the ratio \(r_a\) of inhibitor to enzyme in the enzyme-inhibitor complex for one of the amino acids, L-leucine. A plot of the logarithm of \((v_0/v-1)\) versus the logarithm of inhibitor concentration (95) gave a straight line whose slope was equal to \(r_a\) (Figure 14). The slope of the line for leucine inhibitor was 1.1. This provided direct evidence for the formation of a leucine-enzyme complex in a 1:1 ratio.

Table 13 illustrates that aromatic and aliphatic hydrocarbons were potent inhibitors of aminopeptidase M catalysis. In order to test the hypothesis that such inhibitors are "extracted" from the aqueous phase of the medium to a non-aqueous phase in the enzyme, a plot of the free energy of complex formation versus the molecular surface area of the inhibitor was constructed (Figure 15). Surface areas were obtained from the data of Miles et al. (115) based on the assumption that the molecules were spherical. Examination of Figure 15 shows nonlinearity with a rapid increase in \(-\Delta G^0\) with increasing inhibitor surface area. It is interesting to note that neither aliphatic nor aromatic hydrocarbons inhibited the hydrolysis of Gly-p-NO\(_2\)-anilide (Table 13).
The effect on inhibitors on the aminopeptidase M catalyzed hydrolysis of Leu-p-NO₂-anilide and Gly-p-NO₂-anilide.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>( K_i ) (M⁻¹)</th>
<th>(- ΔG ) (cal)</th>
<th>Inhibition Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leu-p-NO₂-anilide</td>
<td>benzene</td>
<td>5.04x10⁻³</td>
<td>3144</td>
<td>noncomp</td>
</tr>
<tr>
<td></td>
<td>toluene</td>
<td>4.31x10⁻³</td>
<td>3238</td>
<td>noncomp</td>
</tr>
<tr>
<td></td>
<td>ethylbenzene</td>
<td>2.25x10⁻³</td>
<td>3624</td>
<td>noncomp</td>
</tr>
<tr>
<td></td>
<td>propylbenzene</td>
<td>1.21x10⁻³</td>
<td>3993</td>
<td>noncomp</td>
</tr>
<tr>
<td></td>
<td>naphthalene</td>
<td>3.08x10⁻⁴</td>
<td>4805</td>
<td>noncomp</td>
</tr>
<tr>
<td></td>
<td>pentane</td>
<td>5.10x10⁻³</td>
<td>3137</td>
<td>noncomp</td>
</tr>
<tr>
<td></td>
<td>hexane</td>
<td>2.66x10⁻³</td>
<td>3525</td>
<td>noncomp</td>
</tr>
<tr>
<td></td>
<td>L-leucine</td>
<td>1.79x10⁻³</td>
<td>3759</td>
<td>comp</td>
</tr>
<tr>
<td></td>
<td>norleucine</td>
<td>8.35x10⁻⁴</td>
<td>4212</td>
<td>comp</td>
</tr>
<tr>
<td></td>
<td>norvaline</td>
<td>1.48x10⁻³</td>
<td>3873</td>
<td>comp</td>
</tr>
<tr>
<td></td>
<td>aminobutyric acid</td>
<td>3.21x10⁻³</td>
<td>3412</td>
<td>mixed</td>
</tr>
<tr>
<td></td>
<td>alanine</td>
<td>1.76x10⁻²</td>
<td>2399</td>
<td>noncomp</td>
</tr>
<tr>
<td></td>
<td>glycine</td>
<td>3.21x10⁻³</td>
<td>3412</td>
<td>noncomp</td>
</tr>
<tr>
<td></td>
<td>ethylamine</td>
<td>2.19x10⁻³</td>
<td>3641</td>
<td>mixed</td>
</tr>
<tr>
<td></td>
<td>propylamine</td>
<td>2.35x10⁻³</td>
<td>3597</td>
<td>mixed</td>
</tr>
<tr>
<td></td>
<td>butylamine</td>
<td>2.35x10⁻³</td>
<td>3597</td>
<td>mixed</td>
</tr>
<tr>
<td></td>
<td>n-amylamine</td>
<td>2.35x10⁻³</td>
<td>3597</td>
<td>mixed</td>
</tr>
<tr>
<td></td>
<td>n-hexylamine</td>
<td>2.35x10⁻³</td>
<td>3597</td>
<td>mixed</td>
</tr>
<tr>
<td>Gly-p-NO₂-anilide</td>
<td>pentane</td>
<td></td>
<td></td>
<td>no inhib.</td>
</tr>
<tr>
<td></td>
<td>benzene</td>
<td></td>
<td></td>
<td>no inhib.</td>
</tr>
</tbody>
</table>
Figure 14. Graphical method of Johnson et al. (95) for the determination of the molar ratio of inhibitor to enzyme in the enzyme-inhibitor complex ($r_a$). The slope of the line is equal to $r_a$. L-leucine, a competitive inhibitor of aminopeptidase M, was employed in this study.
Figure 15. Plot of free energy of enzyme-inhibitor complex formation vs. molecular surface area of inhibitor for aminopeptidase M.
The implications of this finding are discussed later.

Chemical Modification Studies-Essential Active Site Residues. The time course of the reaction of aminopeptidase M with diazotized sulfanilic acid appears in Figure 16. Initially only 75% of the enzyme activity is lost. The extent of reaction may be explained by decomposition of the inactivating reagent. The inset shows the rate of decomposition of diazotized sulfanilate in the absence of enzyme. A second addition of reagent reduces the enzyme activity to below 90%. Some activity remains, however, even when a large excess of reagent is present. Considerable protection against enzyme inactivation is provided by the presence of a competitive inhibitor, L-leucine.

Aminopeptidase M is rapidly inactivated by TNM. The time course for the inactivation of the soluble aminopeptidase shows a rapid initial decrease in activity with a leveling off at longer times (Figure 17). The immobilized enzyme is inactivated at a slower rate (Figure 17). This experiment was done with the immobilized enzyme supported in a column. Use of the bound enzyme is convenient and allows one to maintain a constant level of a specific substrate as a protecting agent against inactivation.

Continuous replenishing of substrate maintains saturating levels of substrate and negligible concentration of product. Any side reactions of the inactivating reagent with the protection agent are minimized because of the short
Figure 16. Inactivation of aminopeptidase M and aminopeptidase M + L-leucine, a competitive inhibitor, by diazotized sulfanilic acid. The reaction was carried out at 0° in 0.1M Tris-HCl buffer (pH 7.5). The inset shows the decomposition of diazotized sulfanilate at 0° in the same buffer.
DECOMPOSITION OF DIAZOTIZED SULFANILIC ACID

INITIAL ACTIVITY (%)

TIME (min)

AMOUNT REMAINING (%)
Figure 17. Inactivation of unprotected soluble and insoluble aminopeptidase M by tetranitromethane. The graph also shows protection of the insoluble derivative during inactivation by Leu-p-NO₂-anilide, a specific substrate, and L-leucine, a competitive inhibitor. All reactions were at 25° in 0.05M Tris-HCl buffer (pH 8.0).
exposure of one to the other. The enzyme, however, is exposed to the inactivating reagent as long as the feed is continued. To assume that the enzyme is saturated one may calculate the substrate converted from kinetic constants or determine the fraction of substrate transformed by the same method used for assay in the solid-supported enzyme. In this case, 8% of the substrate was converted. L-leucine and Leu-p-NO₂-anilide, a competitive inhibitor and specific substrate, respectively, provide similar protection of the enzyme against inactivation. The concentration of both substrate and inhibitor are at saturating levels.

The dependence of the enzymatic activity on the number of tyrosine residues modified per enzyme molecule is shown in Figure 18. Extrapolation of the initial slope to zero percent activity indicates the modification of 4-6 tyrosine residues per molecule of enzyme. At greater than 50% inactivation, the curve becomes non-linear due to the great reactivity of tetranitromethane and the availability of tyrosine residues at positions other than the active site.

The activity of soluble aminopeptidase M goes to less than 10% in two hours in the presence of 1,000 fold molar excess of acetylimidazole. The immobilized enzyme is also inactivated by this reagent (Figure 19). The activity may be restored to 95% of the original with hydroxylamine. The rates of reactivation would suggest deacetylation of
Figure 18. Inactivation of aminopeptidase M by tetranitromethane as a function of the number of tyrosine residues modified per mole of enzyme.
Figure 19. Inactivation of insoluble aminopeptidase M by N-acetylimidazole. After four hours the enzyme is reactivated by hydroxylamine. Both reactions were in 0.05M Veronal buffer pH 7.5 at 25°.
O-acetyltyrosine.

In the presence of rose bengal, aminopeptidase M is inactivated by light in 90 seconds (Figure 20). The reactions were carried out over a pH range of 5.5 to 8.5. Assuming that the inactivation follows pseudo-first order kinetics, the pseudo-first order rate constants were calculated from the slopes of \( \ln(\% \text{ act}) \) vs t plots shown in Figure 21. The plot of relative rates against pH (Figure 21, inset) would suggest a group with a \( pK_a \) of approximately 6.2-6.4.

The quantitation of histidine residue modification is shown in Figure 22. As is the case with tetranitromethane, extrapolation to complete enzyme inactivation shows the modification of a small number (4-6) of histidine residues. As before, the accessibility of non-active site histidines brings about modification of more residues than are needed for total inactivation.

Table 23 shows a summary of the chemical modification studies which were performed in our laboratory. Reagents directed at COOH groups were without effect. Two diazo reagents were tried with the hope of esterifying necessary COOH groups. One of these, diazoacetyl-D,L-norleucine methyl ester should have considerable specificity since norleucine is a powerful competitive inhibitor of aminopeptidase M. Treatment of the enzyme with a water-soluble carbodiimide and either glycine ethyl ester or leucine amide also had no effect. In order to show definitively that car-
Figure 20. Photochemical oxidation of aminopeptidase M at 18° in 0.1M phosphate buffer (pH 8.0). Rose bengal (0.01%) was used as the photosensitive dye.
Figure 21. Plots of \( \ln (\% \text{ activity}) \) as a function of time at different pH values (0.1M phosphate buffers). The slope of the lines are pseudo-first order rate constants of photochemical inactivation. The slope of the line of pH 8.0 was taken as 100\%. The inset shows the pH profile of the relative rates of inactivation.
Figure 22. Inactivation of aminopeptidase M by photochemical oxidation as a function of the number of histidine residues modified per mole of enzyme.
TABLE 23

CHEMICAL MODIFICATION OF MICROSOMAL AMINOPEPTIDASE

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Inactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diphenyldiazomethane</td>
<td>No</td>
</tr>
<tr>
<td><strong>Diazooacetyl - D, L-norleucine</strong> Methyl ester</td>
<td>No</td>
</tr>
<tr>
<td>Glycine Methyl Ester + Carbodiimide</td>
<td>No</td>
</tr>
<tr>
<td>Sodium Borohydride + Formaldehyde</td>
<td>No</td>
</tr>
<tr>
<td>Sodium Borohydride + L-leucine</td>
<td>No</td>
</tr>
<tr>
<td>Dimethyl (2-hydroxy-5-nitrobenzyl) -Sulfonium Bromide</td>
<td>No</td>
</tr>
<tr>
<td>Photochemical Oxidation</td>
<td>Yes</td>
</tr>
<tr>
<td>N-Acetylimidazole</td>
<td>Yes</td>
</tr>
<tr>
<td>Diazotized Sulfanilic Acid</td>
<td>Yes</td>
</tr>
<tr>
<td>Tetranitromethane</td>
<td>Yes</td>
</tr>
</tbody>
</table>
boxyl groups were modified, radioactive glycine ethyl ester was used to quantitate the reaction. After two hours of modification under the conditions given (see Experimental), the enzyme lost less than 5% of its initial maximal activity. However, 57 moles of glycine ethyl ester were bonded to the enzyme presumably through amide linkages with carboxyl groups.

Reductive alkylation of ε-amino groups of lysine did not inactivate aminopeptidase M. Reduction in the presence of L-leucine, a good competitive inhibitor, had no effect. The reaction of TNBS with primary amino groups provides a means to quantitate the reductive methylation of lysine groups, as illustrated in Figure 24. If TNBS is assumed to react with 100% of the available lysine residues at zero time, the curve indicates that after 30 minutes of modification TNBS can react with only about 20% of the initial number of primary amino groups. This indicates that 80% of the available lysine residues have been alkylated. The inset of Figure 24 shows that the enzyme loses only a small amount of activity during the modification. Amino acid analysis corroborated these results. After identical modification conditions, amino acid data showed that 86% of the available lysine residues were modified compared to a control. These observations tend to rule out amino groups and "active carbonyls" at the active site.

Spectrophotometric determination of the number of
Figure 24. Decrease in $A_{340}$ as a function of time. The absorbance at 340 nm is due to trinitrobenzene substituted primary amino groups. The inset shows the aminopeptidase activity during the time course of modification as followed by the hydrolysis of Leu-p-NO$_2$-anilide.
tryptophan residues modified indicated that approximately 20 indole moieties were alkylated by dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide. Activity was determined at timed intervals during the modification. No decrease in maximal velocity could be observed over the time course of reaction.

**Isotope Effect Studies.** These experiments were done with the hope of defining the catalytic role of the groups implicated by chemical modification studies, and to verify the importance of a proton abstraction step in the mechanism of hydrolysis. Aminopeptidase M catalyzed hydrolysis of Leu-p-NO₂-anilide is retarded in D₂O. Representative double reciprocal plots appear in Figure 25. It is evident that at pH (pD) 7.35, both the maximal activity and the Michaelis parameter were affected by D₂O. The pH (pD) dependence of the isotope effect on both of these parameters was studied, since D₂O can bring about substantial changes in the dissociation constants of ionizable groups on the protein (115-116). A plot of \( k_{\text{cat,H₂O}} / k_{\text{cat,D₂O}} \) vs. pH (pD) (Figure 26) indicated considerable pH dependence of the isotope effect on \( k_{\text{cat}} \). The maximum isotope effect of 1.5 occurred near the pH optimum of the enzyme. The pH (pD) dependence of the isotope effect on the Michaelis parameter \( (K_{M(\text{app})}) \) is shown in Figure 27. At the pH optimum of the enzyme the isotope effect was 1.4. However, the effect was greater than 3 at higher pH values. Furthermore, at pH values lower than 7, an inverse isotope effect on \( K_{M(\text{app})} \) was observed.
Figure 25. Lineweaver-Burk plots for the hydrolysis of Leu-p-NO₂-anilide by aminopeptidase M in 0.1M Tris-D₂O buffer and 0.1M Tris-H₂O buffer, pH (pD) 7.35.
Figure 26. pH (pD) profile of the solvent deuterium isotope effect on the catalytic constant, $k_{cat}$, for Leu-p-NO$_2$-anilide hydrolysis by aminopeptidase M.
Figure 27. pH (pD) profile of the solvent deuterium isotope effect on the apparent Michaelis constant, $K_{M(app)}$, for Leu-p-NO$_2$-anilide hydrolysis by aminopeptidase M.
**Equilibrium Dialysis.** One of the most widely used methods for the study of small ligand binding by macromolecules is equilibrium dialysis. The representation of the binding of L-leucine, a competitive inhibitor, by aminopeptidase M is illustrated in Figure 28. The data were plotted as \( r/A \) vs \( r \), where \( r \) represents the number of moles of ligand bound per mole of enzyme, and \( A \) is the free ligand concentration. The curve was not linear and indicated non-specific binding of the amino acid at high ligand concentration. Frequently, an extrapolation to the \( x \)-axis is used to obtain the number of binding sites \( (n) \). However, Klotz has pointed out the hazards and extreme ambiguities inherent in such extrapolations (117). Consequently, these experiments did not provide a definitive value for \( n \).

**Gel Electrophoresis.** Commercial aminopeptidase M was subjected to gel chromatography on a column of Sephadex G-100 as shown in Figure 29. The protein eluted as a sharp band with a slight trailing edge. The elution profile showed the presence of little, if any, contaminating material. Samples of the eluate containing protein along with aliquots of a commercial preparation of aminopeptidase (before Sephadex treatment) were electrophoresed on 7.5% polyacrylamide gels (Figure 30). Both samples showed a sharp band 0.75 cm from the origin. No contaminating bands of protein were detectable.

Figure 31 shows the polyacrylamide gel (7.5%) electrophoretic patterns of aminopeptidase treated by the methods
Figure 28. Representation of the binding of L-leucine, a competitive inhibitor, by aminopeptidase M. The moles of ligand bound per mole of enzyme is given as r, while A is the free ligand concentration. All binding studies were done in 0.1M phosphate buffer (pH 7.9).
Figure 29. Gel chromatographic purification of commercial aminopeptidase M on a column (2.5 x 30 cm) of Sephadex G-100 equilibrated with 0.1M phosphate buffer (pH 8.0). The column was eluted with 0.1M phosphate buffer (pH 8.0) and 0.5 ml fractions were collected.
Figure 30. Polyacrylamide gel (7.5%) electrophoresis of (a) commercial aminopeptidase M before gel filtration, and (b) aminopeptidase M after gel filtration on Sephadex G-100.
Figure 31. Polyacrylamide gel electrophoresis of aminopeptidase M under several dissociating conditions: (a) 1% SDS + 1% β-mercaptoethanol, (b) guanidine-HCl treatment followed by alkylation with iodoacetamide, (c) performate oxidation + 1% SDS. For the details of these procedures, see Experimental.
described. Regardless of whether the enzyme was subjected to SDS-mercaptoethanol, guanidinium HCl-alkylation, or performic acid treatment, the pattern gave essentially the same three bands. Furthermore, electrophoresis on 5% acrylamide gels after 1% SDS-1% mercaptoethanol treatment gave identical results. To obtain the molecular weights of the subunits indicated by the bands, a plot of log MW versus distance of the band from the origin was constructed using standard protein markers (phosphorylase A, albumin, pepsinogen). The results for both the 7.5% and 5% acrylamide gels are shown in Figure 32. The molecular weights of the three bands obtained by extrapolation were 130,000-140,000, 100,000, and 55,000-60,000.

Reacting Species Sedimentation. At 2 minute intervals during centrifugation, the photoelectric scanner was used to record the absorbance (405 nm) due to the release of p-nitroaniline from the substrate by aminopeptidase M as described in Experimental. When successive traces from the scanner were superimposed on a single frame of reference, the results were similar to a representative run as illustrated in Figure 33. Alternate traces were omitted from the figure for clarity of presentation. Such collections of traces are important for the detection of enzyme inactivation and band spreading due to convection during the course of the run. In all cases, the top and bottom of the traces were relatively flat. This constant plateau level of absorbance indicated that the above mentioned problems associated with
Figure 32. Plot of relative mobilities obtained for aminopeptidase M and protein markers as a function of molecular weight following SDS-acrylamide gel electrophoresis on 5% and 7.5% acrylamide gels. All determinations were made in 0.2M phosphate buffer (pH 7.2) which was 0.2% in SDS. Standard protein markers were phosphorylase A (103,000), serum albumin (68,000), and pepsinogen (41,000). The arrows represent the position of the aminopeptidase subunit bands relative to the standard protein markers (circles).
Figure 33. Analysis of the sedimentation of the reacting species of aminopeptidase M showing successive scanner traces superimposed on a single frame of reference. The hydrolysis of Leu-p-NO₂-anilide was followed at 405 nm with a photoelectric scanner coupled to the Schlieren optical system of a Beckman-Spinco model E analytical ultracentrifuge.
this technique were insignificant during the time of
the run (118).

The logarithm of the radius of the maximum absorbance
deflection was plotted as a function of the time after the
start of the run for a typical experiment (Figure 34). The
plot was linear with very limited scatter in the data. The
slope of the line of best fit was used to calculate the sedi­
mentation coefficient. Observed sedimentation coefficients
were corrected for the viscosity and density of the solvent
in the same manner as in standard sedimentation velocity
studies. The $s_{20,w}$ value obtained by this technique from an
average of three separate runs at different enzyme concen­
trations was 9.45 ± .4. This is identical within experimented
error to the sedimentation coefficient of 9.8 ± .2 obtained
by conventional sedimentation equilibrium studies (79).
Furthermore, no catalytically active species smaller than
this was detected even at the lowest enzyme concentration
allowed by the sensitivity of the instrument (0.8 ug/ml).
Figure 34. Plot of the log of the radius (distance from time zero) of the maximal absorbance deflection at 405 nm as measured by the photoelectric scanner versus the mean time for the sedimentation of the reacting form of aminopeptidase M.
DISCUSSION

Aminopeptidase M covalently bound to an arylamino derivative of porous glass retains 100% of its soluble activity at saturating levels of substrate. This remarkable complete retention of activity is more the exception than the rule for immobilized enzyme systems, but can be explained by arguments based on the accessibility of essential catalytic residues. One explanation postulates the coupling of the support to groups far from the active site to allow unhindered substrate catalysis. Consequently, the active site would have to be on one side of the enzyme molecule, while the majority of binding groups (tyrosine and/or lysine residues) would be on the opposite side. It is interesting to note that Schwabe (119) reported full retention of activity of a noncovalent complex of calcium phosphate gel and dental pulp leucine aminopeptidase. He postulated that a specific orientation of the active site to face the solvent occurred upon interaction of the enzyme with the support. However, this hypothesis suffers from a lack of experimental data, especially the absence of a comparison of kinetic parameters of the two forms of the enzyme. A more likely explanation for complete retention of activity assumes that the active site, and thus the residues necessary for catalytic activity,
are recessed in a hole or crevice. This hypothesis allows
the support to bind to the enzyme relatively close to the active
center, and minimizes the necessity of a rather unlikely enzyme
orientation by the support. On the basis of low angle x-ray
diffraction studies, Kretschmer and Kollin (120) suggested
a toroid structure for leucine aminopeptidase from bovine
eye lens. There is a distinct possibility that the active
site of aminopeptidase M could be situated on the inner
wall of such a structure. One further explanation of ac-
tivity retention must be mentioned in passing. It is possi-
ble, though highly unlikely, that during immobilization one
molecule of enzyme can be inactivated while another molecule
is activated. This combined inactivation-activation effect
would require an extremely precise balancing of the two
processes to account for the identical $V_{\text{max}}$ values of the
soluble and insoluble enzymes. Besides being unlikely from
a probability standpoint, this explanation can probably be
ruled out because of the similarities of the activation
energies and pH dependence of the two enzymic forms.

An increase in the apparent Michaelis constant, $K_{M(\text{app})}$,
upon insolubilization of aminopeptidase M is routinely ob-
served in most other insoluble enzymes. The presence of an
unstirred layer of solvent (Nernst layer) surrounding im-
mobilized enzyme particles in aqueous suspension has been
suggested (121-123). The rate of diffusion of substrates across
this unstirred layer affects the kinetic parameters of im-
mobilized enzymes in a manner analogous to nonenzymatic heterogeneous catalysis (124-125). Because of the resultant concentration gradient of substrate across the Nernst layer, enzyme saturation occurs at higher substrate concentrations than for soluble enzymes. Consequently, an increase in $K_M^{\text{app}}$ is observed. An effect on the kinetic behavior of immobilized enzymes due to steric restrictions brought about by the solid support is often expected. However, in most cases the effect is noticeable only with high molecular weight substrates, such as polypeptides or proteins (2, 6, 126-129).

Identical activation energies of the bound and free form of aminopeptidase M is a necessary condition of coincidental $k_{\text{cat}}$ values. The Arrhenius plots (Figure 8) indicate that thermal inactivation occurs at a lower temperature for bound aminopeptidase than for free enzyme. This phenomenon has been observed for the majority of immobilized enzymes (2, 6, 127, 129, 130), but several cases of improved thermal stability upon insolubilization have been reported (21, 25, 131-134). Katchalski, et al. (6) have attempted to explain this thermal stability decrease on the basis of a decrease in the probability of native enzyme conformation recovery following thermal perturbation.

Enzymes immobilized to solid supports, especially polyelectrolytic carriers, often exhibit shifts in their pH dependencies. The pH profiles of polyanionic enzymic derivatives are usually shifted to the alkaline region (6, 127, 135), while polycationic derivatives exhibit shifts toward the acid
region (6). These observations have been explained on the basis of microenvironmental pH effects induced by the poly-electrolytic carriers (136). In contrast, the pH profile of immobilized aminopeptidase M is virtually identical to the free enzyme. Similar pH dependencies tend to indicate that the essential active site residues of the enzyme are unaffected by the presence of the solid support.

The data illustrate the applicability of immobilized aminopeptidase M in the sequential analysis and total amino acid determination of peptides and proteins. The rates of release of the first four amino acids in the A-chain of insulin are quite well resolved (Figure 9). This illustrates a feature of aminopeptidase M which makes it especially suited for amino acid sequence determination. Except for proline, the amino acids are released at rates which differ by only a few-fold. For a protein containing no proline residues, immobilized aminopeptidase M alone can be used to hydrolyze smaller proteins completely to amino acids. Furthermore, immobilized aminopeptidase M can be used in combination with other immobilized proteases for the total hydrolysis of larger proteins. Theoretically, any protein or peptide can be totally hydrolyzed into amino acids by a combination of immobilized pronase, aminopeptidase M, and a proline-releasing peptidase, such as proline iminopeptidase (137-138) or aminopeptidase P (139). The problems of autolytic digestion and enzymic inactivation during hydrolysis are
minimized by immobilization. Furthermore, separation of proteolytic enzymes from reactants and products is accomplished by simple filtration. Since aminopeptidase M is a relatively expensive enzyme, insolubilization is economically very valuable. Because of the increased storage and operational stability, reuse of the bound enzyme is possible with little loss of activity over extended periods of time.

The study of the effect of inhibitors on aminopeptidase substrate hydrolysis is informative and provides insight into the substrate binding site. The structures of two specific substrates of aminopeptidase M, Leu-p-NO₂-anilide (I) and Gly-p-NO₂-anilide (II), are shown below. The only structural difference between the two is that (I) contains a hydrocarbon side chain while (II) possesses no side chain except a hydrogen atom. Leu-p-NO₂-anilide (I) hydrolysis is strongly inhibited by both aromatic and aliphatic hydrocarbons, with inhibition constants ($K_i$) an order of magnitude greater than the apparent Michaelis parameters ($K_{M(app)}$). In contrast, Gly-p-NO₂-anilide (II) is inhibited neither by aliphatic nor aromatic hydrocarbons. This observation tends to implicate a hydrophobic (apolar) binding site necessary for substrate, and presumably inhibitor, binding. In this light,
It is interesting to note that amino acid anilides with hydrophobic side chains are hydrolyzed at the maximal rate by aminopeptidase M, while anilides possessing hydrophilic side chains are hydrolyzed at a slower rate (70-71). The presence of a hydrophobic region, or at least a region of low polarity, on the enzyme raises the possibility that hydrocarbon inhibitors are "extracted" from the aqueous solution to a non-polar, organic phase in the enzyme (140-141). Canady and Wildnauer (142) have pointed out that the transfer of hydrocarbon inhibitors from an aqueous phase to an organic phase is dependent on the distribution coefficient between water and a liquid apolar phase. This transfer is independent of the polarity of the organic phase. With a series of hydrocarbons, a plot of $G^0$, the free energy of extraction or free energy of complex formation, versus molecular surface area is linear with a slope of 0.12 if an extraction mechanism of inhibitor binding is applicable. Such a linear dependence has been demonstrated for $\alpha$-chymotrypsin (142-143) and yeast alcohol dehydrogenase (143), both of which contain hydrophobic binding sites. The enzymic hydrophobic region is thought to be analogous to the non-aqueous phase in a simple hydrocarbon distribution between water and an organic phase. The data clearly indicate that such an extraction mechanism does not occur during the binding of hydrocarbon inhibitors to aminopeptidase M.

Chemical modification studies can provide very convincing evidence for the necessity of certain amino acid residues in
the catalytic process. Preliminary studies on aminopeptidase M (76-77) using tetranitromethane and diazonium-1-H-tetrazole have suggested that tyrosine and histidine are necessary for enzymatic activity, despite the lack of convincing experimental evidence. No mention was made of the fact that tetranitromethane will also react with sulfhydryl groups (96, 144) methionine residues (145), and tryptophan (146-147). Likewise, diazonium salts can react with tyrosine residues (40), lysine residues, and indole moieties (41-42), as well as with imidazole groups. Furthermore, no protection of the active site by competitive inhibitors or substrates was demonstrated upon chemical modification. Consequently, it was evident that a thorough and systematic chemical modification study of aminopeptidase M would be both relevant and also necessary in light of the sparse experimental data in existence.

The fact that aminopeptidase M retains full activity when coupled to diazonium salts linked to a solid support (diazotized arylamino glass) is extremely significant when compared to the results of modification by diazotized sulfanilic acid. The first conclusion is that the low molecular weight diazonium salt inactivates the enzyme by chemical modification of an amino acid residue, either tyrosine or histidine, at the active site. This rests on the observation that L-leucine, a competitive inhibitor, significantly protects the enzymic active site from modification. Secondly,
the results are consistent with the picture of the active site in a crevice or hole as mentioned earlier. Small diazonium salts are able to penetrate to the active site and inactivate the enzyme, while large, high molecular weight diazonium salts are prevented from reacting with essential residues due to steric hindrance introduced by the solid support (6).

The results of nitration with TNM are similar to Pfleiderer's results (76) and lend considerable support to the necessity of tyrosine for maximal enzymatic activity. Furthermore, protection of the enzyme with a competitive inhibitor and a specific substrate suggest that the tyrosine modified is at the active site. The quantitation of tyrosine nitration is informative in that the non-linearity of the inactivation is consistent with the conception of an enzyme with a number of accessible tyrosine residues on the surface, which are not essential for activity, and one or more tyrosines in a recessed active site. The experiments with N-acetylimidazole support the idea of a tyrosine residue with essential catalytic properties. The enzyme is inactivated by the modification reagent by acetylation of an amino acid residue. The observed rate of deacetylation and reactivation by hydroxylamine provides some evidence for the identity of the modified residue. Even though N-acetylimidazole can acetylate lysine, histidine, and tyrosine residues (148-149), acetyllysine cannot be deacetylated by hydroxylamine,
while the deacetylation of acetylhistidine is instantaneous (150). The rate of enzymatic reactivation by hydroxylamine is relatively slow and suggests the deacetylation of O-acetyl-tyrosine.

The presence of an essential histidine residue at the active site is suggested on the basis of photochemical oxidation experiments. Although photo-oxidation is relatively non-specific, Westhead (151) has shown that histidine is the only amino acid residue which shows a pH dependence of the rate of inactivation at pH values between 5 and 8 similar to the results found in this study. The data (Figure 21) clearly show such a pH dependence, from which a pK of 6.2-6.4 can be estimated for the group being oxidized. The observed pK is consistent with a "normal" pK for an imidazolium group of histidine. The pH dependence of the photo-oxidation, of course, rules out the possibility of oxidation of other amino acid residues, such as tryptophan or methionine, as the cause of enzyme inactivation. The quantitation of histidine oxidation is very similar to the results obtained with TNM modification. The results show not only the existence of a number of accessible histidine residues outside the active site on the enzyme surface, but also the fact that tyrosine and histidine are in a 1:1 ratio at the active site. Extrapolation of the inactivation to zero activity yields the same number of histidine and tyrosine residues modified per molecule of enzyme.
It has been suggested by Femfert and Pfleiderer (152) that the binding of the charged α-amino group of the substrate is facilitated by the presence of a charged carboxyl group at the active site. However, no evidence supporting this assertion is available. The data presented here show that the possibility of a carboxyl at the active site necessary for enzymatic activity may be eliminated in view of the inability of diazonium reagents or carbodiimides to inactivate the enzyme. The introduction of 57 moles of glycine bound to carboxyl groups per mole of enzyme would certainly sterically hinder the approach of the substrate if a carboxyl group was necessary for substrate binding. Similarly, α-amino groups of lysine do not appear to be required for enzymatic activity. Extensive methylation of 80% of the available lysine residues by the use of formaldehyde and NaBH₄ results in a negligible loss of enzymatic activity.

Frohne et al. (153) implied the presence of tryptophan in the active site of bovine eye lens aminopeptidase, an enzyme very similar to aminopeptidase M, based on a 50% inactivation of the enzyme with N-bromosuccinimide (NBS). The present data indicate no inactivation of APM by the action of dimethyl (2-hydroxy-5-nitrobenzyl) sulfonium bromide, a more specific modifying reagent, although 20 tryptophan residues were modified per enzyme molecule. This evidence suggests that the presence of an active site indole moiety necessary for enzymatic activity is highly doubtful. The apparent inactivation of lens aminopeptidase by N-bromosuccinimide
is possibly the result of tyrosine or histidine oxidation (154-155). Furthermore, NBS is readily able to cleave peptide bonds in an intact protein molecule (154, 156-159), possibly bringing about partial inactivation due to structural changes of the native enzymatic conformation.

Solvent deuterium isotope effects on the kinetic parameters, $k_{cat}$ and $K_M(app)$, are informative for the interpretation of the mechanism of aminopeptidase M catalysis. The results indicate that the observed isotope effect on $k_{cat}$ could possibly reflect a proton transfer at or near the rate limiting step in the mechanism. However, the dangers involved in drawing conclusions based on isotope effect studies are pointed out by Jencks (116). Deuterium oxide effects on the protein itself is a distinct possibility. There is direct evidence that a change from H$_2$O to D$_2$O can change the conformation of enzymes, by changing the properties of hydrophobic and hydrogen bonds (160-162). The observed isotope effect on the Michaelis constant is a rather common feature of enzymatic isotope effects (116, 163). This is not surprising, since the Michaelis constant, even for the simplest enzymatic mechanism, is a collection of kinetic constants. An isotope effect on a rate constant will be reflected by an isotope effect on the Michaelis parameter. Furthermore, Kresheck et al. (164) have pointed out the tendency of apolar bonds to be strengthened in D$_2$O. Inspection of the results shows that the $K_M$ of aminopeptidase M in D$_2$O is lower than the $K_M$ in H$_2$O above pH 7.0. If the binding involved in the formation of enzyme-
substrate complex is manifested in the Michaelis parameter, this observation is consistent with the idea of a hydrophobic binding site for aminopeptidase M and corroborates the results obtained by inhibition studies.

One possible mechanism to account not only for the presence of tyrosine and histidine at the active site but also for the isotope effect observed in D$_2$O is proposed as in Figure 35. Attempts to detect a covalent intermediate have been unsuccessful. The author proposes that histidine can function as a general base to abstract a proton from water. Nucleophilic attack on the carbonyl carbon leads to the formation of a tetrahedral adduct. The abstraction of a proton from water by the imidazole group of histidine could possibly be the cause of the small but significant solvent isotope effect when substrate hydrolysis is carried out in D$_2$O. Decomposition of the tetrahedral intermediate may be facilitated by the proton donating ability of the phenolic hydroxyl group of tyrosine. This is reasonable since it is postulated that tyrosine functions as a general acid in at least one enzymatic mechanism (165-167). The binding of substrates is not clear, but evidence presented earlier suggests the presence of a primary apolar binding site. There is evidence which suggests that divalent metal ions are necessary for enzymatic activity (168-169). Lehky et al. (169) have shown the presence of two atoms of Zn$^{2+}$ per molecule enzyme. The function of the metal ion is also unclear at this time. It may participate in substrate binding by
Figure 35. Proposed mechanism of action for aminopeptidase M catalysis. (a) histidine acting as a general base to facilitate nucleophilic attack by water to form a tetrahedral intermediate (b) decomposition of the intermediate aided by tyrosine acting as a general acid.
interacting with the primary α-amino group and carbonyl oxygen as proposed by Klotz and Ming (170). Similarly, Smith and Spackman (171) suggested that the metal ion interacts with the α-amino group and the amide nitrogen, not the carbonyl oxygen. Alternatively, Zn\(^{2+}\) may act as an electron sink to stabilize the charged oxygen of the proposed tetrahedral intermediate. However, the presence of only two zinc atoms per mole enzyme casts some doubt on the necessity of metal ions in the catalytic process.

The future potential of immobilized enzymes for the elucidation of essential active site residues cannot be ignored. For most soluble enzyme modification reactions, it is not possible to use a specific substrate to protect the enzyme active site. Under these conditions, the reaction between enzyme and modification reagent would be much slower than substrate depletion. Substrate catalysis occurs before significant protection of the active site from modification is accomplished. Furthermore, many modification reagents, such as diazonium salts, react not only with groups on the enzyme but also with the inhibitor of substrate protecting reagents. Use of a solid supported enzyme packed in a column eliminates these problems. This technique maintains both modification and protection reagents at constant concentrations. Likewise, reaction between the two or depletion of substrate protecting agent is minimized. Obviously, this approach is valid only if the immobilized and free forms of the enzyme are considerably similar. It has been
amply demonstrated that this is indeed the case for aminopeptidase M.

The determination of the number of active sites per molecule of aminopeptidase M can possibly shed some light on the function of the metal ion. Since L-leucine inhibits the enzyme in a competitive manner and forms a 1:1 ratio with enzyme in the enzyme-substrate complex (see Results), the choice of this amino acid for equilibrium binding studies was obvious. The data, however, indicate rather non-specific binding with a great amount of scatter in the determined parameters. The errors in attempting to draw conclusions on the number of active sites based on extrapolation are enormous (117). Consequently, no accurate value for the number of active sites can be extracted from the data available. A good active site directed irreversible inhibitor for aminopeptidases in general would be valuable. At this time, no such inhibitor exists for any of the aminopeptidases studied. The specific active site directed properties of chloromethyl ketone analogues of D,L-leucine and alanine have been demonstrated by Akhtar and Birch (172) for hog kidney leucine aminopeptidase. Likewise, Lasch et al. (173) studied several butylcarbamides, which proved to be potent inhibitors of bovine eye lens aminopeptidase. However, in all cases, the inhibitors studied were reversible and could not be used to irreversibly inactivate the enzyme.
The data presented here casts doubt on the ten subunit model of aminopeptidase M proposed by Wachsmuth (80). No matter what method was used to disrupt the quaternary structure of the enzyme (see Experimental), the same three-banded pattern was obtained on polyacrylamide electrophoresis. Some methods, such as boiling in 1% SDS-1% mercaptoethanol or denaturation by Gu-HCl followed by alkylation, involved rather drastic treatment of the enzyme. However, no evidence for a species of molecular weight less than 55,000-60,000 was observed. The model of Wachsmuth proposed 10 subunits of molecular weight about 30,000 (80). It is indeed tantalizing to notice that the molecular weights of the protein bands determined by SDS-gel electrophoresis in this study are all multiples of 30,000. This common multiple observation suggests that subunit aggregation is still a factor. However, it is virtually impossible to envision more severe disaggregating conditions than those reported here. Too little information is available at this point to postulate a subunit structure for aminopeptidase M.

In the study of multi-subunit enzymes such as aminopeptidase M, the problem of determining the actual size of the species taking part in the catalysis of substrate hydrolysis is of paramount importance. The sedimentation coefficient of a molecular species, which is a function of size and molecular weight, can be readily determined using conventional ultracentrifugation techniques. However, an enzyme
concentration range between 0.5 and 10 mg/ml is needed to yield accurate results. A great discrepancy exists between the amount of enzyme per unit volume needed for conventional zonal centrifugation and the amount employed in an enzymatic assay. For example, 0.5 to 5 μg of aminopeptidase M per ml assay mixture is routinely used in the assay for enzymatic activity with Leu-p-NO₂-anilide as the substrate. Consequently, the possible problem of subunit dissociation becomes important at these low concentrations. Conversely, enzyme aggregation at higher concentrations is a distinct possibility. It is entirely possible that the size of the catalytically active species at low assay concentrations may be much lower than at the higher concentrations used to determine $s_{20,w}$ values by zonal centrifugation. The results indicate that the sedimentation coefficient of the catalytically active species as determined by the method of Cohen et al. (108-110) is identical within experimental error to the value of $s_{20,w}$ obtained by zonal centrifugation (79). The enzyme concentrations used in the studies presented here were in the same range of concentrations used in a normal aminopeptidase M assay, and not several orders of magnitude higher as in conventional techniques.

Conclusions. In summary, it can be stated that the insoluble porous-glass derivative of aminopeptidase M described in this dissertation is of potential value in the field of primary structure determination of proteins and
peptides. The immobilized enzyme derivative efficiently alleviates the problems associated with the conventional preparation of hydrolysates for amino acid analysis. Furthermore, the amino acid sequence determination of smaller peptides can be accomplished with ease and little ambiguity. In a more fundamental vein, the data presented here allow a direct comparison of the immobilized and soluble forms of the enzyme with regard to kinetic parameters, pH dependence of substrate hydrolysis, and temperature dependence of enzymatic reaction rate.

Several chemical and physical studies have been accomplished on the soluble form of aminopeptidase M, the microsomal aminopeptidase from swine kidney. Valid and convincing evidence strongly suggests that most likely histidine and probably tyrosine are catalytically essential residues located at the active site of the enzyme. This assertion is strongly supported by extensive chemical modification experiments and active site protection studies. No other residues were found to be necessary for enzymatic activity. Along with these findings, evidence has been presented for the existence of a hydrophobic binding site on the enzyme. Furthermore, data from inhibition studies show conclusively that inhibitor binding does not involve an "extraction" mechanism. Preliminary evidence is presented for the number of active sites and subunit structure of the
enzyme. However, it must be stated that more clarity in these areas is necessary before final conclusions can be drawn. In contrast to this data, a definite size of the catalytically active species has been determined at enzyme concentrations normally used in the assay of enzymatic activity. Finally, a proposed, but by no means definitive, mechanism of action has been suggested which incorporates not only the involvement of the catalytically essential active site residues, but also a small but significant solvent deuterium isotope effect detected upon substrate hydrolysis in D₂O.

As in most areas of scientific endeavor, the elucidation of ambiguities and solution of problems provides some measure of intellectual gratification, but, at the same time, raises more questions and provides more suggestions for further study than were heretofor inherent in the original investigation. Much work remains to be done in the field of aminopeptidases, which depend solely on the imagination and ability of the investigator. The areas of possible investigation, including detailed aspects of the mechanism of action, appear enormous; indeed, almost limitless. Nevertheless, it is the author's hope that this dissertation sheds some light, however dimly, on the perplexities of aminopeptidase M, specifically, and enzyme chemistry in general.
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