MAGNETIC RESONANCE STUDIES OF TRYPsin

by

SHYUE YUE KANG

B.Sc. (Hons.), National Taiwan University, China, 1967.

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY in the Department of Chemistry

We accept this thesis as conforming to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

1974
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the Head of my Department or by his representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of CHEMISTRY

The University of British Columbia
Vancouver 8, Canada

Date Feb 5, 1974
Abstract

A great advance in the understanding of the mechanism for enzymatic reactions on a molecular basis has resulted from knowledge of the three dimensional structure of several enzymes from x-ray diffraction methods. It is not possible, however, to determine the enzyme mechanism only by knowing its three dimensional structure. The dynamic aspect of the enzymatic reaction is required to understand its mechanism.

Nuclear magnetic resonance (NMR) and electron spin resonance (ESR) are physical methods which contain both structural and dynamic information. This thesis presents studies of the interactions between the macromolecule, trypsin, and the small molecules, substrate like inhibitors, or ions, Ca$^{++}$, Mn$^{++}$ by magnetic resonance.

The theories of nuclear magnetic relaxation in the presence of chemical exchange, relaxation mechanisms, and the methods of measurement of relaxation are presented in Chapter 1. Here are discussed the equations relating measured relaxation times to chemical exchange rates, chemical shifts and relaxation times of nuclei on small molecules in dynamic chemical exchange to a macromolecular site. Different exchange limits are discussed and means for distinguishing various limits are provided.
In order to understand the effect of divalent ions (Ca$^{++}$, Mn$^{++}$, etc.) on the properties of trypsin, a study of Mn$^{++}$ binding to trypsin is described in Chapter II.

Mn$^{++}$ was chosen as a model for Ca$^{++}$ binding, since Mn$^{++}$ is paramagnetic. Although all previous attempts to use NMR to interpret Mn$^{++}$ binding were based on use of "enhancement factors", I found that a more straightforward and clearer approach was to use the NMR relaxation times directly. The existing theory for effect of chemical exchange on NMR $T_1$'s was extended to the case of three distinct chemical sites with all possible mutual interconversions, and applied the result to the binding of water to free Mn$^{++}$ and Mn$^{++}$:enzyme complex. An exact treatment of the correction for the internal rotation of water at the Mn$^{++}$ binding site is also presented. The main conclusions were that Mn$^{++}$ binds strongly only on active trypsin and at just one site, and that the water bound to Mn$^{++}$ at that site can rotate rather freely, suggesting that the site must lie in an open region of the tertiary structure. These facts are most consistent with the binding of Mn$^{++}$ at Asp 71 and Asp 153 (or Glu 77), where it has been suspected (but not shown) that Ca$^{++}$ may act to hold two loops of the enzyme together.

The next two chapters are devoted to the study of the active site of trypsin by UV and NMR. This basic approach was to choose a homologous series of substrate-like inhibitors of trypsin, and study their binding to trypsin both by steady-
state (uv) kinetics and also by NMR relaxation time measurements. This work would provide for the first time a direct comparison between the strength of binding (as measured by the binding constant from uv data) and the rigidity with which the inhibitor was bound to the enzyme (from NMR data). Any correlation, or lack of it, between these two parameters should provide more insight into theories of enzyme action. For rigorous NMR analysis, it was desirable to have inhibitors with \(-\text{OCH}_3\) groups, to obtain a single, sharp NMR signal well-separated from other parts of the NMR spectrum. This requirement entailed the synthesis of a number of inhibitors, and in most cases, a given synthesis was not in the literature and had to be devised individually. This is described in the experimental section of Chapter III.

In order to locate the binding sites of each inhibitor, and to obtain an accurate dissociation constant for each inhibitor, uv steady-state trypsin assays using D,L-BAPA as substrate were carried out. As a result, five of the interesting inhibitors gave Dixon plots with intersections below the x-axis, a result which cannot be explained by previous trypsin inhibitor work. The difficulty was eventually resolved by taking into account the interaction between the D-BAPA and my inhibitors. Although apparently a complication, the algebraic consequences showed that my seven trypsin inhibitors could be classified according to whether their binding was competitive, repulsive, non-competitive, or cooperative with the binding of D-BAPA. This then gave a rather complete picture of the inhibitor binding. The data definitely showed the
presence of at least one secondary binding site, which is consistent with a number of unpublished X-ray results, and the secondary binding site exhibits some cooperative effect toward binding of a substrate analog. This had been observed on TAME substrate activation at high concentration.

Chapter IV presents the measurements of the bound relaxation time of each of the inhibitors on trypsin by selective pulsed high resolution NMR (The measurement was made on the single sharp line of the methyl protons of the inhibitors). A special-purpose pulse unit is described briefly and the advantages and limitation of selective determination of relaxation time on high resolution NMR was also discussed. The relaxation time for the rigidly bound inhibitor was calculated and a correction for the effect of internal rotation of methyl group was made. The results strongly suggest that for the inhibitors of the same category (from uv), correlation between strength of binding and rigidity of binding can be demonstrated. The resultant implications for theories of enzyme catalysis was also discussed.

In the last chapter, an ESR "spin label" to the active site serine of trypsin, with the intent of using the effect of this spin label on the NMR lineshape of my inhibitors as a "ruler" to determine the distance from the active site to the secondary binding sites. Hopefully, the interaction between the "spin label" and Mn^{++} on the enzyme is strong enough so that the distance between them can also be estimated. The conformational change of
the active site region with different perturbations (pH, Ca\textsuperscript{++}, inhibitors, etc) was also expected to be monitored through the changes of the ESR signal of the attached spin label. In order to ensure the protection of the spin labelled trypsin from autoproteolysis during the NMR experiments, the active trypsin-free, spin labelled trypsin was prepared successfully by a new process using soybean trypsin inhibitor. From the NMR measurements, it can be estimated that the distances between the spin label and the secondary binding sites are all around 9 to 10 Å. With the help of X-ray data, the location of the secondary sites may be speculated and the consequence evaluated. Due to the long distance between the spin label and the Mn\textsuperscript{++} ion binding site, the interaction between these two paramagnetic species cannot be observed. In addition, the ESR signal of the ser-195 spin label was not sensitive to the minor conformational changes induced by the various perturbations added.

Supervisor.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>vii</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xiii</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>xvi</td>
</tr>
<tr>
<td><strong>CHAPTER I</strong> Basis of Magnetic Resonance Methods for Study of Biological Macromolecules in Solution</td>
<td></td>
</tr>
<tr>
<td>A. Introductory Remarks</td>
<td>1</td>
</tr>
<tr>
<td>B. Nuclear Relaxation and Chemical Exchange</td>
<td>3</td>
</tr>
<tr>
<td>C. Chemical Shifts and Chemical Exchange</td>
<td>12</td>
</tr>
<tr>
<td>D. The Mechanism of the Relaxation Process</td>
<td>12</td>
</tr>
<tr>
<td>E. Methods of Measurement</td>
<td>15</td>
</tr>
<tr>
<td>1. Measurement of spin-lattice relaxation time, $T_1$</td>
<td>15</td>
</tr>
<tr>
<td>2. Measurement of spin-spin relaxation time, $T_2$</td>
<td>21</td>
</tr>
<tr>
<td>3. Fourier-Transform NMR</td>
<td>24</td>
</tr>
<tr>
<td>References (Chapter I)</td>
<td>28</td>
</tr>
<tr>
<td><strong>CHAPTER II</strong> Magnetic Resonance Studies of Mn(II) Ions Binding to Trypsin</td>
<td></td>
</tr>
<tr>
<td>A. Introduction</td>
<td>30</td>
</tr>
<tr>
<td>B. Theory</td>
<td>31</td>
</tr>
<tr>
<td>C. Experimental</td>
<td>35</td>
</tr>
<tr>
<td>D. Results and Discussion</td>
<td>39</td>
</tr>
</tbody>
</table>
1. Strength and number of Mn$^{++}$-binding sites on trypsin ................................................. 39
2. Rotational lability of water at the strong Mn$^{++}$-binding site of trypsin ...................... 44
E. Summary ......................................................... 57
References (Chapter II) .............. 58

CHAPTER III  Steady-State Inhibition Kinetics Using Racemic Substrate : A Probe For Cooperative Inhibitor Binding In Trypsin
A. Introduction ................................................. 60
B. Experimental .................................................. 63
C. Theory ......................................................... 66
D. Results and Discussion ................................. 70
E. Summary ......................................................... 78
References (Chapter III) .............. 80

CHAPTER IV  The Studies of Rigidity of Binding of Inhibitors to Trypsin by NMR and its Correlation with the Strength of Binding
A. Introduction ................................................. 82
B. Experimental .................................................. 84
1. Instrument .................................................... 84
2. Method of Measurement ................................. 87
   a. chemical shift ............................................ 87
CHAPTER V Magnetic Resonance Studies of Serine-195-Spin-Labeled Trypsin

A. Introduction .................................................. 111
B. Experimental .................................................. 113
   1. Materials ................................................... 113
   2. Preliminary preparation of gel filtration columns .......... 114
   3. Preparation of active trypsin-free, spin labeled trypsin .......... 114
   4. Method ...................................................... 131
C. Theory ......................................................... 132
D. Analysis of Data .............................................. 134
   1. Determination of T_{2M} and \( \gamma \) ....................... 134
   2. \( \tau_C \) is dominated by \( \tau_Y \) ............................ 136
   3. Justification for the assumption of fast exchange .......... 137
E. Results and Discussion ....................................... 139
   1. Estimation of distance by measuring the induced nuclear relaxation .......... 139
   2. Conformational changes examined by ESR .......... 151
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Various limits for chemical exchange and its determination</td>
<td>9</td>
</tr>
<tr>
<td>2.1</td>
<td>Experimental proton relaxation rates ($\text{sec}^{-1}$) for water protons in the presence and absence of trypsin at different concentration of Mn$^{++}$ solutions</td>
<td>46</td>
</tr>
<tr>
<td>3.1</td>
<td>Interpretation of the significance of the $y$-values at the intersection of two Dixon plots corresponding to two different concentrations of a racemic &quot;substrate&quot;</td>
<td>73</td>
</tr>
<tr>
<td>3.2</td>
<td>Structures, inhibition constants, and interaction coefficients for binding of inhibitors to trypsin (our studies)</td>
<td>74</td>
</tr>
<tr>
<td>3.3</td>
<td>Structures, inhibition constants, and interaction coefficients for binding of inhibitors to trypsin. (Mares-Guia and Shaw, 1965)</td>
<td>76</td>
</tr>
<tr>
<td>4.1</td>
<td>Strength of binding ($K_I$), cooperativity towards binding of substrate ($A$), and rigidity of binding ($1/T_2$) of various inhibitors to trypsin</td>
<td>102</td>
</tr>
<tr>
<td>Table</td>
<td>Title</td>
<td>Page No.</td>
</tr>
<tr>
<td>-------</td>
<td>----------------------------------------------------------------------</td>
<td>----------</td>
</tr>
<tr>
<td>5.1</td>
<td>Experimental parameters used in enzyme intramolecular calculations</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>(at temperature $30^\circ \pm 1^\circ$ C)</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>Experimental parameters used in estimation of the electron-nuclear</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>dipole-dipole contribution to inhibitor proton nuclear relaxation</td>
<td></td>
</tr>
<tr>
<td></td>
<td>due to SL-trypsin at a different temperature</td>
<td></td>
</tr>
<tr>
<td>5.3</td>
<td>Length of inhibitor molecules and the possible location of secondary</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td>sites</td>
<td></td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Computer simulation of the temperature behavior of $T_2$ at 60 MHz, 100 MHz and 220 MHz</td>
<td>10</td>
</tr>
<tr>
<td>1.2</td>
<td>Measurement of $T_1$: Method #1</td>
<td>17</td>
</tr>
<tr>
<td>1.3</td>
<td>Measurement of $T_1$: Method #2</td>
<td>19</td>
</tr>
<tr>
<td>1.4</td>
<td>Measurement of Nuclear $T_2$</td>
<td>21</td>
</tr>
<tr>
<td>1.5</td>
<td>Transient and Fourier-Transformed NMR Responses for an Inhibitor of Trypsin</td>
<td>25</td>
</tr>
<tr>
<td>2.1</td>
<td>ESR Spectrum of $10^{-4}$ M/l Mn$^{++}$ solution at pH 7.1 and 21 ± 0.5° C</td>
<td>37</td>
</tr>
<tr>
<td>2.2</td>
<td>Scatchard plot for determination of strength and number of binding sites for Mn$^{++}$ to trypsin</td>
<td>41</td>
</tr>
<tr>
<td>2.3</td>
<td>The possible structure of Mn$^{++}$ strong binding site on trypsin</td>
<td>52</td>
</tr>
<tr>
<td>2.4</td>
<td>Log-log plot of longitudinal relaxation rate versus macromolecular rotational correlation time</td>
<td>54</td>
</tr>
<tr>
<td>3.1</td>
<td>Dixon plot for inhibitor of trypsin by the inhibitors shown in each plot</td>
<td>71</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1</td>
<td>Computer simulation for $1/T_2$ versus $[E_0]/[I_0] \times 10^3$ at different hypothetical concentration with $[E_0] = 10^{-3} \text{ M/\ell}$</td>
<td>90</td>
</tr>
<tr>
<td>4.2-4.2</td>
<td>$1/T_2$ and $1/T_1$ for the methyl protons of the inhibitors at different concentrations in the presence of constant amounts of trypsin, $[E_0] = 10^{-3} \text{ M/\ell}$, PD = 8.1. Temperature 30 ± 1°C</td>
<td>92-99</td>
</tr>
<tr>
<td>5.1</td>
<td>Separation of trypsin-soybean trypsin inhibitor (STI) complex from a mixture of spin-labeled trypsin, trypsin-STI complex and STI by chromatograph on column (5.0 x 80 cm) of Sephadex G-50</td>
<td>117</td>
</tr>
<tr>
<td>5.2</td>
<td>Separation of $\alpha$ and $\beta$ spin labeled trypsin from a mixture of $\alpha$ and $\beta$ spin labeled trypsin, STI, and inactive (spin labeled) trypsin by ion exchange chromatography on a column (2.6 x 50 cm) of SP-Sephadex C-50</td>
<td>119</td>
</tr>
<tr>
<td>5.3</td>
<td>ESR spectrum of free spin label, spin labeled $\alpha$ and $\beta$ trypsin</td>
<td>121</td>
</tr>
<tr>
<td>5.4</td>
<td>A comparison of the ESR spectra of spin labeled trypsin before and after freeze-drying</td>
<td>123</td>
</tr>
</tbody>
</table>
LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5</td>
<td>The stability of the active trypsin-free, spin-labeled trypsin examined by ESR</td>
<td>125</td>
</tr>
<tr>
<td>5.6</td>
<td>The hydrolysis of spin-labeled trypsin in the presence of commercial trypsin</td>
<td>127</td>
</tr>
<tr>
<td>5.7 A.</td>
<td>ESR spectra of Mn$^{++}$ in the presence of spin labeled trypsin</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B.C.D. ESR spectra of spin labeled trypsin with different perturbations</td>
<td>129</td>
</tr>
<tr>
<td>5.8</td>
<td>Typical Proton High-Resolution Spectrum of an inhibitor of Trypsin</td>
<td>143</td>
</tr>
<tr>
<td>5.9</td>
<td>Typical effect of SL-Trypsin on Proton NMR spectrum of an inhibitor</td>
<td>145</td>
</tr>
</tbody>
</table>
ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my research director, Dr. A.G. Marshall, for his guidance, invaluable advice, and constant encouragement throughout my work.

Thanks are also due to
Dr. B.A. Dunell for his kind permission to use the Bruker NMR pulse Spectrometer.
Dr. L.D. Hall for his kind permission to use the Audio-Frequency Pulse Unit.
Dr. K.A. Walsh for permitting me to work in his laboratory and for his advice in conduction of my trypsin assays.
Dr. D.G. Clark for his advice on my enzyme preparation and his help on various biochemical techniques.
Dr. L. Weiler and his research group for advice and suggestion in the synethetic work.
Dr. D.T. Suzuki for use of his U.V. spectrometer.
Dr. P.D. Bragg for his stimulating suggestions in theory of enzyme kinetics.
My colleagues Dr. P. Griffith, L.G. Werbelow and P.J. Morrod for their friendship and help.

The members of electronic shop for their ideal service on the spectrometers.
CHAPTER I

Basis of Magnetic Resonance Methods for Study of Biological Macromolecules in Solution

A. Introductory remarks

Only two types of spectroscopic methods are capable of detecting individual atoms in macromolecules, x-ray diffraction in the crystalline state and magnetic resonance in the liquid state. Both types of information are required for unravelling the central question in biological chemistry: the relation between molecular structure and function. The exciting success of x-ray diffraction in reconstructing the three dimensional structure of enzymes is already established. A comparative latecomer - magnetic resonance, is just beginning to demonstrate its potentiality.

Sources of information from NMR.
1) Chemical shift (\( \delta \)) - identification of the nuclei in different environments.
2) Coupling constant (J) - detail of molecular structure in conformation.
3) Area under the resonance signal-relative populations of nuclei in different environments.
4) Spin-lattice and spin-spin relaxation time ($T_1$ and $T_2$) - dynamic information such as molecular motions, rate of chemical exchanges, rigidity of binding, and the distance between the interacting nuclei or between the interacting electrons and nuclei.

Similar information is obtainable from ESR hyperfine splitting, peak area, and lineshape; however, it is generally necessary to design and synthesize a specific "spin-label" for binding to a particular macromolecular site.

Direct NMR studies on macromolecules are difficult to conduct because of the weakness of the NMR signal and the complexity of the spectra. The interactions between a small molecule (inhibitor) and a macromolecule (enzyme) provides a means for amplifying the NMR signal by taking advantage of the observable perturbation of the strong NMR signal of a (more concentrated) small molecule in the presence of a (highly dilute) macromolecular solution. In general, it is not easy to directly detect changes in chemical shift or coupling constant of a macromolecular site. In the following sections, discussion will be concentrated on the information obtained from relaxation time measurements ($T_1$ and $T_2$).

The theory of relaxation in the presence of chemical exchange will be reviewed. Some of the limits for chemical exchange will be re-evaluated and discussed and finally some simple physical description of the different techniques involved in measuring $T_1$ and $T_2$ are presented.
B. Nuclear relaxation and chemical exchange

The interactions between small molecules and macromolecules are our main interest in these studies. In general, the small molecule jumps back and forth between an aqueous or free environment "A" and some binding site "B" of the macromolecule

\[ \begin{align*}
  \text{"A"} & \xleftarrow{K_1} \xrightarrow{K_{-1}} \text{"B"} \\
\end{align*} \] (1.1)

"A" usually represents the solvent, dimagnetic environment, etc. and "B" usually refers to the active site of the enzyme, or to the magnetic sphere of influence of paramagnetic ion; \( K_1 \) is the rate constant for the small molecule to jump to "B" state; and \( K_{-1} \) is the rate constant for it jumping back into the solvent.

McConnell\(^1\) modified the Bloch equation to include the process of chemical exchange between two magnetically distinct sites. This treatment was extended to 3 sites and various limits were explored by Swift and Connick\(^2\). When one chemical component was in great excess (as in enzyme kinetics) the assumption that the fraction of the observed molecules at site A is much greater than that of the molecule at site B leads to the following form for the observed transverse relaxation time;

\[ \frac{1}{T_2} = \frac{f_A}{T_{2A}} + \frac{f_B}{T_B} \left[ \frac{(1/T_{2B} + 1/\tau_B)(1/T_{2B} + \Delta W_B^2)}{(1/T_{2B} + 1/\tau_B)^2 + \Delta W_B^2} \right] \] (1.2)

where \( f_A, f_B \) are the fractions of the observed molecules at sites
A and B respectively and $f_A \gg f_B$, $\tau_B = 1/K_1$, $\Delta W_B$ is the difference in chemical shift between sites "A" and "B" in radians/sec., $T_{2A}$, $T_{2B}$ are the transverse relaxation times for the nuclei at sites "A" and "B" respectively.

The corresponding equation for spin-lattice relaxation time has been derived by Luz and Meiboom

$$\frac{1}{T_1} = \frac{f_A}{T_{1A}} + \frac{f_B}{T_{1B} + \tau_B} \quad (1.3)$$

where $T_{1A}$ and $T_{1B}$ are the longitudinal relaxation times for nuclei at sites "A" and "B" respectively.

**case A.** $\Delta W_B \approx 0$ (no chemical "shift" on binding)

Here the chemical shift at site "B" is close or equal to that of site "A". There will be only one observed resonance signal at frequency $W_A$. Equation (2) can then be simplified to

$$\frac{1}{T_2} = \frac{1}{T_{2A}} + \frac{f_B}{T_{2B} + \tau_B} \quad (1.4)$$

with $f_A \gg f_B \quad f_A \approx 1$ (this condition will apply to all succeeding equations)

**limit (a)** slow exchange,

$$\frac{1}{\tau_B} \ll \frac{1}{T_{2A}} \quad \text{or} \quad \tau_B \gg T_{2B}$$

so that

$$\frac{1}{T_2} = \frac{1}{T_{2A}} + \frac{f_B}{\tau_B} \quad (1.5)$$
Rate of chemical exchange is slow compared to magnetic relaxation and the observed line broadening is dominated by exchange rate or lifetime broadening.

**Limit (b) fast exchange**

\[
\frac{1}{\tau_B} \gg \frac{1}{T_{2B}} \quad \text{or} \quad \tau_B \ll T_{2B}
\]

so that

\[
\frac{1}{T_2} = \frac{1}{T_{2A}} + \frac{f_B}{T_{2B}} \quad (1.6)
\]

Rate of chemical exchange is fast compared to magnetic relaxations and the observed line broadening is dominated by the transverse relaxation time at site "B".

The temperature dependence of \(1/T_2\) provides the means of distinguishing between limit (a) and (b)\(^4\). Since \(1/\tau_B\) increases with temperature according to

\[
1/\tau_B = K_{-1} = A e^{(-E_A/RT)} \quad (1.7)
\]

where \(A\) is a constant, \(E_A\) is the energy required for the chemical exchange process, \(R\) is the gas constant, and \(T\) is temperature, \(1/T_2\) should increase logarithmically with increasing temperature in limit (a). On the other hand, since \(1/T_{2B}\) decreases with increasing temperature\(^5\),

\[
1/T_{2B} \propto \tau_c \propto \frac{1}{T} \quad (1.8)
\]
1/T_2 should decrease with temperature in limit (b).

**Case B.** \( \Delta W_B > 0 \) (measurable chemical "shift" on binding)

The situation is greatly complicated by introducing this extra factor, since equation (1.2) can no longer be simplified as in case A. Furthermore, the temperature control experiment used in case A is no longer sufficient to distinguish unambiguously the different exchange limits.

The line broadening could be dominated by any of three variables, \( \Delta W_B, \frac{1}{T_{2B}}, \frac{1}{\tau_B} \). The different limit for chemical exchange had been discussed by Swift and Connick\(^2\). Each of the limits will be re-examined and a means of distinguishing among these limits will be provided. For brevity, let

\[
(\frac{1}{T_2})_{NET} = (\frac{1}{T_2}) - \frac{f_A}{T_{2A}}
\]

**Limit (a)** \( \Delta W_B^2 \gg \frac{1}{T_{2B}}, \frac{1}{\tau_B} \)

so that

\[
(\frac{1}{T_2})_{NET} = \frac{f_B}{\tau_B}
\]

The separation of the two resonance lines at site A and B is much larger than either the exchange rate or the transverse relaxation times. Theoretically, there should be two resonance lines each at resonance frequencies \( W_A, W_B \), it should be remembered that "A" is in great excess, so that only the \( W_A \) resonance
will be visible.

\[
\text{limit (b)} \quad \frac{1}{T_{2B}} \gg \Delta W_B^2, \quad \text{or} \quad \frac{1}{\tau_B}
\]

\[
\left(\frac{1}{T_{2}}\right)_{\text{NET}} = \frac{f_B}{\tau B}
\]  

(1.11)

It will be two broad lines partially collapsed or a single line depending on whether \(\Delta W_B \gg \frac{1}{\tau_B}\) or \(\Delta W_B \ll \frac{1}{\tau_B}\) respectively.

The line broadening in limit (a) and (b) is mainly controlled by exchange rate \(\tau_B\).

\[
\text{limit (c)} \quad \frac{1}{T_{2B}} \gg \Delta W_B^2 \quad \text{and} \quad \frac{1}{\tau_B} < \frac{1}{T_{2B}}
\]

\[
\left(\frac{1}{T_{2}}\right)_{\text{NET}} = f_B \tau_B \Delta W_B^2
\]  

(1.12)

The exchange rate is fast compared to the separation of the two resonances lines at sites A and B. There will be one single line located between \(W_A\) and \(W_B\). \((1/T_{2})_{\text{NET}}\) is controlled by the rate of relaxation through the change in the precession frequency and is field dependent.

\[
\text{limit (d)} \quad \frac{1}{T_{2B} \tau_B} \gg \Delta W_B^2 \quad \text{and} \quad \frac{1}{\tau_B} \gg \frac{1}{T_{2B}}
\]

\[
\left(\frac{1}{T_{2}}\right)_{\text{NET}} = \frac{f_B}{T_{2B}}
\]  

(1.13)

This is the real fast exchange limit. Rate of chemical
exchange is fast compared to either the relaxation times or the differences of chemical shifts between sites "A" and "B". The line broadening is due mainly to the relaxation process at site "B".

Here, the temperature behaviors of the "fast" exchange limit (c) and (d) are qualitatively similar, the measurement of line width at different temperatures is not sufficient to distinguish these two limits.

However, the chemical shift and the relaxation time at site "B", $\Delta W_B$ and $\left(1/T_2\right)_{\text{NET}}/f_B$, can be determined independently from concentration dependence of the observed Net chemical shift and Net line broadening respectively.

Set $\pi \Delta \nu = \frac{(1/T_2)_{\text{NET}}}{f_B}$ for brevity,

if limit (c) holds, then

$$\frac{1}{\tau_B} \ll \frac{1}{T_2 B} \quad \frac{\pi \Delta \nu}{\Delta W_B^2} \gg \frac{1}{\pi \Delta \nu}$$

if limit (d) holds, then

$$\frac{1}{\tau_B} \gg \frac{1}{T_2 B} \quad \frac{\pi \Delta \nu}{\Delta W_B^2} \ll \frac{1}{\pi \Delta \nu}$$

Thus, if the line width as $f_B \to 1$ is larger than the frequency separation of the resonance for the two sites, limit (c) holds; if the line width as $f_B \to 1$ is smaller than $\Delta W_B$, limit (d) holds.

This extra treatment of the data on top of the temperature
control experiment will leave no ambiguity in distinguishing which process dominates the line broadening, and thus will ensure the correct interpretation of the data.

With the above consideration in mind, one can systematically sort out the problem of chemical exchange at different limits, see Table 1.1 and Figure 1.1

Table 1.1

<table>
<thead>
<tr>
<th>cases</th>
<th>$\Delta W_B$</th>
<th>$\Delta \nu$</th>
<th>$T$</th>
<th>process dominated by</th>
<th>limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, (a)</td>
<td>$\approx 0$</td>
<td>$\uparrow$</td>
<td>$\uparrow$</td>
<td>$\tau_B$</td>
<td>$\tau_B &gt;&gt; T_{2B}$</td>
</tr>
<tr>
<td>(b)</td>
<td>$\approx 0$</td>
<td>$\uparrow$</td>
<td>$\uparrow$</td>
<td>$T_{2B}$</td>
<td>$\tau_B &lt;&lt; T_{2B}$</td>
</tr>
<tr>
<td>B, (a) &amp; (b)</td>
<td>$\neq 0$</td>
<td>$\uparrow$</td>
<td>$\uparrow$</td>
<td>$\pi \Delta \nu / \Delta W_B^2 &lt;&lt; \frac{1}{\pi \Delta \nu}$</td>
<td>$\tau_B &gt;&gt; T_{2B}$</td>
</tr>
<tr>
<td>(c)</td>
<td>$\neq 0$</td>
<td>$\uparrow$</td>
<td>$\uparrow$</td>
<td>$\frac{\pi \Delta \nu}{\pi \Delta \nu} &gt; \frac{1}{\pi \Delta \nu}$</td>
<td>$T_{2B}$, $\tau_B &lt;&lt; T_{2B}$</td>
</tr>
<tr>
<td>(d)</td>
<td>$\neq 0$</td>
<td>$\uparrow$</td>
<td>$\uparrow$</td>
<td>$\pi \Delta \nu / \Delta W_B^2 &gt;&gt; \frac{1}{\pi \Delta \nu}$</td>
<td>$T_{2B}$, $\tau_B &lt;&lt; T_{2B}$</td>
</tr>
</tbody>
</table>

Some extra information is provided by the last column of Table 1.1 in regard to an upper or lower limit of $\tau_B$.

For example, we can determine $\tau_B$ through the measurement of line width at different concentrations, even if we cannot determine $T_{2B}$ as in case A(a). $\tau_B$ itself is an upper limit for $T_{2B}$. This could be important for the estimation of correlation time at site "B" or the distance between site "B" and another site which contributes most of the dipole-dipole relaxation between these two sites, as we will see later.
Figure 1.1  Computer simulation of the temperature behavior of $T_2$

60 MHz, 100 MHz, and 220 MHz

With $\Delta \omega_B = 100$ Hz at 23.4 K Gauss

<table>
<thead>
<tr>
<th>Region</th>
<th>Chemical Exchange Limit</th>
<th>Dominant Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>$\Delta \omega_B^2$ or $(\frac{1}{T_{2B}})^2 &gt; (\frac{1}{\tau_B})^2$</td>
<td>$\frac{1}{\tau_B}$</td>
</tr>
<tr>
<td>B</td>
<td>$(\frac{1}{\tau_B})^2 &gt; \Delta \omega_B^2 &gt; \frac{1}{T_{2B}\tau_B}$</td>
<td>$\tau_B \cdot \Delta \omega_B^2$</td>
</tr>
<tr>
<td>C</td>
<td>$(\frac{1}{\tau_B})^2 &gt;&gt; \Delta \omega_B^2$, $\frac{1}{T_{2B}\tau_B}$</td>
<td>$\frac{1}{T_{2B}}$</td>
</tr>
</tbody>
</table>
C. Chemical shifts and chemical exchange

The observed chemical shift in the presence of chemical exchange was derived by Swift and Connick\(^2\) under the condition \( f_B \ll 1 \)

\[
\delta_{\text{obsd}} = \delta_A + \frac{f_B \Delta W_B}{1 + \frac{\tau_B}{\tau_{2B}}} + (\tau_B \Delta W_B)^2
\]

(1.14)

If the exchange rate is much larger than the chemical shift difference at site "A" and "B", and site "A" is in great excess over site "B", the observed chemical shift will be

\[
\delta_{\text{obsd}} = \delta_A + f_B \Delta W_B
\]

(1.15)

This corresponds to cases B(c) and B(d) in Table 1.1. Clearly \( \Delta W_B \) can be estimated without any difficulty.

D. The mechanisms of the relaxation process

A concise and comprehensive description of different relaxation mechanisms can be found in Chapter 11 and 13 of the book by Carrington\(^6\).

For the systems I studied in the absence of paramagnetic species, the intramolecular dipole-dipole interaction whose magnitude is modulated by rotational diffusion will dominate the relaxation process\(^7\). In this case, the relaxation rates are given by

\[
(\frac{1}{\tau})_i = \sum_{ij} \frac{3}{40} \left( \frac{n^2 \gamma_i^4}{r_{ij}} \right) \left\{ 6\tau_c + \frac{10\tau_c}{1+4\tau_c} + \frac{4\tau_c}{1+4\tau_c} \right\}
\]

(1.16)
where \((1/T_2)_i, (1/T_1)_i\) are the transverse and longitudinal relaxation rates respectively.

Proton \(i\) is relaxed by proton \(j\), \(r_{ij}\) is the internuclear distance, \(\gamma\) is the magnetogyric ratio for protons, \(\hbar\) is the plank constant, \(W_0\) is the resonance frequency for the observed nuclei, \(\tau_c\) (rotational correlation time) is a measure of the time it takes for the axis of the proton-carbon chemical bond to reorient the order of a radian, so \(\tau_c\) can be a measure of the flexibility at the small molecule-binding site on a macromolecule.

For systems in the presence of paramagnetic species, the electron-proton dipolar interactions that depend upon the electron-proton distance and the contact hyperfine interaction that depends on the electron spin density at the nucleus will be the two dominant relaxation mechanisms.

The relaxation rates can then be described by the Solomon-Bloembergen equation\(^8, 9\), with the approximation \(W_I \ll W_S\).

In fact, \(W_S = 650 W_I\)

\[
\frac{1}{T_{2M}} = \frac{1}{15} \frac{S(S + 1)\gamma_i^2 g_i^2 B_i^2}{r^6} \left[ 4\tau_c + \frac{3\tau_c}{1 + W_I^2 \tau_c^2} + \frac{13\tau_c}{1 + W_S^2 \tau_c^2} \right] + \frac{1}{3} \frac{S(S + 1)A_i^2}{\hbar^2} \left[ \frac{\tau_e}{1 + W_S^2 \tau_e^2} \right]
\]

\[(1.18)\]
\[
\frac{1}{T_{1M}} = \frac{2}{15} \frac{S(S+1)\gamma_I^2\beta^2}{r^6 \left( 1 + \frac{3\tau_c}{I_1 \tau_c^2} + \frac{7\tau_c}{W_I \tau_c^2} \right)} + \frac{2}{3} \frac{S(S+1)A^2}{h \left( 1 + \frac{\tau_e}{W_S \tau_e^2} \right)}
\]

where \(\tau_c\) and \(\tau_e\) are the correlation times for dipolar and hyperfine interactions respectively; \(S\), the electron spin quantum number; \(\gamma_I\), the nuclear magnetogyric ratio; \(r\), the ion or "spin-label" (unpaired electron) - proton internuclear distance; \(g\), the electronic "g" factor; \(\beta\), the Bohr magneton; \(W_I\) and \(W_S\), the Larmor angular precession frequency for the nuclear and electron spins, respectively; and \(A\), the hyperfine coupling constant. The first term in eq. (1.18) and (1.19) represents the dipolar (throughspace) contribution and second term the scalar (through-bonds) to the relaxation rates.

Depending on the situations, some further simplifications can be made. This will be discussed separately as required in each chapter. In the presence of paramagnetic species, the effective correlation time \(\tau_c\) for the dipolar interaction is determined by the interplay of rotational correlation time, \(\tau_r\), electronic spin-lattice relaxation time, \(\tau_s\), and residence time at site "B", \(\tau_B\)

\[
\frac{1}{\tau_c} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_B}
\]
Whichever is the shortest will dominate the dipolar modulation process, since each process causes a change in magnitude of the dipole-dipole interaction, and thus contributes similarly to relaxation.

The effective correlation time $\tau_e$ for the hyperfine interaction (the interaction that is transmitted through chemical bonds rather than through space) is

$$\frac{1}{\tau_e} = \frac{1}{\tau_s} + \frac{1}{\tau_B}$$

with the same meanings for $\frac{1}{\tau_s}$ and $\frac{1}{\tau_B}$ as above.

A systematic way of determining which correlation time dominates the modulation processes for the relaxation has been reviewed by Cohn.

E. Methods of measurement.

1. Measurement of spin-lattice relaxation time, $T_1$

$T_1$ can be measured by $T_{1\rho}$, adiabatic rapid passage, saturation recovery, progressive saturation and spin-echos. For spin-echos, which were employed here, all techniques for measuring $T_1$ are based on first altering the magnetization which lies parallel to the field direction (+Z). With either one or more rectangular pulses of oscillating magnetic fields of strength, sufficient to cause the magnetization (initially along the z-axis) to process by 90° about the x'-axis, to end up along the y'-axis of a reference frame which rotates at the Larmor frequency about the z-axis (see for discussion of the rotating frame representation). Or, with a 180° pulse which aligns the magnetization antiparallel to the field (-Z), one then observes the re-establishment of the equilibrium.
magnetization in the field direction. Only two methods which have been actually tried or used will be considered here.

(a) \( \pi - (t - 2\pi)n \) or \( \pi - (t - \frac{\pi}{2} \cdot n \cdot \frac{\pi}{2})n \). (Figure 1.2)

Monitoring can be accomplished by a train of "2\( \pi \)" pulses that rotates the magnetization vector one complete revolution in the \( Y'Z' \) plane of the rotating frame of reference. As the magnetization rotates through the \(+Y'\) and \(-Y'\) direction, a signal is induced in the detection coil and at the end of each 360° pulse, the magnetization vector is left parallel to the \( Z \) axis where it continues its longitudinal recovery towards equilibrium. If sufficiently strong radiofrequency field \( H. \) is available, then a number of monitoring pulses can be applied during one relaxation process, and the envelope of the induced signal peaks can be reduced to yield \( T_1 \) from a semilog plot of \( \log(<M_z>) \) vs time.

This method is quite economical in time, but its error is also big, since there will be a cumulative error when each monitoring pulse is not exactly 360°.

(b) \( \pi - t - \frac{\pi}{2} \) \hspace{1cm} (Figure 1.3)

In this case, monitoring is accomplished by applying a \( \frac{\pi}{2} \) pulse at different \( t \) after the \( \pi \) pulse. Following each \( (\pi - t - \frac{\pi}{2}) \) pulse sequence, a waiting period longer than \( 5T_1 \) is required to allow the magnetization, \( <M_z> \), to return to its equilibrium value between experiments. Although this method
Figure 1.2  Measurement of $T_1$: Method #1

Effect of a $180^\circ - (t - 360^\circ)n$ sequence of pulses of oscillating magnetic field ($H_1$) on the magnetization vector, $M_0$, in a rotating frame of reference $X',Y',Z'$. As the magnetization passes through the observation axis ($y'$), a signal is induced in the receiver coil, as shown in the oscilloscope traces below.

A. Equilibrium
B. Initial $180^\circ$ $H_1$ pulse along the $x'$ axis
C. Magnetization return toward $+ z'$ direction
D. & F. A $360^\circ$ (monitoring) pulse along the $x'$ axis to make the magnetization pass through the axis of the detector ($y'$).
E. Further magnetization recovery to $+ z$
G. Complete recovery of $z$-magnetization

H. Diagram showing the exponential recovery of an initially inverted magnetization vector, $M_0$.
I. Oscilloscope photograph of proton nuclear induction for $H_2O$ using a diode (i.e., rectifying) detector. Time base is 0.5 sec/cm. Time between $360^\circ$ pulses are 200 m.s.
\[ M(t) = M_0 \left(1 - 2e^{-t/T_i}\right) \]
Figure 1.3 Measurement of $T_1$: Method #2

Effect of $180^\circ - t - 90^\circ$ $H_1$-pulse magnetic field sequence on the magnetization vector, $M_0$, in a rotating frame of reference $X'$, $Y'$, $Z'$ and the corresponding induced magnetization behaviour appearing on an oscilloscope.

A. Equilibrium

B. Initial $180^\circ$ $H_1$-pulse along the $X'$ axis

C. Recovery of $M_z$ towards $+Z'$ directions

D. $90^\circ$ (monitoring) pulse along $X'$ axis to rotate the magnetization to the axis $(y')$ of the detector

E. Magnetization de-focusing in the $X'$, $Y'$ plane, (free-induction decay) due to inhomogeneity in $H_0$ applied field

F. Diagram showing the signal induced in the detector in the $X'$-$Y'$ plane by a $90^\circ$ pulse along the $X'$-axis varying delay times, $t$, $t'$, $t''$, etc., after an initial $180^\circ$ $H_1$-pulse.

G. Oscilloscope photograph of proton nuclear induction from water in the presence of Mn$^{++}(10^{-3}\text{M/l})$ and trypsin $(10^{-3}\text{M/l})$. The oscilloscope display was triggered immediately after the completion of a $90^\circ$ monitoring pulse.

Time base 1 m.s./cm.
\[ M(t) = M_0 (1 - e^{-t/T_2}) \]
has the drawback of "waiting", the data are much more reliable.

2. Measurement of spin-spin relaxation time, $T_2$

The spin-spin relaxation times of the small molecules in solution are always overshadowed by the contributions from sources like field inhomogeneity, effect of diffusion, etc. These unfavorable contributions can be removed by refocusing the phases of the spins in the $x'$-$y'$ plane with the technique of spin echo. This is necessary, because the intramolecular contribution to $\frac{1}{T_2}$ of methyl group might be only one quarter of the contribution from field inhomogeneity.

There are at least half a dozen ways to determine $T_2$ through the spin echo experiment\textsuperscript{18}. Gill-Meiboom modification of Carr-Purcell sequence is the technique most often used for the studies in liquids.

(a) Carr-Purcell sequence - with Gill-Meiboom modification\textsuperscript{19} (Figure 1.4)

This sequence consists of a $\frac{\pi}{2}$ pulse along the $X'$-axis followed by a series of (90° phase shifted) $\pi$ pulses along the $Y'$ axis of the rotating reference frame. In this way one can minimize the contribution of field inhomogeneity which causes $T_{2A}$ much shorter, and it can also minimize the error on setting the pulse durations. The value of $T_2$ can easily be calculated from the envelope of the echos developed at
Figure 1.4  Measurement of Nuclear T$_2$

Effect of a modified Carr-Purcell pulse sequence on the magnetization vector, M$_0$.
A. equilibrium
B. 90° pulse along the x'-axis
C. Free-induction decay
D. 90°-phase-shifted 180° pulse along the y'-axis causing the refocusing of the field - inhomogeneity - induced "fanning-out" of individual magnetization vectors
E. Echo (culmination of re-focusing)
F. Free-induction decay
G. 180° - pulse along y'-axis
H. echo
I. diagram showing the exponential decay of the amplitude of the echoes in the x'y' plane: this decay envelope represents the "true" inherent T$_2$ for the proton, corrected for inhomogeneity in H$_0$.
J. Oscilloscope photograph of the proton nuclear magnetization for water in the presence of Mn$^{++}$ (10$^{-4}$M/€) and trypsin (10$^{-3}$M/€). Time base is 10 ms/cm. Time between successive 180° pulses 2 ms. The lower trace is the free-induction decay with no attempt to eliminate the contribution from field inhomogeneity (i.e., no 180° pulses).
K. Oscilloscope trace of proton nuclear magnetization of water, using a longer time between 180° pulses of 0.2 sec. Time base is 0.5 sec/cm.
\[ M(t) = M_0 \left(1 - 2e^{-t/T_1}\right) \]
different times.

(b) line width measurement from a continuous-wave absorption signal

If the line width of the sample is much larger than the field inhomogeneity, then $T_2$ can be estimated as $1/\pi \Delta \nu$. $\Delta \nu$ is the full line width of the resonance line in Hz at half height of the resonance line.

Sometimes we will be interested in the changes of the line width introduced by different perturbations, in the absence and presence of enzymes, free radicals, metals, etc. In this case, we can determine the changes in linewidth by employing an internal standard which will not be effected by the perturbations; the non-specific line-broadening can thus be removed by subtracting the line width of internal standard line from that of the line of interest.

3. Fourier-Transform NMR\textsuperscript{20} (Figure 1.5)

A sufficiently strong r.f. pulse will cause the magnetization vectors of all the nuclei over a spectral frequency range of roughly $\gamma H_1/2\pi$ to tip by (for example) $90^\circ$, and then precess in the $X'Y'$ plane, generating a complex beat frequency pattern in the detector coil, which is located in the $x'y'$ plane. This pattern consists of a superposition of all the free induction decays (FIDs) of each of the lines in the spectrum.
Figure 1.5  Transient and Fourier-Transformed NMR Responses For an Inhibitor of Trypsin

A. The decaying pattern of interfering frequencies in the time domain, following application of a $90^\circ H_1$ pulse. It is this pattern which stored in the digital computer.

B. The fourier transform of the transient response yields a spectrum which is equivalent to an ordinary slow-sweep continuous wave absorption spectrum. Sample: p-methoxyl phenylguanidine.HCl.

(0.05 M/l).

Acquisition time 4 sec.
Sweep width 1000 Hz.
Number of transients 1.
Each FID will decay according to its own relaxation time and will have an amplitude proportional to the population of that particular nucleus in the x'y'-plane.

This decaying pattern of interfering frequencies is then stored in a multi-channel digital computer using analog to digital conversion in the time domain. The Fourier transform of this information from the time domain into the frequency domain will result the equivalent of an ordinary slow-sweep continuous-wave absorption spectrum.

The advantages of this technique over continuous wave mode are:

a. information can be accumulated at a much faster rate; so that chemically dilute samples can be studied using time-averaging.

b. undistorted line shapes and true spectral frequencies are obtained.

c. $T_1$ of all the lines in the spectrum can be measured at the same time by carr-purcell sequence.

d. non-specific broadening can be removed almost completely, by inclusion of an inert species in the same sample tube.

In succeeding chapters, the methods introduced in this chapter will be applied to a number of aspects of binding of Mn$^{++}$ or specific inhibitors to the enzyme, trypsin, including: number of binding sites, kinetics and strength of binding,
rigidity at the binding site, and disposition of the binding site relative to other sites.

References

18. Bruker's Lab. Manu., "NMR pulsed spectrometers",
20. T.C. Farrar and E.D. Becker, "Pulsed and Fourier Transform
CHAPTER II

Magnetic Resonance Studies of Mn(II) ions Binding to Trypsin

A. Introduction

The purpose of the work here was twofold: first, to determine the stoichiometry, binding constants, and rigidity of bound water, in the binding of Mn $^{++}$ to active trypsin, as an analogy with the interaction of Ca $^{++}$ with trypsin and second, to find a method for measuring the rotational lability of water bound near a paramagnetic site on a macromolecule which is more direct than use of "enhancement factors" $^*$ 1, 2.

Calcium(II) ions have a number of effects on the properties of trypsin: Ca $^{++}$ stabilizes trypsin against autolysis, 3 increases enzyme stability toward acid, base, or urea, 4 promotes the formation of active trypsin from trypsinogen, 5 and enhances

* NOTE: Eisinger et al. 2. The observed enhancement in the relaxation rates $1/T_1$ and $1/T_2$ in terms of parameters $\epsilon_1$ and $\epsilon_2$, is defined as follows:

Contribution of Mn(II) ions, in the presence of macromolecules to the spin relaxation rate of water protons $\epsilon = \frac{\text{Contribution of an equal concentration of Mn(II) ions, in the absence of macromolecules, to the spin relaxation rate of the water protons.}}$
the efficiency of catalysis toward benzoyl-L-arginine ethyl ester (BAEE). These effects may also be produced by the divalent metals, Mn, Cd, Co, and by trivalent Nd. Among these, Mn++ possesses the dual advantages that it has an observable electron spin resonance (ESR) spectrum and, in addition, has a well-described effect on the nuclear magnetic relaxation of protons in water molecules. In this paper, we use the EPR peak height combined with a Scatchard analysis to determine the number of strong and weak Mn++-binding sites on trypsin and their respective binding constants; the nuclear magnetic resonance (NMR) data may then be analyzed to yield the rotational correlation time for bound water, a measure of the degree of immobilization of water at the Mn++-binding site. Since the effect of Mn++ in enhancing catalysis of BAEE by trypsin is virtually identical to that of Ca++, the present results should be directly applicable toward understanding of the effect of Ca++ on trypsin.

B. Theory

The most convincing derivation of the strength and number of binding sites for Mn++ on a macromolecule using electron spin resonance data is by means of a Scatchard plot. This procedure has been used independently by Birkett et al. as described in a recent review by Dwek, and is described in Results and Discussion, part 1.
Reduction of the NMR data is far less straightforward. Water molecules in the present studies may reside at any of three distinct sites: free water (A), hydrated free Mn$^{++}$ ion (B), and water bound to the Mn:enzyme complex (C), and a given water molecule may jump from any site to any other site, although all previous analyses\textsuperscript{10} have been based on a simplified mechanism in which one of the exchange processes is ignored:\textsuperscript{11}

\[
\begin{align*}
A & \xleftarrow{k_1} B \\
A & \xrightarrow{k_{-1}} C
\end{align*}
\] (2.1)

Swift and Connick's calculations of $T_2$ for the system, (2.1)\textsuperscript{12} have recently been generalized by Degani and Fiat to a true three-site situation and since theoretical interpretation of $T_1$ is much simpler than that of $T_2$ when paramagnetic species are present,\textsuperscript{13} we now present the derivation of $T_1$ for the three-site system, (2.2). For the system, (2.2) and (2.3), McConnell's equations may be written,\textsuperscript{14}

\[
\begin{align*}
A + M & \xleftarrow{k_1} B \\
B + E & \xrightarrow{k_2} C \quad \text{where M is metal ion and} \\
B + E & \xleftarrow{k_{-2}} C \\
A + ME & \xrightarrow{k_3} C \quad \text{E is enzyme, and ME} \\
A + ME & \xleftarrow{k_{-3}} C \quad \text{is metal-enzyme complex,}
\end{align*}
\] (2.2)
\[
\frac{dM_A}{dt} = -\left(\frac{M_A - M_o}{T_1}\right) - (k_1[M] + k_3[ME])M_A + k_{-1}M_B + k_{-3}M_C
\]

\[
\frac{dM_B}{dt} = -\left(\frac{M_B - M_o}{T_1}\right) + k_1[M]M_A - (k_{-1} + k_2[E])M_B + k_{-2}M_C
\]

\[
\frac{dM_C}{dt} = -\left(\frac{M_C - M_o}{T_1}\right) + k_3[ME]M_A + k_2[E]M_B - (k_{-2} + k_{-3})M_C
\]

(2.4)

By solving the system, (2.4), subject to the initial condition of a 180° pulse, the following relation may be derived, a more detailed derivation is presented in Appendix A.

\[
\frac{1}{T_1} = \frac{1}{T_1 A} + \left\{1- \frac{1}{T_1 C} + k_{-2} + k_{-3}ightharpoonup \left(\frac{1}{T_1 B} + k_{-1} + k_2[E]\right)(\frac{1}{T_1 C} + k_{-2} + k_{-3}) - k_{-2}k_2[E]\right\} k_1[M]
\]

\[
+ \left\{1- \frac{k_{-2}}{T_1} \left(\frac{1}{T_1 B} + k_{-1} + k_2[E]\right)(\frac{1}{T_1 C} + k_{-2} + k_{-3}) - k_{-2}k_2[E]\right\} k_3[ME].
\]

(2.5)
There are two conditions under which the general expression, (2.5), is rendered tractable. First, if the $A\leftrightarrow B$ and $A\leftrightarrow C$ processes are both fast compared to the largest of $(1/T_1^A)$, $(1/T_1^B)$, and $(1/T_1^C)$, then the observed longitudinal relaxation is independent of the $B\leftrightarrow C$ process, and

\[
(1/T_1)_{obs} = (1/T_1^A) + f_B(1/T_1^B) + f_C(1/T_1^C)
\]  

(2.6)

where $f_i$ is the fraction of water molecules at each site and $f_A = 1$ for free water because it is present in such large excess. On the other hand, if the $B\leftrightarrow C$ process is slow compared to $A\leftrightarrow B$ and $A\leftrightarrow C$, then

\[
(1/T_1)_{obs} = (1/T_1^A) + f_B/(T_1^B + \tau_B) + f_C/(T_1^C + \tau_C)
\]  

(2.7)

where $\tau_i$ is the lifetime for water at the $i$'th site.

It is in fact likely that both simplifying conditions apply in the present experiments: the exchange rate for water solvated to Mn$^{++}$ is fast and shows only slight dependence on the degree of substitution about the Mn$^{++}$ atom; moreover, the rate of dissociation of Mn$^{++}$ from the Mn:enzyme complex is probably slower than the dissociation of water from an Mn$^{++}$ ion. These views will be supported in the discussion.
C. Experimental

Analytical grade MnCl₂·6H₂O was obtained from British Drug Houses. Twice lyophilized and salt-free trypsin was purchased from Worthington Biochemical Corporation and used without further purification. Trypsin activity was determined by titration with p-nitrophenyl, p'-guanidinobenzoate;¹⁶ the enzyme was found to be 50% active.

Mn⁺⁺ solutions were made up to concentrations of [Mn⁺⁺] = 10⁻¹, 10⁻², 5x10⁻³, 10⁻³, 5x10⁻⁴, 2.5x10⁻⁴, and 10⁻⁴ M, with each solutions 0.05M in tris-maleate buffer, pH 7.1. For measurements in presence of enzyme, 12 mg of trypsin was dissolved in 0.5ml of each of the Mn⁺⁺ solutions.

ESR measurements. A Varian model E-3 spectrometer operating at 9.5 GHz provided all ESR data (Figure 2.1). Free [Mn⁺⁺] concentration was determined from the amplitude of a particular Mn ESR transition, calibrated from samples of known Mn⁺⁺ concentration. [The amplitude of the ESR signal for Mn bound to enzyme will be negligible.]¹⁹ To ensure uniformity of sample size, a group of tubes of equal diameter were selected from a batch of approximately 1.5 mm melting-point capillary tubes. Each ESR peak height represents the average of at least 6 separate determinations.
Figure 2.1

E.S.R. spectrum of $10^{-4}$ M/l Mn$^{++}$ solution at pH 7.1 and $21 \pm 0.5^\circ$C. The spectrum was recorded at 10 mW (microwave) power and 2.5 Gauss modulation amplitude. The resonance line peak height used for estimation of free Mn$^{++}$ concentration was the third line from the right.

- Magnetic Field: 3400 G.
- Microwave power: 10 mW
- Modulation amplitude: 2.5 G.
- Receiver Gain: $1.25 \times 10^5$
- Recorder time constant: 1 sec.
- Scan time: 8 min
- Scan range: $10^3$ Gauss
NMR measurements.

(a) Bruker pulse spectrometer. This spectrometer contains a
basic 1 MHz quartz Oscillator with a frequency stability lying
between 0.01-0.001 Hz. Three separate pulse channels are
available to gate the high frequency in the oscillator unit.
The basic 1 MHz frequency from the main oscillator is taken into
a frequency synthesizer, where half of the resonance frequency
is produced. This half high frequency is then fed to the three
channels after amplification. The first channel is gate
channel I where the high frequency signal is fed after being
phase shifted, doubled and amplified. In the second channel,
which is gate channel II, the high frequency is fed directly
after being doubled and amplified. In the third channel, the
reference channel, the high frequency fed serves as a phase
coherent reference frequency for the phase sensitive detector.
The gate channel I is opened only by pulse I and the gate
channel II is opened by pulses II and III. The high frequency
in the form of pulses after passing through the gates opened
up by d.c. pulses I, II, and III is led through a fine stage
amplifier to the transmitter coil (single coil) into the probe
where it excites the NMR frequency signal. This NMR frequency
signal combined with the signal produced by r.f. pulses is
passed through a preamplifier and after attenuation is detected
by the receiver either by diode or phase sensitive detection.

The maximum band width of the receiver in the spectrometer
is 1 MHz and it can be reduced to 100 KHz. The dead time of the receiver after an r.f. pulse is approximately 5-6 μsec. The experiments in this study were done using a band width of 1 MHz.

The magnet used for polarizing magnetic field $H_0$ was Varian DP-60, 12 inch diameter pole gap, high-resolution electromagnet. The signal amplitudes were recorded on a Tektronix Type 549 storage oscilloscope (band width 30 MHz) with type 1A1 Dual Trace plug in unit.

(b) **Measurement of relaxation time.** $T_1$ was measured by a $180° - \tau - 90°$ pulse sequence and $T_2$ by the Gill-Meiboom modification of a Carr-Purcell sequency. A short review on these techniques was provided in chapter 1. Each reported $T_1$ or $T_2$ represents an average of at least 3 independent determinations. Temperature control to $21 \pm 0.5°C$ (room temperature) was achieved with a Bruker temperature control unit, B-ST 100/700. Flat-ended 8mm sample tubes were used to reduce inhomogeneity.

D. **Results and Discussion**

1. **Strength and number of Mn$^{++}$-binding sites on trypsin.**

Since the electron paramagnetic resonance (EPR) signal for free Mn$^{++}$ is narrow and easily observed, while the EPR signal for Mn$^{++}$ bound to a macromolecule is broadened beyond detection, the EPR peak height for Mn$^{++}$ in the presence of trypsin provides
a measure of the concentration of free manganous ion, \([\text{Mn}]_f\). The total \([\text{Mn}]_0\) concentration and total enzyme \([\text{E}]_0\) are known, so that the EPR data can be combined to give the concentration of "bound" manganese, \([\text{Mn}]_b\). With the help of 3 dimensional structure of D1P-trypsin\(^{20}\) a direct and rigorous determination of the number and strength of binding sites for metal to enzyme can be furnished by a Scatchard\(^{8,9}\) reduction of EPR peak height data for a set of solutions of varying \([\text{Mn}]_0\) in the presence of a constant enzyme level, \([\text{E}]_0\), as follows. The non-linear behaviour of the data in a Scatchard plot (Figure 2.2) clearly shows that there exists at least two kinds of binding sites on trypsin and the stronger binding site has an intercept on the x-axis of about 0.5. Since NPGB\(^{16}\) active site titration showed that 50% of the trypsin was fully active it can be concluded that only the active trypsin can have a strong binding site.

For simplicity, we assume that there are just two types of Mn\(^{++}\)-binding sites, with respective maximum occupancy \(n_1\) and \(n_2\) and respective binding constant \(K_1\) and \(K_2\). With this assumption, the EPR peak height data provide concentration values for fitting to the equation,

\[
\frac{\nu}{[\text{Mn}]_f} = K_1(n_1 - \nu) + K_2(n_2 - \nu), \quad (2.8)
\]

where

\[
\nu = \frac{[\text{Mn}]_b}{[\text{E}]_0}. \quad (2.9)
\]
Figure Captions

Figure 2.2 Scatchard plot for determination of strength and number of binding sites for Mn$^{++}$ to trypsin. Solid line is a non-linear least-squares fit to concentration data derived from EPR peak height measurements (see text); the two dotted lines represent a decomposition of the binding into two types of sites with maximum occupancy given by the x-intercept for each line and (negative) binding constant given by the slope of each line.
with \( n_1 = 0.5, 3 \leq n_2 \leq 4.5 \) (from 3 dimension DLP-trypsin\(^{20}\), see below), \( \nu \) and \([\text{Mn}]_f\) are values determined by experiments.

The computer is readily programmed to perform non-linear least-squares fit* of eq. (2.8) to the concentration data derived from EPR peak heights, \( \nu \) gave the result shown as the solid curve in Figure 2.2. The x-intercept for each dotted line gives the maximum number of Mn\(^{++}\) at that site, while the y-intercept for each line gives the value of \( nK \) for that site. The two straight (dotted) lines in Figure 2.2 thus represent the decomposition of the results into the two separate terms on the right-hand-side of eq. (2.8).

The best fit was found for \( n_2 = 4.5, K_1 = 2900 \text{ liter mole}^{-1}, \) and \( K_2 = 53 \text{ liter mole}^{-1}. \) Inspection of the x-ray structure of DLP-trypsin\(^{20}\) shows that the most likely location for the strong binding site would be between Asp 153 and Asp 71. For the native trypsin, weak binding sites are likely at nearby Glu 77 or the remote Glu 186. For the inactive enzyme, the strong binding site is no longer present, suggesting that the Asp 153 and Asp 71 are no longer proximal; in addition, it is now possible that the additional residues Asp 90, Asp 194, and Asp 189 are exposed, leading to a total of somewhere between 4 and 7 sites for weak binding of Mn\(^{++}\). Keeping in mind that the observed results will

* see BMD-X85 non linear least square fit, computing center, U. B. C.
represent the sum of active and inactive trypsin,, it seems likely that the average number of weak binding sites will be somewhere between 3=((2+4)/2) and 4.5=((2+7)/2). The computer was thus asked to search for fits using values of 3 \leq n_2 \leq 4.5. So, trypsin possesses one strong binding site, with \( K_1 = 2900 \text{ liter mole}^{-1} \), and several \( (n_2 \approx 4.5) \) weak binding sites of average binding constant. \( K_2 = 53 \text{ liter mole}^{-1} \). At very high \([\text{Mn}^{++}]\) levels even more binding sites may be populated, as shown by the position of the right-most point in Figure 2.2.

2. Rotational lability of water at the strong Mn\(^{++}\)-binding site of trypsin.

Although eq. (2.6) is expected to apply to the present experiments, the data was analyzed according to the more general eq. (2.7) as follows. In a set of control experiments, \( T_1 \) and \( T_2 \) were measured for solutions containing buffer and Mn\(^{++}\) but no enzyme: the appropriate form for \( T_1 \) is given by

\[
(1/T_1)_{\text{obs}} = (1/T_1^A) + \frac{[\text{Mn}^{++}] \cdot 6}{(T_{1B} + \tau_B) \cdot 55.6}, \quad (2.10)
\]
where 6 is the hydration number for water around Mn\(^{++}\). In a second control experiment, enzyme was added to buffer solution with no Mn\(^{++}\) present. While the solution viscosity increased somewhat, as shown by the \(T_2\)-increase, the effect on \(T_1\) was negligible. The principal measurements of \(T_1\) and \(T_2\) were then carried out on solutions which contained constant amounts of buffer and enzyme, with varying concentrations of Mn\(^{++}\) (see Table 2.1). According to the equation,

\[
\frac{1}{T_1} \times \text{obs} = \frac{1}{T_1^A} + \frac{[[Mn]]_o - [Mn:trypsin] \cdot 6}{(T_1^B + \tau_B) \cdot 55.6}
\]

\[
+ \left( \frac{[Mn:trypsin]}{55.6} \right) \cdot \left( \frac{q}{T_1^C + \tau_C} \right)
\]

(2.11)

where \(q\) is the number of water molecules coordinated to bound Mn\(^{++}\).

 Subtracting eq. (2.10) from eq. (2.11) then yields,

\[
\frac{1}{T_1} \times \text{obs} - \frac{1}{T_1} \times \text{obs} = \left\{ \frac{[Mn:trypsin]}{55.6} \right\} \left\{ \frac{q}{T_1^C + \tau_C} - \frac{6}{T_1^B + \tau_B} \right\}
\]

(2.12)

An equation of the same form is valid for \(T_2\) also.
Table 2.1

Experimental proton relaxation rates (sec\(^{-1}\)) for water protons in 0.05M tris-maleate buffer, pH 7.1, in the presence (*) or absence ( ) of trypsin (12 mg in 0.5 ml), for the concentrations of [Mn\(^{++}\)] listed. Relaxation rates are the average of at least three runs.

<table>
<thead>
<tr>
<th>Solution</th>
<th>((1/T_1)_{obs})</th>
<th>((1/T_1)^*)</th>
<th>((1/T_2)_{obs})</th>
<th>((1/T_2)^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A [Buffer only]</td>
<td>0.38</td>
<td>0.38</td>
<td>1.2</td>
<td>1.9</td>
</tr>
<tr>
<td>A + 10(^{-5}) M [Mn(^{++})]</td>
<td>0.42</td>
<td>0.45</td>
<td>2.09</td>
<td>1.94</td>
</tr>
<tr>
<td>A + 10(^{-4}) M [Mn(^{++})]</td>
<td>1.04</td>
<td>1.11</td>
<td>8.8</td>
<td>6.3</td>
</tr>
<tr>
<td>A + 10(^{-3}) M [Mn(^{++})]</td>
<td>6.8</td>
<td>7.35</td>
<td>75.0</td>
<td>62.0</td>
</tr>
<tr>
<td>A + 10(^{-2}) M [Mn(^{++})]</td>
<td>64.5</td>
<td>67.0</td>
<td>735.0</td>
<td>690.0</td>
</tr>
</tbody>
</table>
Now the left-hand-side of eq. (2.12) is obtained from experiment, and the second term on the right-hand-side is obtained from corresponding control experiments. A value for the binding constant for Mn$^{++}$ to trypsin is now guessed, and the computer performs a non-linear least-squares fit of eq. (2.12) to experimental $T_2$-data, and lists the standard deviation for the choice of $[q/(T_2^C + \tau_C)]$ which gave the best agreement. The procedure is then repeated for a range of other values for the binding constant, and the best fit was found for $(1/K) = 1.3 \pm 1.0 \times 10^{-3}$ M. Due to the contact interaction involved in the relaxation process for $T_2$, no further use was made of $T_2$-data in analysis of molecular motion of water.

$T_1$ data can be treated the same way to give the terms $q/(T_1^M + \tau_C)$ and $q/(T_2^M + \tau_C)$. By treating the data this way one will have a better estimate of the exact paramagnetic contribution to the spin-lattice relaxation time and spin-spin relaxation time, than that from discussion based in terms of enhancement factor, $e$.

To test the consistency of the approach, a second set of computer fits was performed with the assumption that the stoichiometry of the Mn:trypsin complex was 2:1 rather than 1:1. These fits showed ten times larger standard deviation (poorer fits) than for the 1:1 assumption. Thus, the NMR relaxation data do provide accurate stoichiometry, but the binding constant is obtained much more reliably from EPR peak height data,
in contrast to the example quoted by Dwek\textsuperscript{10}.

The next step is to determine whether $T^M_1$ or $\tau_M$ is the main contributor for the longitudinal relaxation. Since $\tau_{ex}$ for water on aqueous Mn\textsuperscript{++} is about $10^{-7}$ sec\textsuperscript{21} and since the lifetime for water on Mn\textsuperscript{++} is about the same or even a little shorter in the presence of ligation of the Mn\textsuperscript{++}\textsuperscript{15} it seems very likely that the lifetime for water on the Mn:trypsin complex should be of the order of $10^{-7}$ sec. Thus, if the denominator of the first term of eq. (2.12) were dominated by $\tau_C$, that term should be of the order of $10^7$. Since the determined magnitude of that term is only about $10^4$, we can be confident that the term is dominated by $T^C_1$, not by $\tau_C$. We are now in a position to discuss rotational motion of water from analysis of $T^C_1$.

With $6/(T^B_1 + \tau_B) = 3.56 \times 10^5$ obtained from the least square fit of the data without enzyme, equation (2.12) can be written as

$$(-\frac{1}{T_1})^{*}_{obs} - (-\frac{1}{T_1})^{*}_{obs} = \left\{ \frac{[\text{Mn:trypsin}]}{55.6} \right\} \left[ \frac{q}{T^C_1} - 3.56 \times 10^5 \right]$$

(2.13)

Since the determination of binding constants by EPR is more accurate than by NMR, the results of Figure 2.2 may now be used

*NOTE: $\tau_C$ = residence time at site C, not rotational correlation time in this chapter.*
to obtain a better value for \( \frac{q}{T_i} \), provided that the contribution from the weak binding sites can be taken into account. For the NMR data, then, the high-[Mn\textsuperscript{++}] solutions will exhibit a substantial contribution to \( T_i \) from the weak binding sites, but the low-[Mn\textsuperscript{++}] solutions show such a small change in \( T_{1\text{obs}} \) that accurate \( T_i \) cannot be obtained. A compromise is provided by the \( T_i \) data for intermediate [Mn\textsuperscript{++}] = 10\textsuperscript{-3}M. Even at this concentration, there is some contribution to \( T_i \) from the weak binding sites: upper and lower limits for this effect may be found from supposing that the water \( T_i \) at the weak binding site is the same as at free manganese ion, or that \( T_i \) at the weak binding site is the same as at the strong binding site.

With a little algebra, equation (2.8) and (2.9) can be rearranged to

\[
[Mn]_b = [Mn:trypsin] = \left\{ \frac{n_1 K_1 [Mn]_F}{1 + K_1 [Mn]_F} \right\} E_0 + \left\{ \frac{n_2 K_2 [Mn]_F}{1 + K_2 [Mn]_F} \right\} E_0 \tag{2.14}
\]

the first term represents the number of Mn\textsuperscript{++} bound at strong binding sites, the second term will be the number of Mn\textsuperscript{++} at weak binding sites.

So, the term [Mn:trypsin] in equation (2.13) will be either equal to the first term only or equal to both terms in equation (2.14) depending on whether \( T_i \) at the weak binding sites is the same as \( T_i \) at free manganese, or \( T_i \) at the weak binding site is
the same as at the strong binding site.

\( n_1, n_2, K_1, K_2 \) have been determined by a Scatchard plot of the ESR data (Figure 2.2); \([\text{Mn}]_F\) is the measurable quantity from ESR thus \([\text{Mn:trypsin}]\) can be calculated for each case by using eq. (2.14). \( q/(T_1^C) \) can be found by equation (2.13) from \([\text{Mn:trypsin}]\) calculated for these two extremes.

The final determination of \( T_1 \) for the strong binding site is then obtained from the observed \( T_1 \) as

\[
4.18 \times 10^5 \leq \frac{q}{T_1^C} \leq 4.46 \times 10^5 \text{ sec}^{-1} \quad (2.15)
\]

To anticipate what will shortly be shown, the correlation time calculated from the \( T_1^C \) of (2.15) must be \( 2.5 \times 10^{-10} \) sec or shorter. Since this correlation time is given by

\[
\left( \frac{1}{\tau_C} \right) = \left( \frac{1}{\tau_{\text{rot}}} \right) + \left( \frac{1}{\tau_S} \right) , \quad (2.16)
\]

and since \( \tau_S \) is \( 2 \times 10^{-9} \) sec,\(^{22}\) it is evident that \( \tau_C = \tau_{\text{rot}} \), and will be so interpreted from here on.

B.M. Fung has recently calculated the effect of internal rotation\(^{23}\) on the electron-nuclear dipole-dipole interaction, with results that closely resemble an earlier calculation for the nuclear-nuclear dipole-dipole case:\(^{24}\)

\[
\frac{1}{T_1} = \left\{ \frac{2y_s^{2}y_s^{2}S(S+1)}{r^6} \right\} \left\{ \frac{7}{30} \left[ \frac{(\cos^2 \theta)^2}{1 + W_s^2 \tau_1^2} \right] + \frac{3(\sin^2 \theta)\tau_2}{1 + W_s^2 \tau_2^2} + \frac{3(\sin^4 \theta)\tau_3}{1 + W_s^2 \tau_3^2} \right\}
\]
\[
\frac{1}{10} \left\{ \frac{(3\cos^2 \theta - 1)^2 \tau_1}{1 + W_1^2 \tau_1^2} + \frac{3(\sin^2 2\theta) \tau_2}{1 + W_1^2 \tau_2^3} + \frac{3(\sin^4 \theta) \tau_3}{1 + W_1^2 \tau_3^2} \right\}, \tag{2.17}
\]

where \((1/\tau_1) = (1/\tau_p)\) in this case, and \((1/\tau_2) = (1/\tau_1) + (1/\tau_{\text{int}})\), and \((1/\tau_3) = (1/\tau_1) + (4/\tau_{\text{int}})\), where \(\tau_r\) is the rotational correlation time for the complex as a whole and \(\tau_{\text{int}}\) is the correlation time for internal rotation of water in the complex, and \(\theta\) is the angle between the proton-proton vector in water and the axis about which internal rotation occurs. For example, for \(\text{Mn(H}_2\text{O)}_6^{++}\) (Figure 2.3), \(\theta = 90^\circ\) for the most likely internal rotation axis through the oxygen atom, and if the previously calculated correlation time for water rotation is taken as \(3 \times 10^{-11}\) \(\text{sec}\), one obtains from the present data for \((1/T_1^B) = 5.9 \times 10^4 \text{ sec}^{-1}\), that the rotational correlation time for the \(\text{Mn(H}_2\text{O)}_6^{++}\) complex as a whole is \(4.8 \times 10^{-11} \text{ sec} = \tau_1\), as shown graphically in Figure 2.4. For the Mn:trypsin complex, assuming that the bound water rotates freely (\(\tau_{\text{int}} = 3 \times 10^{-11} \text{ sec}\)), the figure shows that the correlation time for rotation of the Mn-binding site is \(8 \times 10^{-11} \text{ sec}\). If the bound water is assumed rigidly held to the Mn:trypsin site, then the binding site is even more flexible. These calculations are based on assumption of \(q = 4\) water molecules per bound Mn\(^{++}\). If \(q\) is taken as 2, or 3, then the correlation time for rotation of the Mn-binding site will be \(2.5 \times 10^{-10}\) or \(1.4 \times 10^{-10}\) respectively. Thus, the Mn\(^{++}\) bound to trypsin has a surprisingly large degree
Figure 2.3  The possible structure of Mn$^{++}$ strong binding site on Trypsin.

The angle between the axis of internal rotation and the axis connecting the two protons of water is seen to be 90°. Here it is assumed that two water molecules are replaced by two Aspartic residues of trypsin (see text).
Log-log plot of longitudinal relaxation rate versus macromolecular rotational correlation time, using eq. (2,17) of the text. Relaxation rates are calculated for a water molecule coordinated to Mn^{++}, with proton-Mn^{++} distance of 2.8 Å, \( \omega_i = 2\pi \times 10^8 \text{ sec}^{-1} \), and internal rotation rate fixed by \( \tau_{\text{int}}^{-1} = 3 \times 10^{-11} \text{ sec} \). (bottom curve). The upper curve represents the same calculation, but with no internal rotation present, that is \( \tau_{\text{int}} \to \infty \) in eq. (2,17). Solid-line intercepts refer to \((1/T_1)\) and \(\tau_1\) for the Mn(H_2O)_6^{++} complex; dotted-line intercepts are for water bound to the Mn:trypsin complex (see Discussion).
of rotational mobility: if the Mn$^{++}$ were rigidly bound to the 
trypsin "backbone", its rotational correlation time would be 
about two orders of magnitude slower.

Most previous studies on Mn$^{++}$-induced water nuclear relaxa-
tion have been based on ratios of relaxation rates or "enhancements" 
rather than on differences in relaxation rates as in the present 
case. As demonstrated recently for nuclear-nuclear dipole-dipole 
relaxation, whenever internal motion is present, the information 
obtained from ratios of relaxation times offers a questionable 
mixture of the rotational rate of the macromolecules as a 
whole and the internal rotational rate. Since the only cases for 
which Mn$^{++}$-induced proton relaxation offers rotational information 
are when the rotational "effective" correlation time is shorter 
than the electron $T_1$ of about $2 \times 10^{-9}$ sec, and since typical 
enzyme rotational correlation times are $10 - 100 \times 10^{-9}$ sec, it 
is clear that the typical situation in which Mn$^{++}$ is a useful 
nuclear magnetic probe of enzyme local motion is one for which 
internal rotation is significantly faster than macromolecular 
reorientation as a whole. We, therefore, suggest that future 
NMR relaxation studies of water in the presence of Mn$^{++}$ complexes 
with macromolecules should base their interpretaitons on differ-
ence in relaxation rate in presence and absence of enzyme, rather 
than on ratios, or "enhancements" of relaxation rates.
E. Summary

The strength and number of binding sites of Mn\textsuperscript{++} to trypsin have been determined by a Scatchard reduction of electron paramagnetic resonance peak height data. Active trypsin possesses one strong binding site with binding constant 2900 l mole\textsuperscript{-1}; there are 4-5 binding sites with a much weaker binding constant of 53 l mole\textsuperscript{-1}. Binding seems most likely to involve coordination to Asp 153 and Asp 71. Inactive trypsin has no strong Mn\textsuperscript{++}-binding site.

Flexibility at the binding site has been determined from analysis of proton longitudinal magnetic relaxation rates for water at the strong Mn\textsuperscript{++}-binding site. Using differences (rather than ratios) between relaxation rates in presence and absence of enzyme, the flexibility of the binding site itself is determined to have a rotational correlation time of 8 x 10\textsuperscript{-11} sec or shorter, so that there is appreciable local motion at the strong Mn\textsuperscript{++}-binding site.
References


CHAPTER III

Steady-State Inhibition Kinetics Using Racemic Substrate:
A Probe For Cooperative Inhibitor Binding In Trypsin

A. Introduction

Accumulating evidence from x-ray crystallography of chymotrypsin\textsuperscript{1} and other enzymes indicates that many enzymes possess one or more secondary binding sites which will attract various inhibitors to the enzyme. While these sites in chymotrypsin are probably an evolutionary coincidence connected to homologies in structure with other serine esterases, secondary binding sites have central significance in the mechanism of operation of allosteric enzymes\textsuperscript{2}. In solution, an enzyme inhibitor is most often categorized as "competitive", "non-competitive", etc., by the form of a Dixon plot\textsuperscript{3} of reciprocal reaction initial rate, \((1/v)\), versus inhibitor which shows "competitive" inhibition as judged by a Dixon plot is generally presumed to bind as the same site as the native substrate, and thus the strength of binding of such an inhibitor can be correlated with the shape of the substrate-binding site of the enzyme.

However, most enzymes act on optically active substrates in nature, and it may be inconvenient to study inhibition by using
a pure "L" or "D" optical isomer artificial substrate; in such cases, of which trypsin is one, a racemic substrate is used. The initial complication here is that only one of the optical isomers will in general be a "good" substrate, and the other may very well be a competitive inhibitor, as with the present example. The importance of this fact is that an unknown inhibitor may appear to be "competitive" when assayed by such a racemic substrate, when in fact the unknown inhibitor does not prevent binding of substrate.

The apparent complication of a racemic substrate, however, can be turned to advantage, to provide a means for classifying inhibitors according to whether they are purely competitive with substrate, repulsively interact with substrate, are purely non-competitive (have no effect on substrate binding), or activate substrate binding. It should be noted that no additional experimental data beyond the ordinary Dixon plot is required in this analysis.

In this chapter the above considerations are applied to the analysis of several arginine or lysine analogs which are inhibitors of trypsin, and it is also shown that some other inhibitors previously classified as "competitive" do not in fact prevent binding of substrate and thus must bind elsewhere than at the substrate "pocket".

Trypsin shows a very narrow specificity for catalyzing the hydrolysis of bonds involving the carboxyl group of arginine or lysine⁴,⁵. A series of guanidine or lysine analogs which each possessed a -CH₃ or -OCH₃ group was collected and/or synthesized to provide for simple interpretation of NMR results as well as
conventional steady-state inhibition kinetics. The U.V. studies reported here will provide the necessary information to establish where the inhibitor binds to the enzyme, and thus facilitate the interpretation of the NMR experiment in the next chapter. This system would offer for the first time a comparison between the strength of binding (from inhibition kinetics) and rigidity of binding (from NMR), and the trypsin system was chosen for its specificity to assure that all the inhibitors should bind competitively at the active site.

Although no X-ray evidence for secondary binding sites on trypsin has yet appeared, a variety of other work (besides the present experiments) points to additional binding. It has been observed that the substrate, TAME (p-toluenesulfonylarginine methyl ester) is self-activating at high concentrations, suggestive of an auxiliary binding site. More recently it has been reported that even neutral compounds can bind to trypsin, resulting in cooperative interaction with substrate. Although Mares-Guia and Shaw reported that all seven of the inhibitors they studied were competitive, a re-analysis of their results (see Discussion) shows that two of these were not truly competitive, but acted to restrict, rather than prevent access of substrate to its binding site. Finally, since Ca\(^{++}\) is known to stabilize trypsin against autolysis, and also against acid, base, and urea, calcium ions was present in all assays, and its effect will be discussed in the Results section.
B. Experimental

Bovine trypsin, twice-recrystallized, salt-free, lyophilized preparation (TRL) from Worthington Biochemical Corp., Freehold, N.J., was used without further purification. Other compounds were examined by NMR for impurity peaks.

3-methoxypropylamine (Eastern Organic Chemicals). The technical grade compound was stirred overnight with sodium carbonate and fractionally distilled afterwards. B.P. 116-117°C.

Acetamidine-HCl (J.T. Baker Chemical Co.) was recrystallized twice from ethanol to give a white powder, m.p. 174°C.

p-methoxybenzylamine (J.T. Baker Chemical Co.) was used without further purification.

m-anisidine-HCl (Eastman Organic Chemicals) was crystallized twice from absolute ethanol to give a white powder, m.p. 176-178°C.

p-anisidine (Eastern Organic Chemicals) was crystallized twice from water to give light brown plates, m.p. 57°C.

m-methoxyphenylguanidine hydrochloride was synthesized by a modification of a previous method\(^1\). 1-guanyl-3,5-dimethylpyrazole nitrate (8g, 0.04 moles) and m-anisidine (50g, 0.4 moles) were mixed in 160 ml H\(_2\)O, and the solution refluxed until the GDPN had disappeared (as gauged by its NMR spectrum). The mixture was allowed to cool and then extracted eight times with 40 cc portions of ether. The aqueous layer was decolorized with active carbon, and then put through a Dowex 2-X8 ion exchange resin (Cl\(^-\) form) to convert the nitrate salt to a chloride salt. After freeze-
drying, the solids were dissolved in a small amount of ethanol, and passed through a preconditioned silica gel column \(^{12}\) (100 mesh), eluted with benzene:ethanol mixtures varying from 4:1 to 1:4 composition. The guanidine product was identified by Sakaguchi test \(^{13}\) and also Weber test \(^{14}\). Tubes with positive reactions were pooled, solvent evaporated under vacuum, then dissolved in absolute ethanol, followed by addition of a few drops of concentrated acetic acid and then ethylacetate until precipitation was initiated.

The solution was then stored in a freezer overnight to give a maximum yield of precipitate, identified by NMR. A portion of the m-methoxyphenylguanidine hydrochloride was converted to the base by addition of NaOH in ice cold water, followed by extraction with ether and evaporation of the ether with a water pump. The base was then dissolved in CDCl\(_3\), and NMR gave a ratio of guanidine to methyl protons of 4-5 to 3. The uv absorption band of m-methoxyphenylguanidine hydrochloride was shifted to low wavelength compared to m-anisidine in methanol, and the uv spectrum was similar to the known phenylguanidine-HCl. m.p. was 115-116°C and thin-layer chromatography showed no other impurities:

Analysis calcd. for C\(_8\)H\(_{12}\)N\(_3\)OCl:  C 47.64  H 5.99  N 20.84  
Found:  C 46.82  H 5.93  N 20.33

p-methoxyphenylguanidine•HCl was synthesized and tested as for the meta-compound, and showed m.p. 137-139°C. Thin-layer chromatography showed no other impurities:
Analysis calcd. for C_{8}H_{12}N_{3}OCl:  C  47.64  H 5.99  N 20.84

Found:  C  46.87  H 5.82  N 19.96

The difficulties in the above syntheses lie in the instability of the product (hydrolysis at high temperature or basic solution), and because a small amount of impurity (possibly biguanidine) seems to prevent crystallization.

**Trypsin assays** pH of all buffers was measured with an Orion Model 401 pH meter; a Gilford uv spectrophotometer with recorder was employed for absorbance measurements, and temperature was controlled by a Haake circulator-thermostat. Enzyme solution was prepared by dissolving 2 mg trypsin in a 5 ml volumetric flask which contained 0.1 ml of 0.25M CaCl\textsubscript{2} and the remainder of 0.001M ice-cold HCl. Substrate solutions, D,L-benzoylarginine-p-nitroanilide [D,L-BAPA], 1 x 10\textsuperscript{-4} M, 2x10\textsuperscript{-4} M, and 3 x 10\textsuperscript{-4} M, were prepared fresh every time, because sometimes even at low concentration, D,L-BAPA would start to crystallize at the bottom of the volumetric flask (30°C). Inhibitors were prepared at concentrations appropriate to their potency for inhibition, but usually about 0.05M in 0.05M tris buffer, pH 7.65, with 0.02M CaCl\textsubscript{2}. Spectrophotometric assays were conducted with D,L-BAPA as substrate\textsuperscript{15}. Tryptic activity was measured by adding 0.1 ml of trypsin solution (4 mg in 10.00 cc of 0.001N HCl) at zero time to 2.5 cc of substrate and 0.4 cc buffer [or 2.5 cc substrate plus x cc inhibitor and (0.4 - x) cc buffer] in the cuvette, with prior thermal equilibration in the cell compartment of the spectrophotometer (30°C). After addition of enzyme, the change in absorbance at 4100 Å was recorded for 5 minutes;
in all experiments, the rate was obtained in triplicate and the reported value is an average. The rate, in mole liter\(^{-1}\) sec\(^{-1}\) was obtained by multiplying the measured absorbance per minute by \([1/(60\times8800)]\), where 8800 is the molar extinction coefficient of p-nitroaniline at 4100 Å.

C. Theory

The present experiments involve a racemic substrate whose L-form is a substrate but whose D-form is a competitive inhibitor: these species will be denoted as S and I\(_1\). The racemic substrate is then used to assay an unknown inhibitor, I\(_2\), and the appropriate scheme is shown below:

\[
\begin{align*}
K_1 & \quad E \quad EI_1 \quad EI_1I_2 \\
K_2 & \quad E \quad EI_2 \\
K_S & \quad E \quad ES \\
\alpha K_2 & \quad EI_1 \quad EI_1I_2 \\
\gamma K_2 & \quad EI_2 \quad EI_2S \\
\gamma K_S & \quad ES \\
\end{align*}
\]

(3.1)

where

\[
\begin{align*}
K_1 &= \frac{[E][I_1]}{[EI_1]} , \quad K_2 = \frac{[E][I_2]}{[EI_2]} , \\
K_S &= \frac{[E][S]}{[ES]} , \quad \alpha K_2 = \frac{[EI_1][I_2]}{[EI_1I_2]} , \\
\alpha K_1 &= \frac{[EI_2][I_1]}{[EI_1I_2]} , \quad \gamma K_S = \frac{[EI_2][S]}{[EI_2S]} , \\
\gamma K_2 &= \frac{[ES][I_2]}{[EI_2S]} .
\end{align*}
\]

(3.2)
If $\alpha = \infty$, then $I_2$ is pure competitive with respect to $I_1$; if $\gamma = \infty$, then $I_2$ is pure competitive with $S$. $\alpha = 1$ and $\gamma = 1$ are the corresponding pure non-competitive cases, and so forth. The general steady-state solution for the effect of two inhibitors on an enzyme-substrate system is known; with the present special case, the resultant general rate expression can be simplified as follows.

$$\left(\frac{1}{v}\right) = \frac{K_S}{V_{\text{max}}} \left\{ \left(\frac{1}{[S]}\right) + \left(\frac{[I_1]}{K_1[S]}\right) + \left(\frac{1}{K_S}\right) \right\}$$

$$+ \frac{[I_2]}{K_2} \left\{ \left(\frac{1}{[S]}\right) + \left(\frac{[I_1]}{\alpha K_1[S]}\right) + \left(\frac{1}{\gamma K_S}\right) \right\},$$

where $v$ is the initial rate of formation of product at the initial concentrations of $S$, $I_1$, and $I_2$ for the run, $V_{\text{max}}$ is the maximum initial rate for that value of $I_2$, and is given by the usual

$$V_{\text{max}} = k[E]_{\text{total}}.$$  

(3.4)

Now in the present experiments, racemic substrate is used, so that

$$[I_1] = [S]$$  

(3.5)

at all times. With this simplification, eq. (3.3) may be reduced to
Thus a Dixon-type plot of $(1/v)$ versus $[I_2]$ will still be a straight line at a given substrate concentration. If the experiment is repeated at a second (fixed) substrate level, then the two Dixon plots can readily be shown to intersect at the point,

$$[I_2] = -K_2,$$

$$\frac{(1/v)}{V_{\text{max}} \left( \frac{1}{[S]} + \frac{1}{K_1} + \frac{1}{K_S} \right) \left( \frac{1}{[S]} \frac{1}{\alpha K_1} + \frac{1}{\gamma K_S} \right)}.$$  \hfill (3.6)

From eqs. (3.7), it is clear that the Dixon plots will intersect above the x-axis when $I_2$ is pure competitive ($\alpha, \gamma = \infty$) or shows repulsive interaction ($1 < \alpha, \gamma < \infty$) with $I_1$ and $S$; the intersection will occur on the x-axis when $I_2$ is pure non-competitive ($\alpha, \gamma = 1$) with $I_1$ and $S$; and the intersection will lie below the x-axis when $I_2$ activates ($\alpha, \gamma < 1$) binding of $I_1$ and $S$.

Independent extraction of the interaction coefficients, $\alpha$ and $\gamma$, would require an unprofitably large number of rate measurements. However, a parameter offering nearly as much information may be defined by
The parameter, \( A \), provides a weighted average of the interaction of the unknown inhibitor, \( I_2 \), with the two racemates, \( I_1 \) and \( S \). When, as in the present case, \( K_1 \approx K_s^{15} \), \( \alpha \) and \( \gamma \) are probably similar in magnitude anyway, so that \( A \) should be a good measure of either effect. Parameter \( A \) is readily extracted from the slopes of two Dixon plots of \((1/v)\) versus \([I_2]\) corresponding to two values for \([S] = [I_1] \). If the slopes of the two lines are designated as \((\text{Slope})_1\) and \((\text{Slope})_2\), and their ratio is defined as

\[
R = \frac{\text{Slope}_1}{\text{Slope}_2} = \frac{K_S}{V_{\text{max}}} \left( \frac{1}{[S]_1} + \frac{1}{\alpha K_1} + \frac{1}{\gamma K_S} \right) \left( \frac{1}{[S]_2} + \frac{1}{\alpha K_1} + \frac{1}{\gamma K_S} \right) \tag{3.9}
\]

then parameter \( A \) is readily obtained from

\[
\frac{1}{A} = \left( \frac{1}{(R - 1)((1/K_1) + (1/K_S))} \right) \left( \frac{1}{[S]_1} - \frac{R}{[S]_2} \right). \tag{3.10}
\]
Since
\[ K_S = 9.39 \times 10^{-4} \text{ M for L-BAPA}, \quad (3.11) \]

and
\[ K_I = 8.00 \times 10^{-4} \text{ M for D-BAPA}, \quad (15) \]

the parameter, \( A \), is easily obtained from the Dixon plots shown in the Figures for the Results and Discussion.

As a useful guide to the presentation, the significance of Dixon-intersection points and \( A \)-values is listed in Table 3.1 for reference.

D. Results and Discussion

Values for the inhibition constant, \( K_2 \), and interaction coefficient, \( A \) (see Theory) for each of the seven inhibitors of the present study are listed in Table 3.2. Values for \( K_2 \) and \( A \) were determined from the Dixon plots shown in Figure 3.1. The self-consistency of the determination of \( A \) is evidenced by the fact that although \( A \) was determined from the ratio of the slopes of the two Dixon plots for a given inhibitor, the value of \( A \) corresponds exactly to the correct \( (1/v) \)-value at the intersection of the plots (i.e., above or below the x-axis) for each inhibitor, as predicted in the Theory section and summarized in Table 3.1.

The data in Table 3.2 show that only two of the seven compounds tested are true competitive inhibitors of trypsin; the remaining compounds actually activate the binding of substrate.
Figure 3.1 Dixon plots for inhibition of trypsin by the inhibitor shown in each plot. Ordinate gives initial reaction rate in liter mole$^{-1}$ sec x $10^7$; abscissa is molar concentration of the appropriate inhibitor. Concentration of (racemic) substrate D,L-benzoylarginine-p-nitroanilide was: $0.833 \times 10^{-4}$M (upper line for each plot); $1.67 \times 10^{-4}$M (lower line for each plot); and $2.5 \times 10^{-4}$M (lowest curve for m-methoxyphenylguanidine). Trypsin concentration was $5.5 \times 10^{-7}$ M, in 0.05M tris buffer, pH 7.65, temperature 30.0°C, at Ca$^{++}$ concentration of 0.02 M.
- 72 -

3-METHOXYPROPYLAMINE, $10^3 M$

m-METHOXYPHENYL GUANIDINE-HCl, $10^4 M$

P-METHOXYPHENYL GUANIDINE-HCl, $10^4 M$

m-ANISIDINE-HCl, $10^3 M$

P-ANISIDINE-HCl, $10^3 M$

ACETAMIDINE, $10^3 M$

P-METHOXYBENZYLAMINE, $10^4 M$
Table 3.1

Interpretation of the significance of the y-value at the intersection of two Dixon plots corresponding to two different concentrations of a racemic "substrate". The "substrate" itself consists of one isomer which is a true substrate and the other isomer is a competitive inhibitor. (see Theory). The A-value defined by eq. (3,8), with experimental determination given as eq. (3,10), indicates the degree of cooperativity between binding of an unknown inhibitor, \( I_2 \), with the racemic "substrate", S and \( I_1 \).

<table>
<thead>
<tr>
<th>A-value</th>
<th>Value of ((1/v)) at intersection of two Dixon plots</th>
<th>Mechanistic Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>( A = \infty )</td>
<td>positive</td>
<td>( I_2 ) is purely competitive with ( I_1 ) and S.</td>
</tr>
<tr>
<td>( 1 &lt; A &lt; \infty )</td>
<td>positive</td>
<td>( I_2 ) restricts, but does not prevent binding of ( I_1 ) and S.</td>
</tr>
<tr>
<td>( A = 1 )</td>
<td>zero</td>
<td>( I_2 ) has no effect on binding of ( I_1 ) and S.</td>
</tr>
<tr>
<td>( A &lt; 1 )</td>
<td>negative</td>
<td>( I_2 ) facilitates binding of ( I_1 ) and S.</td>
</tr>
</tbody>
</table>
Table 3.2 Structures, inhibition constants, and interaction coefficients for binding of inhibitors to trypsin. For interpretation of parameter $A$, see Table 3j. Experiments were carried out at pH 7.65 at 30°C, in the presence of 0.02M CaCl₂ and 0.05M tris buffer.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>Structure</th>
<th>$K_2$ (M)</th>
<th>$A$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>acetamidine</td>
<td><img src="image1.png" alt="Structure 1" /></td>
<td>$3.5 \times 10^{-2}$</td>
<td>0.75</td>
</tr>
<tr>
<td>2</td>
<td>3-methoxypropylamine</td>
<td><img src="image2.png" alt="Structure 2" /></td>
<td>$2.2 \times 10^{-2}$</td>
<td>$\infty$</td>
</tr>
<tr>
<td>3</td>
<td>p-methoxybenzylamine</td>
<td><img src="image3.png" alt="Structure 3" /></td>
<td>$6.1 \times 10^{-4}$</td>
<td>$\infty$</td>
</tr>
<tr>
<td>4</td>
<td>p-methoxyphenylguanidine</td>
<td><img src="image4.png" alt="Structure 4" /></td>
<td>$2.3 \times 10^{-3}$</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>m-methoxyphenylguanidine</td>
<td><img src="image5.png" alt="Structure 5" /></td>
<td>$5.0 \times 10^{-4}$</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>p-anisidine</td>
<td><img src="image6.png" alt="Structure 6" /></td>
<td>$1.7 \times 10^{-2}$</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>m-anisidine</td>
<td><img src="image7.png" alt="Structure 7" /></td>
<td>$9.4 \times 10^{-2}$</td>
<td>0.17</td>
</tr>
</tbody>
</table>
Published Dixon plots for five additional compounds have also been subjected to the same analysis, with the results given in Table 3.3. Failure to include the species, $E_{1}I_{2}$ and $E_{1}S$ in the kinetic scheme led those authors to conclude that the cyclohexyl compounds were competitive inhibitors when, as the present analysis conclusively shows, those two inhibitors act to restrict, but not prevent access of substrate to its binding site. It is to be emphasized that the present analysis requires no additional experiments beyond the ordinary Dixon plot at two different substrate concentrations, but offers the additional parameter, $A$, which provides a measure of the cooperativity between binding of inhibitor $I_{2}$ and binding of the racemic substrate $I_{1}$ and $S$.

Attention will now be turned to the specific conclusions about trypsin which may be gathered from the results in Tables 3.2 and 3.3. First of all, it is clear that at least one secondary binding site exists, since seven inhibitors in the Tables can bind to the enzyme without preventing binding of substrate. Comparison of the binding of compounds 4 and 5 of Table 3.2 shows that the charged meta-derivative shows the stronger binding, in contrast to the behavior for chymotrypsin, where NMR results have shown that similar para-derivatives are more rigidly held than the corresponding meta-inhibitors. Although there is reason to expect that a neutral inhibitor of trypsin might bind at a secondary site, as with compounds 6 and 7 of Table 3.2, it
Table 3.3 Structures, inhibition constants, and interaction coefficients for binding of inhibitors to trypsin.

For interpretation of parameter A, see Table 3.1. A-values were calculated from published Dixon plots (Mares-Guia and Shaw, 1965), for experiments carried out at pH 8.15 at 15°C in the absence of Ca^{2+}.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>Structure</th>
<th>$K_2$ (M)</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>p-aminobenzamidine</td>
<td><img src="image1" alt="Structure" /></td>
<td>$8.25 \times 10^{-6}$</td>
<td>$\infty$</td>
</tr>
<tr>
<td>2</td>
<td>phenylguanidine</td>
<td><img src="image2" alt="Structure" /></td>
<td>$7.25 \times 10^{-5}$</td>
<td>$\infty$</td>
</tr>
<tr>
<td>3</td>
<td>benzamidine</td>
<td><img src="image3" alt="Structure" /></td>
<td>$1.84 \times 10^{-5}$</td>
<td>$\infty$</td>
</tr>
<tr>
<td>4</td>
<td>cyclohexylcarboxamidine</td>
<td><img src="image4" alt="Structure" /></td>
<td>$4.27 \times 10^{-4}$</td>
<td>18</td>
</tr>
<tr>
<td>5</td>
<td>cyclohexylguanidine</td>
<td><img src="image5" alt="Structure" /></td>
<td>$1.54 \times 10^{-3}$</td>
<td>18</td>
</tr>
</tbody>
</table>
is somewhat surprising that a number of other charged inhibitors should be attracted to secondary sites.

It is now acknowledged that even highly purified trypsin exhibits some chymotrypsin-like activity toward aromatic ester hydrolysis \(^{18,19,20,21}\), indicating the presence of a binding site for aromatic residues close to the catalytic site. In this context, it is interesting to note that all but one of the aromatic compounds in this study showed binding at a secondary site, and the binding was in each case capable of affecting the binding of substrate.

The Dixon plot behavior for m-methoxyphenylguanidine offers additional evidence of secondary binding (Figure 3.1). When a third assay was conducted at a concentration of racemic substrate, D,L-BAPA of \(2.5 \times 10^{-4}\) M, it was observed that the Dixon plot curved downward at high concentration of m-methoxyphenylguanidine. The experiment was repeated several times and the effect was reproducible. The downward deflection could be due to enhanced binding of substrate or to an activation of the hydrolysis step itself, and must arise from the presence of an appreciable amount of species, \(\text{EI}_2\)s.

A final aspect of the present results is the apparent effect of Ca\(^{++}\) ion on inhibitor binding. Most reported trypsin inhibitors to date have appeared to be purely competitive\(^{8,22,23,24,25,26,27,28,29,30}\), but most studies of trypsin inhibition have been conducted in the
absence of Ca\(^{++}\). In the present study, our value for the inhibition binding constant, \(K_{2}\), for the small molecule, acetamidine (see Table 3.2) is nearly the same as obtained in the absence of calcium ion\(^8\). In contrast, binding of the aromatic compounds 3-7 in Table 3.2 is much weaker than the binding of analogous inhibitors in the absence of Ca\(^{++}\) found in Table 3.3. Now Ca\(^{++}\) has been suggested to bind to trypsin so as to hold together two "loops" in the enzyme\(^3^1\) binding of Ca\(^{++}\) does induce a conformational change resulting in a more compact structure\(^3^2\) with an accompanying enhancement in the rate of hydrolysis of substrate\(^3^3\). The behavior of aromatic inhibitors in Table 3.2 suggests that the conformational change induced by Ca\(^{++}\) both restricts access of these inhibitors to the substrate-binding pocket (only compound 3 could gain access), and also favors binding at secondary sites. A similar effect may account for the stabilization of trypsin against autolysis in the presence of Ca\(^{++}\), by making the substrate-binding site more selective.

E. SUMMARY

When a racemic "substrate" is used in assay of an unknown inhibitor of an enzyme, it may well be that only one optical isomer of the "substrate" is a true substrate, and the other a competitive inhibitor, as in the present example. The situation may be exploited as follows. Detailed kinetic analysis shows that initial velocity
measurements may be used to construct a Dixon plot in the usual way, and the intersection of two plots corresponding to two (racemic) "substrate" concentrations will still yield the inhibition constant for the unknown inhibitor. In addition, however, the ratio of the slopes of the two plots provides a measure of the interaction (competitive, repulsive, non-competitive, or attractive) of the unknown inhibitor toward the "substrate".

The importance is that an inhibitor may show a positive $(1/v)$-value at the intersection point of the Dixon plots, when binding of that inhibitor still allows access of substrate to its binding site, in contrast to the usual interpretation of a Dixon plot intersection. This point has been demonstrated using inhibitors modeled after arginine and lysine, and in addition, the method has been used to provide direct evidence for secondary binding sites on trypsin. The presence of $\text{Ca}^{++}$ appears to favour binding of aromatic inhibitors to these secondary sites, and several inhibitors actually activate the binding of "substrate".
References

1. A. Tulinsky, private communication.


33. N.M. Green and H. Neurath, J. Biol. Chem. 204, 379 (1953).
A. Introduction

As detailed in a number of recent reviews 1 - 6, there is considerable interest and disagreement about the relative importance of various effects which contribute to the efficiency and specificity of enzyme catalysis. While chemical precedents from physical organic studies have provided insight toward such aspects as "proximity" effect\(^7\), acid-base catalysis\(^8\), and solvent effects\(^9\), the role of selective orientation of substrate in enhancement of catalysis is more difficult to evaluate. Present discussion of enzyme function centers around the relative importance of strain, whether geometric\(^10\) or electronic\(^11,12\), versus orientation or alignment of the reactants by the enzyme--if the orientation requirement of a reaction is sufficiently narrow, then it is possible to account for the entire rate enhancement without invoking any strain in the reactant molecules\(^13\). Such emphasis on the importance of orientation\(^14\) has been criticized\(^15\) on the basis
of shallow minima for energy as a function of conformation from both experiment and theory for transition state bonds, but no direct evidence is available.

It is becoming increasingly clear that a major degree of strain results on binding of substrates to most enzymes. X-ray diffraction of lysozyme in the presence of substrate analogs suggested that ring "D" might be distorted from a chair to a half-chair conformation on binding of the hexamer, \((\text{N-acetylglucosamine: N-acetylmuramic acid})_3\), to the enzyme. Nuclear magnetic resonance (NMR) stopped-flow measurement of the scalar coupling constant for the "D" ring of the analogous tetrameric substrate has recently shown definitively that there is a distortion of the "D"-ring to a half-chair on binding to lysozyme in solution. Along similar lines, it is often the case that an enzyme may change its own conformation on binding of substrate, as illustrated by X-ray crystallography of carboxypeptidase A, and as demonstrated in solution by proton NMR relaxation work with creatine kinase.

Regarding the orientation question, it has long been recognized that magnetic resonance relaxation times can provide direct information about motional lability for a molecule; however, the small changes of \(T_2\) of the sharp resonance line (such as methyl group) upon adding enzyme is difficult to estimate by conventional line width measurement, since the contribution to the line width from magnetic field inhomogeneity can be several
times larger than that from either the molecular transverse relaxation time or the change induced by the enzyme.

Only recently, however, have experimental\textsuperscript{20} and theoretical\textsuperscript{21} methods become available for measurement and interpretation of relaxation times for individual high-resolution proton NMR signals in liquids. In this study, a series of trypsin inhibitors has been collected and/or synthesized (described in Chapter III), such that each inhibitor possesses a single, sharp (unsplitted) proton NMR signal from a methyl group to give unambiguous interpretation—NMR relaxation for inhibitor in the presence of enzyme then provides a measure of the rigidity with which that inhibitor is bound to the enzyme. To help identify the binding site, complementary steady-state kinetics studies were conducted—by using a racemic substrate (see Chapter III). Finally, by correlating rigidity of binding with strength of binding, and binding strength with catalytic efficiency, one can compare rigidity of binding with catalytic enhancement to clarify the role of rigid orientation in enzyme action.

B. Experimental

1. Instrument

Pulsed High Resolution NMR Spectrometer

A varian HA-100 NMR spectrometer had been modified to perform this selective determination of relaxation time
according to Freeman's procedure.

The lock channel frequency is variable and is taken from a digital frequency synthesizer (model 51106) driven by a 1 MHz master oscillator. The lock signal of ca. 2.5 KHz was obtained by digital division of a radiofrequency in the MHz range and filtering to obtain a sine wave at (1 v.p.p.) whose frequency can be set with accuracy and which can be switched to replace the ordinary manual oscillator (card 910868).

In the observation channel, the 2.5 KHz audio-modulation frequency required for detection of resonances was derived by digital division of the 1 MHz master oscillator and is fixed. Hence the lock-in amplifier (Princeton applied research-121) was permanently tuned to this frequency. Pulse I and pulse II are the exact same frequency 2.5 KHz, except pulse II can have a phase shift (90°, 180°) relative to pulse I introduced manually. The pulses I and II are gated according to switch settings on the front panel to give any sequence desired. These pulse sequences are amplified through a Hewlett-Packard 465 A amplifier, and they fed to the D.C. Modulation Coils into the probe. The demodulated output of the receiver coil (demodulated in V-4511 receiver unit) is fed to the lock in amplifier (PAR 121). The signal was recorded on a Tektronix (R 564 B) storage scope and photographed by a Polaroid Camera. The procedure and the limitations of these methods have been
thoroughly described elsewhere$^{20,21}$. Here only some additional experiences of my own will be reported.

(a) In spite of refocusing nature of the spin echo pulse sequences, it is still necessary, prior the pulsing experiment, to adjust the field homogeneity, phasing, and other controls such that a symmetrical signal is obtained on-sweeping through resonance from either up-or down-field in conventional continuous-wave operation.

(b) The concentration of the internal standard (for locking the field frequency ratio) should not be too high. Otherwise, the receiver will be saturated and lose the "lock".

(c) No NMR line should be closer than 30 Hz to the pulsed line; also, the farther the lock signal from the observed line, the better the results.

(d) A S/N of at least 3:1 was required to record a reliable Carr-Purcell sequence spin-echo train.

(e) For the case, $T_2 \leq 1$ sec, the pulse power needed to generate a $\pi$ or $\pi/2$ pulse could saturate the receiver coil. The result will be an unsteady "lock" and a wandering magnetic field, so the trace of the relaxation on the oscillo-scope will not be reproducible.

One can then measure the decay of the peak of interest and the contribution of the field inhomogeneity can be estimated from the free induction decay of an internal standard, usually
tertiary-butyl alcohol present in the same sample tube.

2. Method of Measurement

a. Chemical Shift

For the measurement of chemical shifts, a capillary containing tetramethyl silane as reference standard was inserted into the sample tubes. The capillary was held concentric with the NMR tube and magnetic field was "locked" by using this external standard; chemical shifts were measured with reference to the methyl protons of an internal standard, usually tertiary butyl alcohol or acetone. The sweep rate for chemical shift measurements was 0.2 Hz/sec or less, to satisfy the slow sweep condition necessary for accurate chemical shift determination. The frequency of the peak of interest and also that of the internal standard was obtained by interpolation between two frequency markers on both sides of the peak to be measured. The frequency of the marks was read accurately from a frequency counter (Hewlett-Packard 3734 A).

b. Relaxation Time

Transverse magnetic relaxation time, \( T_2 \), was determined for the methyl proton NMR signal for each inhibitor by the usual Gill-Meiboom modification of a Carr-Purcell pulse sequence. Measurements of longitudinal relaxation time, \( T_1 \), were performed by a \( \pi - t - \pi/2 \) pulse sequence, and viscosity corrections to
$T_2$ were shown to be negligible.

Each reported $T_1$ or $T_2$ represents an average of at least 6 independent determinations. The temperature of the probe was determined directly by having a thermistor (YSI model 4256 Tele-Thermometer) in the NMR tube thermally equilibrated within the sample "probe"; it was $30 \pm 1^\circ$C. In order to fulfill the 30 Hz-spacing condition set by the pulse unit mentioned early in the instrumental section, the typical 0.05M TRIS buffer was sometimes replaced by 0.1M borate buffer to avoid any strong signal within 30 Hz of the observed resonance. Magnetic field was stabilized by locking onto an HDO signal or tertiary butyl alcohol signal depending on which signal was farther from the observed signal. Deuterated methanol was added (less than 15%), whenever it was needed to bring enough inhibitor into the stock solution.

All inhibitor solutions are prepared in the following way. Prepare a concentrated (≈ 0.2M) inhibitor stock solution as well as concentrated buffer solution, CaCl$_2$ solution, etc. The proper amount of different concentrate stock solutions was mixed and titrated to pH=7.65 ($\mathrm{pD} = 8.05 - 8.1$), the titrated solution was then brought to the volume in a volumetric flask with D$_2$O. 12 mg TRYPsin was weighed in a 5 ml beaker and dissolved in 0.5 ml of the above prepared stock solution. The dissolved solution was then transferred into the NMR tube and equilibrated in a constant temperature bath (31°C) for 4 minutes.
and was equilibrated again in the spectrometer for at least 3 minutes. The time between dissolving of the enzyme in the inhibitor solution and the time for complete recording the data, was less than 25 minutes. This procedure minimizes danger of autolysis of trypsin (enzyme still has 95% of original activity).

C. Results and Discussion

For each inhibitor, plots of reciprocal relaxation time, \((1/T_1), (1/T_2)\), versus initial concentration ratio, \(([E_0]/[I_0])\), of enzyme to inhibitor are shown in Figure 4.2 to 4.8. For some inhibitors, the plot curves downwards at high \([E_0]/[I_0]\) ratio due to a relatively weak binding constant. A computer simulation of the predicted changes of \(1/T_1, 1/T_2\) at different \([E_0]/[I_0]\) ratio for inhibitors with various binding constants is presented in Figure 4.1; one can see that the "plots" do not in general yield a straight line as one usually assumed in the literature\(^{25,26}\). The plotted lines for a weak inhibitor (dissociation constant is larger than \(10^{-3}\)) will curve downward, whereas the plot for a strong inhibitor is more or less a straight line. Since the binding constant estimated from the NMR experiment is based on the curvature of the plotted lines, it is evident that the estimation of binding constant by NMR will be inaccurate for the strong inhibitors. This is further evidenced when the binding constant for each of the inhibitors was estimated by a non-linear least square fit (BMD - x 85) to the NMR data.
Figure 4.1

Computer simulation for $1/T_2$ versus $[E_0]/[I_0] \times 10^3$ at different hypothetical concentration with $[E_0] = 10^{-3}$ M/l.

case A : $K_I = 10^{-4}$
case B : $K_I = 10^{-3}$
case C : $K_I = 10^{-2}$
case D : $K_I = 10^{-1}$

$$\frac{1}{T_2} = \frac{1}{T_{2A}} + \frac{f_B}{T_{2B}}$$

With the assumption that the enzyme inhibitor complex is 1:1, $\frac{1}{T_{2A}} = 0.5$ sec$^{-1}$, $\frac{1}{T_{2B}} = 60$ sec$^{-1}$ (rigidly bound inhibitor) $f_B$ can be calculated for cases A, B, C, and D, and $\frac{1}{T_2}$ can then be evaluated and plotted against $[E_0]/[I_0] \times 10^3$. 
Figure 4.2 to Figure 4.8

$1/T_2$ and $1/T_1$ for the methyl protons of the inhibitors at different concentrations in the presence of constant amounts of trypsin, $[E_0] = 10^{-3}$ M/l, pH = 8.1. Temperature 30 ± 1°C. Relaxation times were measured with HA-100 equipped with a pulse unit (see experimental section).

- $1/T_2$
- $1/T_1$
Figure 4.2

\[ \frac{1}{T_1}, \frac{1}{T_2} \text{ (SEC}^{-1}) \]

\[ [E]_0/[I]_0 \times 10^3 \]

\[ \text{ACETAMIDINE} \]

\[ \text{H}_2\text{N} \]

\[ + \]

\[ \text{C} \]

\[ \text{H}_3\text{C} \]

\[ \text{H}_2\text{N} \]
Figure 4.3

\[ \frac{1}{T_1}, \frac{1}{T_2} \text{(SEC)} \]

\[ [E]/[I] \times 10^3 \]

2 3-METHOXYPROPYLAMINE + \( \text{H}_3\text{NCH}_2\text{CH}_2\text{CH}_2\text{OCH}_3 \)
Figure 4.4

\[
\frac{1}{T_1}, \frac{1}{T_2} \quad (\text{SEC}^{-1})
\]

\[
\frac{[E]_o}{[I]_o} \times 10^3
\]

3 p-Methoxybenzylamine + H_3NH_2C-OCH_3
Figure 4.5

\[ \frac{1}{T_1}, \frac{1}{T_2} \text{(SEC}^{-1}) \]

\[ [E]_0 / [I]_0 \times 10^3 \]

4. p-methoxyphenylguanidine
Figure 4.6

\[ \frac{1}{T_1}, \frac{1}{T_2} \text{ (SEC}^{-1}) \]

\[ \frac{[E]_o}{[I]_o} \times 10^3 \]

5. m-methoxyphenylguanidine

\[ \text{H}_2\text{N} \quad \text{H} \quad \text{H} \]

\[ \text{H}_2\text{N} \quad \text{N} \quad \text{C} \]

\[ \text{H} \quad \text{OCH} \]
Figure 4.7

\[ \frac{1}{T_1}, \frac{1}{T_2} \text{ (SEC}^{-1} \text{)} \]

\[ [E]_o/[I]_o \times 10^3 \]

6  p-anisidine

\[ \text{H}_2\text{N} \quad \text{OCH} \text{H} \quad \text{H} \quad \text{H} \]
Figure 4.8

\[ \frac{1}{T_1}, \frac{1}{T_2} (\text{SEC}^{-1}) \]

\[ \frac{[E]_o}{[I]_o} \times 10^3 \]

7 m-anasidine

\[ \text{H}_2N-\text{H} \]

\[ \text{H} \]

\[ \text{OCH}_3 \]
Theoretically, $1/T_1$ data will yield the same information as $1/T_2$ data, for dipole-dipole relaxation processes and thus would be a good double check for the experiment. But as can be seen in the Figures 4.2-4.8 the changes in $1/T_1$ is rather too small for this approach to be feasible.

No chemical shift was observed for any of the inhibitors studied, thus the observed relaxation time can be described as in chapter I, case A.

$$\frac{1}{T_2} = \frac{1}{T_{2A}} + \frac{f_B}{T_{2B} + \tau_B}$$  \hspace{1cm} (4.1)$$

When the temperature control experiment was conducted at a lower temperature (15°C), $(1/T_2)$ of all the inhibitors become larger: this shows that the fast exchange limit applies, so that the relaxation time at the bound site dominates the transverse relaxation time,

$$\frac{1}{T_2} = \frac{1}{T_{2A}} + \frac{f_B}{T_{2B}}$$  \hspace{1cm} (4.2)$$

In principle, $K_I$ and $1/T_{2B}$ can be estimated by the non-linear least square fit (BMDX-85). But it was soon found that the data can be fit with wide range values of $K_I$ for a strong inhibitor ($K_I \leq 10^{-3}$). Thus the bound relaxation times were calculated using the binding constant from uv studies in chapter III. For comparison, some of the "less precise" NMR based $K_I$-values are also shown in Table 4.1.
To set the scale for the $(1/T_2)$-values in the Table 4.1 one can calculate the value which would be expected if the inhibitor molecule were rigidly bound to the "backbone" of trypsin, using a proton-proton distance of 1.8 Å for a methyl group, a rotational correlation time of $1.6 \times 10^{-8}$ sec for trypsin, Larmor frequency of $2\pi \times 10^8$ sec$^{-1}$, and the appropriate formula to give a rigidly bound value of $(1/T_2) = 240$ sec$^{-1}$. More realistically, it is reasonable to suppose that even a rigidly held inhibitor will still exhibit rapid internal rotation for the -CH$_3$ or -OCH$_3$ group itself--this will result in a reduction of the apparent $(1/T_2)$-value by a factor of $[(3\cos^2(\pi/2) - 1)/2]^2 = (1/4)$ to produce an apparent "bound" $(1/T_2)$ of about 60 sec$^{-1}$, or just about what is observed for the most rigidly bound inhibitors in Table 4.1.

From the fact that the competitive inhibitors, 3-methoxypropylamine and p-methoxybenzylamine, are still rotationally labile (Table 4.1) about the long axis of each inhibitor within the active site, it can be deduced that any conformational change induced by the formation of the enzyme-inhibitor complex along the specific binding pocket is not so much as to totally immobilize the inhibitor. The size and the shape of the competitive inhibitor is very similar to the specific residues, arginine or lysine, for which an ester or peptide bond of a synthetic or natural substrate is hydrolyzed. Thus, it appears that complete immobilization of substrate is not a critical requirement for trypsin to carry out the hydrolysis.
Table 4.1

Strength of binding ($K_I$), cooperativity toward binding of substrate ($A$), and rigidity of binding ($1/T_2$) of various inhibitors to trypsin.

$K_I = [E][I]/[EI]$. $A$ has the following interpretation:

- $A = \infty$, inhibitor is competitive with substrate;
- $1 < A < \infty$, inhibitor restricts but does not prevent binding of substrate;
- $A = 1$, inhibitor binding has no effect on substrate binding;
- $A < 1$, inhibitor facilitates binding of substrate. A larger value of ($1/T_2$) corresponds to more rigid binding (see text):

$$\frac{1}{T_2} = \pi \Delta\nu,$$

where $\Delta\nu$ is the nuclear magnetic resonance line-width at half height for the methy protons of that inhibitor.

$K_I^*$ and ($1/T_2^*$) are obtained from NMR data only (see Text).
<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Structure</th>
<th>$K_I$</th>
<th>$A$</th>
<th>$(1/T_2)$</th>
<th>$K_I^*$</th>
<th>$(1/T_2)^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>acetamidine</td>
<td><img src="image" alt="Acetamidine Structure" /></td>
<td>$3.5 \times 10^{-2}$ M</td>
<td>0.75</td>
<td>&lt;5 sec$^{-1}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3-methoxypropylamine</td>
<td><img src="image" alt="3-Methoxypropylamine Structure" /></td>
<td>$2.2 \times 10^{-2}$</td>
<td></td>
<td>12 ± 3</td>
<td>(9.3 4.4)</td>
<td>18.5 ± 4x10$^{-3}$</td>
</tr>
<tr>
<td>p-methoxybenzylamine</td>
<td><img src="image" alt="PMethoxybenzylamine Structure" /></td>
<td>$6.1 \times 10^{-4}$</td>
<td></td>
<td>36 ± 5</td>
<td>(7.7 3.4)</td>
<td>44 ± 9x10$^{-3}$</td>
</tr>
<tr>
<td>p-methoxyphenylguanidine</td>
<td><img src="image" alt="PMethoxyphenylguanidine Structure" /></td>
<td>$2.3 \times 10^{-3}$</td>
<td>0.5</td>
<td>56 ± 7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-methoxyphenylguanidine</td>
<td><img src="image" alt="MMethoxyphenylguanidine Structure" /></td>
<td>$5.0 \times 10^{-4}$</td>
<td>0.3</td>
<td>67 ± 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-anisidine</td>
<td><img src="image" alt="PAnisidine Structure" /></td>
<td>$9.4 \times 10^{-2}$</td>
<td>0.17</td>
<td>68 ± 22</td>
<td>(7.7 5 )</td>
<td>56 ± 20x10$^{-3}$</td>
</tr>
<tr>
<td>m-anisidine</td>
<td><img src="image" alt="MAnisidine Structure" /></td>
<td>$1.7 \times 10^{-2}$</td>
<td>0.1</td>
<td>36 ± 4</td>
<td>(1.6 0.1)</td>
<td>55 ± 8x10$^{-2}$</td>
</tr>
</tbody>
</table>
Before drawing any conclusions about the rigidity of binding and strength of binding, let us reconsider the meaning of the binding constant obtained from the uv steady-state studies. The binding constant from uv studies only tells us the strength of binding of the sites which will interfere with the hydrolysis, whereas binding which may have no influence on the hydrolysis is not reflected in the uv studies. In contrast, NMR reflects the statistically averaged binding of all sites no matter whether the bound molecule has any form of interaction with the substrate at the active site or not. Only for a truly competitive inhibitor (which binds only at the active site of the enzyme) will these two physical techniques provide the same binding constant.

For the two purely competitive inhibitors (compound 2 and 3)

\[
2 \quad \text{3-methoxypropylamine} + \text{H}_3\text{NCH}_2\text{CH}_2\text{CH}_2\text{OCH}_3
\]

\[
3 \quad \text{p-methoxybenzilamine} + \text{H}_3\text{NH}_2\text{C} - \text{OCH}_3
\]
having different lengths but similar over all symmetry, it is seen that stronger binding correlates directly with greater rigidity at the binding site for both sets of data (uv and NMR) (Table 4.1). A similar conclusion applied to the charged but not purely competitive inhibitors (compound 1 and homologs 4 and 5).

1 acetamidine

\[
\begin{align*}
\text{acetamidine} & \quad + \quad \text{CH}_3 \\
\text{H}_2\text{N} & \quad \text{N} \\
\text{H}_2 & \quad \text{C} \\
\end{align*}
\]

4 p-methoxyphenylguanidine

\[
\begin{align*}
p\text{-methoxyphenylguanidine} & \quad + \quad \text{OCH}_3 \\
\text{H}_2\text{N} & \quad \text{N} \\
\text{H} & \quad \text{C} \\
\end{align*}
\]

5 m-methoxyphenylguanidine

\[
\begin{align*}
m\text{-methoxyphenylguanidine} & \quad + \quad \text{OCH}_3 \\
\text{H}_2\text{N} & \quad \text{N} \\
\text{H} & \quad \text{C} \\
\end{align*}
\]

A reverse situation was found for the neutral inhibitors (compound 6 and 7)

6 p-anisidine

\[
\begin{align*}
p\text{-anisidine} & \quad + \quad \text{OCH}_3 \\
\text{H}_2\text{N} & \quad \text{N} \\
\text{H} & \quad \text{H} \\
\end{align*}
\]

7 m-anisidine

\[
\begin{align*}
m\text{-anisidine} & \quad + \quad \text{OCH}_3 \\
\text{H}_2\text{N} & \quad \text{N} \\
\text{H} & \quad \text{H} \\
\end{align*}
\]
Meta-anisidine, a weaker inhibitor, is nevertheless bound more rigidly than P-anisidine, a stronger inhibitor (Table 4.1). It was suggested that the binding sites for neutral compounds is different from the binding sites for the charged compounds\textsuperscript{29,30,31}. Perhaps the geometry of neutral homolog binding sites stops the meta-isomer from rotating due to the steric hindrance\textsuperscript{*} and results the reversed situation.

Although the factor of steric hindrance may play a big role in the immobilization of the small molecule at the binding site\textsuperscript{*}, nevertheless, the general trend revealed by the NMR relaxation results for the charged inhibitor is that the stronger binding correlates directly with greater rigidity at the binding site. Since the mechanism of catalysis by trypsin and $\alpha$-chymotrypsin is believed to be the same\textsuperscript{11}, and for chymotrypsin, the relative magnitude of the binding constant is independent of the magnitude of the rate enhancement\textsuperscript{32}. More to the point, a smaller amount of data for trypsin substrates\textsuperscript{33,34} shows that binding constant and catalytic efficiency are unrelated for trypsin also. It would thus appear that rigid immobilization may not be a major factor in enzyme catalysis, at least for trypsin in these preliminary studies.

\* It should be noted that even for a series of truly competitive inhibitors, each of the same binding constants, a direct correlation between the strength of binding and the rigidity of the bound species could be modified by the presence of steric hindrance. For example, a rod-shaped molecule in a cleft could have a rotation about it's long axis, but the addition of an extra side chain group might stop it rotating completely, but still permit binding.
D. Summary

Several arginine or lysine analog inhibitors of trypsin have been subjected to nuclear magnetic resonance measurements. The u.v. assays provide determination of the binding constant of inhibitor to enzyme and indicates the type of binding. The NMR results can be analyzed to yield a quantitative measure of the degree of motional freedom at the binding site of the inhibitor. Strength of binding is found to correlate directly with rigidity of binding for the charged groups and since strength of binding of substrate does not correlate with catalytic efficiency, the results suggest that rigid immobilization of substrate is not a major factor in trypsin catalysis.
References


34. T. Yamamoto and N. Izumiya, Arch. Biochem. Biophys. 120, 497 (1967).
CHAPTER V

Magnetic Resonance Studies of Serine-195-Spin-Labeled Trypsin

A. Introduction

A spin label is a synthetic paramagnetic organic free radical, usually having a molecular structure and/or chemical reactivity that results in its attachment or incorporation at some particular site, covalently or noncovalently in a biological macromolecule, or assemblage of macromolecules. This type of probe was first introduced by McConnell\(^1\) and was successfully applied to studies of membranes\(^2\) as well as studies of the conformational changes in macromolecules\(^3\). Several excellent review articles are now available\(^4,5\).

Recently, several papers have exploited the large magnetic moment associated with an unpaired electron, to enhance the dipolar relaxation of nuclei at distances of as much as 10 Å away. Thus the detection of the dipolar interaction between the nitrooxide spin label and the nucleus of interest permits the estimate of the distances over a relatively large range. The estimate of such a large distance cannot usually be obtained through the weak dipolar interaction between a pair of distant nuclei.

Mildvan and Weiner\(^6\) have used a spin-labeled analog of Nicotinamide Adenine Dinucleotide to perturb the line width of ethanol and some other
substrates in Liver Alcohol Dehydrogenase (LAD). The relative position on LAD of the appropriate coenzymes and the substrates may then be reconstructed.

Experiments using both paramagnetic metal ions and nitroxide spin labels to enhance nuclear relaxation rates and to estimate intramolecular distances in enzyme complexes in solution have also been described\(^7,^8,^9\).

Recently, distances of up to 20 Å between either bound saccharides or Histidine-15, and Tryptophan-123 on lysozyme in solution have been estimates from the spin-label induced line broadening of the proton of interest by Wien et al\(^10\).

In the last chapter of this thesis, this powerful spin label technique will be applied to help demonstrate the existence of the secondary binding site which had been suspected from earlier indirect evidence\(^11,^12,^13\) and from the UV studies (chapter III) in this thesis.

Since the diameter of trypsin is only about 40 to 50 Å, even if the dipolar relaxation induced by the unpaired electron of the nitroxide spin label is not detectable, the result will at least limit the number of possible locations for the secondary site. If the line broadening induced by the nitroxide spin label is indeed observable, then distances between each inhibitors and the nitroxide of the spin label at Serine-195, determined by nuclear relaxation measurements, and with the help of the X-ray three dimensional structure of trypsin should enable one to make a resonable guess as to where the secondary site on trypsin could be.

It is known that the ESR spectra of spin lebels are very sensitive
to the rate at which the label is able to reorient rotationally\textsuperscript{4,14}, that is, the ESR spectrum of the label will reflect the degree of rotational mobility allowed in the environment of the label.

Since the nitrooxide of the spin label was attached to the Serine-195 at the active site\textsuperscript{15}, one would expect that the free radical at the active site will provide a good probe to monitor the conformational changes at the active site. Thus it should be possible to observe any conformational changes induced upon adding the Ca\textsuperscript{++} or Mn\textsuperscript{++} ion, as well as to examine the interaction between the spin label attached to the Serine-195 and the inhibitors bound to the secondary binding sites, by monitoring the ESR spectrum of the free radical in the active site.

Finally, in order to get rid of the reported undesired autoproteolysis caused by the presence of trace amount of trypsin in the spin labeled trypsin, a preparation of active trypsin-free spin labeled trypsin was designed and carried out as described in the experimental section of this chapter.

B. Experimental

1. Materials

Spin label 4-Hydroxy-2,2,6,6.-tetramethyl piperidino oxyl monoethylphosphoro fluoride (ester) was obtained from Synvar. Sephadex G-25, G-50 and ion exchange Sp-C50 were from Pharmacia Fine Chemicals. The rest of the chemicals are all obtained from the same source as in previous chapters.
2. Preliminary preparation of gel filtration columns

Sephadex G-50 was hydrated in 0.05 M TRIS-Chloride buffer (pH = 7.1), containing 0.02 M CaCl₂, at room temperature for 12 hours with frequent decantations to remove fine material, and degassed for three hours on a water aspirator. It was then poured into a column (5.0 x 80 cm) and operated with the solvent reservoir typically below the head of the column to avoid packing. About two to three bed volumes of buffer was eluted through the column to achieve equilibrium at 50°C.

The same procedure was employed for the preparation of Sephadex G-25 (5.0 x 85 cm), Sp-Sephadex C-50 columns (2.6 x 50).

3. Preparation of active trypsin-free, spin labeled trypsin

Trypsin was covalently labeled at Serine-195 with spin label inhibitor by a modification of the method described by Berliner 16. 25 mg of the spin label inhibitor (4-Hydroxy-2,2,6,6-tetramethylpiperidinoxy1 monoethylphosphorofluoridate (ester)) from Synvar was dissolved completely in 1 ml dioxane. Half of the spin label was added to a solution containing 20 ml of 0.05 M Tris-Chloride buffer (pH = 7.7, 0.02 M CaCl₂) and 5 ml of dioxane. After
these two solutions were completely mixed, 250 mg of trypsin were added, the pH was adjusted to 7.8 with 1 M NaOH, and the solution were stirred slowly. About 30 minutes later, the rest of the spin label was poured into the reaction mixture. The reaction was allowed to proceed for about another two hours at room temperature. The organic solvent serves to help dissolving the spin label as described in Berliner's work\textsuperscript{16}. It was also shown that 50\% (V/V) dioxane does not effect enzyme activity\textsuperscript{17}. The labeled enzyme was then passed through a column (5.0 x 85 cm) of Sephadex G-25 equilibrated with 0.1 M NaCl and 0.01 M acetic acid, pH 3.3 at 5\textdegree C. The last few tubes containing protein were checked by ESR to ensure complete removal of free unreacted spin label. Then fractions of the effluent which contained protein without free spin label were pooled together and 100 mg soybean trypsin inhibitor was added, to prevent attack on spin-label trypsin by (unreacted) active trypsin. After 7 volumes exchange with the 0.05 M TRIS-Cl buffer (pH = 7.1, CaCl\textsubscript{2}, 0.02 M) by continuous ultrafiltration (UM-10 membrane from Amicon Corp.), the solution was concentrated down to 40 ml. A small amount of the insoluble material which formed during concentration was removed by centrifugation and the supernatant solution was applied to a column (5.0 x 80 cm) of sephadex G-50 which had been equilibrated with the buffer being exchanged before. The column was eluted at a flow rate of 30 ml per hour and the eluate was collected in 8 ml fractions (Figure 5.1). The second peak was pooled together and was concentrated down to 40 ml by ultrafiltration. The concentrate was examined by ESR again to identify the compound. In Figure 5.1, the first peak
represents trypsin - soybean trypsin inhibitor complex, which is excluded from the gel due to its high molecular weight (45,000), and the third peak represents autolyzed polypeptide. This process removes almost all of the unreacted active trypsin in the form of STI - T complex, because the dissociation constant the trypsin - soybean trypsin inhibitor is smaller than $10^{-8}$ at pH 7.1. 40 ml of the concentrate from the 2nd peak was then applied to a Sp-Sephadex C-50 column (2.6 x 50), to get rid of the inactive trypsin. A typical elution diagram is shown in Figure 5.2. The α and β trypsin fractions were pooled separately and dialyzed against 0.01 M acetic acid in the cold. A small fraction of each pooled and the dialized solution was concentrated and ESR spectra were obtained (Figure 5.3). The rest of the dialyzed solution was lyopholized, and the lyopholized sample was also examined by ESR (Figure 5.4). The spin labeled β-trypsin (before freeze drying) was left at room temperature (25° C) pH = 7.1 and there were no changes in the ESR spectrum for at least 3 days (Figure 5.5). Thus we can be confident that there was no active trypsin in this spin-labeled trypsin preparation. Because the isoelectric point of soybean trypsin inhibitor (STI) is 4.5, STI will not bind to a cation-ion-exchanger at neutral pH. Hence the excess STI should be eluted out first (along with inactive trypsin) from the SP - C 50 column (Figure 5.2). The broad ESR signal of spin-labeled β-trypsin sharpens to a narrow signal after adding commercial trypsin (Figure 5.6), as observed by Berliner.
Figure 5.1.

Separation of trypsin - Soybean trypsin inhibitor (STI) complex from a mixture of spin labeled trypsin, trypsin-STI complex and STI by chromatograph on a column (5.0 x 80 cm) of sephadex G-50, in 0.05 M tris-chloride buffer, pH 7.1, 0.02 M CaCl$_2$ at 4°C. The flow rate was 30 ml per hour; 8 ml were collected per tube.
Figure 5.2.

Separation of $\alpha$ and $\beta$ spin labeled trypsin from a mixture of $\alpha$ and $\beta$ spin labeled trypsin, STI, and inactive (spin labeled) trypsin by ion exchange chromatography on a column (2.6 x 50 cm) of Sp-Sephadex C-50 in 0.05 M Tris-Chloride buffer, pH 7.1, 0.02 M CaCl$_2$, at 4°C. The flow rate was 15 to 20 ml per hour; 8.5 ml were collected per tube.
Excess STI + Inactive Trypsin

ABSORBANCE AT 280 nm

TUBE NUMBER

0 40 80 120 160 200 240
Figure 5.3. ESR spectrum of free spin label, spin labeled α and β trypsin.

A. ESR spectrum of free spin label (4-Hydroxy-2,2,6,6-tetramethylpiperidinooxyl monoethylphosphorofluoridate ester) in 0.05 M TRIS-Chloride buffer, PH = 7.1.

B and C. ESR spectra of spin labeled α-trypsin (spectrum B) and β-trypsin (spectrum C) in 0.05 M TRIS-Chloride buffer PH = 7.1 0.02 M CaCl₂ before freeze drying.

The spectrum were unchanged even after the solutions were separately dialized against 0.01 M acetic acid (PH 3.3) for 24 hours at 4°C.

E-3 ESR SPECTROMETER SETTINGS.

Field Set : 3380 G
Modulation Amplitude : 0.5 G
Scan time : 16 min
Receiver Gain : 6.2x10⁵
Scan range : 100 G
Figure 5.4.

A comparison of the ESR spectra of spin labeled-trypsin before and after freezedrying.

A. ESR spectra of spin-labeled-β trypsin in 0.05 M Tris-Chloride buffer, pH 7.1, 0.02 M CaCl₂.

B. A small percentage of spin-labeled-β trypsin was denatured by the freezedrying process. The denaturation was indicated by the appearance of a sharp signal at the high field resonance line.

C. The ESR spectra of spin-labeled-β trypsin which has been incubated in the NMR probe (40°C) for 30 min: no noticeable change is observed. This ensures that denaturation during the NMR experiment can be neglected.
Figure 5.5.

The stability of the active trypsin-free, spin-labeled-trypsin is shown by the invariant ESR spectrum of spin-labeled-β trypsin in Tris-Chloride buffer PH 7.1, 0.02M CaCl₂ at room temperature (20-25°C) for three days.

A. immediately after being concentrated from Amicon cell.

B. 2 days later.

C. 3 days later.
The hydrolysis of spin-labeled trypsin on addition of a small amount of commercial (active) trypsin in 0.05 M Tris-Chloride buffer pH 7.1, 0.02 M CaCl$_2$.

A. 30 min after the addition of trypsin.

B. 12 hours after the addition of trypsin.

C. 3 days after the addition of trypsin.
Figure 5.7

A. E.S.R. spectra of $5 \times 10^{-4} \text{Mn}^{++}$ solution (0.05 MTRIS-Chloride buffer Ph = 7.1) in the presence of $1.26 \times 10^{-4} \text{M/L S.L. TRYSIN}$ except the E.S.R. spectra of the spin label trypsin was superimposed on top of the center of the Mn$^{++}$ spectrum.

B. The expansion of the center portion of A. The E.S.R. signal of the spin labelled trypsin is now visible, and no obvious change can be detected.

C. E.S.R. spectrum of $1.26 \times 10^{-4} \text{M/L S.L—TRYSIN}$ in the presence of 0.025 M p-methoxyphenyl Guanidine.HCl (good inhibitor) and 0.02 M CaCl$_2$.

D. E.S.R. spectrum of the $1.26 \times 10^{-4} \text{S.L—TRYSIN}$ in the presence of $2.4 \times 10^{-3} \text{MTAME}$ and 0.02 M CaCl$_2$. 
4. Method

Spin labeled trypsin stock solution. 21 mg of spin labeled trypsin was dissolved in 2 ml of D$_2$O Borate buffer (pDH = 8.1), containing 0.02 M CaCl$_2$. The insoluble material was removed by centrifugation. The concentration of the protein in the supernatant was determined on a Carl-Zeiss PMQ-II by measuring the absorption at 280 nm using an extinction coefficient of 0.651 mg ml$^{-1}$ (OD unit)$^{-1}$ and assuming a molecular weight of 24,000$^{22}$. This solution was stored in ice bath for later use.

Samples for NMR measurement were prepared by adding 0.2 ml of the spin-labeled trypsin stock solution and 0.2 ml of the inhibitor stock solution. Before insertion into the NMR probe, the sample was prewarmed in a constant temperature bath at 30° C for 3 minutes. The NMR probe temperature was monitored through a thermister (YSI Model 42SC Tele-Thermometer) in a NMR tube and was controlled to be 30 ± 1° C by running N$_2$ gas through a cooling coil immersed in a water-and-ice mixture. The Varian temperature-control unit and the coolent N$_2$(l) were not used because the temperature variation was too big ± 3 - 5° C and the heat capacity of N$_2$(l) is too small that it is necessary to refill the thermoflask every hour. The refilling process usually will disturb the temperature at the probe as well as the stability of the spectrometer.

All the line width studies were done on a varian XL-100-15 NMR spectrometer with a FT-8K on line computer. The expansion of the interesting portion of the spectrum can be done by the teletype connected to the computer. The field homogeneity was optimized by
first locking on the $^1\text{H}$ resonance of $\text{HDO}$, then switching to the $^2\text{H}$ lock using the $\text{D}_2\text{O}$ peak. Field homogeneity control was left undisturbed while the pulsed F.T. NMR measurement was carried out.

The pH of solutions was measured at room temperature with a Copenhagen Radio-meter equipped with a Beckman 39030 combination electrode. All reported pH values have been subjected to the correction $(\text{pH} = \text{meter reading} - 0.4)^{23}$ for solutions in $\text{D}_2\text{O}$.

C. Theory

The conditions for fast exchange can be found in Chapter 1 case B(d);

$$\left(\frac{1}{\tau^*_{\text{EI}}} \gg \left(\frac{1}{T_2}\right)^{\text{EI}}\right)$$

$$\left(\frac{1}{\tau^*_{\text{EI}}} \left(\frac{1}{T_2}\right)^{\text{EI}} \gg \Delta \omega_{\text{EI}}^2 \right)$$

where

$$\left(\frac{1}{T_2}\right)^{\text{EI}} = \left(\frac{1}{T_{2B}} + \frac{1}{T_{2M}}\right)$$

Here, $\tau^*_{\text{EI}}$ is the life time for the inhibitor bound to the enzyme; $(T_2)^{\text{EI}}$ and $T_2^A$ are the transverse nuclear relaxation times of the proton of the inhibitor for EI complex and free inhibitor respectively. $T_{2B}$ and $T_{2M}$ are contributions from nucleus-nucleus dipole-dipole interaction and electron-nucleus dipole-dipole interaction for EI complex respectively. $\Delta \omega_{\text{EI}}$ is the difference of the resonance frequencies of the bound and free inhibitors.
Under the above conditions of fast exchange, the observed transverse relaxation time will be

\[ \frac{1}{T_2} = \frac{f_A}{T_{2A}} + f_{EI} \left( \frac{1}{T_{2B}} + \frac{1}{T_{2M}} \right) \]  \hspace{1cm} (5.3)

where \( f_{EI} \) is the fraction of inhibitors bound to the enzyme; \( f_A = 1 - f_{EI} \); also \( f_A \gg f_{EI} \) thus \( f_A = 1 \). From Chapter I equations (1.18) the transverse relaxation induced by the unpaired electron of spin labels can be described as

\[ \frac{1}{T_{2M}} = \frac{1}{15} \frac{S(S+1)\gamma I g^2 \beta^2}{r^6} [4\tau_c + \frac{3\tau_c}{1 + \frac{W_{1\tau_c}^2}{1 + W_{2\tau_c}^2}} + \frac{13\tau_c}{1 + W_{2\tau_c}^2 - 13\tau_c}] \]

\[ + \frac{1}{3} \frac{S(S+1)A^2}{2} \left[ \frac{\tau_e}{1 + W_{2\tau_e}^2} \right]. \]  \hspace{1cm} (5.4)

However, in the present experiment, the unpaired electron of the nitroxide and the proton of the inhibitor, are well separated in the EI complex. Thus we may omit the second term corresponding to isotropic spin-exchange interaction. Furthermore, when the inhibitor binds to the enzyme \( \tau_c \) increases and to a good approximation \( W_{2\tau_c}^2 \gg 1 \). Under these conditions, the nuclear transverse relaxation time can be simplified to

\[ \frac{1}{T_{2M}} = \frac{S(S+1)\gamma I g^2 \beta^2}{15r^6} \left[ 4\tau_c + \frac{3\tau_c}{1 + W_{1\tau_c}^2} \right] \]  \hspace{1cm} (5.5)

so, if \( T_{2M} \) and \( \tau_c \) are known, the distance between the unpaired electron and the proton of interest can be determined.

As will be discussed later, \( \tau_c \) in this equation is essentially
equal to the rotational correlation time of the enzyme.

A convenient expression for \( r(\text{Å}) \) in terms of \( T_{2M} \) (in sec) is

\[
r(\text{Å}) = 485[T_{2M}(4\tau_c + \frac{3\tau_c}{1 + W_I^2\tau_c})]^{1/6}
\]

(5.6)

where \( \tau_c = 1.6 \times 10^{-8} \) sec

\( 2^4 \) and \( W_I = 100 \) MHz

D. Analysis of data

I. Determination of \( T_{2M} \) and \( r \)

In the absence and presence of the spin-labeled enzyme, the observed transverse relaxation time for the inhibitor will be

\[
\left(\frac{1}{T_2}\right) = \frac{1}{T_{2A}} + \frac{1}{T_{2H}}
\]

(5.7)

\[
\left(\frac{1}{T_2}\right)^* = f_A\left(\frac{1}{T_{2A}} + \frac{1}{T_{2V}} + \frac{1}{T_{2C}} + \frac{1}{T_{2H}^*}\right) + f_{EI}\left(\frac{1}{T_{2B}} + \frac{1}{T_{2M}}\right)
\]

(5.8)

where \( f_A = 1 \) if \([I] \gg [EI]\)

\( f_{EI} \) is the fraction of bound inhibitors, can be calculated from \( K_I \) (Chapter IV). \( \frac{1}{T_{2A}} \) is the transverse relaxation time of free inhibitors.

where \( \frac{1}{T_{2V}}, \frac{1}{T_{2C}}, \frac{1}{T_{2H}} \), represent the contribution from viscosity change by the presence of enzyme, the contact interaction between the unpaired electron on the spin label and the protons of inhibitor and the field inhomogeneity at the time of measurement, respectively.
The relaxation time for an internal standard (which will not bind to the enzyme) in the absence and presence of enzyme will be

\[
\left(\frac{1}{T_2}\right)_s = \left(\frac{1}{T_{2A}}\right)' + \left(\frac{1}{T_{2H}}\right)' \tag{5.9}
\]

\[
\left(\frac{1}{T_2}\right)_* = \left(\frac{1}{T_{2A}}\right)' + \frac{1}{T_{2V}} + \frac{1}{T_{2C}} + \left(\frac{1}{T_{2H}}\right)' \tag{5.10}
\]

By using a multichannel pulse NMR spectrometer, the line width of both the internal standard and the inhibitor can be measured at the same time; thus

\[
\frac{1}{T_{2H}} = \left(\frac{1}{T_{2H}}\right)' \quad \text{and} \quad \left(\frac{1}{T_{2H}}\right)_* = \left(\frac{1}{T_{2H}}\right)'.
\]

Now, the term \(\frac{1}{T_{2M}}\) can easily be found as follows:

\[
\frac{1}{T_{2M}} = \frac{1}{T_{EI}} \left\{ \left[\left(\frac{1}{T_2}\right)^* - \left(\frac{1}{T_2}\right)_s\right] - \left[\left(\frac{1}{T_2}\right) - \left(\frac{1}{T_2}\right)_s\right] \right\} - \frac{1}{T_{2B}}
\]

\[
= \frac{\pi}{T_{EI}} x (\text{Net broadening}) - \frac{1}{T_{2B}} \tag{5.11}
\]

Here \(\left(\frac{1}{T_2}\right)^*\), \(\left(\frac{1}{T_2}\right)_s\), \(\left(\frac{1}{T_2}\right)\) and \(\left(\frac{1}{T_{2B}}\right)_s\) can be determined experimentally and \(\left(\frac{1}{T_{2B}}\right)\) has been determined previously in Chapter 4. \(\frac{1}{T_{2M}}\) values for each of the inhibitors are given in table 5.1 (30°C) and table 5.2 (40°C). With the assumptions (to be proved later) that the fast exchange limit is valid and \(\tau_c\) is dominated by \(\tau_r\), the distance between the free radical and the methyl group of each inhibitors can be calculated by substituting \(\frac{1}{T_{2M}}\) for each inhibitors into equation (5.6). The results can be found in the last column of table 5.1.
2. \( \tau_C \) is dominates by \( \tau_R \)

As in Chapter 1 equation (1.20), the correlation time, \( \tau_C \), which characterizes the rate process that modulates the dipolar interactions is given by

\[
\frac{1}{\tau_C} = \frac{1}{\tau_r} + \frac{1}{\tau_s} + \frac{1}{\tau_{EI}}
\]  

(5.12)

where \( \tau_r \); rotational correlation time, \( \tau_s \); electronic spin lattice relaxation time, \( \tau_{EI} \); residence time for the inhibitors on the enzyme. The correlation time is determined by the fastest rate process; that is, which ever time is shortest - \( \tau_r \), \( \tau_s \) or \( \tau_{EI} \). Since the molecular weight of trypsin (23.800) is about the same as chemotrypsin (23.200), we would expect the rotational correlation time for trypsin will be about the same as that of chemotrypsin, i.e, \( 1.6 \times 10^{-8} \) sec.

The exchange lifetime, \( \tau_{EI} \), may be estimated from \( K_I \) and \( k_1 \) (the rate of formations of the enzyme inhibitor complex) as following; since the typical values for \( k_1 \) is smaller or equal to \( 10^9 \) sec\(^{-1}\), and the \( K_a \) for the inhibitors studied here are larger or equal to \( 10^2 \) M\(^{-1}\) (Chapter III)

\[
k_{-1} = \frac{k_1}{K_a} = \frac{10^9}{10^2} = 10^7 \text{ sec}^{-1}
\]

(5.13)

and

\[
\frac{1}{\tau_{EI}} = k_{-1}
\]

(5.14)

thus \( \tau_{EI} \) should either close to or larger than \( 10^{-7} \), and this is about one order of magnitude slower than the rotational correlation
time \((10^{-8})\). Clearly \(1/\tau_{E_i}\) can not dominate the rate process.

The electronic relaxation time, \(\tau_s\), for Fremy's salt a solid nitroxide species), is \(3.4 \times 10^{-7}\) sec. Also analysis of the saturation behavior of 3-SL-His-15-lysozyme has been reported and the electronic relaxation time, \(\tau_s\), for spin-labeled lysozyme is about \(3.5 \times 10^{-7}\) sec. Hence, it would be expected that \(\tau_s\) for the nitroxide SL-Ser 195 trypsin will be the same order of magnitude. An effective shortening of \(\tau_s\) due to spin exchange at high concentration of nitroxide \((\geq 10^{-3}\text{M})\) was negligible in the present experiment since SL-trypsin concentration was about 1/10 the concentration used in ref 10. Now, since the rotational correlation time for SL-Trypsin is about one order of magnitude smaller than that of the electronic relaxation time of the nitroxide on the enzyme it can be sure that the dominant dipolar modulation process will be the rotational motion of the enzyme-inhibitor complex, i.e. \(\tau_c \leq 1.6 \times 10^{-8}\) sec.

3. Justification for the assumption of fast exchange (see APPENDIX B)

Although the measurement of transverse relaxation time at different temperatures in Chapter 3 shows that all the seven inhibitors are on the fast exchange limit, this can not be the criterion for fast exchange in this experiment because the line width of an spin labeled trypsin-inhibitor complex is much greater than the line width of a trypsin-inhibitor complex due to the electron-nuclei dipole interaction. With this precaution in mind, a control experiment was conducted at 40°C, and the results are
shown in table (5.2). Without exception, \( \frac{1}{T_{2M}} \) for each of the seven inhibitors at 40°C is smaller than the corresponding \( \frac{1}{T_{2M}} \) at 30°C. However, since \( \frac{1}{T_{2M}} \) at 40°C is not too much smaller than \( \frac{1}{T_{2M}} \) for the m-arrisidine, this inhibitor may fall into the intermediate range, i.e., between a "fast" exchange and "slow" exchange limit.

Since no chemical shift is observed on binding this will be case A in Chapter I and

\[
\frac{1}{T_2} = \frac{1}{T_{2A}} + \frac{f_{EI}}{T_{2EI} + \tau_{EI}}
\]

Let \( \Delta(\frac{1}{T_2}) = \frac{1}{T_2} - \frac{1}{T_{2A}} \) for brevity.

For the different exchange limits:

- **Fast exchange**: \( \Delta(\frac{1}{T_2}) = \frac{f_{EI}}{T_{2EI}} \), \( \tau_{EI} < T_{2EI} \)

- **Intermediate**: \( \Delta(\frac{1}{T_2}) = \frac{f_{M}}{T_{2M} + \tau_{M}} \), \( \tau_{EI} < T_{2EI} \)

- **Slow exchange**: \( \Delta(\frac{1}{T_2}) = \frac{f_{M}}{\tau_{M}} \), \( \tau_{EI} > T_{2EI} \)

and equation (5.6) \( r_t = \text{const} (T_{2M})^{1/6} \)

so

- **Fast exchange**
  \[ r_{\text{obs}} = r_t \] (\( r \) is determined)

- **Intermediate**
  \[ r_{\text{obs}} = \text{const} (T_{2M} + \tau_{M})^{1/6} > r_t \] (given upper limit)

- **Slow exchange**
  \[ r_{\text{obs}} = \text{const} (\tau_{M})^{1/6} = r_t \] (given upper limit)
whatever limit applies one can always extract some distance information from this kind of measurement.

E. Result and Discussion

1. Estimation of distances by measuring the induced nuclear relaxation. The results of experiments utilizing the enhanced nuclear resonance line widths to calculate distances between the unpaired electron of spin label and the methyl group of each bound inhibitors are given in table 5.1 and the results of temperature control experiments at 40°C are given in table 5.2. Examples of the observed spectrum of one of the inhibitors in the presence and absence of SL-Trypsin are given in figure 5.8 and 5.9 respectively.

From the three dimensional structure at the active sites of DIP-trypsin (diisopropyl fluorophosphate inhibited trypsin)\textsuperscript{28}, it seems that the spin label covalently bound to the \(\gamma\)-oxygen of Ser-195 on trypsin could have two extreme orientations, one with the piperidinyl ring projecting out into the solvent, and the other with the ring pointed inward and extending into hydrophobic specific binding pocket.

If the nitroxyl ring was projecting outward into the solvent, then the ethyl group of the phosphate ethyl ester may partially block the entrance of the specific binding pocket. Since the DIP-trypsin can still bind Benzamidine (Stroud private communication)\textsuperscript{29} and since the isopropyl group in that experiment is larger than the ethyl group in this experiment, also compound (3-methoxypropylamine) is definitely smaller than Benzamidine (space filling models),
Table 5.1

Experimental parameters used in enzyme intramolecular calculations (at temperature 30° ± 1°C).

a. Given in hertz
b. Given as M$^{-1}$ and determined by u.v. steady-state kinetics (Chapter III).
c. Bound relaxation time in the presence of trypsin (Chapter IV)
d. $f_B$ is a fraction of inhibitor bound to enzyme
e. Electron-nuclear dipole-dipole contribution to nuclear relaxation of inhibitor protons in presence of SL-trypsin
f. Distance from \(-\text{OCH}_3\) (or \(-\text{CH}_3\)) of inhibitor to unpaired electron of SL-trypsin

Table 5.2

Experimental parameters used in estimation of the electron-nuclear dipole-dipole contribution to inhibitor proton nuclear relaxation due to SL-trypsin at a different temperature.

a. Given in hertz
b. Inhibitor:enzyme binding constants, given as M$^{-1}$ and determined by u.v. kinetic (Chapter III). With the assumption $K_a$ will not vary too much in the range of temperature 30°C to 40°C.
c. Bound relaxation time in the presence at trypsin, with the assumption that the temperature variation of $(1/T_{2B})$ is small compared to the variation of $(1/T_{2M})$ on increasing the temperature from 30°C to 40°C.
<table>
<thead>
<tr>
<th>Inhibitor Name</th>
<th>Conc. of Inhibitor (M/\text{\AA})</th>
<th>Conc. of Enzyme (M/\text{\AA})x10^4</th>
<th>Observed NMR Line width \textsuperscript{a} \text{-OCH}_3, \text{-CH}_3 of inhibitor</th>
<th>Observed NMR Line width \textsuperscript{a} \text{-CH}_3 of (CH\textsubscript{3})\textsubscript{3}C-OH</th>
<th>(K\textsubscript{a})</th>
<th>(\frac{1}{T\textsubscript{2B}})</th>
<th>(f\textsubscript{B}x10^3)</th>
<th>(\frac{1}{T\textsubscript{2M}})</th>
<th>(r\textsubscript{\text{\AA}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acetamidine</td>
<td>0.025</td>
<td>0</td>
<td>0.36</td>
<td>0.38</td>
<td>29</td>
<td>&lt; 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>1.26</td>
<td>0.44</td>
<td>0.52</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2. 3-methoxypropylamine</td>
<td>0.03</td>
<td>0</td>
<td>0.44</td>
<td>0.44</td>
<td>45.5</td>
<td>12</td>
<td>2.42</td>
<td>323</td>
<td>10.7</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>1.26</td>
<td>0.76</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3. p-methoxybenzylamine</td>
<td>0.015</td>
<td>0</td>
<td>0.48</td>
<td>0.3</td>
<td>1640</td>
<td>36</td>
<td>8.1</td>
<td>766</td>
<td>9.3</td>
</tr>
<tr>
<td></td>
<td>0.015</td>
<td>1.26</td>
<td>2.64</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4. p-methoxyphenyl-</td>
<td>0.025</td>
<td>0</td>
<td>0.62</td>
<td>0.5</td>
<td>435</td>
<td>56</td>
<td>4.62</td>
<td>869</td>
<td>9.1</td>
</tr>
<tr>
<td>guanidine</td>
<td>0.025</td>
<td>1.26</td>
<td>1.83</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5. m-methoxyphenyl-</td>
<td>0.025</td>
<td>0</td>
<td>0.48</td>
<td>0.28</td>
<td>2000</td>
<td>67</td>
<td>5.0</td>
<td>574</td>
<td>9.8</td>
</tr>
<tr>
<td>guanidine</td>
<td>0.025</td>
<td>1.26</td>
<td>1.56</td>
<td>0.36</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6. p-anisidine</td>
<td>0.025</td>
<td>0</td>
<td>0.36</td>
<td>0.32</td>
<td>59</td>
<td>36</td>
<td>3.0</td>
<td>550</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>1.26</td>
<td>1.0</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7. m-anisidine</td>
<td>0.025</td>
<td>0</td>
<td>0.41</td>
<td>0.4</td>
<td>11</td>
<td>68</td>
<td>1.1</td>
<td>1087</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>0.025</td>
<td>1.26</td>
<td>0.92</td>
<td>0.4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 5.1**
<table>
<thead>
<tr>
<th>Inhibitor Name</th>
<th>Conc. of inhibitor (M/l)</th>
<th>Conc. of SL-Trypsin (M/l) x 10^4</th>
<th>Observed NMR Line width of inhibitors</th>
<th>Observed NMR Line width of (CH_3)J-C=OH</th>
<th>(Ka)</th>
<th>(1/T_2B)</th>
<th>c</th>
<th>(fB x 10^3)</th>
<th>d</th>
<th>(1/T_2M)</th>
<th>e</th>
<th>(1/T_2M)_{400}</th>
<th>f</th>
<th>(1/T_2M)_{300}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Acetamidine</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td>-</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>2. 3-methoxypropylamine</td>
<td>0.03</td>
<td>1.57</td>
<td>0.66</td>
<td>0.46</td>
<td>45.5</td>
<td>12</td>
<td></td>
<td>3.02</td>
<td></td>
<td>196</td>
<td></td>
<td>323</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>3. p-methoxybenzylamine</td>
<td>0.015</td>
<td>1.57</td>
<td>2.04</td>
<td>0.3</td>
<td>1640</td>
<td>36</td>
<td></td>
<td>10.00</td>
<td></td>
<td>423</td>
<td></td>
<td>766</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>4. p-methoxyphenyl- guanidine</td>
<td>0.025</td>
<td>1.57</td>
<td>1.92</td>
<td>0.4</td>
<td>435</td>
<td>56</td>
<td></td>
<td>5.75</td>
<td></td>
<td>765</td>
<td></td>
<td>869</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>5. m-methoxyphenyl- guanidine</td>
<td>0.025</td>
<td>1.57</td>
<td>1.52</td>
<td>0.32</td>
<td>2000</td>
<td>67</td>
<td></td>
<td>6.15</td>
<td></td>
<td>444</td>
<td></td>
<td>574</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>6. p-anisidine</td>
<td>0.025</td>
<td>1.57</td>
<td>1.0</td>
<td>0.4</td>
<td>59</td>
<td>36</td>
<td></td>
<td>3.74</td>
<td></td>
<td>434</td>
<td></td>
<td>550</td>
<td></td>
<td>-</td>
</tr>
<tr>
<td>7. m-anisidine</td>
<td>0.025</td>
<td>1.57</td>
<td>0.84</td>
<td>0.36</td>
<td>11</td>
<td>68</td>
<td></td>
<td>1.32</td>
<td></td>
<td>1050</td>
<td></td>
<td>1087</td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

Table 5.2
Figure 5.8  Typical Proton High-Resolution
Spectrum of an Inhibitor of Trypsin

Proton NMR spectrum of p-methoxyphenylguanidine (0.025 M)
in 0.1 M Borate buffer, PH 7.65, 0.02 M CaCl₂, spectral width
400 Hz; Acquisition time = 5 sec. 10 points/Hertz.
Spectrum represents the Fourier transform of the sum of 4
transients; field-frequency lock based on D₂O. [no trypsin present]
Insert: The Methyl group of the inhibitor and the internal
standard (tertiary butyl alcohol) are shown on a
20X - expanded horizontal scale.
Proton NMR spectrum of p-methoxyphenylguanidine (0.025 M) in the presence of $1.26 \times 10^{-3}$ M SL TRYPSIN. The buffer is the same as in Figure 5.8; spectral width is 400 Hz; acquisition time 5 sec, 10 points/Hertz; 25 transients, D$_2$O lock.

Insert: The horizontal scale for the spectrum of the methyl groups of the inhibitor and of the internal standard (tertiary butyl alcohol) are expanded 10 and 20 times, respectively.
compound 2 should have no difficulty in finding its way into the specific binding pocket. Now compound 3 (p-methoxybenzylamine) is also a competitive inhibitor (shown by U.V. in Chapter III), and is about 2Å longer than benzamidine, so whether 3 can bind to the specific binding pocket or not will depend on the disposition of the end methyl group of the spin label relative to methoxy group at the para position of Compound 3. Although some steric hindrance may be present, but still binding of 3 to the specific pocket can not be ruled out completely. Due to their longer length and bent shape, compounds 4 and 5 (P- and m-methoxylphenyl guanidine.Hcl) are unlikely be bound in the specific binding pocket. Compounds 6 and 7 (p- and m-anisidine) are neutral, and thus their chance to be bound in specific pocket will be slim.

Suppose the piperidinyl group is pointed towards the specific binding pocket and sits snugly in the pocket. Then none of the inhibitors studied here will be able to bind in the specific binding pocket. Hence the observed line broadening of compound 2 and 3 could only be explained by supposing that both of them can also bind to a secondary site. This is possible because compounds 2 and 3 are not that different in size from compounds 4 and 5; if the secondary sites are in an exposed region of the enzyme surface, then the requirement of steric specificity will not be so severe as that of the specific binding pocket. The reason why compounds 2 and 3 did not show any similar behavior in the U.V. studies (Chapter III) would then be that compounds 2 and 3 must be bound at the secondary site in such a way that the access of substrate
(D.L. BAPA) to the specific binding pocket is prevented. Whereas the binding of compounds 4, 5, 6 and 7 at the secondary site acts to facilitate the binding of D.L-BAPA (Chapter III), perhaps this was due to the extra range of hydrophobic environment furnished by these bound inhibitors.

From the U.V. results, the distances measured for the inhibitors might be expected to be classified into three kinds, competitive, noncompetitive (charged and neutral). The surprisingly similar inhibitor-spin label distances (all around 10 Å) found here highly suggest that there is only one secondary binding site.

Since the E.S.R. spectrum of the S.L. Trypsin did not change in the PH range of 3 to 8 while at the same time the activity of trypsin varies by many orders of magnitude, no major conformational change at the active site seems likely.

The substrate activation and the facilitated binding behavior shown by some of the inhibitors (Chapter III) can all be explained by having secondary binding site close to the specific binding pocket. Extra evidence to support this model is that thionine with a large size, is also a competitive inhibitor. It may well be bound to the secondary site and at the same time act to block the entrance of the primary site, thus showing apparent competition inhibition behavior.

Further distance information can be found in table 5.3. The sum of the length of the inhibitor (on the longer axis) and the distances estimated here are all given around 20.5 ± 0.4 Å for the charged inhibitors, and 19.1 ± 1.1 Å for the neutral inhibitors.
Table 5.3  Length of inhibitor molecules and the possible location of secondary binding sites on trypsin.

Shown in the Table are: the molecular length of each inhibitor (L); the distance (r) between the spin-label unpaired electron and the inhibitor-CH$_3$ or -OCH$_3$ group, estimated from spin label-induced nuclear relaxation; and the sum of these two terms. See text for discussion.
<table>
<thead>
<tr>
<th>Compound</th>
<th>Name</th>
<th>Structure</th>
<th>L(Å)</th>
<th>r(Å)</th>
<th>L + r(Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>acetamidine</td>
<td><img src="image1" alt="Structure" /></td>
<td>5.8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>3-methoxypropylamine</td>
<td><img src="image2" alt="Structure" /></td>
<td>9.5</td>
<td>10.7</td>
<td>20.2</td>
</tr>
<tr>
<td>3</td>
<td>p-methoxybenzylamine</td>
<td><img src="image3" alt="Structure" /></td>
<td>11.4</td>
<td>9.3</td>
<td>20.7</td>
</tr>
<tr>
<td>4</td>
<td>p-methoxyphenylguanidine</td>
<td><img src="image4" alt="Structure" /></td>
<td>11.8</td>
<td>9.1</td>
<td>20.9</td>
</tr>
<tr>
<td>5</td>
<td>m-methoxyphenylguanidine</td>
<td><img src="image5" alt="Structure" /></td>
<td>10.8</td>
<td>9.8</td>
<td>20.6</td>
</tr>
<tr>
<td>6</td>
<td>p-anisidine</td>
<td><img src="image6" alt="Structure" /></td>
<td>10.4</td>
<td>9.8</td>
<td>20.2</td>
</tr>
<tr>
<td>7</td>
<td>m-anisidine</td>
<td><img src="image7" alt="Structure" /></td>
<td>9.6</td>
<td>8.4</td>
<td>18.0</td>
</tr>
</tbody>
</table>

*Note: Values marked with an asterisk (*) are estimated.*
For longer inhibitors, the distance between the methyl group of the inhibitor and the unpaired electron of the spin label is shorter, while the converse situation applies to the shorter ones. It seems highly unlikely that there could be more than one secondary site at around 10Å from the spin label. From the two-published color stereo diagrams on DIP-tryps in, it is rather difficult to speculate as to the location the secondary binding site. With the real three dimensional structure, one should be able to narrow the possibilities and locate the secondary site.

The discrepancy of the binding constants (discussed in Chapter 4) measured by U.V. and by NMR should not pose too much trouble. Even if the binding constant difference between these two methods were about one order of magnitude, the NMR estimated distance $(r)$ would only change by about 2Å. For example, for m-anisidine, if one uses the binding constant obtained from NMR concentration data, the distance turns out to be 11.1Å, thus bring the only anomalous result back into the pattern: i.e., the sum of the length of this inhibitor and the estimates distances for all the inhibitors will now come out to be about $20.5 \pm 4Å$.

2. **Conformational changes examined by ESR**

From the control experiment, the section on preparation of SL-trypsin, an active trypsin-free, spin-labeled trypsin has been prepared (figure 5.5). This SL-trypsin will be hydrolized by adding commercial trypsin; (Fig 5.6) also some of it will be denatured during the process of lyopholization (Fig. 5.4). But the
denatured SL-trypsin will not constitute more than a few per cent of the total protein. The ESR spectra of SL-trypsin, after incubates 30 - 45 minutes in the NMR probe at 40°C, was not changed as shown by comparison to the ESR spectra of the lyopholized SL-trypsin (Figure 5.4). This is another good indication of lack of autoproteolysis.

The fact that Ca$^{++}$ can enhance the efficiency of the catalysis of trypsin towards certain substrates has been suggested to arise from conformational changes at the active site. Thus by comparing the ESR spectrum of the labeled trypsin in the presence and the absence of Ca$^{++}$ or Mu$^{++}$, one might hope to detect such divalent metal ion induced conformational changes.

Also the substrate activation reported by LasKowski and the interaction among the inhibitors observed by our U.V. steady state kinetic studies in Chapter 3. All suggest the existence of a secondary binding site. This was proved by the NMR line width studies in this chapter. The distances between the nitroxide free radical and the inhibitors bound to the secondary site was estimated to be around 10Å. Now if any changes had appeared in the E.S.R spectrum of the spin-labeled enzyme upon adding the inhibitor or substrate, one would have additional insight into the effect of inhibitor bound at the secondary site on the conformation on at the active site.

Unfortunately a careful examination of the spectra (Figure 5.7) showed no detectable changes upon any of the above-listed perturbations. The only conclusion to be drawn here is that the
spin label ESR line shape is insensitive to the small conformational changes or weak interactions with bound inhibitors.

F. Summary

The active trypsin-free, S.L. trypsin was prepared successfully. The fast exchange limit for the spin label experiment was re-examined and discussed. Through the induced line broadening of the Methyl group of the bound inhibitors, the distance between the methyl group of the inhibitors at the secondary site and the unpaired electron of the spin-label at serine 195 was estimated to be around 10Å. Its implications was discussed with the help of 3 dimensional structure of DIP- and BA-trypsin. E.S.R spectrum of the S.L. trypsin with different perturbations was studied, and found that minor conformational changes will not produce an observable change in the ESR spectrum.
References


5. I.C.P. Smith, Biological Application of electron spin resonance spectroscopy, Wiley interscience N.Y.


12. T. Inagami and S.S. York, Biochem. 7, 4045 (1968)


29. R.M. Strond, (Specific binding of trypsin), private Communication.


Appendix A. The Longitudinal Relaxation Time $T_1$ for a Three Site System.

The longitudinal relaxation time $T_1$ for a three site system can be derived as follows

\[
\begin{align*}
A + M & \xrightarrow{k_1/k_1} B \\
B + E & \xrightarrow{k_2/k_2} C \\
A + ME & \xrightarrow{k_3/k_3} C
\end{align*}
\]

For this system, McConnell's equations may be written,

\[
\begin{align*}
\frac{dM}{dt} &= -\left(\frac{M - M_0}{T_1}A\right) - (k_1[M] + k_3[ME])M_Z^A + k_1M_Z^B + k_3M_Z^C \\
\frac{dM}{dt} &= -\left(\frac{M - M_0}{T_1}B\right) + k_1[M]M_Z^A - (k_1 + k_2[E])M_Z^B + k_2M_Z^C \\
\frac{dM}{dt} &= -\left(\frac{M - M_0}{T_1}C\right) + k_3[ME]M_Z^A + k_2[E]M_Z^B - (k_2 + k_3)M_Z^C
\end{align*}
\]
From the concentration conditions that \([A] \gg [B] \) or \([C] \) it follows that \(M^A_Z \gg M^B_Z\) or \(M^C_Z\). Then

\[
\frac{dM^B_Z}{dt} = \frac{dM^C_Z}{dt} = 0 \quad (A.4)
\]

For brevity, let

\[
M^A_Z = A, \quad M^B_Z = B, \quad M^C_Z = C, \quad M^A_0 = A_0, \quad M^B_0 = B_0, \quad M^C_0 = C_0
\]

two simultaneous equations result:

\[
- \left( \frac{B_Z - B_0}{T_1} \right) - (k_{-1} + k_2[E])B_Z + k_1[M]A_Z + k_{-2}C_Z = 0 \quad (A.5)
\]

\[
- \left( \frac{C_Z - C_0}{T_1} \right) - (k_{-2} + k_{-3})C_Z + k_3[ME]A_Z + k_2[E]B_Z = 0 \quad (A.6)
\]

\(B_Z\) and \(C_Z\) can then be solved in terms of \(A_Z\) and substituted into the first equation of \((A.3)\).

A first order differential equation can then be obtained,

\[
\frac{dA_Z}{dt} = \left\{ - \frac{1}{T_1}A - k_1[M] - k_3[ME] + \frac{k_{-1}}{F} \left( \frac{k_1[M]}{C} + k_{-2}k_3[ME] \right) \\
+ \frac{k_{-3}}{k_{-2}} \left( \frac{1}{T_B} \cdot \frac{1}{T_C} \cdot \frac{1}{F} \cdot k_1[M] - k_1[M] + \frac{k_{-2}k_3[ME]}{T_B \cdot F} \right) \right\} A_Z \\
+ \left\{ \frac{A_0}{T_1} + \frac{k_{-1}}{F} \left( \frac{f}{T_B} + \frac{f'k_{-2}}{T_C} \right) \\
+ \frac{k_{-3}}{k_{-2}} \left( \frac{f}{T_B} \frac{T_C}{T_1} - \frac{f}{T_B} + \frac{f'k_{-2}}{T_1} \right) \right\} A_0 \quad (A.7)
\]

where
\[ \frac{1}{k_B} = \left( \frac{1}{T_1} + k_{-1} + k_2 \right) \]

\[ \frac{1}{k_C} = \left( \frac{1}{T_1} + k_{-2} + k_{-3} \right) \]

\[ B_0 = fA_0, \quad C_0 = f' \]

and

\[ F = \frac{1}{k_Bk_C} - k_{-2}k_2 \]

\[ (A.8) \]

For the first order differential equation

\[ \frac{dA}{dt} = -pA + Q \]

\[ (A.9) \]

the solution is

\[ A = (\text{const})e^{pt} + \frac{Q}{p} \]

\[ (A.10) \]

Since \( T_1 \) is the time constant for the relaxation along the \( z \) axis,

\[ A = (\text{const})e^{-t/T_1} + \frac{Q}{p} \]

\[ (A.11) \]

and

\[ p = -\frac{1}{T_1} \]

equations (A.12) can then be obtained:

\[ \frac{1}{T_1} = \frac{1}{T_A} + \left[ 1 - \frac{\frac{1}{T_1} + k_{-2} + k_{-3}}{\left( \frac{1}{T_B} + k_{-1} + k_2 \right) \left( \frac{1}{T_C} + k_{-2} + k_{-3} \right) - k_{-2}k_2} \right] \]
This general solution for three site system can be reduced to three simple cases.

Case 1. only A is present

\[
\frac{1}{T_1} = \frac{1}{T_1^A}
\]  
(A.13)

Case 2. only A and B (or A and C) are present, then

\[
\frac{1}{T_1} = \frac{1}{T_1^A} + \frac{k_1[M]}{1 + k_{-1}T_1^B}
\]  
(A.14)

with

\[
k_{-1} = \frac{1}{\tau_B}, \quad k_1[M] = \frac{[B]}{[A]} = f_B
\]

Thus

\[
\frac{1}{T_1} = \frac{1}{T_1^A} + \frac{f_B}{T_1^B + \tau_B}
\]  
(A.15)
Case 3. the exchange rates between B and C are extremely slow, i.e. $k_2 = k_{-2} = 0$

$$\frac{1}{T_1} = \frac{1}{T_1^A} + \left( \frac{1}{1 + T_1 B k_{-1}} \right) k_1 [M] + \left( \frac{1}{1 + T_1 C k_{-3}} \right) k_3 [M E]$$

(A.16)

With (A.16) and $k_{-3} = \frac{1}{\tau_C}$, $\frac{k_3}{k_{-3}} = \frac{[C]}{[A]} = f_C$, then result the equation (2.7)

$$\frac{1}{T_1} = \frac{1}{T_1^A} + \frac{f_B}{T_1 B + \tau_B} + \frac{f_C}{T_1 C + \tau_C}$$

(A.17)
Appendix B. Comments on fast exchange limit for spin label enhancement of nuclear relaxation.

The inequality (7) employed in Wien's work$^1$ on spin-labeled lysozyme for the condition of fast exchange is not generally true:

$$\frac{1}{\tau} \gg \frac{f_A}{T_{2A}}, \quad \frac{f_M}{T_{2M}}$$

Algebraically, one can not get to equation (9) of ref. 1 by utilizing the two inequalities (7) and (8) in that paper.

In order to obtain the equation (9) in their work, the necessary and sufficient condition is$^2$

$$\frac{\tau_M}{T_{2M}}, \quad \frac{\tau_A}{T_{2A}} \gg 1 \quad \text{and} \quad \frac{1}{\tau_M} \gg \Delta w_m$$

for the first exchange limit to be true.

A control experiment$^3$ at different temperature for each of the inhibitors studied as we did in chapter 4 should be able to resolve which process dominates the NMR line broadening.

Even in some favorable cases, inequalities (7) and (8) of ref. 1 could lead to a fast exchange limit. But for the inhibitors NAG, di-NAG of lysozyme the temperature studied$^4$ showed that the chemical exchange dominated the transverse relaxation time i.e. in the slow exchange limit

$$\frac{1}{T_{2A}}, \quad \frac{1}{T_{2M}} \gg \frac{1}{\tau_A}, \quad \frac{1}{\tau_M}$$
or \[ \tau_A > \tau_{2A}, \quad \tau_M > \tau_{2M}. \]

With spin-labeled lysozyme, the terms \[ \frac{1}{\tau_{2A}}, \quad \frac{1}{\tau_{2M}} \] can only be made larger by the dipolar interaction and contact interaction in the presence of free radicals in the solution.

If one assumes as those authors did, that the association constant (as well as off rate of each inhibitor) are the same for spin-labeled lysozyme and ordinary lysozyme, then fast exchange limit cannot result from an increased \[ \frac{1}{\tau_{2M}} \] in going from ordinary lysozyme to spin-labeled lysozyme. Thus the line broadening for SL-lysozyme in ref. 1 is due mainly to the process of chemical exchange, rather than the "bound" \[ \tau_2 \], in contrast to the claim of ref. 1.

For slow exchange, \[ \tau_M > > \tau_{2M} \], the distance estimated from the line broadening should provide a crude higher limit to the corresponding distance rather than the crude lower limit as the author claimed.

REFERENCES

1. R.W. Wien, J.D. Morrisett, and H.M. McConnell, \_1\_3707 (1972)