CRYOENZYMOLICAL AND $^{13}$C-NMR STUDIES OF PAPAIN

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A Thesis Presented for the Degree of Doctor of Philosophy
University of Edinburgh
1983
This thesis is the original composition of the author's work, unless stated otherwise, and has not been submitted for any other degree. Certain results included in this thesis have already been published.
Acknowledgments

I would especially like to thank Dr. Paul Malthouse, Professor A. I. Scott, and Dr. R. L. Baxter for their help and guidance during the course of this work.

I would also like to thank Dr. N. E. Mackenzie and Mr. W. U. Primrose for the synthesis of substrates and inhibitors, Dr. A. S. F. Boyd for his NMR expertise, Dr. R. P. Ambler for the amino acid analyses, and Dr. K. Brocklehurst and Mr. E. Salih for the stopped-flow analysis.
Abstract

The feasibility of using $^{13}$C-NMR for the elucidation of the mechanism of enzyme-catalysed reactions has been evaluated. The main limitation of applying $^{13}$C-NMR to the study of enzyme-bound species is its low sensitivity which requires that the enzyme-bound species be present for long periods of time (0.5 to 24 h) at a concentration of ~1 mM. This presents no problem for stable inhibitor complexes but is a major problem for the detection of enzyme-substrate intermediates since for efficient catalysis intermediates should not accumulate and turnover should be rapid. To help overcome these problems, cryoenzymological techniques in combination with $^{13}$C-NMR have been used to slow down the enzyme-catalysed reactions at low temperatures. $^{13}$C-NMR evidence is presented for an acyl-papain formed in the reaction of N-benzoylimidazole with papain under cryoenzymological conditions. A distinct resonance, typical of a thioester, was detected. This resonance would not be detectable at room temperature.

The structure of a papain-aldehyde inhibitor complex was examined by $^{13}$C-NMR. Two resonances due to tetrahedral hemithioacetals were detected. These were assigned to the two possible diastereoisomeric hemithioacetals which can be formed by attack of the papain thiolate ion on the aldehyde carbonyl. The detection of these two hemithioacetal resonances shows that hemithioacetal formation is not stereospecific. This also demonstrates the potential of $^{13}$C-NMR for determining the stereochemistry of enzyme adducts.

The irreversible inhibition of papain by CMK substrate analogues was studied by $^{13}$C-NMR and an unusually low-field resonance was observed for the inhibitor-enzyme complex. This resonance, which is typical of cyclic ketones, may be due to enzyme distortion and/or cyclization of the CMK bound to the enzyme.
The cryoenzymological investigations were extended to specific substrates. None of the ester substrates examined were found to be amenable to a cryoenzymological-$^{13}$C-NMR investigation. Even at temperatures down to -50°C, the reactions in DMSO-water cryosolvent were too rapid for detection by $^{13}$C-NMR. Lower temperatures slowed the reactions but also caused a dramatic increase in the viscosity of the solutions which precluded further investigations by both UV and NMR at low temperatures.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Abbreviations</td>
<td>vi - vii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1 - 61</td>
</tr>
<tr>
<td>Acyl Intermediate</td>
<td>5</td>
</tr>
<tr>
<td>Tetrahedral Intermediate</td>
<td>17</td>
</tr>
<tr>
<td>Additional Intermediates</td>
<td>24</td>
</tr>
<tr>
<td>pH Dependence</td>
<td>26</td>
</tr>
<tr>
<td>Cryoenzymology</td>
<td>28</td>
</tr>
<tr>
<td>$^{13}$C-NMR Spectroscopy</td>
<td>46</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>62 - 88</td>
</tr>
<tr>
<td>Instrumentation</td>
<td>63</td>
</tr>
<tr>
<td>Materials</td>
<td>66</td>
</tr>
<tr>
<td>Methods</td>
<td>71</td>
</tr>
<tr>
<td>Results and Discussion</td>
<td>89 - 214</td>
</tr>
<tr>
<td>Purification of Papain</td>
<td>90</td>
</tr>
<tr>
<td>Papain and N-Benzoylimidazole</td>
<td>94</td>
</tr>
<tr>
<td>Papain and N-Acetyl-L-phenylalanylglycinai</td>
<td>132</td>
</tr>
<tr>
<td>Papain and Nα-CBZ-L-lysine-chloromethyl ketone</td>
<td>161</td>
</tr>
<tr>
<td>Absorption Spectra in Cryosolvent at Low Temperature</td>
<td>173</td>
</tr>
<tr>
<td>Papain and Nα-CBZ-L-lysine-p-nitrophenyl ester</td>
<td>184</td>
</tr>
<tr>
<td>Other Ester Substrates:</td>
<td>210</td>
</tr>
<tr>
<td>References</td>
<td>215 - 230</td>
</tr>
<tr>
<td>Publication</td>
<td>231</td>
</tr>
<tr>
<td>List of lectures and seminars</td>
<td>235</td>
</tr>
</tbody>
</table>
**List of Abbreviations**

- $A_0$ - absorbance at time = 0
- $A_\infty$ - absorbance at the completion of the reaction
- $A_x$ - absorbance at a particular wavelength 'x'
- $\text{Ac}$ - acetyl
- BAA - $\text{Na-}^\alpha\text{-benzoyl-argininamide}$
- BAEE - $\text{Na-}^\alpha\text{-benzoyl-arginine ethyl ester}$
- BAPNA - $\text{Na-}^\alpha\text{-benzoyl-arginine p-nitroanilide}$
- CBZ - carbobenzyloxy-, $\text{Ph-CH}_2\text{-O}$
- CD - circular dichroism
- CMK - chloromethyl ketone
- CSA - chemical shift anisotropy
- $\delta$ - chemical shift parameter (in ppm)
- DMF - N, N-dimethylformamide
- DMSO - dimethylsulphoxide
- DTT - dithiothreitol
- EDTA - ethylenediamine tetraacetic acid
- $\varepsilon_x$ - molar extinction coefficient at a particular wavelength 'x'
- $\Delta\varepsilon$ - difference molar extinction coefficient
- FID - free-induction decay
- $\nu$ - gyromagnetic ratio
- I - nuclear spin quantum number
- Im - imidazole (or ImH$^+$ for imidazolium)
- $J_{CH}$ - coupling constant between $^{13}$C and $^1$H
- $\lambda$ - wavelength
- LW - linewidth
- NMR - nuclear magnetic resonance
- NOE - nuclear Overhauser effect
- $pH^*$ - pH in aqueous-organic solvent
- pNP - p-nitrophenol
r.f. - radiofrequency
RNA - ribonucleic acid
s. d. - standard deviation
SH - thiol group (or $S^-$ for thiolate ion)
S/N - signal-to-noise ratio
STI - soybean trypsin inhibitor
$T_1$ - spin-lattice relaxation time
$T_2$ - spin-spin or transverse relaxation time
THI - tetrahedral intermediate
TMS - tetramethyl silane
$\tau_R$ - rotational correlation time
Tris - tris(hydroxymethyl)aminomethane
INTRODUCTION
Introduction

Papain (EC 3.4.22.2), one of the proteolytic enzymes from the tropical fruit tree "carica papaya L", has undergone extensive study since first isolated. Papaya latex contains many proteolytic and other enzymes among which are the chymopapains, papaya peptidases, and lysozyme. Balls et al. (1937), and Balls and Lineweaver (1939), developed procedures for isolating crystalline papain from fresh papaya latex. Later this procedure was modified for dried papaya latex by Kimmel and Smith (1954) and both this method, and a similar procedure by Arnon (1970), have been widely used for the isolation of the enzyme. Recently, Baines and Brocklehurst (1979) have further modified the procedure to produce chymopapain-free papain from spray dried latex. Using this procedure, 80% active papain contaminated only by inactive and unactivatable papain can be prepared.

Further purification can be achieved using various affinity chromatography methods. Sluyterman and Wijdenes (1970) used a column of p-aminophenylmercuric acetate bound to sepharose. Proteins containing thiol groups are separated from other proteins and stored as the mercuri-derivative. Blumberg et al. (1970), and Burke et al. (1974), use a glycy1-glycyl-(O-benzyl)-L-tyrosyl-L-arginine tetrapeptide inhibitor attached to sepharose. Brocklehurst et al. (1973, 1974), and Stuchbury et al. (1975), have modified sepharose with a mixed disulphide moiety which reacts with the thiol group on the enzyme by thiol-disulphide exchange and releases 2-thiopyridone which allows the reaction to be monitored. At low pH, only the papain thiolate will be highly reactive and other thiol containing molecules can be separated. Papain containing one catalytic site per molecule of protein can be isolated.
Papain consists of a single polypeptide chain of 212 amino acid residues (MW = 23406) whose sequence has been determined (Husain and Lowe, 1969, 1970; Mitchell et al., 1970). There are seven cysteine residues present six of which form internal disulphide linkages and the seventh residue (Cys-25) is an essential part of the active site.

The three dimensional structure of papain has been determined by x-ray crystallography (Drenth et al., 1967, 1968, 1970, 1971; Glazer and Smith, 1971). The papain molecule is ellipsoidal with rough dimensions 50x37x37 Å. The molecule is binuclear, composed of two lobes each containing a hydrophobic core with hydrophilic residues at the surface. The lobes are connected by three sections of the peptide chain which crosses from one lobe to the other at residues 11, 111, and 208. Hydrogen bonding, electrostatic, and hydrophobic interactions at the edge of the lobes maintain the position of one lobe relative to the other. Along the cleft between the lobes lies the active site region of the enzyme.

The active site is approximately 20 Å long and contains Cys-25, His-159, and Asp-158 charged residues. The N-1 of His-159 is 3.4 Å away from the sulphur of Cys-25 on opposite sides of the cleft as shown by x-ray crystallography. This distance is the van der Waals or hydrogen bonding distance. Husain and Lowe (1968) show that this close proximity is maintained in the solution by connecting the residues through the reaction of 1, 3-dibromoacetone. Further analysis of the crystal structure shows the side chain carboxyl of Asp-158 is 6.7 Å away from the imidazole of His-159. His-159 is hydrogen bonded to the side chain of Asn-175 and in the hydrophobic environment of Trp-177. The fluorescence spectrum of papain is dominated by the contribution from the Trp-177 residue (Steiner, 1971; Lowe and Whitworth, 1974).

Arguments supporting the relevance of the crystal structure to the enzyme in solution are presented by Sluyterman and De Graaf (1969). Crystals from 66% methanol-water solutions were cross-linked
and placed water. The methanol is exchanged out and the thin crystals showed the same catalytic activity as the enzyme in solution.

The active site of papain has been "mapped" by studying the interaction of substrates and inhibitors of increasing length and varying stereochemistry (Berger and Schechter, 1970; Schechter and Berger, 1967, 1968). Using diastereoisomeric peptides of alanine from Ala$_2$ to Ala$_6$, Schechter and Berger (1967) concluded that papain has an active site approximately 25 Å long containing seven subsites capable of accommodating a single amino acid residue of the substrate (Scheme 1). The subsites are located on both sides of the catalytic site; four toward the amino end of the substrate and three toward the carboxyl end.

Scheme 1. Representation of the active site region of papain with subsites located on both sides of the catalytic site ($\uparrow$). Also shown are the corresponding sites on the substrate

Schechter and Berger (1968) showed that peptides with phenylalanine third or further from the C-terminal were hydrolysed particularly well and that the S$_2$ subsite on the enzyme shows a strong preference for phenylalanine residues. This defined a specificity represented -Phe-X-$\downarrow$Y- where the bond of the residue next to Phe is hydrolysed. N-Benzoyl, and N-benzoyloxycarbonyl residues are also effective at promoting cleavage by papain at the peptide bond of the adjacent amino acid residue
(Bergmann et al., 1935, 1936). The structural similarity of these groups to phenylalanine is responsible (Lowe, 1970).

The S_1 subsite cannot be occupied by valine, possibly due to its branched side chain (Berger and Schechter, 1970), but substrates with arginine and lysine residues in S_1 prove to be good substrates (Kimmel and Smith, 1957; Lowe, 1970; Williams et al., 1972b).

There is also evidence that binding of the leaving group in S_1' is an important factor in determining enzyme specificity. The S_1' subsite is stereospecific for L-amino acid residues (Berger and Schechter, 1970), and shows a preference for hydrophobic residues, particularly isoleucine and tryptophan (Alecio et al., 1974). Although the concept of specific substrate-subsite interactions discussed above implies a rigid active-site, there is also evidence of some conformational freedom of both the substrate and the enzyme residues forming the active-site. This will be discussed in a following section.

The mechanism of papain-catalysed hydrolysis has been the subject of extensive investigations. Results consistent with proposed acyl and tetrahedral intermediates have been presented along with suggestions emphasizing the importance of conformational changes to the catalytic mechanism. Unambiguous detection of productive intermediates along the catalytic pathway is difficult. The following sections contain a brief review of some of the earlier work concerned with the elucidation of the mechanistic pathway of papain-catalysed hydrolysens.

Acyl Intermediate

The formation of a thioacyl intermediate in the papain-catalysed hydrolysis of peptides was proposed after some of the earliest investigations into the reaction pathway (Weiss, 1937; Smith et al., 1955a; Stockell and Smith, 1957). Smith and
co-workers (Smith and Parker, 1958; Stockell and Smith, 1957; Smith et al., 1955a) determined the kinetic constants for a number of N-acylamino acid esters and amides as a function of temperature and pH and analysed the data according to the simple Michaelis-Menten scheme (1).

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_{\text{cat}}} E + P
\]  
\[
K_m = \frac{k_{-1} + k_{\text{cat}}}{k_1}
\]  

Based on this work, Stockell and Smith (1957) concluded that \(k_{\text{cat}}\) represents a combination of rate constants meaning that a more complex mechanism than that implied by the simple Michaelis-Menten scheme (1) is involved. The rate constants for the papain-catalysed hydrolysis of N-\(\alpha\)-benzoyl-L-argininamide (BAA) (Stockell and Smith, 1957) and the corresponding ethyl ester (BAEE) (Smith and Parker, 1958) were found to be similar and it was suggested that this was due to the rate-limiting deacylation of a common acyl enzyme (Smith, 1958).

After a re-investigation into the papain-catalysed hydrolysis of BAA and BAEE, Whitaker and Bender (1965) also presented a three step mechanism. They observed that the two substrates possessed identical \(k_{\text{cat}}/K_m\) - pH profiles but different \(k_{\text{cat}}\) - pH profiles. This is not consistent with the two step catalytic process of the Michaelis-Menten model (1), and a more complex scheme was proposed. The three step mechanism is the minimal kinetic scheme for the papain-catalysed hydrolysis of esters and amides:

\[
E + S \xrightarrow{k_1} ES \xrightarrow{k_2} EA + P_1 \xrightarrow{k_3} E + P_2
\]
EA represents the acyl-enzyme and ES the Michaelis-complex. The rate constants in scheme (3) can be related to the Michaelis-Menten constants in scheme (1) by the following.

\[
K_{m(app)} = \frac{k_3(k_1 + k_2)}{k_1(k_2 + k_3)}
\]  

(4)

\[
k_{cat} = \frac{k_2 k_3}{k_2 + k_3}
\]  

(5)

Whitaker and Bender (1965) determined the individual rate constants and found that deacylation \(k_3\) was rate determining for the ester while acylation \(k_2\) was rate determining for the amide. This result was questioned by Sluyterman (1968) in his studies on the inactivation of papain with chloroacetic acid in the presence of BAEE. These conflicting results have been extensively studied (Brocklehurst et al., 1968; Whitaker, 1969; Allen et al., 1978; Yuthavong and Suttimool, 1978) with the general conclusion that the papain-catalysed hydrolysis of BAEE is influenced significantly by both the acylation and deacylation steps. For this review it is of primary interest that in all of these studies the kinetics are interpreted with respect to a mechanism containing an acyl enzyme intermediate. Deacylation is found to be the only rate determining step with esters containing good leaving groups such as p-nitrophenol (Yuthavong and Suttimool, 1978).

Similar \(k_{cat}\) values have been observed for the papain-catalysed hydrolysis of esters of amino acids with varying leaving groups. If these different substrates generate the same acyl-intermediate whose breakdown is rate determining, then they all should have the same catalytic activity, \(k_{cat}\). Kirsch and Ingelström (1966) observed such similarities in the \(k_{cat}\) values for the \(o-, m-, p\)-nitrophenyl and ethyl esters of Na-CBZ-glycine in contrast to the rate constants for alkaline hydrolysis of these esters which
varies 100-fold. Similar results were obtained by Lowe and Williams (1965b) with aryl and alkyl esters of hippuric acid and Lucas and Williams (1969) using a variety of esters. Bender and Brubacher (1966) also proposed a common acyl enzyme after studying the papain-catalysed hydrolysis of p-nitrophenyl, benzyl, and methyl esters of Na-CBZ-L-lysine over a wide pH range. They also observed that \( k_{\text{cat}} \) which approximates to \( k_3 \) at pH values \( \geq 5 \) becomes influenced by \( k_2 \) at lower pH values.

If deacylation of the thioacyl enzyme is rate limiting, then \( k_2 \gg k_3 \), and from (5):

\[
k_{\text{cat}} = k_3
\]

And if \( k_{-1} \gg k_2 \) (Lucas and Williams, 1969) then equation (4) becomes:

\[
K_m = \frac{k_{-1} k_3}{k_1 k_2} = \frac{k_3}{K_s} \frac{k_1}{k_2}
\]

Conversely, if acylation is rate limiting, \( k_3 \gg k_2 \), and the following equations are valid:

\[
k_{\text{cat}} = k_2
\]

\[
K_m = \frac{k_{-1}}{k_1} = \frac{k_1}{K_s}
\]

For both cases:

\[
\frac{k_{\text{cat}}}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2}
\]

And if \( k_{-1} \gg k_2 \):

\[
\frac{k_{\text{cat}}}{K_m} = \frac{k_1 k_2}{k_{-1}} = \frac{k_2}{K_s}
\]
While the evidence presented is consistent with an acyl-intermediate mechanism it can also be interpreted using other schemes. Rate determining conformational changes, non-enzymatic substrate reactions, or rate determining release of product $P_2$ are possible alternative explanations of these results.

The effect of added amine or alcohol nucleophiles on papain-catalysed reactions has been used in the detection of intermediates as well as providing information on the nature of the rate determining step. For example, if the rate determining step in a papain-catalysed reaction is formation of an acyl enzyme, and if the nucleophile reacts with the acyl enzyme after this rate determining step, it will not increase the rate of reaction. If the rate limiting step is hydrolysis of the acyl enzyme, added nucleophiles would increase the breakdown of the acyl enzyme and therefore the overall rate of the reaction (Fersht, 1977). Since papain catalyses transesterification reactions (Glazer, 1966), then addition of an alcohol to a solution containing an acyl enzyme should increase the rate of deacylation. Partitioning of the acyl enzyme as expressed by the ratio of the rate of hydrolysis to the rate of alcoholysis should then be independent of the nature of the leaving group of the ester substrate. Henry and Kirsch (1967) demonstrated this for the papain-catalysed hydrolysis-transesterification of p-nitrophenyl and ethyl hippurate in 10% ethanol. The ratio of the second order rate constants $k_{\text{EtOH}} / k_{\text{H}_2\text{O}}$ was similar for both substrates. Hinkle and Kirsch (1971) took advantage of the fact that rates of deacylation are increased by added nucleophiles to change the rate determining step for the hydrolysis of several esters from deacylation to acylation. The initial rates of formation of product ($P_1$) increase as the concentration of added amine nucleophile increases. The rates finally reach a plateau level at high concentrations of added amine where the rate is independent of the nature of the nucleophile or its concentration. At
this point the rate constant for deacylation is greater than that for acylation. At high concentrations of nucleophile where acylation is rate determining, it was then possible to obtain values of $k_2$ and $K_s$ under steady-state conditions. Previously these constants were directly obtainable only by using stopped-flow methods. Similar experiments with added nucleophiles were used to determine the rate limiting steps in the BAEE hydrolysis studies mentioned earlier (Allen et al., 1978; Yuthavong and Suttimool, 1978). All of these experiments with added nucleophiles provide convincing indirect evidence for the existence of an acyl intermediate.

Experiments using C\(^{18}\)O labelled ethyl hippurate as a substrate for papain showed that hydrolysis occurred without \(^{18}\)O exchange (Kirsch and Katchalski, 1965). These results are consistent with the formation of an acyl enzyme but not a free tetrahedral intermediate. The results may also be explained by a sterically hindered enzyme-substrate complex which prevents exchange or a concerted process involving nucleophilic attack and protonation of the alkoxide leaving group.

The assumption that there is a rapid accumulation of an acyl-intermediate and a subsequent slow turnover rate has been used in active-site titrations of papain (Bender et al., 1966). If acylation is much faster than deacylation, a suitable substrate may react with the enzyme to form stoichiometric amounts of the acyl enzyme and product. If deacylation is negligible, a measure of the product formed ($P_1$ in equation (3)) is easily related to the enzyme concentration. More often deacylation is not negligible and formation of product as shown by equation (3) under steady-state conditions with $[S] >> [E]$ is described by equation (12) (Bender et al., 1966).
\[
[P_1] = \frac{k_{\text{cat}}[E]_o [S]_o}{[S]_o + K_m(\text{app})} t + [E]_o \left( \frac{k_2}{1 + \frac{K_m(\text{app})}{[S]_o}} \right)^2 x \\
\left( 1 - \exp \left( -\frac{(k_2 + k_3) [S]_o + k_3 K_s}{K_s + [S]_o} t \right) \right) 
\]

(12)

In practice, the absorbance of a suitable chromophore liberated as \( P_1 \) is measured as a function of time. Such a "burst" titration is represented below.

Under these conditions a pre-steady-state corresponding to acylation may first be observed followed by steady-state deacylation. The exponential portion of equation (12), describing acylation, may be too fast to observe, but as "t" increases this term approaches zero and the product concentration is described by a linear function. The linear portion can be extrapolated to zero time to obtain the magnitude of the burst, \( \pi \), which is related to the enzyme concentration.

\[
\pi = [E]_o \left( \frac{k_2}{k_2 + k_3} \right)^2 \left( 1 + \frac{K_m(\text{app})}{[S]_o} \right) 
\]

(13)
In order to observe a burst equal to the enzyme concentration, two conditions must be met; \( k_2 \gg k_3 \) and \([S] \gg K_m(\text{app})\). If either of these conditions is not met the magnitude of the burst, which represents the amount of acyl enzyme present, will be dramatically reduced due to the squared relationship in equation (13). Bender et al. (1966) observed an initial burst of p-nitrophenol in the papain-catalysed hydrolysis of Na-CBZ-L-tyrosine and Na-CBZ-L-tryptophan p-nitrophenyl esters. In the presence of 30% methanol the rate of turnover increased two-fold but no measurable effect on the burst was observed. This indicated that the term \( k_2/(k_2 + k_3) \) in equation (13) is close to unity and that \( k_2 \gg k_3 \). The observation of burst kinetics with papain presents further evidence for the acyl enzyme intermediate.

Stopped-flow techniques have also been used for the detection of burst kinetics with papain and Na-CBZ-L-lysine p-nitrophenyl ester (Holloway and Hardman, 1973). Under conditions where \([S]_0 \gg [E]_0\), a burst proportional to enzyme concentration is observed. With \([E]_0 \gg [S]_0\), unexpected spectral changes occurred. The authors concluded that although an acyl enzyme does exist, deacylation may not be the rate limiting reaction.

Direct evidence for a thioacyl intermediate in papain-catalysed hydrolyses has been presented by Lowe and Williams, (1964,1965a), Bender and Brubacher (1964), and Brubacher and Bender (1966) with the spectrophotometric observation of dithioacyl and thioacyl papains.

Lowe and Williams (1964,1965a) utilized a substrate that would exhibit a characteristic UV absorption distinguishable from that of the enzyme upon formation of an acyl enzyme. The papain- and ficin-catalysed hydrolysis mixtures of methyl thionohippurate displayed transient absorptions at 313 and 315 nm respectively. This absorption was assigned to the dithioester formed upon reaction of the thiol group of the enzyme with the thionohippurate (see scheme (14)).
Upon denaturation both absorption maxima shifted to 309 and 313 nm for papain and ficin respectively. The $\lambda_{\text{max}}$ values reported are close to that observed for ethyl dithioacetylacetate (305 nm) supporting the postulate of a dithioacyl intermediate.

A comparison of Michaelis-Menten parameters for methyl thionohippurate and methyl hippurate showed that the thionohippurate is a specific substrate for papain and ficin. The observed catalytic constant $k_{\text{cat}}$, which represents deacylation of the enzyme, differ by a factor of ten for the two substrates, but the ratio $k_{\text{cat}}/K_m$, which represents binding and acylation are similar. The experimental evidence presented by Lowe and Williams (1964, 1965a) supports the view that methyl thionohippurate reacts at the same site on the enzyme as does methyl hippurate.

Bender and Brubacher (1964), and Brubacher and Bender (1966) prepared trans-cinnamoyl-papain by reaction of an excess of the non-specific substrate trans-cinnamoylimidazole with papain at acid pH. After gel-filtration, an absorption maximum of 326 nm was observed and assigned to trans-cinnamoyl-papain although the model compound S-trans-cinnamoylcysteine shows a significantly different $\lambda_{\text{max}}$ of 306 nm. Only upon denaturation of the enzyme did the spectrum attributed to trans-cinnamoyl-papain shift to a $\lambda_{\text{max}}$ of 301 or 309 nm (in the presence or absence of the denaturing agent respectively). Deacylation proved to be first-order in acyl enzyme and equal to the rate of appearance of trans-cinnamic acid. When BAEE was added to a solution of trans-cinnamoyl-papain, activity towards the substrate reappears at
the same rate as the acyl enzyme deacylates. These studies support the conclusion that the non-specific substrate reacts at the same site which catalyses the hydrolysis of a specific substrate. Added amine and alcohol nucleophiles increased the rate of deacylation of the acyl enzyme. The 700-fold increase in reactivity of methyl amine to trans-cinnamoyl-papain as compared to trans-cinnamoyl-α-chymotrypsin is consistent with the formation of a thiolester as these have been shown to be more susceptible to attack by nitrogen nucleophiles than the corresponding esters (Bruice, 1961; Bruice and Benkovic, 1966).

Similar results were obtained by Hinkle and Kirsch (1970) with furylacryloyl- and indolylacryloyl-papains. Both acyl enzymes were prepared, isolated and extensively studied over a wide range of pH, temperature, and varying concentrations of organic cosolvents. The acyl enzymes are all reported to be characterized by red-shifted absorption maxima as compared to the corresponding S-acylcysteine model compounds and the denatured acyl enzymes. The pK_a for hydrolysis of furylacryloyl-papain was determined to be 4.63 as compared to 4.7 for trans-cinnamoyl-papain by Brubacher and Bender (1966). The effect of organic solvents on the rate of deacylation of the furylacryloyl-, indolylacryloyl, and trans-cinnamoyl-papains was much greater than that observed for the specific Na-CBZ-glycyl and Na-CBZ-L-lysyl papains. These results suggested an improvement in the orientation of the acyl group with respect to the enzyme upon addition of organic solvent which increases the rate of deacylation. The more reactive Na-CBZ-glycyl and Na-CBZ-L-lysyl papains presumably can assume an optimal orientation which is less subject to improvement by added solvents. The acyl papains prepared were also observed to be much more stable to variations in temperature and pH as compared to the native enzyme.

Zannis and Kirsch (1978) used the same methods to study the effects of substituents on the rates of deacylation of substituted
benzoyl-papains. At low pH a series of meta- and para-substituted benzoylimidazoles in excess were reacted with papain. The acyl papains isolated slowly recovered activity to Na-CBZ-glycine p-nitrophenyl ester with a rate constant equal to that determined by the decrease in absorbance of the acyl enzyme. The deacylation rates are greatly increased by electron withdrawing groups on the benzene ring. The rate of deacylation of benzoyl-papains are related to the pH of the solution by equation (15):

\[ k_3 = \frac{k_{a}^{\text{lim}}}{K_a + [H^+]} \]  

For N-benzoylimidazole \( k_3^{\text{lim}} = 0.271 \text{ min}^{-1} \) and \( pK_a = 4.3 \).

Carey et al. (1976, 1978) used resonance Raman spectroscopy in experiments designed to observe the acyl enzyme intermediate directly. Resonance Raman spectra attributed to furylacryloyl- and derivatives of cinnamoyl-papain were observed. Upon acylation an intense double-bond resonance Raman peak at an unusually low frequency appears. The unusual Raman spectra were at first suggested to be due to a side-chain conformational change upon acylation (Carey et al., 1976). Further investigation suggested that electron polarization through the \( \pi \) electron system present in the side-chains of each of the acyl moieties might be responsible (Carey et al., 1978). A major criticism of these experiments is that the resonance Raman features observed with these substrates and acyl enzyme intermediates are associated with vibrational resonances of bonds spatially removed from the bonds undergoing hydrolysis. This problem is overcome in later work by Carey and co-workers in similar experiments to those of Lowe and Williams (1964, 1965a). In these experiments, Storer et al. (1979) and Ozaki et al. (1982) prepared dithioacyl-papains from a series of thionoesters. The advantage of using dithioacyl
derivatives is that the dithioacyl group formed in the reaction is observable by resonance Raman techniques. Transient resonances associated with the C=S and C-S stretching frequencies of a dithioacyl-papain intermediate were observed. Ozaki et al., (1982) concluded that the vibrational properties of the dithioester centre is perturbed. This perturbation is suggested to arise from an intramolecular interaction between the N-acyl group and the dithioester:

\[
\begin{array}{c}
\text{HN} \\
\text{C}=\text{O} \\
\text{S} \\
\text{R}
\end{array}
\quad \begin{array}{c}
\text{CH}_2 \\
\text{C}=\text{S}
\end{array}
\]

They also suggested that this interaction is present in a majority of the dithioacyl-papain molecules but a small population of the acyl enzyme is in a conformation where this interaction is absent. The intramolecular interaction is believed to arise from dipole-dipole forces or amide \(\pi\) electrons interacting through space with sulphur \(\pi\) orbitals and does not involve simple hydrogen bonding or enethiol tautomerism. As in the studies of Lowe and Williams (1964,1965a) the results obtained with the dithioacyl-papains may or may not be relevant to the investigations concerning an actual acyl intermediate in the papain-catalysed hydrolysis of ester and amide substrates.

Fink and co-workers have applied cryoenzymological techniques to the study of papain-catalysed hydrolyses (see Cryoenzymology section). Fink and Angelides (1976) and Angelides and Fink (1978) studied the papain-catalysed hydrolysis of the methyl and p-nitrophenyl esters of Na-CBZ-L-lysine at low temperature and in aqueous-dimethylsulphoxide cryosolvents. Both reports claim direct observation and trapping of Na-CBZ-L-lysyl-papain. In addition, in the hydrolysis of the methyl ester,
evidence for the detection of two reactions preceding formation of the acyl enzyme are reported. (Fink and Angelides, 1978). A more extensive discussion of this work is presented in a following section.

There is an abundance of evidence in support of an acyl enzyme intermediate in papain catalysis. Most of the indirect evidence is very convincing but suffers an intrinsic limitation in that it is indirect. Direct evidence, such as observation of the dithioacyl intermediate by both UV and resonance Raman spectroscopy is also important, but thionohippurates are chemically distinct from amide and ester substrates. Direct UV observation of acyl enzymes from specific or non-specific substrates unfortunately provides little information regarding the specific atomic and/or molecular interactions of particular interest at the active site. The fact that there is a red shift of the absorption maxima of the acyl enzymes as compared to the models which is not fully explained indicates that UV is not a good diagnostic probe of thioester formation. The results do provide a wealth of information regarding the catalytic mechanism of papain-catalysed hydrolyses but also emphasize the need for further investigations.

Tetrahedral Intermediate

The reaction pathway (3) previously presented is a relatively simple summation. There is also evidence of other intermediates involved in the reaction pathway. Tetrahedral intermediates, THI's, have been proposed in the non-enzymatic hydrolysis of amides and esters (Bender, 1960). The possibility that the remarkable catalytic efficiency of papain in hydrolysing peptide bonds is a result of specific stabilization of a THI has received widespread interest. In the OH⁻ catalysed hydrolysis of aryl esters and anilides, formation of the THI is rate limiting for the aryl esters (Bunton and Spatcher, 1956) while breakdown is rate limiting for the anilides
(Bender and Thomas, 1961).

The search for evidence for the involvement of a THI in the hydrolytic mechanism of the serine proteases has been most extensive. Caplow and co-workers proposed a mechanism involving rate limiting breakdown of a THI which accumulates in a pre-equilibrium reaction prior to acyl enzyme formation in the chymotrypsin-catalysed hydrolysis of anilides, (Caplow, 1969; Lucas and Caplow, 1972) and hydrazides (Lucas et al., 1973). Fersht and Requena (1971), Fersht (1972), and Fastrez and Fersht (1973), also presented mechanisms involving THI's for the chymotrypsin-catalysed hydrolyses but in this case they conclude that the intermediate is present only in low steady-state concentration and is not detectable. They also suggest that breakdown of the THI is rate determining at high pH and formation is rate determining at low pH. This is consistent with the results of O'Leary and Kluetz (1972) who observed a change in the nitrogen isotope effect with changes in pH in the chymotrypsin-catalysed hydrolysis of Na-Ac-L-tryptophanamide.

Evidence for a tetrahedral adduct had been presented in x-ray diffraction studies of the crystalline complexes formed between trypsin and soybean trypsin inhibitor (Sweet et al., 1974) and pancreatic trypsin inhibitor (Ruhlmann et al., 1973). These inhibitors are thought to act like true substrates but do not completely hydrolyse due to conformational constraints. Later studies using refined x-ray (Huber et al., 1974; Huber and Bode, 1978) and $^{13}$C-NMR (Hunkapillar et al., 1979, Baillargeon et al., 1980; Tschesche and Wüthrich, 1980) techniques have shown no evidence for a covalent fully tetrahedral adduct. The $^{13}$C-NMR studies found no evidence for the significant upfield shift of the NMR resonance expected in changing from an sp$^2$ hybridized carbonyl carbon to the sp$^3$ hybridized carbon of a tetrahedral adduct. Such a shift was observed in the binding of a pepstatin inhibitor analogue to porcine pepsin (Rich et al., 1982).
Direct observation of accumulated THI's in the serine protease catalysed hydrolysis of specific substrates has also been claimed. Hunkapillar et al. (1976) followed the α-lytic protease and elastase catalysed hydrolysies of Na-Ac-L-Ala-L-Pro-L-Ala-p-nitroanilide by stopped-flow spectrophotometry and observed burst kinetics. This observation is attributed to the rapid formation of a THI and its subsequent rate limiting breakdown. Petkov (1978) obtained similar results with Na-Ac-L-lysine-p-nitroanilide and trypsin. Both observations of burst kinetics were based on the assumption that the THI has an absorption spectrum similar to p-nitroaniline (Robinson, 1970; Lowe and Yuthavong, 1971b; Fastrez and Fersht, 1973).

Cryoenzymological techniques have also been used in the study of the hydrolysis of specific anilide substrates by the serine proteases. Claims for detection and accumulation of a THI in the elastase (Fink and Meehan, 1979) and trypsin (Compton and Fink, 1980) catalysed hydrolysies of specific anilide substrates have been presented. Both reports showed evidence of burst kinetics purportedly due to the accumulation of a THI. These papers are discussed further in the Cryoenzymology section.

Markley et al. (1981) reinvestigated the claims for burst kinetics observed with the specific substrates just outlined above. In each case, the authors presented alternative results inconsistent with THI formation and accumulation and offered explanations for the observation of artefactual burst kinetics in the previous studies. For example, The non-linear kinetics reported by Hunkapillar et al. (1976) and Fink and Meehan with Na-Ac-L-Ala-L-Pro-L-Ala-p-nitroanilide can be explained by cis, trans heterogeneity about the Ala-Pro peptide bond. Markley et al. (1981) conclude that there is no spectroscopic evidence for the existence of a THI in amide hydrolysis by the serine proteases and reiterated the conclusions of Fastrez and Fersht (1973), that if a tetrahedral species is involved it is a transition state or an intermediate that
does not accumulate appreciably.

Detection of a THI with a thiol protease such as papain may be favoured over that of a serine protease due to the favourable equilibrium constants of thiolates for formation of tetrahedral addition intermediates. It has been observed (Bender and Ginger, 1955) that there is considerable carbonyl oxygen exchange in the alkaline hydrolysis of amides as compared to esters:

\[
R\text{-}C\text{-}NHR + OH^- \rightleftharpoons R\text{-}C\text{-}NHR (R\text{-}COO^-) + NH_2R
\] (16)

This is explained by the fact that the amine is a poorer leaving group than an alcohol. In enzyme catalysed reactions, acylation is also found to be the rate limiting step in amide or anilide hydrolysis (Inagami and Sturtevant, 1964; Zerner and Bender, 1964). Any factor which increases the equilibrium concentration of THI should favour detection and accumulation of that intermediate. The thiol protease catalysed hydrolysis may be represented as below:

\[
R\text{-}C\text{-}NHR + E-S^- \xrightleftharpoons[k_1]{k_{-1}} R\text{-}C\text{-}NHRE-S^- \xrightarrow[k_2]{k_{-2}} \text{Acyl enzyme}
\] (17)

Formation of the THI for sulphur nucleophiles should be enhanced due to the high carbon basicity of sulphur as compared to oxygen nucleophiles (Hine and Weimar, 1965; Hupe and Jencks, 1976, Lienhard and Jencks, 1966). This should shift the equilibrium in favour of the THI for sulphur relative to oxygen nucleophiles. Although relative nucleophilicities are measured using
low molecular weight compounds it is reasonable to assume that these results will be significant when applied to enzymatic systems.

Equilibria in favor of THI accumulation would be influenced by the ability of an enzyme to stabilize such an intermediate in the active site. Poulos et al. (1976) has proposed that a chloromethyl ketone (CMK) derivative of a peptide substrate inhibits subtilisin-BPN’ by forming a stabilized tetrahedral adduct covalently attached to two amino acid residues in the active site. X-ray crystallographic data obtained for the complex formed when subtilisin-BPN’ is inactivated with L-Phe-L-Ala-L-Lys-CMK suggests that the inhibitor forms a stabilized hemiketal with the serine side chain and is covalently bonded to the nearby histidine residue:

\[
\begin{align*}
\text{R-C-CH}_2\text{Cl} & \quad \text{O}^-
\text{O-H} \quad \text{N-H} \quad \text{N} \quad \text{N} \\
\text{Ser} & \quad \text{His} & \quad \text{Ser} & \quad \text{His}
\end{align*}
\]

The oxygen atom from the inhibitor carbonyl is held in an oxyanion hole by hydrogen bonds to the side chain amino of an asparagine residue and backbone -NH- of the enzyme. Re-examination of the x-ray data of Robertus et al. (1972) is found to be consistent with this interpretation. Recently, Malthouse et al. (1983) have presented C-NMR results showing that a stabilized hemiketal is formed between trypsin and a CMK inhibitor.

Unlike the serine proteases, papain when inhibited with CMK's is alkylated only at the essential thiol group and not the active-site histidine (Husain and Lowe, 1965; Bender and Brubacher, 1966). A detailed x-ray crystallographic study of papain inhibited
by chloromethyl ketone substrate analogues has been presented by Drenth et al. (1976). CBZ-L-Phe-L-Ala-CMK, CBZ-Gly-L-Phe-CMK, and Ac-L-Ala-L-Ala-L-Phe-L-Ala-CMK were prepared, reacted with papain, and crystallized. The authors suggested that the binding of CMK's to papain is a good model for substrate binding. The ketone carbonyl is found to be adjacent to a backbone -NH- of Cys-25 and pointing toward the amino group of Gln-19 although it is not located optimally for hydrogen bonding. The phenylalanine side chain of the inhibitor lies near Val-133 and Val-157 explaining the specificity of papain for a hydrophobic residue at P$_2$ (Berger and Schechter, 1970). A theoretical model for the acyl enzyme was constructed by removing the extra methylene group and forming the thioester linkage. This manipulation was predicted to cause only a slight perturbation of the rest of the inhibitor. The carbonyl group remains in the oxygen binding site in the region containing the two potential hydrogen bonding groups. From this acyl enzyme a model for a THI may be obtained by converting the carbonyl carbon to a tetrahedrally arranged carbon atom. In this model, the hypothetical leaving group is positioned optimally for protonation by His-159 leading to acylation of the enzyme. The model suggests that papain can potentially accommodate and stabilize a THI which may be the important factor in being able to accumulate and detect this intermediate.

Westerik and Wolfenden (1972) theorize that if catalysis by papain is due to stabilization of a transition state or intermediate resembling a THI, then the stability of adducts similar to this intermediate should be enhanced. The formation of a hemithioacetal resembling a THI has been proposed in the reversible inhibition of papain with aldehydes (Westerik and Wolfenden, 1972; Lewis and Wolfenden, 1977b; Bendall et al. 1977; Frankfater and Kuppy, 1981). Deuterium isotope effects (Lewis and Wolfenden, 1977b) and $^1$H-NMR results (Bendall et al., 1977; Clark et al., 1977)
are consistent with formation of a hemithioacetal between the thiol group of Cys-25 and the aldehyde:

\[
\begin{align*}
\text{Enzyme-SH} + \text{R-C-H} & \rightleftharpoons \text{S-Enzyme} \\
& (19)
\end{align*}
\]

There is evidence consistent with formation of a THI in the papain-catalysed hydrolysis of specific substrates. Lowe and Williams, (1965b) studied the acylation reaction of papain with a series of p-substituted phenyl esters of hippuric acid and calculated a Hammett \( \rho = +1.2 \) for the process. This is interpreted to mean that acylation is subject to nucleophilic, or general-base catalysis. Later, Lowe and Yuthavong (1972b) studied the effects of substituents in the leaving groups on the acylation of papain by hippuryl anilides. A Hammett \( \rho = -1.04 \) is obtained suggesting that the rate limiting process is electrophilic or general-acid catalysed for the anilides. Both observations are consistent with a mechanism involving a THI. The formation of the intermediate is rate determining for the esters in a general-base catalysed step, and breakdown is rate determining for the anilides in a general-acid catalysed step.

The papain-catalysed hydrolysis of \( \text{Na-benzoyl-L-argininamide} \) was shown to exhibit a nitrogen isotope effect close to the upper limit indicating that the rate limiting step involves breaking of the carbon to nitrogen bond (O'Leary et al., 1974; O'Leary and Kluetz, 1971). The isotope effect observed for papain is larger than that previously observed for chymotrypsin (O'Leary and Kluetz, 1972). This is interpreted in terms of partitioning of the THI. For chymotrypsin \( k_2 \approx k^{-1} \) (equation (20)) so there is a smaller nitrogen isotope effect as compared to papain where
carbon-nitrogen bond breaking is entirely rate determining (i.e. 
\( k_1 > k_2 \)).

\[
\begin{align*}
E + S & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \quad \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} \text{THI} \quad \overset{k_3}{\underset{P_1}{\rightarrow}} \text{Acyl-Enzyme} \quad (20)
\end{align*}
\]

A concerted process may not be ruled out and the observation
that the isotope effect is pH independent is consistent with this
possibility.

Angelides and Fink (1979a, b) claim to have detected and
accumulated a THI in the papain-catalysed hydrolysis of Na-CBZ-
L-lysine-p-nitroanilide using both cryoenzymological and rapid
reaction techniques. In both cases burst kinetics were observed
with the magnitude of the burst reported to be proportional to
the enzyme concentration. Using cryoenzymological techniques
it was claimed that the intermediate can be accumulated with 1:1
stoichiometry with active enzyme at high pH and stabilized indefinitely
at subzero temperatures. Formation of the THI was observed to
be rate limiting at low pH whereas breakdown is rate limiting
at high pH. This result will be discussed in more detail in the
Cryoenzymology section.

In contrast to the evidence in support of an acyl intermediate
in papain-catalysed reactions, there is little evidence for a discrete
THI. This re-emphasizes the conclusions of Fastrez and Fersht
(1973) that there may be a THI in enzyme catalysed reactions but
it is present only in low steady-state concentrations and is
undetectable.

Additional Intermediates

There is also evidence for other intermediates which may
be present in papain-catalysed hydrolyses. Henry and Kirsch (1967) suggest that departure of the leaving group may be an important step in enzyme catalysed hydrolyses. Many other investigators have also concluded that there is interaction of substrate leaving groups with the enzyme (Brubacher and Bender, 1966; Bender and Brubacher, 1966; Berger and Schechter, 1970; Lowe and Yuthavong, 1971a; Williams et al, 1972b; Mole and Horton, 1973; Alecio et al., 1974).

Stopped-flow experiments of the papain and ficin-catalysed hydrolysis of Na-CBZ-L-lysine p-nitrophenyl ester under conditions with $[E]_0 > [S]_0$ indicated an extra step in addition to the three step process (3) (Holloway and Hardman, 1973). This extra step is proposed to be a conformational change in the enzyme-substrate complex occurring prior to release of p-nitrophenol. They also propose that different substrates are hydrolysed by different pathways determined by the leaving group. Similar suggestions have been made by Mole and Horton (1973). Lowe and Yuthavong (1971a) predicted conformational changes in the enzyme-substrate complex. They suggested a model for an enzyme-substrate complex which implies a mutual distortion of the substrate and the enzyme. The distortion facilitates binding and the stronger binding facilitates distortion.

Similar conclusions suggesting enzyme-substrate conformational changes after binding were presented as the result of steady-state and pre-steady-state fluorescence experiments (Lowbridge and Fruton, 1974; Mattis and Fruton, 1976). In these experiments, a fluorescent probe on the amino terminus of peptide substrates provided information on binding interactions. Later studies using aldehyde derivatives as reversible competitive inhibitors also showed evidence of a conformational change (Mattis et al., 1977; Henes et al., 1979).

Drenth et al. (1976) predicted a widening of the active-site groove upon binding from the x-ray crystallographic study of a
papain-CMK complex. In addition, rotation of the imidazole of His-159 in the active-site, from its position found in the crystal structure, upon substrate binding is proposed.

Angelides and Fink (1978) presented a detailed mechanism also involving movement of the active-site imidazole. Two conformational states of the enzyme are proposed; one with the imidazole hydrogen-bonded to Asn-175 (UP conformation), and the other involving protonated imidazole interacting with the carboxylate of Asp-158 and thiolate of Cys-25 (DOWN conformation). This mechanism was put forward after observation of three intermediate reactions prior to the acyl enzyme in the papain-catalysed hydrolysis of Na-CBZ-L-lysine methyl ester at low temperature in fluid aqueous cryosolvent.

These intermediates outlined in this section are proposed in addition to the more commonly suggested acyl and tetrahedral intermediates. Detection of these other intermediates is extremely difficult since many of the transformations involved are associated with non-covalent interactions of enzyme and substrate. These interactions are not limited to a specific site on the enzyme as is the case of tetrahedral and acyl intermediates which may occur at a single catalytic centre.

pH Dependence

Papain is sufficiently stable to allow complete kinetic analysis over a wide range of pH values (Stockell and Smith, 1957). The pH dependence of the Michaelis-Menten parameters have been determined for several substrates and the results obtained are useful in the determination of the catalytic mechanism (Whitaker and Bender, 1965; Bender and Brubacher, 1966; Williams and Whitaker, 1967; Lowe and Yuthavong, 1971b). From these studies, the bimolecular acylation constant, $k_{cat}/K_m = k_2/K_s$ shows bell-shaped pH dependence with apparent $pK_a$'s of
approximately 4.2 and 8.2. These $pK_a$ values represent ionization of the His-159 and Cys-25 residues at the active site. The ionic forms of papain with both histidine and cysteine protonated, represented by $[-\text{SH} \text{His}^+]$, is inactive at low pH, and the non-protonated form represented $[-\text{S}^- \text{Im}^-]$ has low activity at high pH. The catalytically active form at neutral pH is one of the monoprotonated forms; $[-\text{SH} \text{Im}^-]$, $[-\text{S}^- \text{His}^+]$, or an intermediate form $[-\text{S}^+ \text{H} \text{Im}^-]$ (Brocklehurst and Little, 1970, 1972, 1973; Shipton et al., 1975; Shipton and Brocklehurst, 1978; Lewis et al., 1976; Creighton et al., 1980). The interaction of thiol and imidazole may be further affected by another group with a $pK_a \approx 4$ (Shipton et al., 1975; Bendall and Lowe, 1976; Shipton and Brocklehurst, 1978; Lewis et al., 1978). The other group is assumed to be the carboxyl group of Asp-158. The deacylation step shows sigmoidal pH dependence with an apparent $pK_a$ around 4 depending upon the nature of the acyl group. This $pK_a$ is usually attributed to the His-159 residue which has been suggested to act as a general-base catalyst in deacylation (Brubacher and Bender, 1966).

Brocklehurst and co-workers illustrated the interaction of the active site histidine, cysteine, and aspartic acid residues through the use of two-protonic state reactivity probes; in particular 2, 2'-dipyridyl disulphide (2PDS). This two-protonic state electrophile is also useful as an active-site titrant since papain exhibits high reactivity towards this irreversible inhibitor over a range of pH from 3 to 9 (Brocklehurst and Little, 1970; 1973). The protonated form of the electrophile, present at low pH, is most reactive which enables titration of the reactive papain thiol in the presence of less reactive thiol groups in either low molecular weight compounds or denatured papain. 2-PDS has also been used in detecting chymopapain A and B contaminants commonly found in some preparations of papain (Baines and Brocklehurst, 1978). The thiol of papain reacts with 2-PDS by thiol disulphide exchange.
to form the blocked enzyme and release a 2-thiopyridone chromophore:

\[
\begin{align*}
&\text{S}^- \text{HIm}^+ \\
&\text{S} - \text{S} - \\
&\text{S}^- \text{HIm}^+ \\
&\xrightarrow{+} \\
&\text{S} - \text{S} - \\
&\text{S}^- \text{HIm}^+ \\
&\text{S} - \text{S} - \\
&\text{S}^- \text{HIm}^+ \\
&\text{S} - \text{S} - \\
&\text{S}^- \text{HIm}^+ \\
&\text{S} - \text{S} - \\
&\text{S}^- \text{HIm}^+
\end{align*}
\]

\[
\begin{align*}
\epsilon_{343} &= 8080 \text{ M}^{-1} \text{cm}^{-1} \\
\text{Stuchbury et al. (1975)}
\end{align*}
\]

The 2-thiopyridone released is stoichiometric with active enzyme.

Cryoenzymology

Enzymes, by their nature, are extremely efficient catalysts with the majority having turnover numbers in the $10^3$ to $10^6$ s$^{-1}$ range (Fersht, 1977). This in turn means that a given enzyme-substrate complex or transient intermediate must have a lifetime of milliseconds or less. The existence and structure of such short-lived intermediates, often present in very low concentrations, must be observed and characterized before a detailed mechanism can be proposed. An elucidation of the catalytic mechanism should help explain the great catalytic efficiency of the enzyme.

Enzymes catalyse the transformation of a substrate into product along a reaction pathway involving a series of intermediates. Transformation of one intermediate to the next requires energy in excess of the free energy of activation for the particular reaction. The free energy of activation, $\Delta G^\pm$, comprises both entropic and enthalpic terms:

\[
\Delta G^\pm = \Delta H^\pm - T \Delta S^\pm
\]

The rate of a chemical reaction is related to the temperature of the system. The relation between the rate constant for the reaction and the temperature is represented by the Arrhenius
equation (23):

\[ k = A e ^\frac{-E}{RT} \]  

(23)

Where \( E_a \) is the activation energy which is related to the enthalpy of activation by the equation:

\[ \Delta H^\ddagger = E_a + RT \]  

(24)

A decrease in temperature can therefore cause a dramatic decrease in the reaction rate as illustrated in the table below (Fink, 1976b).

<table>
<thead>
<tr>
<th>Temp</th>
<th>( \Delta H^\ddagger ) (kcal/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>298 K</td>
<td>1 1 1 1 1 1</td>
</tr>
<tr>
<td>223 K</td>
<td>5.9x10^-2 1.1x10^-2 2.0x10^-3 2.1x10^-4 1.3x10^-5</td>
</tr>
<tr>
<td>173 K</td>
<td>2.4x10^-3 6.7x10^-5 1.8x10^-6 1.4x10^-8 3.5x10^-11</td>
</tr>
</tbody>
</table>

The table shows the theoretical reduction in the relative reaction rates (compared to 298 K) with the decrease in temperature for different values of \( \Delta H^\ddagger \). The higher the value of \( \Delta H^\ddagger \) for a particular reaction the greater the rate reduction upon lowering the temperature.

A hypothetical energy diagram for an enzyme catalysed reaction involving several intermediates is shown (Fink, 1976b).
Starting with enzyme and substrate and using a suitably low temperature, intermediates such as $I_2$ and $I_4$ could not be accumulated although it may be possible to accumulate $I_4$ by the reverse reaction of enzyme with excess product. Cryoenzymology, the use of low temperature techniques in the study of enzyme mechanisms, has been developed to exploit the enthalpy differences of the individual steps of an enzyme-catalysed reaction in order to accumulate and characterize the intermediates along the catalytic pathway. P. Douzou and A. L. Fink have been instrumental in developing and utilizing this technique and the subject has been extensively reviewed (Douzou, 1973, 1974, 1977, 1979; Fink, 1976b, 1977; Fink and Geeves, 1979; Fink and Cartwright, 1981; Fink and Petsko, 1981; Makinen and Fink, 1977).

Accumulation of an intermediate, $I_i$, in high concentration with negligible breakdown,

$$k_i \overset{k_{-i}}{\underset{k_{i+1}}{\rightleftharpoons}} I_i$$

should be possible using cryoenzymological techniques if $k_i \gg k_{-i} + k_{i+1}$.

The potential to accumulate intermediates in high concentration and with relatively long lifetimes is a major advantage of cryoenzymology over rapid reaction techniques. Detailed structural information provided from high resolution techniques such as x-ray diffraction and nuclear magnetic resonance require samples of high concentration and long lifetimes which are unobtainable in rapid reaction experiments. Previously, much of the structural information on enzyme-substrate complexes or intermediates was obtained from the results of experiments using inhibitors (e.g. Drenth et al., 1976; Poulos et al., 1976). These studies provided information on non-productive species which may be significantly different from the structures actually found with
specific substrates. Cryoenzymology also has the potential of allowing investigation of a single step in the catalytic pathway using enzyme specific substrates. Also of particular interest is the possibility of detecting and accumulating intermediates at low temperatures which are undetectable at ambient temperatures (Fink, 1976b). For example, if an intermediate is formed by a reaction with an energy of activation of 10 kcal/mole and breaks down with an energy of activation of 15 kcal/mole, and at 25°C both rates are equal, at -100°C the rates would differ by approximately 500. The intermediate should accumulate since the slower step is breakdown of the intermediate.

Obviously for an enzyme and substrate to interact at low temperature a suitable cryosolvent, fluid at low temperature, must be utilized. This is a major disadvantage of cryoenzymology as there is a distinct possibility that the cryosolvent may bring about changes in the structure of the enzyme and/or changes in the catalytic pathway. There is also the possibility that the rate limiting step in the overall reaction may be so positioned that very few intermediates are detectable. Some of the techniques and applications of cryoenzymology are discussed below.

A suitable cryosolvent must be selected which has no adverse effects on the catalytic or structural properties of the enzyme. Douzou and co-workers have carried out extensive physicochemical studies on the properties of many cryosolvent systems (Douzou et al., 1976; Hui Bon Hoa and Douzou, 1973; Travers et al., 1975; Douzou, 1977,1979,1974; Larroque et al., 1976).

The types of cosolvents commonly in use include alcohols, polyols, DMSO, and DMF. Viscosity and freezing point data have been determined for many of these cryosolvent systems (Douzou, et al., 1976). Due to its low viscosity even at low temperatures, methanol is preferentially used in many solvent systems. Unfortunately many enzymes denature in the presence of this cosolvent and it
may not be desirable in reactions where it can compete with water for an enzyme-bound species (e.g. as with the hydrolytic enzymes). Polyols tend to be extremely viscous at low temperatures and addition of an alcohol to produce a ternary solvent system may overcome this problem (Travers et al. 1975). DMSO is a particularly useful solvent since solutions of 50 to 70% (v/v) can be supercooled (see table (28), Travers and Douzou, 1974; Douzou, et al., 1976).

<table>
<thead>
<tr>
<th>DMSO % solvent (v/v)</th>
<th>Freezing point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>-3</td>
</tr>
<tr>
<td>20</td>
<td>-12</td>
</tr>
<tr>
<td>30</td>
<td>-19</td>
</tr>
<tr>
<td>40</td>
<td>-41</td>
</tr>
<tr>
<td>50</td>
<td>supercooled (28)</td>
</tr>
<tr>
<td>60</td>
<td>supercooled</td>
</tr>
<tr>
<td>70</td>
<td>supercooled</td>
</tr>
<tr>
<td>80</td>
<td>-38</td>
</tr>
<tr>
<td>90</td>
<td>-7</td>
</tr>
<tr>
<td>100</td>
<td>+19</td>
</tr>
</tbody>
</table>

DMSO is also a very good solvent and has often been used in cryosolvents although the viscosity of the solutions may create problems below -50°C.

The apparent pH in aqueous-organic cryosolvent, designated pH*, will vary with temperature and cosolvent concentration. The pH* may be measured directly using a conventional pH meter and glass electrode over a range of temperatures above 0°C and extrapolated to a given subzero temperature since the pH* is linearly related to 1/T. More accurate determinations are obtainable with the modified electrode system of Larroque et al. (1976) or a spectrophotometric method using a series of indicators (Hui Bon Hoa and Douzou, 1973). Generally the pH* increases with decreasing temperature, and increasing cosolvent concentration (Douzou, 1974). Usually the pK_a of a buffer will increase in the less polar aqueous-
organic solvent system.

The dielectric constant can affect many biochemical processes since it influences the interaction between charged species. Addition of most cosolvents lowers the dielectric constant but a decrease in temperature is found to cause an increase (Travers and Douzou, 1974). These effects may compensate and dielectric constants for various solvent systems over a wide range of temperatures have been determined (Douzou et al., 1976; Travers and Douzou, 1974).

Once an appropriate cryosolvent is selected the effect on the conformational and catalytic properties of the enzyme must be assessed. A wide range of enzymes are found to be active in aqueous-organic solvent systems and have been studied using cryoenzymological techniques (see Fink and Geeves, 1979; Fink and Cartwright, 1981). Fink and Geeves (1979) summarize the steps involved in a complete cryoenzymological study which can be applied to a variety of enzyme systems:

1) Preliminary tests (e.g. freezing point, viscosity) to identify possible cryosolvent(s).

2) Determine the effects of cosolvent and subzero temperatures on the catalytic and structural properties of the enzyme.

3) Detection of intermediates by initiating the enzyme-catalysed reaction at subzero temperatures.

4) Kinetic, thermodynamic and spectral characterization of any detected intermediates.

5) Correlation of the low temperature results with those results obtained in water at ambient temperatures.

6) High resolution structure determination of the trapped intermediates.

Since cryoenzymological studies have been applied to many diverse enzyme systems, a review of this protocol as applied to some serine and thiol proteases is most relevant.

α-Chymotrypsin has been found to be active at low temperatures in methanol (Bielski and Freed, 1964), DMSO
(Fink, 1973a, b), and ethylene glycol (Kraicsovits and Douzou, 1973) containing cryosolvents; trypsin in DMSO (Fink, 1974b), 1,2-propanediol and ethylene glycol (Maurel et al., 1975); papain in DMSO and ethanol (Fink and Angelides, 1976); and elastase in DMSO and methanol (Fink and Ahmed, 1976) based cryosolvents.

The effects of the cosolvent on the structure of the protein can be determined by observing the intrinsic spectral properties of the enzyme (Fink, 1973b, 1971b; Fink and Angelides, 1976). Changes in the UV difference, circular dichroism, and fluorescence emission spectra have all been shown to be useful in detecting conformational changes in the enzyme as well as solvent perturbation of the exposed amino acid chromophores (for a review see Timasheff, 1970). Chymotrypsin (Fink, 1973b), trypsin (Fink, 1974b), and papain (Fink and Angelides, 1976) all exhibited an increase in absorption intensity linearly related to an increase in DMSO concentration. These results suggest that only solvent effects on the exposed chromophoric side-chain residues are observed. These effects would be expected to cause linear or smooth monotonic changes with increasing DMSO concentration. Structural perturbations cause sharp deviations with increasing cosolvent. This is illustrated with papain in that high concentrations of DMSO (>60% v/v) cause sharp deviations in absorbance changes which corresponded with the loss of catalytic activity (Fink and Angelides, 1976). Below 60% DMSO no sharp deviations were observed and the circular dichroism and fluorescence emission spectra also showed no evidence consistent with a structural change. With papain and chymotrypsin there was no evidence for changes in the tyrosine to tryptophan energy transfer (excitation at 260 nm, emission at 330-340 nm), as a function of solvent concentration, indicating that the relative positions of these groups is unchanged. A sharp break occurs in the plot of the relative fluorescence emission of papain as a function of methanol concentration at about 10 M (50% v/v) cosolvent making this solvent unsuitable for
cryoenzymological experiments at that concentration. Subzero temperatures similarly were found not to effect the structural integrity of the enzymes. In each case, the transition involving thermal denaturation of the proteins occurs at a lower temperature. Papain was shown to reversibly denature above +5 °C in 60% (v/v) DMSO at pH 3.5 by a sharp change in the UV difference spectrum. Chymotrypsin is stable at temperatures <10 °C in 65% DMSO and trypsin at temperatures <6 °C or at low pH. All of these enzymes must therefore be maintained at low temperature when in cryosolvent solutions.

The most sensitive tests for determining the effects of added cosolvent on the enzyme are those involving its catalytic activity. Since enzymes require precise orientation and interaction of its amino acid residues for catalysis to occur, minor structural changes at the active site can produce significant effects on the catalytic parameters.

Papain (Fink and Angelides, 1976) and trypsin (Fink, 1974b) catalysed hydrolysates of $\text{Na-CBZ-L-lysine p-nitrophenyl ester}$ both showed a decrease in $k_{\text{cat}}$ with an increase in DMSO concentration. The same results were obtained for chymotrypsin and $\text{Na-Ac-L-tryptophan p-nitrophenyl ester}$ (Fink, 1973a,b). In each case deacylation, the rate determining step, depends upon water and the decrease in rate was attributable to the decrease in the water concentration. Linear plots of $\log(k_{\text{cat}})$ vs. $1/T$ were obtained over the temperature ranges investigated. This is suggested to indicate that there is no change in the rate determining step with decrease in temperature. Extrapolation of the linear plots to 25 °C, and allowing for the decreased water concentration, resulted in calculated values of $k_{\text{cat}}$ in reasonable agreement with those obtained in the absence of DMSO at +25 °C. These results suggested that the DMSO cosolvent had no adverse effect on $k_{\text{cat}}$.

In contrast, dramatic increases in the values of $K_m$ were
observed with an increase in DMSO concentration. With all three enzymes mentioned above, an exponential increase in $K_{m(app)}$ occurs with increasing DMSO concentration. Since in each case a p-nitrophenyl ester was used, $K_{m(app)} = \frac{k_s^2}{K_3 k_2}$, and the exponential increase could be due to an increase in $K_s$ and/or a decrease in $k_2$. The interaction of a competitive inhibitor, proflavine, with chymotrypsin in DMSO solutions indicated that the predominant effect is upon $K_s$ (Fink, 1974a). The increase was suggested to result from a combination of competitive inhibition and dielectric effects on substrate and inhibitor binding. Maurel (1978) studied the effects of various cosolvents on three enzyme-substrate interactions and concluded that the effect of cosolvents on $K_m$ is due to the hydrophobic interactions in the binding step. The general rule proposed is that the larger the contribution of hydrophobic interactions to the binding process, the larger the effect an organic solvent will have upon $K_m$. This is illustrated by the observation that $K_m$ for the interaction of ribonuclease and RNA, where formation of the Michaelis-complex is predominately due to electrostatic and not hydrophobic forces, is unaffected by added cosolvent. With chymotrypsin, which relies on hydrophobic interactions in the binding process, the values of $K_m$ are significantly affected. Often the increased solubility of a substrate in the cryosolvent will lower its affinity for the active-site which increases the $K_m$. The results imply that changes in the dielectric constant have less of an effect upon $K_m$.

Temperature was shown to have little effect on proflavine-chymotrypsin binding (Fink, 1974a). The effect of temperature on $K_m$ is less well defined. Values of $K_m$ for papain and Na-CBZ-L-lysine p-nitrophenyl ester are reported to remain unchanged at least over the range 0 to -20°C (Fink and Angelides, 1976). With trypsin and the same substrate there is a significant decrease with decreasing temperature.

Exponential increases in $K_{m(app)}$ are of course undesirable
since increased concentrations of substrate are then required to saturate the enzyme and make detection of an enzyme-bound intermediate easier. Douzou and Balny (1977) have shown that the addition of water soluble polyelectrolytes to enzyme solutions offers some protection against cosolvent effects on the kinetic parameters. For example, $K_{m(app)}$ for the hydrolysis of L-BAEE by trypsin in 50% DMSO is reduced 10-fold by the addition of 10 μg of RNA. The strong electrostatic potential of these polyelectrolytes is thought to cause clustering of water molecules at the expense of the organic solvent and preferentially solvating the enzyme.

The evidence presented is consistent with the conclusion that the structure and catalytic mechanism of the enzyme is not significantly changed in some aqueous-organic cryosolvents. Although $K_{m(app)}$ is significantly affected, there is no evidence that the catalytic pathway has been altered.

In light of the previous section on intermediates in the reactions catalysed by the serine and thiol proteolytic enzymes, cryoenzymology has found widespread application to the detection and accumulation of such intermediates. After concluding that α-chymotrypsin is stable and catalytically active in aqueous DMSO and methanol solutions at low temperature, Fink has claimed to have detected and isolated an acyl-chymotrypsin intermediate (Fink, 1973a, b). The presumed Na-Ac-L-tryptophanyl-α-chymotrypsin was isolated by Sephadex LH-20 chromatography in the cryosolvent at low temperature. Using similar procedures, Fink et al. (1979) have claimed the isolation of Na-furylacryloyl-L-tryptophanyl-α-chymotrypsin. Acyl enzymes from α, δ, ψ, chymotrypsins are all reported to be detected in high yields in both DMSO and methanol cryosolvents at low temperatures (Fink and Ahmed, 1976). The authors also used crystalline enzymes in cryosolvent and at low temperature and have claimed to have isolated high yields of crystalline acyl enzymes with negligible
turnover rates. Acylation for crystalline \( \gamma \)-chymotrypsin was found to be fifteen times slower than for the dissolved enzyme with no deacylation observable over two to four day periods at temperatures below \(-40^\circ C\). These experiments show the versatility of the cryoenzymological technique and support the conclusion that the catalytic pathway of chymotrypsin remains unchanged in cryosolvent.

Trypsin was found to be stable at low temperature in 65% DMSO and an acyl enzyme is reported to be accumulated with negligible turnover below \(-45^\circ C\) (Fink, 1974b). Little spectroscopic evidence or information is presented for the acyl enzyme. A later study indicated that 44% of the enzyme is acylated at \(-33^\circ C\) (Fink and Ahmed, 1976). Supposedly a higher proportion of enzyme is acylated in studies with crystalline trypsin and the same substrate. In the same report, rates of acylation and deacylation for elastase dissolved in cryosolvent and in the crystalline state are also presented. High concentrations of a proposed acyl enzyme are reported for the elastase-catalysed hydrolysis of Na-CBZ-L-Ala-p-nitrophenyl ester and Na-Ac-Ala\( \_3 \) methyl ester in 70% methanol cryosolvents. At temperatures below \(-45^\circ C\), deacylation is not observed. The rate of acylation in the crystalline enzyme is 20 times slower than in solution and deacylation may be slowed to an even greater extent. Crystallographic studies of the acyl-elastase intermediate have reported (Alber et al., 1976).

Na-CBZ-L-alanine p-nitrophenyl ester substrate is dissolved in the methanol cryosolvent and diffused into the crystal of elastase mounted in a flow-cell designed for use in a single crystal diffractometer. Acylation was complete in one day and collection of a complete set of three-dimensional data to 3.5 Å of the crystalline acyl enzyme was completed in two days. An electron density map is interpreted to show the close proximity of the acyl group to the active-site Ser-195. No density due to the p-nitrophenyl portion of the substrate is evident. (Alber et al., 1976).
Although a detailed structural interpretation is not presented, this demonstrates the potential for the application of the cryoenzymological techniques to high resolution structural determinations.

Cryoenzymological investigations of the \( \alpha \)-chymotrypsin catalysed hydrolysis of Na-Ac-L-Phe methyl ester (Fink and Wildi, 1974), and the trypsin catalysed hydrolysis of Na-Ac-L-lysine methyl ester (Fink, 1974b) provided evidence for a conformational change in addition to formation of the acyl enzyme intermediate in the catalytic pathway. The \( \alpha \)-chymotrypsin catalysed reaction was studied more extensively. The reaction was initiated at \(-90^\circ C\) in 65\% DMSO apparently without viscosity problems. Raising the temperature allowed observation of three distinct reactions attributed to substrate binding, acylation, and deacylation. The product of the acylation reaction is reported to undergo a significant decrease in the fluorescence emission along with a blue shift in \( \lambda_{\text{max}} \) which is suggested to be due to a perturbation of a tryptophan residue at the active-site. A less detailed study with trypsin showed UV absorbance changes suggesting a similar perturbation.

The detection of a possible tetrahedral intermediate is an especially useful application of cryoenzymological techniques. Considering the results of Fastrez and Fersht (1973) who concluded that a THI exists only at low concentrations at room temperature, cryoenzymology may be particularly suitable since as previously mentioned, intermediates which may be at low concentration at room temperature, may be accumulated at higher concentrations at reduced temperatures.

Evidence supporting the presence of stabilized THI's in the elastase catalysed hydrolyses of Ac-L-Ala-L-Pro-L-Ala-p-nitroanilide, Ac-L-Pro-L-Ala-p-nitroanilide, and succinyl-L-Ala\(_3\)-P-nitroanilide at low temperature in aqueous methanol cryosolvents has been presented (Fink and Meehan, 1979).
The absorption spectrum of the intermediate is similar to the product p-nitroaniline ($\lambda_{\text{max}} = 381$ nm) but blue-shifted approximately 20 nm to 359±2 nm. Both are significantly different from the starting p-nitroanilide substrates which have absorption maxima around 320 nm. The rate of formation of the reported THI followed first-order kinetics and exhibited a sigmoidal pH dependence with $pK_a^* = 7.0$ at $-39^\circ C$. The amount of THI trapped is reported to increase with $pH^*$ to a maximum value around $pH^* = 9.4$ and increase with decreasing temperature. Several arguments were presented in favour of identifying this intermediate as a THI: (i) The reaction of the p-nitroanilide substrates is much slower than with the corresponding p-nitrophenyl esters. Since deacylation is rate limiting for the esters, the rate limiting step for the anilides must be prior to deacylation. (ii) An increase in enzyme concentration increased the rate of reaction attributed to turnover and the magnitude of the absorbance change associated with formation of a THI. (iii) Increasing the substrate concentration increased the rate of formation of the intermediate but not the turnover reaction.

The authors also report the detection of an additional intermediate with a UV spectrum resembling the substrate but formed after the Michaelis-complex. It was suggested that this intermediate arises from a conformational change involving movement of the active-site imidazole. Balny and Bieth (1977) also reported evidence of a transient intermediate preceding turnover in the elastase catalysed reaction of succinyl-L-Ala$_3$-p-nitroanilide at subzero temperatures in aqueous ethylene glycol. The cryo-enzymological results compare favourably with the stopped-flow results of Hunkapiller et al. (1976) at room temperature (see THI section).

Similar results are obtained with trypsin and Nε-CBZ-L-lysine-p-nitroanilide (Compton and Fink, 1980). The authors report that burst kinetics are observed in the formation of a THI with a $\lambda_{\text{max}}$ of 355±2 nm. Formation of this intermediate is
negligible at pH* values ≤6 and at temperatures higher than -40°C. Kinetic parameters at room temperature in aqueous solution are reported and $K_m = 1.5$ mM. Burst kinetics corresponding to 80% of the active enzyme are observed under conditions of -45°C, 65% DMSO, and with $[S]_0 = 280$ μM which would correspond to a situation where $[S]_0 < K_m$. No kinetic parameters at low temperature or in aqueous cryosolvent are presented. Although these reports present some of the first direct evidence for detection of a THI, the results are not as yet unambiguous. This is especially true when the results of Markley et al. (1981) are taken into account.

Markley et al. (1981), as discussed previously, reported that burst kinetics observed by Fink and Meehan (1979) for proline containing substrates may be due to cis, trans heterogeneity about the alanyl-prolyl peptide bond. The authors argue that elastase hydrolyses the cis-isomer more rapidly than the trans-isomer which leads to observation of a burst due to different rates of hydrolysis of different substrates and not a THI. This however does not explain the results obtained with succinyl-L-Ala_3-p-nitroanilide substrate. Markley et al. (1981) point out the difficulty in interpreting the data of Fink and co-workers as essential kinetic parameters such as values of $K_m$ and magnitudes of bursts have not been reported. Inclusion of this data would form a more complete and convincing argument in support of the results. Markley et al. (1981) emphasize the need for careful experimental technique since confusing spectral changes may be due to incomplete mixing, (especially possible in viscous solutions at low temperature) thermal gradients, and substrate heterogeneity. The results indicate the applications and problems of cryoenzymology as applied to the serine protease-catalysed reactions.

After extensive CD, UV, fluorescence and kinetic studies, Fink and Angelides (1976) concluded that papain is not adversely affected by low temperature or aqueous-DMSO or aqueous-ethanol
cryosolvents. Methanol in solutions at concentrations greater than 10 M and DMF containing cryosolvents were found to be unsuitable. The acyl enzyme formed from the reaction of Na-CBZ-L-lysine p-nitrophenyl ester and papain is reported to be accumulated and trapped at temperatures below -50°C in 60% DMSO cryosolvents. Some of the data presented is inconsistent with such a conclusion. Fink and Angelides (1976) have reported experimental details for a typical acylation experiment with $[E_0] = 14 \mu$M and $[S_0] = 1$ mM. The amount of p-nitrophenol released in the acylation reaction is reported to be approximately stoichiometric with enzyme and no subsequent release of p-nitrophenol is detected. The $K_m$ in 60% DMSO at 0°C is observed to be 10 mM and is stated to be invariant between 0 and -20°C. Although data is not provided for $K_m$ at lower temperatures, a significant drop must occur between -20 and -70°C for the enzyme to be saturated and a stoichiometric burst to occur. Such a significant effect is not mentioned. The values of $k_{cat}$ used in an Arrhenius plot are obtained in 60% DMSO also with $[S_0] = 1$mM (i.e. $K_m >>> [S_0]$). Furthermore, a similar 1:1 stoichiometry of p-nitrophenol released to enzyme concentration is observed in 12 M aqueous ethanol at -70°C which is below the freezing point of such a solution (CRC Handbook of Chemistry and Physics).

Cryoenzymological studies of the papain-catalysed hydrolysis of Na-CBZ-L-lysine methyl ester detected maximal changes at 276 nm in the UV (Fink and Angelides, 1978). At low temperature, where turnover is reported to be negligible, the reaction exhibits three phases. The first two of these three phases could also be detected in the fluorescence emission of the enzyme. The Trp-177 residue at the active-site, which is responsible for a large part of the fluorescence emission of the enzyme, is greatly influenced by the interaction of the adjacent imidazole ring of His-159. Based on the results of this investigation and those of earlier workers, a detailed mechanism for the action of papain is proposed by
The first reaction, corresponding to a rapid decrease in UV absorption and an increase in fluorescence emission,
involves substrate binding to E to form $ES_1$. The substrate preferentially binds to the so-called UP form E rather than $E'$. According to space-filling models, access to the active-site in the latter form would be sterically hindered. The $pK_a$'s of the thiol group and the histidine imidazole are dependent upon the enzyme conformation. The fluorescence increase in the first reaction may be due to changes in the Trp-177 environment on going from $E'$ in the DOWN conformation with imidazolium to $ES_1$ in the UP conformation with imidazole free base. Imidazolium ion is known to be an effective quenching agent in tryptophan emission (Shinitzky and Goldman, 1967). Reaction 2 is assigned to the transformation of $ES_1$ to $TI'$ via $ES_1'$ at neutral pH. At low pH, $ES_1$ is converted to $ES_1''$ via $ES_1'$. Both cases are suggested to involve the rate limiting $ES_1$ to $ES_1'$ transformation. The quenching of the Trp-177 fluorescence emission is consistent with the proposed step. Reaction 3, the slowest of those observed, in the intermediate pH range is concluded to be the conversion of species $TI'$ to acyl enzyme $EA'$ through $EA''$. At high pH, species $TI$ should be formed in reaction 2 and accumulate since the general-acid catalysis by the imidazole, which is required to form the acyl enzyme, is not possible. Although much of the mechanism proposed is purely hypothetical, it does appear to explain many of the apparent differences in $pK_a$ values observed in papain-catalysed reactions.

In an investigation similar to those previously reported with the serine proteases, Angelides and Fink (1979a, b) studied the papain-catalysed hydrolysis of Na-CBZ-L-lysine-p-nitroanilide. They reported the detection of four different reactions spectrophotometrically prior to the turnover reaction. Each of these reactions are separately characterized with respect to pH, temperature and enzyme and substrate concentration. The slowest reaction in the sequence was suggested to be acylation. The first of the four reactions is considered to be formation of
the Michaelis-complex, coupled with a slower reaction attributed to a conformational change between two forms of the enzyme prior to binding. These results are consistent with the mechanism proposed earlier. The third reaction is reported as a conformational change in the ES complex similar to the change observed with the methyl ester, i.e. ES$_1$ to ES$_1'$. The fourth reaction, the product of which has a similar spectrum to the final product p-nitroaniline, is suggested to be formation of a tetrahedral intermediate. The absorption spectrum of this intermediate displayed a $\lambda_{max}$ of 365 nm in contrast to a $\lambda_{max}$ of 320 nm for the substrate and 380 nm for the product in 60% DMSO. The rate of formation of the product of reaction 4 shows bell-shaped pH dependence with $pK_a$'s of 4.3 and 8.6. At low pH*, formation of the intermediate is rate determining, whereas at high pH* breakdown is rate determining. The concentration of the accumulated intermediate was shown to be pH* dependent with a concentration of intermediate ranging from 3% of the enzyme concentration at a pH* of 3 to a value of 98% of enzyme-bound intermediate at pH* 9.3. The catalytic mechanism at low temperature in cryosolvent was reported to be consistent with that found in aqueous solution at 21.5°C by the reported observation of this same intermediate by stopped-flow experiments. These results are claimed as the first direct observation of a THI in the papain-catalysed reactions. The greatest limitation of this investigation is the small amount of structural information available from the UV spectrum. The actual identity of the intermediates must be inferred from the spectral changes.

The preceding review demonstrates one of the most valuable attributes of cryoenzymology, namely its applicability to many types of enzymes and its adaptability to a wide range of chemical and physical analytical techniques. Less ambiguous results are still required and these may be obtainable by using high resolution techniques such as x-ray diffraction and nuclear
magnetic resonance (NMR). NMR techniques have the advantage in that they can be applied to enzymes in solution without giving rise to many of the artefacts which may easily occur using spectro-photometric techniques.

\[ ^{13} \text{C-NMR Spectroscopy} \]

The preceding sections have outlined the possible intermediates involved in certain enzyme-catalysed reactions and the physical techniques commonly applied to the analysis of enzyme interactions with various substrates and inhibitors. The most commonly used physical technique applied in enzymology is UV-visible spectrophotometry. Due to the large number of atoms involved in a typical enzyme-substrate interaction, there are an enormous number of electronic states present which produce broad absorption peaks in the UV-visible region. These broad peaks contain only a limited amount of diagnostic information. NMR spectroscopy on the other hand, is one of the most important physical techniques available to the organic chemist for molecular analysis. NMR has the potential to not only characterize the functional groups of the molecules of interest, but may also provide structural, stereochemical, and conformational information. This is invaluable if it can be successfully applied to the detection and characterization of intermediates in enzyme-catalysed reactions. A brief introduction to the theory of NMR will illustrate the wealth of information available by utilizing this technique. The special applications of \(^{13}\text{C-NMR}\) methods to enzyme chemistry will then be considered. The basic theory of NMR has been presented in several books and the following section on theory is a short summary of the relevant theory more extensively presented in these texts (Jackman and Sternhell, 1969; Shaw, 1976; Stothers, 1972; Wehrli and Wirthlin, 1976).
Nuclei of certain isotopes possess an intrinsic spin due to the number of nucleonic particles (i.e. protons, neutrons) which are present. The nuclear spin quantum number, \( I \), is a characteristic constant for any given nucleus. Spinning of a positively charged nucleus produces a magnetic field with magnetic moment, \( \mu \), directed along the axis of spin. Organic chemists are primarily interested in carbon and hydrogen isotopes which possess such a spin and therefore the spectral characteristics of the \(^1\text{H}\) and \(^{13}\text{C}\) nuclides with \( I = 1/2 \) have been extensively studied in a large number of systems.

When placed in a static, uniform magnetic field of strength \( B_0 \), the proton or \(^{13}\text{C}\) nucleus is restricted to two possible orientations which are characterized by energies dependent on the magnitudes of \( \mu \) an \( B_0 \). The nuclei are either aligned with (low energy), or against (high energy) the magnetic field. The energy difference between the two states is comparatively small (\( \Delta E = \hbar \nu B_0 \), where \( \nu \), the gyromagnetic ratio, is a characteristic constant of a particular nuclide) as are the population differences which are described by a Boltzmann distribution. Fortunately, there exists a slight excess of nuclei in the low energy state. Application of a particular frequency of electromagnetic radiation in the radio-frequency range results in a net absorption of energy. Restoration of the populations to thermal equilibrium is achieved by a number of relaxation processes which will be discussed in more detail.

Unfortunately, NMR is a relatively insensitive technique as compared to the optical techniques commonly used. This is due to a combination of the low number of nuclei in excess in the lower energy level and the small energy differences between these levels. The long lifetime in the excited states (\( 10^{-3}\) to \( 10^{-3}\) sec.) as compared to electronic transitions (\( 10^{-8}\) to \( 10^{-9}\) sec., Jaffe, Orchin, 1962) contributes to the insensitivity. The low gyromagnetic ratio of \(^{13}\text{C}\), approximately one-fourth that of \(^1\text{H}\), and the fact that the natural abundance of the \(^{13}\text{C}\) isotope is 1.1%, makes \(^{13}\text{C}\)-NMR
approximately 1/5800 as sensitive as $^1$H-NMR. Development of various techniques, in particular pulse and Fourier transform methods, have overcome these difficulties and have made $^{13}$C-NMR a powerful analytical tool.

After perturbation of the spin-state equilibrium by the application of a radiofrequency pulse, the process of returning a nucleus from a high to low energy state takes place by relaxation processes. Two processes are most important, the spin-lattice, and the spin-spin relaxation.

a) **Spin-lattice relaxation.** This process is due to the interaction of the nuclei with rapidly fluctuating local magnetic fields in its surroundings, or lattice. The fluctuations have a component at the resonance frequency which enables a nucleus in the upper spin state to relax to the lower state. The energy lost is transferred to the lattice as extra rotational or translational energy. The time constant for this exponential process is designated $T_1$. The magnitude of $T_1$ depends upon the nature and rapidity of the local magnetic fluctuations which are predominately dependent on the molecular motions. This relaxation process is responsible for the excess of nuclei in the low energy orientation at equilibrium. A large value of $T_1$ indicates an inefficient relaxation process.

The $^{13}$C spin-lattice relaxation process is itself composed of various relaxation mechanisms (Wehrli and Wirthlin, 1976). Two of these mechanisms are especially important when considering the $^{13}$C-NMR of proteins (Allerhand, 1979). Dipole-dipole relaxation results from rotational motions of C-H vectors. This is the predominant relaxation mechanism for protonated carbons and contributes to the total spin-lattice relaxation in non-protonated carbons at low magnetic field strength (Norton et al., 1977). An important contributor to the spin-lattice relaxation in non-protonated carbons is chemical-shift anisotropy (CSA). If a
C-X bond is placed with its axis perpendicular to the external magnetic field the carbon shielding by the electrons will be different from that found if the axis is parallel to the field. Rotational motion then produces a fluctuating magnetic field and relaxation may occur by this mechanism. CSA is found to be the predominant relaxation mechanism for non-protonated sp\textsuperscript{2} carbons at high magnetic field (>40 kG) (Levy and Edlund, 1975; Norton et al., 1977; Allerhand, 1979).

b) **Spin-spin relaxation.** This process is operative when two nuclei in close proximity are able to interact. The magnetic field generated by a spinning nucleus has a component perpendicular (transverse) to the main field which is ideal for inducing transitions in an adjacent spinning nucleus. This process results in spin-spin or transverse relaxation which is also an exponential process with time constant designated $T_2$. The magnetic field produced by one nucleus will also cause a variation in the field experienced by another nucleus in close proximity. This will cause a spreading of the resonance frequency leading to broadening of the resonance lines in the spectrum.

Also dependent on the relaxation processes is the nuclear Overhauser effect (NOE). A $^{13}$C-NMR spectrum obtained with simultaneous proton irradiation often yields an improvement in the signal-to-noise ratio (S/N) of the carbon resonance in addition to that expected from removal of $^{13}$C-$^1$H scalar coupling. The NOE phenomenon is due to a favourable redistribution of the $^{13}$C energy level populations caused by irradiation of nearby protons (Lyerla and Grant, 1972) and therefore depends upon the influence of these protons on the relaxation mechanism.

All of these processes affect the resolution and the intensity of the $^{13}$C-NMR resonances.

As mentioned previously, the advent of pulse and Fourier transform methods enabled $^{13}$C-NMR spectroscopy to become routinely used. Briefly, a strong pulse of radio-frequency energy
when applied to a sample is capable of exciting all resonances at once. Since there was an excess of nuclei in a low energy state originally there is a net magnetization parallel to the applied magnetic field along the z-axis:

Application of a pulse causes rotation of the net magnetization away from the z-axis toward the x'-y' plane. After termination of the r.f. pulse, the nuclei relax to obtain thermal equilibrium with an exponential time constant related to the relaxation mechanisms involved. If a receiver coil (RC in the figure) has an axis perpendicular to the field, the nuclei will induce an A.C. signal in the coil which decays exponentially. This signal, known as the free-induction decay (FID), represents the time development of the magnetization \( M(t) \). This is mathematically related to a conventional spectrum, where the magnetization is a function of frequency, \( M(\omega) \), by a Fourier transformation. A sequence of pulses may then be applied to a sample and the responses to each pulse accumulated as an FID and stored. Application of a single pulse will usually not yield the desired S/N and a summation of the responses to a number of pulses will directly increase the intensity of a signal while the signal due to random noise will increase at the square-root of the number of pulses accumulated. This summation process leads to a dramatic increase in the sensitivity of pulse-Fourier transform NMR spectroscopy.

The signal induced in a receiver coil after a single pulse is
greatest for a pulse which rotates the magnetization \(90^\circ\) from the \(z\)-axis. Often it is more practical to use a pulse (or flip-angle) much less than \(90^\circ\). The relaxation times (i.e. \(T_1, T_2\)) will affect the optimal pulse angle and the pulse repetition time (or relaxation delay) needed for the best results. Usually, the relaxation times are not known, so a compromise between the flip-angle, relaxation delay, and number of pulses must be sought in order to obtain the most information in the shortest possible time.

The most useful parameter obtainable from an NMR spectrum is the chemical shift. The chemical shift is the difference in frequencies between a particular signal and a standard (usually the resonance of tetramethylsilane, TMS). This frequency value is directly proportional to the applied magnetic field \(B_0\). Spectra from different spectrometers may be compared by using the chemical shift parameter \(\delta\), which is obtained from the following equation:

\[
\delta \text{(in parts per million)} = \frac{(v_{\text{sample}} - v_{\text{TMS}}) \text{ in Hz}}{\text{spectrometer frequency in MHz}}
\] (29)

This chemical shift value is influenced by the distribution of electrons about the nucleus which reflects the nature and conformation of the surrounding groups. The magnetic field experienced by a nucleus is not equal to the applied field \(B_0\) because the nucleus is shielded to some extent by its own electrons and electrons of neighbouring nuclei. A secondary magnetic field is generated by the circulating electrons which opposes the applied field. The greater the electron density, the greater the affect, and the further upfield (lower frequency, lower ppm) the resonance occurs. The nature and orientation of neighbouring groups can affect the shielding and therefore the chemical shift. Several important contributions to shielding in \(^{13}\text{C}\)-NMR are outlined below (Wehrli and Wirthlin, 1976).
1. The **hybridization** of the nucleus is an important factor in determining the chemical shift.

2. **Inductive effects** such as electronegativity effects which removes electron density from the nucleus.

3. **Steric effects** which cause distortion of the electronic distribution.

4. **Electric field effects** may be generated for example, by an ionized group resulting from protonation.

5. **Mesomeric effects** are due to delocalization of charge across a π system. This is particularly relevant to the carbonyl carbon. The carbonyl carbon bears a partial positive charge which accounts for its low field chemical shift.

6. **Heavy atoms** such as F, Cl, Br, I, contain a large number of electrons which may influence electronic shielding in the observed nucleus.

Slight differences in the electronic shielding due to one or several of the above effects allows observation of single distinct resonances assignable to carbons in different functional groups and/or conformations. Of particular significance are the changes in chemical shift due to medium effects. A change of temperature, ionic strength, and solvent modify the above effects to a greater or lesser extent. For example, the chemical shift of the carbonyl resonance in benzoic acid changes from 172.6 ppm in CDCl₃ (Johnson and Jankowski, 1972), to 168.5 ppm in acetone-d₆.

In most organic compounds, the carbon nucleus under observation is in close proximity to other nuclei possessing a spin. This is usually a ¹H as ¹³C makes the ¹³C-¹³C coupling insignificant. These spins may be coupled so that combinations of the spin-states differ in energy causing splitting of the resonance signal. Most ¹³C spectra are obtained with simultaneous irradiation of the proton resonances in order to saturate these resonances and eliminate the coupling. This leads to a large increase in the signal of protonated carbons due
to NOE, and an increase in non-protonated carbon resonances depending upon the contribution of the dipolar relaxation mechanism to these carbons. The signals of non-protonated quaternary carbons may be selectively observed while the resonances of protonated carbons may be reduced or eliminated by using an NMR technique called low-power noise decoupling (Sadler, 1973). In this method, broad band decoupling at a very low power level (0.05 to 0.25 W) is applied at the proton resonances. This results in inefficient decoupling of the proton-bearing carbons which spreads the resonance frequencies for these carbons and results in broad, low intensity signals. The quaternary carbons on the other hand, are completely decoupled since the long-range coupling effects on quaternary carbons are much smaller. This means that the NOE contribution to the intensity of the quaternary carbons is retained and splitting due to long range coupling is eliminated.

Application of $^{13}\text{C}$-NMR to the study of proteins is a fairly recent development. Allerhand (1979) presented an extensive review of the techniques used for the observation of individual resonances in the $^{13}\text{C}$-NMR spectra of proteins. The $^{13}\text{C}$-NMR spectrum can be divided into regions depending upon the types of carbon nuclei present. The aliphatic region extends from approximately 0 to 100 ppm, the aromatic region (also containing the $C_6$ of arginine and tyrosine) from 100 to 160 ppm, and the carbonyl region from 160 to 210 ppm (Allerhand, 1979).

The resolution of carbon resonances is greatly affected by the freedom of rotation of the carbons under observation. Allerhand et al. (1971) have shown that sharp resonance lines (long $T_1$ and $T_2$) originate from carbons with a large degree of motion. The backbone carbons of proteins tend to exhibit broad resonances, whereas relatively free side-chain carbons yield sharp lines. This is consistent with the much sharper lines observed for denatured proteins where the backbone carbons have
a much greater degree of motion (Allerhand et al., 1973; Glushko et al., 1972; Nigen et al., 1973a). The increased resolution obtained with the denatured protein is also due to the elimination of chemical shift non-equivalence caused by folding of the polypeptide chain. Sharp resonance lines in protein spectra also arise from non-protonated carbon resonances as compared to protonated carbons because of the inherently longer $T_1$ values (Oldfield et al., 1975a). A decrease in resolution is also observed with an increase in molecular weight of the protein which causes a slower tumbling of the protein molecule resulting in a longer rotational correlation time $\tau_R$, which is the time taken for a molecule to reorient itself with respect to the magnetic field. The decrease in resolution is also caused by an increase in the number of non-equivalent resonances in a larger molecule (Oldfield et al., 1975a).

Specific assignments of individual resonances in high resolution spectra of proteins has also been accomplished (Oldfield et al., 1975b; Allerhand et al., 1977; Glushko et al., 1972). Of particular interest are the well-separated resonances at 155 and 160 ppm assignable to the $C^6$ of tyrosine and arginine residues.

In these studies, high resolution spectra were obtained using a relatively low field (14 kG) instrument. A comparison of the spectra obtained at both low and high field (63 kG) of hen egg-white lysozyme indicates that the resolution of the protonated aliphatic and methine carbons are much more greatly enhanced than the carbonyl and non-protonated aromatic resonances by an increase in field strength (Norton et al., 1977). This may be explained by the contribution of CSA to the relaxation mechanism for non-protonated unsaturated carbons at magnetic field strengths above 40 kG. The contribution from $^{13}$C-$^1$H dipolar relaxation at 63 kG is low for non-protonated carbons. The $T_1$ value for a carbonyl carbon was experimentally determined as 1.6 seconds at
this field strength. A calculated value of 7.9 second is obtained for a dipolar relaxation mechanism and 1.9 seconds when CSA effects are also taken account (Norton et al., 1977).

Allerhand et al. (1977) demonstrated the effect of the change in pH on the chemical shifts of carbons associated with an ionizing group. The behavior of a $^{13}$C resonance of such a carbon is affected by the rate of exchange between the protonated and deprotonated states. If the rate of exchange is fast compared to the difference in chemical shifts of the two states (in Hz), a single, exchange averaged resonance is observed. The chemical shift of the single resonance is a weighted average of the values for the protonated and deprotonated forms. As the pH is changed, the chemical shift of the resonance will also change. If however, the exchange between the two states is slow, two distinct resonances due to both the protonated and non-protonated species may be observed. Intermediate exchange rates may exhibit two broad peaks or one broad resonance (Allerhand, 1979). In this way the predominant tautomeric state of specific histidine residues in hen egg-white lysozyme (Allerhand et al., 1977), ribonuclease A (Walters and Allerhand, 1980) and myoglobin (Wilbur and Allerhand, 1977a) have been determined. Similarly the pK$_a$'s of tyrosine (Wilbur and Allerhand, 1976) and the terminal glycine (Wilbur and Allerhand, 1977b) of myoglobin were obtained. Shindo and Cohen (1976) determined the pK$_a$'s of a number of carboxyl groups of hen egg-white lysozyme by $^{13}$C-NMR.

In order to increase the sensitivity and observe specific resonances, many enzymes have been covalently modified with enriched $^{13}$C containing residues. Often this will provide valuable information concerning the environment of the particular residue and has also been used to verify the existence of molecular interactions in solution proposed from a study of the crystal structure. Multiple $^{13}$C labelling of amino groups of proteins by reductive methylation has allowed extensive study of the environment of the
labelled residues (Jentoft and Dearborn, 1979; Jentoft et al., 1979, 1980, 1981; Gerken et al., 1982). Ionization of a nearby carboxyl group causes a perturbation of the $pK_a$ for the $^{13}$C-methylated amino group and comparison with x-ray structures allows specific assignments to be made.

Enzymes carboxymethylated with enriched bromoacetate have provided a great deal of information concerning the behaviour of covalently bound species. Multiple carboxymethylation of myoglobin and mono-carboxymethylation of ribonuclease A is observed when these enzymes are reacted with $[2-^{13}$C$]$-bromoacetate (Nigen et al., 1972, 1973b). By comparison with model compounds, the multiple resonances observed with the myoglobins can be assigned to carboxymethylation of histidine, lysine, and terminal $-\text{NH}_2$ groups. Cytochrome c has also been carboxymethylated at several residues with $[2-^{13}$C$]$-bromoacetate and studied by $^{13}$C-NMR (Eakin et al., 1975). Khalifah and co-workers utilized $[1-^{13}$C$]$-bromoacetate as an active-site probe of carbonic anhydrase (Strader and Khalifah, 1976; Khalifah, 1977; Khalifah et al., 1977). Bromoacetate mono-carboxylates the His-200 residue found in close proximity to the essential zinc metal in the active site. The $pK_a$ of the carboxymethylated-histidine is greatly affected by the binding of inhibitors to the metal.

The binding of $^{13}$C enriched carbon monoxide to various haemoglobins and myoglobins has been widely studied (Moon and Richards, 1972, 1974; Moon et al., 1977; Vergamini et al., 1973; Matwiyoff et al., 1973; Antonini et al., 1973; Morgan et al., 1976; Giacometti et al., 1976; Banerjee et al., 1976; Banerjee and Lhoste, 1976; Choc and Caughey, 1981). Differences in the environment experienced by the $^{13}$CO in the $\alpha$ and $\beta$ chains are easily discernible as distinct resonances. Information on the interaction of $^{13}$CO$_2$ and haemoglobin has also been obtained (Morrow et al., 1973, 1974, 1976). Resonances due to formation of carbamino residues on the protein chain are clearly observable
along with resonances attributed to bicarbonate, carbonate, and dissolved $^{13}\text{CO}_2$. Carbonic anhydrase interaction with $^{13}\text{CO}_2$ (Stein et al., 1977; Koenig et al., 1973) and $^{13}\text{CN}^-$ (Feeny et al., 1973) has also been studied by $^{13}\text{C}$-NMR. Cyanide labelled with $^{13}\text{C}$ has been utilized in the modification of a specific cysteine residue in glutamate aspartate transaminase (Boettcher and Martinez-Carrion, 1975). Observation of the binding of $^{13}\text{C}$ labelled bicarbonate to essential metal atoms in enzymes by $^{13}\text{C}$-NMR has been useful in obtaining structural and mechanistic information for the enzymes carbonic anhydrase (Yeagle et al., 1975), and ribulose 1, 5-diphosphate carboxylase (Miziorko and Mildvan, 1974).

The complex between chymotrypsin and the virtual substrate N-Ac-L-tryptophan labelled at the acetyl $^{13}\text{C}=\text{O}$, was studied by $^{13}\text{C}$-NMR (Rodgers and Roberts, 1973) A decrease in the coupling constant between $^{13}\text{C}$ and the $\alpha$-proton upon binding was interpreted as evidence for a conformational change undergone by the substrate. Conversely, Robillard et al. (1974) found no evidence for strain in the Michaelis-complex formed between chymotrypsin and N-Ac-L-tyrosine-semicarbazide labelled at the tyrosine carboxyl.

The inhibition of trypsin with soybean trypsin inhibitor has been the subject of both x-ray and $^{13}\text{C}$-NMR investigations. It was first proposed from x-ray evidence that a fully tetrahedral covalent bond is formed between the Arg-63 carboxyl of STI and the serine active-site residue of trypsin (Sweet et al., 1974). Later x-ray evidence was interpreted to show a distorted carbonyl with some pyrimidal character (Huber and Bode, 1978). Baillargeon et al. (1980) and Hunkapiller et al. (1979) enriched the carboxyl carbon of Arg-63 of STI and studied its interaction with trypsin by $^{13}\text{C}$-NMR. Enriched STI by itself has a resonance typical of the amide region of the $^{13}\text{C}$-NMR spectrum at 173.3 ppm. Upon reaction with trypsin, the resonance moves slightly downfield to 174.7 ppm. The slight downfield shift would not be expected if formation of a covalent
tetrahedral adduct occurred. Baillargeon et al. (1980) predicts an upfield shift of approximately 55 ppm upon formation of a tetrahedral carbon from a carbonyl carbon based on values obtained with model compounds. For example, the carbonyl resonance of DMF shifts from 162.4 ppm to 110.6 ppm on formation of the diethyl acetal. The slight downfield shift observed in the inhibitor studies may be due to the different environment of the active-site without formation of a covalent bond, or to formation of an acyl enzyme as proposed by Hunkapiller et al. (1979). The distortion of the trigonal carbon towards a tetrahedrally oriented form as proposed by Huber and Bode (1978) cannot be ruled out from the data obtained. Richarz et al. (1980) in a similar experiment with an enriched modified basic pancreatic trypsin inhibitor, also found no evidence of a covalent tetrahedral carbon formed on interaction with trypsin.

A resonance attributed to an adduct formed between pepsin and a pepstatin analogue has been presented in the $^{13}$C-NMR work of Rich et al. (1982). A ketone analogue of pepstatin enriched at the carbonyl carbon displays a single resonance at 204.2 ppm typical of a ketone carbonyl in CDCl$_3$/CD$_3$OD. When one equivalent of the inhibitor is added to porcine pepsin in D$_2$O, a new signal at 99 ppm appears which is consistent with formation of a tetrahedral adduct. Malthouse et al., (1983) have shown that a tetrahedral adduct is formed between the Ser-195 of trypsin and the inhibitor Nα-CBZ-L-lysine-chloromethylketone. Both the neutral and anionic forms of this adduct were shown to be stabilized by the enzyme by pH titration ($pK_a$ =8).

Niu et al., (1977) detected a resonance attributed to a transient acetyl-chymotrypsin intermediate by $^{13}$C-NMR. In this study, chymotrypsin was reacted with the non-specific substrate p-nitrophenyl [1-$^{13}$C] acetate. Signals due to the substrate at 170.4 ppm and the product acetic acid at 175.1 ppm are detected along with a resonance at 174.0 ppm assigned to an acetyl-chymotrypsin. The intensity of this signal decreased as the acetic acid
signal increased and enzyme activity returned. The resonance at 174.0 ppm had a sufficiently long lifetime to allow determination of $T_1$. A value of $T_1$ for this resonance of 2.6 seconds is reported as compared to a value of 2.9 seconds for backbone carbonyls. This suggests that the carbonyl of the acetyl group is similarly restricted in motion. The results demonstrate the potential of $^{13}\text{C-}N\text{MR}$ for detection of transient enzyme-bound species.

Ghisla et al. (1978) present results of a particularly interesting experiment. These investigators provide evidence for detection of an intermediate in the bacterial luciferase catalysed oxidation of flavin mononucleotide (FMN) by $^{13}\text{C-NMR}$ at low temperature. Evidence obtained by absorption spectrophotometry for the enzyme intermediate has proven unreliable and lacks diagnostic information. The authors used $^{13}\text{C-NMR}$ spectroscopy combined with low temperature enzymology in an attempt to provide more useful information on the transient intermediate. FMN was specifically enriched in the C-4a carbon as this has been proposed as the site of oxidation by the enzyme. A narrow resonance at 137 ppm assigned to the free oxidized FMN substrate in solution was observed superimposed upon a broad signal attributed to the same species bound to the enzyme. Upon reduction, the signal splits into two resonances at 103 and 104 ppm. Addition of ethylene glycol cosolvent and reduction of the temperature further broadened the signal due to the bound-reduced FMN to a linewidth of 50 Hz. Broadening of the signal was probably due to the increased viscosity resulting in decreased molecular motions. Upon addition of oxygen at $-15^\circ\text{C}$, the resonances at 103 and 104 ppm disappeared and reoxidized FMN appeared at 137 ppm and a new broad (50 Hz) resonance at 74 ppm appears. The resonance at 74 ppm disappeared on warming and resulted in observation of oxidized FMN at 137 ppm. The results can be interpreted as evidence for specific oxygenation of the C-4a carbon in FMN catalysed by bacterial luciferase. With the signal-to-noise ratio obtained in this study, the interpretation of the resonances is not entirely convincing. However, these results illustrate the
type of information which may be obtained utilizing $^{13}$C-NMR in
the analysis of low temperature enzyme-catalysed reactions.

The versatility of $^{13}$C-NMR is well illustrated since it
can be used with a wide variety of enzymes for various experiments
ranging from the determination of a microscopic $pK_a$ to the
detection of specific enzyme-bound intermediates.

When combined with low temperature enzymological tech-
niques, $^{13}$C-NMR has the unique potential of supplying a vast amount
of structural, conformational, and kinetic information on transient
enzyme intermediates and inhibitor complexes. The application of
these techniques to the study of intermediates along the pathway
for papain-catalysis is especially interesting. Papain is a well
studied enzyme with a large amount of kinetic and structural information
available from previous investigations using other more conventional
techniques. This is important since we need to develop this new
combination of $^{13}$C-NMR and cryoenzymology methods on such a
familiar system rather than starting with a relatively unstudied
enzyme. Of course the complete mechanistic pathway of papain-
catalysed reactions is still in question and some of the possible
intermediates outlined previously should provide distinct changes in
the $^{13}$C-NMR spectra of enriched substrates. A thioester intermediate
should be well separated downfield from the enzyme background
resonances and a proposed tetrahedral intermediate should show
a dramatic upfield change in chemical shift in transforming from
an $sp^2$ to and $sp^3$ hybridized carbon. Papain has also been previously
studied by cryoenzymological techniques which is a great advantage
to this work since a new system may not be amenable to either
cryoenzymology or $^{13}$C-NMR. Yet some inconsistencies in the
cryoenzymological results for papain require further investigation.

The first stage in the development of this method is to find
the capabilities and requirements of the system. This is done by
varying the concentrations of reactants, the solvent mixtures, and
finding optimal acquisition parameters on the NMR. In this way, the feasibility of cryoenzymology combined with $^{13}$C-NMR spectroscopy for the detection of enzyme intermediates and structural elucidation of enzyme-inhibitor complexes can be evaluated.
MATERIALS

AND

METHODS
Instrumentation

UV-Visible Spectrophotometry

Low temperature absorption spectrophotometry was performed on a Varian Cary 210 UV-Visible spectrophotometer modified for low temperature work using a design similar to that described by Maurel et al. (1974). Dry, gaseous nitrogen is passed through a copper heat exchanging coil immersed in liquid nitrogen. The cooled gas is delivered to the sample compartment through glass vacuum tubing and the temperature of the gas is regulated by passage over electrical resistance heating coils controlled by an Oxford Instruments DTC 2 digital temperature controller. The cooled N₂ is then circulated through two brass sample cell holders (for the sample and reference cells) designed to accommodate 1 cm quartz cells. The nitrogen outlet is directed onto the cell walls to prevent condensation and ice formation. The exiting cooling gas provides a positive pressure of nitrogen in the sample compartment to prevent condensation on the optical lenses of the spectrophotometer. Gas and sample temperatures were measured using a Comark 5000 digital thermometer fitted with Cu/Cu-Ni thermocouples. The sample temperature was measured directly using a plastic coated thermocouple in the solution. Temperatures down to -90°C can be routinely maintained using this apparatus.

Absorption spectrophotometry at 25°C was performed on a Varian DMS-90 UV-visible spectrophotometer with a Varian Model 9176 recorder. Constant temperature was maintained with a thermostatted circulating water bath (Grant Instruments (Cambridge) Ltd., Model SE 10).
**pH Measurement**

pH measurements were determined with a Beckman Model 3500 digital pH meter or Radiometer pHM 82 standard pH meter with Beckman combination electrode 39505 or Radiometer electrode GK2401c. The pH meters were standardized using Radiometer buffer solutions S1306 pH 1.68, S1316 pH 4.01, Beckman buffer solutions S3581 pH 7.00, 3506 pH 4.00, 3009 pH 9.18, 3505 pH 10.01 at 25°C.

**Titrimetry**

Titrimetric assays were performed using a Radiometer pH meter 25 with Titrator Model 11, Titrigraph Model SBR2c, and syringe burette Type SBU1a.

The reaction mixture was maintained at 25°C using a water-jacketed reaction vessel connected to a Shandon thermostatted circulating water bath.

**Conductance Measurements**

Buffer gradient for ion-exchange chromatography was determined using a Portland Electronic Conductivity Meter Model P310 fitted with a Pye-Unicam Conductivity Cell. Samples were diluted 1 in 10 in water.

**Ion-exchange Chromatography**

Fractions were collected during ion-exchange chromatography on an LKB 2070 Ultrorac II and a constant flow rate was provided by an LKB 2132 Microperplex peristaltic pump.
$^{13}\text{C-NMR Spectrometry}$

$^{13}\text{C-NMR}$ spectra were obtained on a Bruker WM-300 wide-bore NMR spectrometer at 75.47 MHz for $^{13}\text{C}$-nuclei. Samples from 0.5 to 15 mls were used in 5, 10, or 20 mm sample tubes. Temperatures were maintained with a Bruker VT-1000 variable temperature control unit using cooled gaseous nitrogen for low temperature work. Probe and sample temperatures were measured with a Comark digital thermometer with Cu/Cu-Ni thermocouples.
Materials

Papain

Dried papaya latex (Papain P. S. I) was obtained from Powell and Scholefield, Ltd., 38 Queensland St., Liverpool, L7 3JG.

Substrates

The following substrates were obtained from Sigma London, Chemical Co., Ltd., Fancy Road, Poole, Dorset, BH17 7NH.
- Na-CBZ-L-lysine-p-nitrophenyl ester
- Na-CBZ-glycine-p-nitrophenyl ester
- Na-CBZ-L-tyrosine-p-nitrophenyl ester
- Na-CBZ-L-alanine-p-nitrophenyl ester
- Na-Benzoyl-DL-arginine-p-nitroanilide (DL-BAPNA)
- Na-Benzoyl-L-arginine-p-nitroanilide (L-BAPNA)
- Na-Benzoyl-L-arginine ethyl ester (L-BAEE)

The following substrates were synthesized by Dr. N. E. Mackenzie, Texas A and M University, College Station, Texas, USA.

- N-benzoylimidazole (prepared by the method of Gerngross, 1913)
- $^{13}$C=O-N-benzoylimidazole (prepared as above using $^{13}$C-benzoic acid.
- N-acetyl-L-phenylalanylglycine-p-nitrophenyl ester (prepared by the method of Lowe and Yuthavong, 1971 a)

Inhibitors

The following inhibitors were synthesized by Dr. N. E. Mackenzie and others as listed.
N-acetyl-L-phenylalanylglycinal dimethyl acetal  (with Dr. R. A. Russell by the method of Mattis et al., 1977)

\[ \text{N-acetyl-L-phenylalanyl-}[1-^{13}\text{C}]-\text{glycinal dimethyl acetal} \]

(with W. U. Primrose by the procedure outlined below).

\[ [1-^{13}\text{C}]-\text{glycine was fused with phthalic anhydride at 145-150}^\circ \text{C}. \] The \([1-^{13}\text{C}]-\text{N-phthalyl-glycine is reacted with thionyl chloride in benzene with heating for 2 hours. The acid chloride is converted to the aldehyde by hydrogenation with 5}\% \text{ Pd on BaSO}_4 \text{ in toluene at 100}^\circ \text{C with a trace of quinoline present.} \]
The aldehyde was converted to the dimethyl acetal by treatment with trimethyl orthoformate, with toluene sulphonylic acid in benzene with heating for 30 minutes. The N-phthalyl group was removed by treatment with 64\% hydrazine hydrate in pure ethanol with heating over 2 hours. The \([1-^{13}\text{C}]-\text{glycinal dimethyl acetal was coupled to N-acetyl-L-phenylalanine using dicyclohexylcarbodiimide in dichloromethane at room temperature over 2 days.} \]

\[ \text{Na-CBZ-L-lysine-chloromethyl ketone (1-chloro-3-(carbo-} \]

\[ \text{benzyloxyamino)-7-aminoheptan-2-one) was synthesized by the method of Coggins et al. (1974) and Bezas and Zervas (1961).} \]

\[ [2-^{13}\text{C}]-\text{Na-CBZ-L-lysine chloromethyl ketone was synthesized by the same methods using [1-^{13}\text{C}]-L-lysine.} \]

Other \(^{13}\text{C}-\text{labelled Compounds} \]

\[ [1-^{13}\text{C}]-\text{benzoic acid was obtained from Stohler Isotope Chemicals, Waltham, Massachusetts, USA (90}\% ^{13}\text{C-enriched)} \]

\[ [1-^{13}\text{C}]-\text{D, L-lysine, 90 atom } \% ^{13}\text{C, was obtained from Merck, Sharp, and Dohme, Canada, Ltd.} \]

\[ [1-^{13}\text{C}]-\text{L-lysine (90}\% ^{13}\text{C enriched) was obtained from Stohler Isotope Chemicals.} \]
$^{13}$C=O]-benzoic anhydride-synthesized by Dr. N. E. Mackenzie by the reaction of [1-$^{13}$C]-benzoic acid with [1-$^{13}$C]-benzoyl chloride.

$^{13}$C=O]-phenylthiobenzoate-synthesized by Dr. N. E. Mackenzie using the method of Schiller and Otto (1876) using [1-$^{13}$C]-benzoic acid.

Other Compounds

2,2'-dipyridyl disulphide (Aldrithiol-2, 98% was obtained from Aldrich Chemical Co., The Old Brickyard New Road, Gillingham, Dorset SP8 4JL.

Ribonucleic Acid (Yeast) Highly Polymerized, Calbiochem-Behring, C.P. Laboratories, Bishops Stortford, Herts, CM22 7RQ.

Sodium Dodecyl Sulphate (Specially purified for biochemical work), BDH Chemicals, Ltd., Poole.

Methyl methane thiosulphonate, DL-dithiothreitol, N-acetyl-L-cysteine, L-cysteine (free base), mercaptoethanol, glutathione, were obtained from Sigma.

EDTA dipotassium salt was from BDH.

Benzoic acid anhydride, 98% and p-nitrophenol were obtained from Aldrich Chemical Co.

Organic Solvents

Dimethyl sulphoxide (Analytical Reagent grade) from Fisons Scientific Apparatus, Loughborough, Leics, was distilled from
calcium hydride under reduced pressure at 35°C. The purified DMSO was separated into 25 ml fractions and stored in a dessicator at 4°C. When required, the solid DMSO was melted at room temperature and stored in a dessicator.

Methanol, acetonitrile, 1,4-dioxane (all 'Spectrograde') were obtained from Fisons Scientific Apparatus and used without further purification.

Acetone (analytical reagent) from Fisons was distilled before use.

**Deuterated Solvents**

Deuterium Oxide, 99.8% D, was obtained from Goss Scientific Instruments, Ltd.

Dimethyl Sulphoxide-d₆, 99.9 atom % D, Gold Label, was from Aldrich Chemical Co.

**Chromatographic Materials**

Sephadex G-25 (Medium), Sepharose 2-B, SP-Sephadex C-50 cation exchange resin were obtained from Pharmacia Fine Chemicals, AB, Uppsala, Sweden.

Thin-layer chromatography was carried out on Polygram Silica Gel Sil G/UV 254, 0.25 mm pre-coated plastic sheets, from Macherey-Nagel, 516 Duren, Werkstrasse, 6-8, Postfach 307, West Germany.

Sepharose-(glutathione-2-pyridyl disulphide) gel was prepared by the method of Brocklehurst et al. (1973).
NaOH and HCl

NaOH and HCl solutions used were volumetric solutions (4M and 5M respectively) obtained from BDH Chemicals, Ltd.

Glass distilled, deionised water was used throughout.

All other solvents, salts, and buffers were of the highest purity available.
Methods

Isolation of Papain

Papain was isolated from the dried latex by the method of Baines and Brocklehurst (1979). Further purification was achieved by chromatography on sepharose-(glutathione-2-pyridyl disulphide) covalent gel by the method of Brocklehurst et al. (1973). Typically, 0.2 to 0.6 g of covalent gel purified papain can be isolated from 100 g of dried latex. The papain was stored as the mercuric salt by dissolving the enzyme in 200-300 mls of water ([E] = 100 to 200 μM) and dialyzing overnight at 4°C against 5-6 litres of an equal concentration of HgCl₂ in water. The Hg-papain was stored in solution at 4°C.

For the ¹³C-NMR experiments, the papain from the covalent gel preparation was immediately redissolved and concentrated before activation. Each NMR experiment required the enzyme from at least one complete preparation (i.e. 100 g latex).

Activation of Papain

Papain stored as a mercuric salt was activated in 0.1 M Tris/HCl buffer, 1 mM EDTA, pH 8.2 with either 30 mM cysteine or 5 mM DTT. Activation with cysteine required stirring for 1/2 hour, whereas DTT activation required only 1 to 2 minutes in order to avoid denaturation by enzyme disulphide bond cleavage. Low molecular weight activating agents and mercuric salts were removed by gel-filtration.

Concentrated papain solutions for ¹³C-NMR experiments were activated by adjusting the pH to 8.2 using 1 M Tris/HCl and slowly adding DTT to a concentration of 5 mM through a syringe.
Rapid addition of DTT caused precipitation of the enzyme. In a typical experiment, 5 ml of 50 mM DTT was added to a rapidly stirred solution of 50 ml of 300 μM papain, pH 8.2 over a period of 2 minutes. After the addition was complete, the activated enzyme was gel-filtered.

**Gel-Filtration**

Low molecular weight compounds were separated from protein solutions by gel-filtration on Sephadex G-25. Small samples were applied to a 3 x 20 cm column and eluted with 10 mM KCl. Larger samples (for example, when preparing enzyme for a $^{13}$C-NMR experiment) were divided and applied to two 5 x 20 cm columns and eluted with 10 mM KCl. Fractions were collected and the protein was detected by its absorbance at 280 nm ($\varepsilon_{280} = 56000 \text{ M}^{-1} \text{cm}^{-1}$, Baines and Brocklehurst, 1979).

**Preparation of 2-PDS Solutions**

Saturated solutions of 2-PDS (~1.5 mM) in water were prepared by stirring solid 2-PDS (~20-30 mg) in 25 ml of water for 30-60 minutes at room temperature. Excess solid was removed by filtration. The concentration of 2-PDS was determined by UV-spectrophotometry ($\varepsilon_{281} = 10200 \text{ M}^{-1} \text{cm}^{-1}$, Shipton and Brocklehurst, 1978).

**Thiol Determinations**

The reactive thiol content of papain solutions was determined by the spectrophotometric method of Baines and Brocklehurst (1978, 1979). The samples were prepared as follows:
Sample cell  
0.75 ml of B  
1.75 - x ml water  
x ml Papain solution

Reference cell  
0.75 ml of B  
1.75 ml water

Solution B was either 0.4 M acetate, pH 4.1, or 0.4 M Tris/HCl, pH 8.2, with both buffers containing 0.4 M KCl and 1 mM EDTA.

The spectrophotometer was balanced at 343 nm and 0.5 ml of saturated 2-PDS solution was added. Thiol concentration was calculated from the absorbance of the 2-thiopyridone released ($\epsilon_{343} = 8080 \text{ M}^{-1} \text{cm}^{-1}$, Stuchbury et al., 1975). Equal A$_{343}$ values at pH 4.1 and 8.2 indicated no contamination with chymopapain or denatured enzyme. Papain isolated by the salt precipitation method of Baines and Brocklehurst (1979) usually possessed 0.65 to 0.75 thiol/mole of protein. Enzyme further purified by covalent gel chromatography (Brocklehurst et al., 1973) had between 0.85 to 0.95 thiol/mole.

Concentration of Enzyme

Concentration of papain solutions, before and after activation, was carried out with Amicon ultrafiltration cells Models 52 or 402 with PM 10 membranes. Smaller samples (~5-10 ml) were concentrated in Minicon-B clinical sample concentrators from Amicon Corporation. Papain solutions up to 3 mM were obtained with no precipitation at room temperature. Papain solutions > 200 µM crystallize at 4°C.

Preparation of Enzyme in Cryosolvent

For low temperature work, papain solutions were prepared in DMSO cryosolvents. Papain in 25% DMSO solvents were stable
at room temperature with no loss of thiol as estimated by 2-PDS titration at pH 4 over 8 hours. Papain solutions containing greater than 25% DMSO were maintained at \( \leq 2^\circ C \) to avoid denaturation.

To prepare enzyme solutions in cryosolvent, DMSO was added slowly to a concentration of about 10% at room temperature. The solution was then cooled to 0-2\(^\circ\)C in an ice-bath and the concentration of DMSO was slowly increased. The addition of DMSO caused the temperature to rise and the solution was allowed to cool between additions such that the temperature did not rise above 5\(^\circ\)C. The concentrated papain solutions in cryosolvent were stored at or below 0\(^\circ\)C. The activity of papain in 50% DMSO solutions remained constant for days (see enzyme purity section). Papain solutions up to 2.5 mM were found to be stable in 25 and 50% DMSO solutions at low temperatures. (All DMSO concentrations used in this thesis are calculated on a volume to volume basis)

**Buffers**

The following buffer systems were used in the pH ranges indicated.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>0-5%</th>
<th>25%</th>
<th>40%</th>
<th>50%/60%</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCl/KCl</td>
<td>1.0-2.0</td>
<td>1.0-2.0</td>
<td>3.0-3.5</td>
<td>3.0-3.5</td>
</tr>
<tr>
<td>Glycine/HCl</td>
<td>2.0-2.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Formic acid/NaOH</td>
<td>3.0-4.3</td>
<td>3.6-4.5</td>
<td>4.0-4.5</td>
<td>4.3-6.0</td>
</tr>
<tr>
<td>Acetic acid/NaOH</td>
<td>4.0-5.5</td>
<td>4.5-6.0</td>
<td>5.5-6.5</td>
<td>6.0-7.0</td>
</tr>
<tr>
<td>KH(_2)PO(_4)/NaOH</td>
<td>6.0-7.5</td>
<td>7.0-8.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cacodylic acid/NaOH</td>
<td>6.0-7.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris/HCl</td>
<td>8.0-9.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Boric acid/NaOH</td>
<td>9.0-10.0</td>
<td>9.5-10.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbonic acid/NaOH</td>
<td>10.0-11.0</td>
<td>-11.0-12.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Buffer concentrations ranged from 10 mM to 0.2 M, all
buffers contained 1 mM EDTA unless otherwise noted. KCl concentrations ranged from 0 to 0.2 M.

The buffers in cryosolvent were prepared by adding DMSO to the buffer solutions at 0°C. The pH* (the pH in aqueous-organic solvents) was determined using a standard pH electrode at 0-2°C. The pH* values determined at 0°C were uncorrected for the change in sensitivity of the electrode in cryosolvent at 0°C (determined to be 0.1 pH unit for the Radiometer electrode). Buffer pK*a's in the cryosolvents and the pH* values at low temperature were estimated from the data of Douzou (1973, 1977). Unless otherwise indicated the pH* values given are the pH* values determined at 0°C.

**Papain-catalysed hydrolysis of L-BAEE**

The activity of papain toward L-BAEE was determined by observing the initial rates of hydrolysis by titrimetric assay at pH 6. Papain (final concentration in the cell of 0.5 to 1 μM) was added to the titration cell containing L-BAEE (5 to 100 mM), 0.1 M KCl, 1 mM EDTA, 10 mM cysteine. The titrant used was 0.1 M NaOH which was standardised using 1.0 ml of 20.0 mM HCl.

For the relative activity of papain (20 μM) stored in 60% DMSO, 0°C, the L-BAEE concentration in the cell was 20 mM, enzyme in the cell was 0.6 μM. The control experiment contained the same concentration of enzyme in aqueous solution without DMSO.

**N-Benzoylimidazole-aqueous hydrolysis**

The rate of non-enzymatic hydrolysis of N-benzoylimidazole was determined in 0.1 M buffer, I = 0.1, by following the decrease in absorbance at 245 nm. 0.05 ml of 6.34 mM N-benzoylimidazole (1 in 1000 dilution of neat in DMSO) was added to 2.95 ml of buffer and the decrease in absorbance was followed to completion (A∞).

For the hydrolysis in 26.7% DMSO, each buffer contained
25% DMSO and the pH\textsuperscript{*} was measured at 2\textdegree{}C. Ionic strength was maintained at 0.1 M using KCl.

A plot of \(\ln(A_t - A_\infty)\) vs time produced straight lines for \(\geq 95\%\) of the hydrolysis. The half-life for the reaction was calculated from \(t_{1/2} = \frac{0.693}{k_{\text{obs}}}\).

**Papain and N-Benzoylimidazole**

A solution of concentrated papain (concentration between 0.1 and 2 mM, with or without DMSO) was adjusted to the appropriate pH with 1 M formate (to a final concentration of 0.1 M formate). A twenty-fold excess of N-benzoylimidazole in DMSO was added and the mixture was stirred for 1 to 2 minutes. For reactions below 0\textdegree{}C, N-benzoylimidazole was added and stirred at 0\textdegree{}C before lowering the temperature. An aliquot of the reaction mixture was removed and added to a cell containing 2-PDS in 0.1 M formate, pH 3.8 at 25\textdegree{}C. To compensate for the absorption of the large excess of N-benzoylimidazole, a similar solution containing N-benzoylimidazole and DMSO without papain was added to the reference cell. The molar extinction coefficients of N-benzoylimidazole and the hydrolysis products benzoic acid and imidazole are similar and contribute a small amount to the overall absorbance (N-benzoylimidazole, \(\epsilon_{343} = 20 \text{ M}^{-1}\text{cm}^{-1}\); benzoic acid + imidazole, \(\epsilon_{343} = 18 \text{ M}^{-1}\text{cm}^{-1}\)). The deacylation was followed at 343 nm and the rate was determined from the log plot of the first-order curve.

Aliquots of the papain-N-benzoylimidazole mixture were removed at various time intervals and added to the 2-PDS titration cells at 25\textdegree{}C. The burst of 2-thiopyridone at \(t = 0\) was equal to the free, unacylated enzyme in solution. The \(A_0\) value was best determined from the log-plot extrapolated to \(t = 0\). The burst and subsequent deacylation is complete (\(A_\infty\)) in 25-30 minutes at pH 3.8.
The fraction of enzyme unacylated is equal to \( \frac{A_o}{A_\infty} \). A plot of 
\[ \ln (\text{acylated}) = \ln \left( 1 - \frac{A_o}{A_\infty} \right) \] versus time produced a straight line with a slope equal to the deacylation rate.

In each titration, the 2-PDS concentration was in at least a ten-fold excess over enzyme concentration. N-benzoylimidazole itself had no activity toward 2-PDS.

Slight errors are introduced when pipetting cold DMSO-water solutions when adding aliquots to the titration cell, but over an 80° range (20°C to -60°C), this error is less than 5% (for 50% DMSO solutions, Douzou and Balny, 1978). The pH of the solution at -10°C can be estimated to be approximately 0.1 pH unit higher than at 0°C (Maurel et al., 1975; Douzou, 1973).

In the \(^{13}\)C-NMR experiments, the \(^{13}\)C=O-N-benzoylimidazole was added and the solution stirred at 0°C for 2 minutes before the temperature of the sample was rapidly lowered to -7°C in an alcohol bath for 2 minutes before placing the sample in the spectrometer. A 0.5 ml sample was removed and stored at -7°C for assay by 2-PDS during the NMR time-course experiments.

**Papain and Na-CBZ-L-lysine-p-nitrophenyl ester**

The solutions of Na-CBZ-L-lysine-p-nitrophenyl ester were prepared by dissolving a known weight of solid substrate in DMSO. The concentration was determined by adding 0.05 ml of the substrate in DMSO to 2.95 ml of 1M NaOH and determining the absorbance of the p-nitrophenolate at 400 nm (\( \epsilon_{400}^{\text{(pNP)}} = 18900 \text{ M}^{-1} \text{ cm}^{-1} \), Goren and Fridkin, 1974). This value was usually within 10% of the value determined from the weight of the solid compound dissolved in the DMSO.

The buffers used in these experiments varied in concentration from 0.01 to 0.2 M with KCl concentrations from 0 to 0.2 M. All buffers contained 1 mM EDTA except in the RNA experiments where 25 \( \mu \)M EDTA was used.
Low temperature techniques varied slightly depending upon the temperature required for the experiment. At +3 to 0°C, the enzyme solution in water was added directly to the pre-cooled buffer in the sample cell. The usual procedure was to record a baseline (from 250-500 nm) with only cryosolvent in the spectrophotometer cells. Enzyme was added, the solution mixed, and the spectrum scanned again. The spectrometer was then set at a single wavelength and an absorbance versus time spectrum was recorded during the substrate addition. The substrate was added after dilution with 1 mM HCl solution (10-50% 1 mM HCl) to prevent freezing (DMSO, f. p. +18°C) and substrate decomposition at higher pH. The mixing of enzyme and substrate was performed with a glass paddle stirrer driven by an electric motor. Complete stirring was usually accomplished in about 1 minute. The mixing was checked by a repeat stirring after a 2-3 minute interval. Properly stirred mixtures show no abrupt changes in absorption after a repeat stirring, but vigorous mixing produces bubbles which will cause fluctuations in absorbance measurements and therefore must be avoided. In most of the experiments, the enzyme was added first to the buffer solutions to allow full equilibration of the enzyme at the pH* and the temperature of the reaction.

For lower temperatures, enzyme in water could be added at 0°C and the temperature of the enzyme in the buffer solution was lowered, or the enzyme in the cryosolvent was added at the desired low temperature. The second procedure was often preferred when attempting several runs at low temperature since continually raising and lowering the temperature and waiting for the system to equilibrate is a time consuming process. Addition of substrate in cryosolvent at low temperature required precooling of the substrate solution so that the addition did not cause dramatic temperature variations. Pre-cooling of the paddle stirrer was also found to be necessary at very low temperatures (< -30°C). Some change in the temperature cannot be avoided after the addition and stirring but the sample
generally returns to a constant temperature after 2 to 3 minutes. During this time interval, the temperature is frequently recorded. The temperature of the sample is usually stable to ± 0.2°C with the apparatus used.

In 50 and 60% DMSO solution at temperatures below -50°C, the solutions became extremely viscous. Mixing problems which produce air bubbles or temperature inhomogeneity were clearly evident in the experiments as fluctuations in the absorbance versus time curves. The presence of air bubbles or a precipitate in the cell could be detected by scanning the spectrum at long wavelengths (500-600 nm) where no species present absorbs light but air bubbles and precipitates cause perturbations.

The extinction of p-nitrophenol in these solutions was checked upon completion of the hydrolysis by adding a small amount of a standard solution of p-nitrophenol to the cell. After completion of the low temperature experiments, the cell contents were warmed to 0°C and the pH of the solution was checked. Cells containing high concentrations of the substrate required 0.2 M buffer solutions.

An initial rate method was also used to determine the effects of temperature on $k_{cat} / K_m$. For example, in 60% DMSO solutions, substrate was added to the enzyme solution at -45°C to a concentration of 200-300 μM. The initial rate of hydrolysis of the first 5% of the substrate was observed and the temperature raised five or ten degrees to get another determination from the same sample. The substrate concentration at the higher temperature can be obtained from the difference extinctions and $k_{cat} / K_m$ can be calculated. Three or four determinations can be obtained over a twenty degree temperature range. Over a greater range, the reaction may become too fast at higher temperatures since the enzyme concentration is constant.

The effect of DMSO on the kinetic parameters was determined in 1.7 to 50% DMSO solutions. In this case, 0.1 M acetate buffer
with 1 mM EDTA was used in each determination without correcting for the variation in ionic strength. Using formate buffer with a different pK$_a$ in 50% DMSO had no effect on the parameters obtained. The effect of RNA on the kinetic parameters was determined by preparing 10 mM acetate buffer with 10 mM KCl, 25 μM EDTA, pH 5.9, 50% DMSO with varying amounts of RNA (0 to 4 mg) in 3 ml. A stock solution of concentrated RNA was added to each cell. The molecular weight of RNA was estimated as 50000 g/mole (Douzou and Balny, 1977).

Other p-nitrophenyl ester substrates

Cryoenzymological investigations with CBZ-glycine-p-nitrophenyl ester and N-acetyl-L-phenylalanyl-p-nitrophenyl ester were performed using identical techniques to those described in the papain-CBZ-L-lysine-p-nitrophenyl ester section.

Interpretation of Kinetic Data

Values of [S] and v were obtained from complete curves by the method of Waley (1981). The data was fitted to the Michaelis-Menten equation by the method of Wilkinson (1961). Each determination from a single complete progress curve listed in the results section has a standard error computed by this method.

The activation energies were obtained by non-weighted linear regression of ln(k$_{obs}$) vs (1/T) data.

Papain and Na-CBZ-glycine-p-nitrophenyl ester at 25°C

This substrate was used to determine the relative activity of papain solutions. The activity was determined in 0.1 M acetate, pH 5.0. The concentration of substrate in the cell was approximately
100 µM and the enzyme concentration varied from 0.1 to 0.5 µM. The hydrolysis was monitored by the increase in absorption at 340 nm ($\Delta e_{340} = 6230 \text{ M}^{-1} \text{ cm}^{-1}$).

**Papain inhibition with N-acetyl-L-phenylalanylglycinal**

N-acetyl-L-phenylalanylglycinal dimethyl acetal was hydrolysed in either 0.05 or 0.2 M HCl (10 mg of dimethyl acetal in 5 ml of acid) under argon or nitrogen with rapid stirring. The hydrolysis was monitored by thin layer chromatography (CHCl$_3$:MeOH, 9:1; Acetal $R_f = 0.63$, Aldehyde $R_f = 0.38$). In 0.05 M HCl, the hydrolysis was complete in 18 hours, and in 0.2 M HCl in 5 hours. The concentration of aldehyde in solution was estimated from the phenylalanine absorbance ($\varepsilon_{258}$(Phe) = 200 M$^{-1}$ cm$^{-1}$; Greenstein and Winitz, 1961).

The inhibition constant $K_i$ for this competitive inhibitor was determined using CBZ-glycine-p-nitrophenyl ester as a substrate and the method of Henderson (1972). At pH 7, a constant amount of enzyme was mixed with varying amounts of inhibitor ([I]$_0$ = 0.1 to 16 µM). The substrate was added and the initial rate was observed at 340 nm. The substrate concentration was held constant for each [I]. The substrate concentration is then changed and the process repeated ([S]$_0$ = 10 to 60 µM). The rate of the uninhibited reaction ($v_o$) was compared to the rate of hydrolysis with inhibitor present ($v_1$). For each substrate concentration, a linear plot of ($v_o / v_1$) vs $I/(1-(v_o / v_1))$ was obtained. A plot of the slopes of these lines against substrate concentration yields a line with a y-intercept of $K_i$. The $K_i$ determination was performed in duplicate.

The stoichiometry of inhibition was determined by the method of Morrison (1969) using L-BAPNA as substrate. This substrate allowed the enzyme concentration to be varied over a wide range without the reactions becoming too rapid for observation. At
constant \([S]\) (1 mM), \([E]\) was varied from 0.05 to 1.6 \(\mu M\) for different values of \([I]\). Inhibitor concentrations varied between 0.03 and 0.3 \(\mu M\). A plot of \([E]\) vs rate produced a series of curves for each inhibitor concentration. The asymptote of the curves intersected the x-axis at \([E] = [I]\), showing 1:1 stoichiometry.

In the \(^{13}\text{C}\)-NMR experiment, acetal hydrolysis was followed in \(D_2O\) solution containing 5\% 4M HCl by both \(^{13}\text{C}\)-NMR and thin-layer chromatography. After addition of the aldehyde to the enzyme and accumulation of the spectra, solid 2-PDS was added to the inhibition mixture and stirred for 1.5 hours. The mixture was centrifuged at room temperature to remove the excess 2-PDS. The 2-PDS concentration in the sample was estimated by scanning the spectrum of the sample at pH 8.2 and obtaining the \(A_{343}\). 30 mM cysteine was added and the spectrum rescanned. The amount of 2-thiopyridone released upon addition of cysteine was calculated from \(A_{343}\) and is double the amount of 2-PDS originally in the sample.

**BAPNA Hydrolysis**

For rate assays using BAPNA, a standard 1 mM solution was prepared by dissolving 43.5 mg BAPNA-HCl (MW=434.9 g/mol) in 1 ml of DMSO with slight warming. This was diluted to 100.0 ml in 0.1 M phosphate, pH 7.0 containing 10 mM cysteine at 25\(^\circ\)C.

0.1 ml of enzyme solution was added to 2.9 ml of the BAPNA solution ([E] = 1 \(\mu M\)) and the initial rate of hydrolysis was obtained at 410 nm (\(\Delta_{410} = 8800 \text{ M}^{-1}\text{ cm}^{-1}\); Mole and Horton, 1973). L-BAPNA without cysteine was used in the aldehyde inhibition studies and D, L-BAPNA elsewhere.

**Papain inhibition with N\(_2\)-CBZ-L-lysine-CMK**

CBZ-L-lysine-CMK hydrochloride was dissolved in 1 mM HCl.
and stored at 4°C. The concentration of material was calculated from the weight dissolved. CMK solution was added to papain in phosphate buffer pH 7.0 and stirred for 15 minutes. Aliquots of this mixture were removed and the activity to CBZ-glycine-pNP, BAPNA, or 2-PDS was determined. After reaction with CMK, low molecular weight material was removed by gel-filtration.

**Denaturation of Papain**

Papain blocked with CBZ-L-lysine-CMK was denatured by adding 0.2 ml of 33% sodium dodecyl sulphate (by weight) to 10 ml of rapidly stirring papain solution from the $^{13}$C-NMR experiment at pH 7.1. The enzyme immediately began to precipitate out of solution and 0.025 ml of 10 M HCl was added to bring the pH to 2. The precipitate immediately redissolved and was used in the $^{13}$C-NMR experiment.

**Preparation of Papain-S-S-CH$_3$**

Papain blocked as a disulphide with an (-S-CH$_3$) group was prepared by the method of Smith et al. (1975). A two-fold excess of methyl methanethiolsulphonate was added to a stirred solution of papain pH 7.8. After treatment, activity to BAPNA was negligible. The blocked papain was eluted down a G-25 column to remove the excess methyl methanethiolsulphonate and CH$_3$SO$_2$H. The enzyme may be unblocked with cysteine or 2-mercaptoethanol by stirring at pH 8 with the recovery of full activity.

**$^{13}$C-NMR spectral parameters**

In general, $^{13}$C-NMR spectra were recorded over a spectral width of ~230 ppm (~17000 Hz) with an acquisition time of around 0.24 seconds which gave 8 K time domain data points. The spectral
width and acquisition time varied slightly depending on the experiment. A 10 µs pulse width was used in all experiments. A 45 µs pulse width corresponds to a flip-angle of 90° which varies ±15% depending upon the solution used in 20 mm sample tubes.

The spectral references were either DMSO-d₆ (39.5 ppm from TMS) or D₂O with shifts referenced to external TMS at 0.0 ppm.

These spectral parameters were used throughout the experiments with variations in the decoupler power used.

For low-power noise-decoupled spectra (see Sadler, 1973), broad-band decoupling at a very low decoupling power level (0.4 W) is applied at the proton resonances. This acts to broaden non-quaternary carbon resonances due to partial decoupling thereby reducing their intensity. Quaternary carbon resonances however retain NOE. This technique was used for observation of carbonyl resonances.

To retain NOE in non-decoupled spectra, low-power (0.4 W) broad-band decoupling was applied only immediately prior to the pulse for approximately one-fourth of the acquisition time. This allows for retention of the favourable NOE effects which decay slowly, but the spectrum is acquired after the decoupler has been switched off (See sequence below). This allows acquisition of a non-decoupled spectrum.

![Diagram of pulse sequences](image-url)
For fully decoupled spectra, high-power (6.4 W) broadband decoupling was used. Application of high-power radio frequencies to solutions of high ionic strength results in dielectric heating of the solution. By switching off the decoupling power for short periods of time, the solution has a chance to cool before a second application. This is illustrated below.

\[\text{\^{1}H-decoupler} \quad \text{on} \quad \text{off} \]

\[\text{\^{13}C-rf pulse} \quad \text{on} \quad \text{off} \]

The decoupler power is switched on during the pulse and acquisition time. The decoupler is switched off immediately after acquisition and the delay before the second pulse is 2 to 5 acquisition times. The exact length of the delay is determined by the solutions used and preliminary trials to establish the length of the delay which produces an acceptable amount of heating in the sample. The longer the delay between pulses, the longer it takes to accumulate a spectrum with reasonable signal to noise.

FID's were Fourier transformed (usually without zero-filling) to give 4 K real data points on the spectra. The sensitivity was increased by line broadening each spectral line by 10 Hz. The linewidths were calculated by an iterative process which fitted the spectral line to a pure Lorentzian lineshape (DISNMRP Manual, 1982, Bruker Instruments).

**Determination of Extinctions in Cryosolvents**

The molar extinction coefficients of p-nitrophenol in cryosolvent
at various temperatures were determined by repetitively scanning the spectrum. The baseline was recorded with cells containing the cryosolvent at room temperature. A standard solution of p-nitrophenol (3.93 or 30.45 mM, standardized in fully aqueous solution by the $A_{400}$ in 1 M NaOH) was added to the cells and the spectrum rescanned. The temperature was lowered $10^\circ$ and the spectrum was scanned again. This process was repeated down to $-50$ to $-60^\circ$C allowing time for the cell temperature to equilibrate before recording each spectrum. The extinction coefficient was calculated by dividing the absorbance by the concentration of pNP in the cell.

The extinctions of the substrates were determined in a similar way except that the substrate was added to cold cryosolvent ($0^\circ$C) to slow the aqueous hydrolysis.

The difference extinction $(\Delta \epsilon)$ was obtained by $(\epsilon_{\text{product}} - \epsilon_{\text{substrate}})$ and used in kinetic analyses.

**Preliminary investigation of alternative cryosolvents**

To screen possible cryosolvents for a cryoenzymological investigation, a simple cooled sample holder was constructed. A 1.5 x 12 cm test-tube was wrapped in Tygon tubing (OD 3/32", ID 1/32") through which gaseous nitrogen, cooled in liquid nitrogen, was passed. The temperature of the sample in the test-tube was measured with a plastic coated thermocouple and the temperature could be rapidly raised and lowered by adjusting the flow rate of the gaseous nitrogen. Temperatures down to $-100^\circ$C can easily be obtained using this apparatus. For preliminary tests, the cryosolvent was placed in the sample tube with a glass paddle stirrer and the temperature was lowered at a constant rate. The viscosity of the sample was judged by manually stirring the solutions and observing the speed at which air bubbles introduced into the sample are dispersed. As mixtures freeze, the decrease in temperature of the system stops. Many supercooled mixtures become viscous
and are unusable at low temperatures. After this preliminary testing, the samples were placed in the spectrophotometer and p-nitrophenol was added at low temperature. The ease of mixing to obtain a homogeneous solution free of air bubbles could be more accurately determined by the stability of the absorbance versus time curves.

The following cryosolvents were tested and approximate freezing points or low temperature limit for cryoenzymological experiments are given.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Approximate f. p. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% acetone-50% water</td>
<td>-22</td>
</tr>
<tr>
<td>25% acetone-25% DMSO-50% water</td>
<td>-45 to -50</td>
</tr>
<tr>
<td>25% CH₃CN-25% DMSO-50% water</td>
<td>-30 to -40</td>
</tr>
<tr>
<td>50% CH₃CN-50% water</td>
<td>&gt; -25</td>
</tr>
<tr>
<td>40% DMSO-10% acetone-50% water</td>
<td>-50</td>
</tr>
<tr>
<td>25% DMSO-25% dioxane-50% water</td>
<td>-26</td>
</tr>
<tr>
<td>50% dioxane-50% water</td>
<td>-15</td>
</tr>
</tbody>
</table>

The following solvents were fluid down to the -80 to -90°C range. However some were more suitable than others for cryoenzymological experiments. The temperatures given are an estimate of the lowest practical temperatures for use in an enzyme experiment.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Approximate f. p. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40% DMSO-40% water-20% MeOH</td>
<td>-70 to -75</td>
</tr>
<tr>
<td>40% DMSO-40% water-20% acetone</td>
<td>-65 to -70</td>
</tr>
<tr>
<td>40% DMSO-30% water-30% MeOH</td>
<td>-75 to -80</td>
</tr>
<tr>
<td>35% DMSO-35% water-30% acetone</td>
<td>-65 to -70</td>
</tr>
<tr>
<td>25% DMSO-25% EtOH-50% water</td>
<td>-50</td>
</tr>
<tr>
<td>30% DMSO-30% EtOH-40% water</td>
<td>-70 to -80</td>
</tr>
</tbody>
</table>
Ion-exchange chromatography

The homogeneity of papain purified by covalent gel chromatography was determined using ion-exchange chromatography by the method of Baines and Brocklehurst (1979). A 1.5 x 20 cm column of SP-Sephadex C-50 in 0.1 M acetate, 1 mM EDTA, pH 5.0 was used. Approximately 90 mg of papain was applied to the column (8 ml of 0.46 mM) and washed with 25 ml of 0.1 M buffer. A linear gradient from 0.1 acetate to 1.0 M acetate was applied at a flow-rate of 15 ml/hour and was complete after 30 hours. 10 ml fractions were collected. The absorbance at 280 nm was measured, and the relative activity to BAPNA. A single protein was isolated with maximum activity corresponding to the highest concentration of protein.

Stopped-flow experiments

A sample of the enzyme used in the N-acetylphenylalanyl-glycinal $^{13}$C-NMR experiments was analysed by stopped-flow spectrophotometry by Mr. E. Salih and Dr. K. Brocklehurst of the Department of Chemistry and Biochemistry at St. Bartholomews Medical College, University of London.

Amino acid analysis

The amino acid analysis of salt-precipitated papain and covalent gel purified papain were carried out by Dr. R. P. Ambler of the Department of Molecular Biology, Edinburgh University.
RESULTS

AND

DISCUSSION
Purification of Papain

Since contamination by other enzymes or proteins could give rise to anomalous or misleading results it was essential for this work that the purity of the papain used for kinetic and $^{13}$C-NMR studies be rigorously ascertained. Papain purified by the method of Baines and Brocklehurst (1979) showed no sign of chymopapain contamination by the spectrophotometric method of Baines and Brocklehurst (1978). The papain purified by this method usually contained between 0.65 and 0.75 thiol/mol of protein with equal amounts of 2-thiopyridone released at pH 4.1 and 8.2. However, an amino acid analysis of the preparation (Table 1) showed contamination by another protein. Several residues are in excess of that expected from the amino acid composition determined by Glazer and Smith (1971). Most noticeable is the appearance of methionine which is absent in papain. Also listed in Table 1 are the amino acid compositions of papaya lysozyme (Howard and Glazer, 1967; Smith et al., 1955b), and chymopapain B (Kunimitsu and Yasunobu, 1970). The results suggest that this preparation may be contaminated by papaya lysozyme as a small amount of this enzyme would result in a methionine content of 0.9 residues. A comparatively large contamination by chymopapain B would be required to produce this amount of methionine and would be detected by the 2-PDS titration method of Baines and Brocklehurst (1978). The number of glutamic acid and aspartic acid residues listed in Table 1 results from both the acids (Glu, Asp) and the amides (Gln, Asn). Covalent gel purification by the method of Brocklehurst et al. (1973) was used to remove contaminating lysozyme, and the amino acid analysis confirms the high purity of this preparation (2-PDS titration of covalent gel purified papain usually showed between 0.85 to 0.95 thiol/mole).
<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Papain (Glazer and Smith)</th>
<th>Covalent-gel purified</th>
<th>Salt-precipitate (Baines and Brocklehurst, 1979)</th>
<th>Papaya lysozyme</th>
<th>Chymopapain B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>10</td>
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<td>12.5</td>
<td>10</td>
<td>27</td>
</tr>
<tr>
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<td>1.9</td>
<td>2.6</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Arginine</td>
<td>12</td>
<td>12.1</td>
<td>9.4</td>
<td>13</td>
<td>10</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>6</td>
<td>18.6</td>
<td>16.6</td>
<td>22</td>
<td>29</td>
</tr>
<tr>
<td>Asparagine</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>8</td>
<td>8.8</td>
<td>9.5</td>
<td>13</td>
<td>17</td>
</tr>
<tr>
<td>Serine</td>
<td>13</td>
<td>12.7</td>
<td>12.7</td>
<td>16</td>
<td>24</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>8</td>
<td>19.5</td>
<td>15.7</td>
<td>11</td>
<td>30</td>
</tr>
<tr>
<td>Glutamine</td>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>10</td>
<td>9.5</td>
<td>13.8</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>Glycine</td>
<td>28</td>
<td>27.7</td>
<td>23.8</td>
<td>26</td>
<td>41</td>
</tr>
<tr>
<td>Alanine</td>
<td>14</td>
<td>13.3</td>
<td>13.3</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Valine</td>
<td>18</td>
<td>16.1</td>
<td>12.4</td>
<td>8</td>
<td>27</td>
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<tr>
<td>Methionine</td>
<td>0</td>
<td>0.09</td>
<td>0.9</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>12</td>
<td>10.2</td>
<td>8.1</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Leucine</td>
<td>11</td>
<td>11.2</td>
<td>10.6</td>
<td>12</td>
<td>16</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>19</td>
<td>17.9</td>
<td>13.6</td>
<td>13</td>
<td>20</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4</td>
<td>4.2</td>
<td>6.1</td>
<td>12</td>
<td>12</td>
</tr>
</tbody>
</table>
Covalent gel purified papain was used in the hydrolysis of several synthetic substrates and in all cases, the kinetic parameters compare well with previous investigations (for CBZ-glycine-pNP and CBZ-lysine-pNP, see appropriate sections). The hydrolysis of L-BAEE has been well studied and the values obtained from two initial rate investigations compare favourably with other values (see Table 2).

Table 2. Hydrolysis of L-BAEE by papain, pH 6.

<table>
<thead>
<tr>
<th></th>
<th>K_m (mM)</th>
<th>k_cat (s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1*</td>
<td>19.4 ± 1.1</td>
<td>26.5 ± 0.5</td>
</tr>
<tr>
<td>Trial 2*</td>
<td>22.2 ± 1.1</td>
<td>29.0 ± 0.6</td>
</tr>
<tr>
<td>Brocklehurst et al. (1973)</td>
<td>18.2 ± 0.1</td>
<td>16.4 ± 0.5</td>
</tr>
<tr>
<td>Baines and Brocklehurst (1979)</td>
<td>13 ± 2</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>Blumberg et al. (1970)</td>
<td>18</td>
<td>28.5</td>
</tr>
<tr>
<td>Sluyterman and Wijdenes (1970)</td>
<td>18</td>
<td>26</td>
</tr>
</tbody>
</table>

*(± Standard Error)

All of the results in Table 2 were obtained using fully active papain preparations. Brocklehurst and Baines (1979) reported that papain contaminated with other enzymes from papaya latex had a higher K_m (20 mM) and lower k_cat (V_m/SH = 2.2 s⁻¹) than the purified enzyme.

The homogeneity of papain prepared by covalent chromatography was confirmed by ion-exchange chromatography on SP-Sephadex C-50 by the method of Baines and Brocklehurst (1979). The relative activity to the hydrolysis of BAPNA coincided with the protein fractions determined by the A_280.

A sample of the papain used in the N-acetyl-L-phenylalanyl-glycinal ¹³C-NMR experiments was used in a stopped-flow analysis.
of the 2-PDS reaction (see Materials and Methods). This showed a monophasic reaction ($k_2 = 15000 \, M^{-1} \, s^{-1}$) at pH 4 indicating no chymopapain impurities.

All of the results indicate that covalent gel purified papain is free from enzyme and protein contaminants.

The stability of a solution of papain (20 μM) in 60% DMSO, at 0°C, pH 8.8 was tested in relation to a control experiment not containing DMSO. The activity to L-BAEE was tested over a period of 5 days. The results are listed below in Table 3. The times listed are the elapsed time from preparing the enzyme in the cryosolvent.

Table 3. The stability of papain in 60% DMSO, 0°C.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>96%</td>
</tr>
<tr>
<td>1150</td>
<td>93%</td>
</tr>
<tr>
<td>2320</td>
<td>92%</td>
</tr>
<tr>
<td>4290</td>
<td>91%</td>
</tr>
<tr>
<td>5180</td>
<td>95%</td>
</tr>
<tr>
<td>6970</td>
<td>85%</td>
</tr>
</tbody>
</table>

The results show little effect of storage in the cryosolvent on enzyme activity and no evidence for more than 10% irreversible enzyme inactivation over 5 days.
The presence of an acyl intermediate in the papain-catalysed hydrolysis of ester and amide substrates has long been inferred from indirect evidence but relatively little direct evidence is presented in the literature (see introduction). The resonance Raman observations of Storer et al. (1979), and Ozaki et al. (1982), provide direct evidence for dithioacylpapains but the direct evidence for the catalytically relevant thioacyl intermediate is less conclusive. Bender and Brubacher (1964), Brubacher and Bender (1966), and Hinkle and Kirsch (1970), have presented results for direct observation of thioacyl papains by UV-spectrophotometry. In each case studied, the absorption spectrum of the acyl papain is red-shifted from the S-acylcysteine model compounds. Only upon denaturation are the absorption maxima in reasonable agreement with those of the model compounds. This implies that the denatured enzyme exists as a thioacyl derivative but some doubt must remain about the structure in the intact enzyme.

$^{13}$C-NMR has the potential to observe individual nuclei and provide structural information which is crucial to the direct detection and unambiguous classification of an enzyme-bound intermediate. In order to detect an acyl intermediate by $^{13}$C-NMR, the concentration of the intermediate must be high ($\sim 1$ mM), and the lifetime relatively long ($t_{1/2} \sim 1$ hour), for detection. To decrease the rate of deacylation, it is necessary to lower the temperature of the system. However, papain solutions at concentrations $> 200 \mu$M rapidly crystallize at $+4^\circ C$ so it was necessary to add a cosolvent to the papain solutions to ensure high enzyme solubility below $25^\circ C$. DMSO was found to be an excellent cosolvent for papain and Fink and Angelides (1976) have determined that solutions of DMSO up to 60% (v/v) have no adverse effect on the structural or catalytic properties of papain. Addition of DMSO to 25% (v/v) ensures high solubility (2 to 3 mM) of papain down to the freezing-point (-15$^\circ$C) of the cryosolvent. The DMSO therefore
serves two purposes; as a good solvent for papain, and as a cryosolvent if it becomes necessary to lower the temperature.

The slow hydrolysis of the non-specific substrate N-benzoylimidazole by papain has previously been studied by Zannis and Kirsch (1978) using UV-spectrophotometry and was chosen for the initial investigation of an acyl papain intermediate by $^{13}$C-NMR. N-Benzoylimidazole resembles an amide substrate and has been proposed to acylate the Cys-25 thiol of papain to form a thioacyl papain. The reaction of N-benzoylimidazole with papain at low pH may be represented by the following scheme:

$$
\text{H}^+ + \text{Enzyme-S-C} \xrightleftharpoons{\text{H}_2\text{O}} \text{Enzyme-S}^- + \text{H-N}^+\text{N-H} + \text{O-C-OH}
$$

Zannis and Kirsch (1978) monitored the rate of deacylation of benzoyl-papain by observing the decrease in absorbance at 277 nm. The rate of deacylation of benzoyl-papain was found to decrease significantly with a decrease in pH according to the following equation:

$$
k_3 = \frac{k_{\text{lim}} K_a}{K_a + [H^+]} \quad (30)
$$

Benzoyl-papain is prepared by reacting a large (20-fold) excess of N-benzoylimidazole with papain at low pH. In order to monitor the rate of deacylation at 277 nm, Zannis and Kirsch (1978) found it necessary to remove the excess N-benzoylimidazole ($\epsilon_{277} = 9000 \text{ M}^{-1}\text{cm}^{-1}$) present by gel-filtration. The excess of N-benzoylimidazole...
is required to ensure a high level of acylation of papain by the non-specific substrate which rapidly hydrolyses at low pH.

Smith (1976) and Choi and Thornton (1974) determined the rate of non-enzymatic hydrolysis of N-benzoylimidazole in aqueous solution over a pH range from 1 to 10. The resulting experimentally observed rate constants lie on a curve described by equation (31) using the parameters listed below in Table 4.

\[
k_{\text{obs}} = \frac{K_a}{[H^+] + K_a} \left( \frac{k_{H^+}[H^+]}{K_a} + k_{H_2O} + \frac{k_{OH^-}K_w}{[H^+]} \right)
\]  

The constants \( k_{H^+} \) and \( k_{OH^-} \) are the rate constants for the acid and base catalysed reactions respectively and \( k_{H_2O} \) is the rate constant for water hydrolysis. \( K_a \) is the acid dissociation constant for the N-benzoylimidazolium ion. The rate constants in Table 4 from Smith (1976) are the values obtained when extrapolated to zero buffer concentration.

The high concentrations of papain (~1 mM), and N-benzoylimidazole (~20 mM), needed for the \(^{13}\)C-NMR experiments require the presence of a high concentration of buffer (0.1 M), and for lower temperatures, 25% DMSO. The effect of these additional components on the non-enzymatic hydrolysis were investigated. The rate of hydrolysis of N-benzoylimidazole was determined by monitoring the decrease in absorption at 245 nm in 0.1 M buffer, \( I = 0.1 \). The \( \lambda_{\text{max}} \) of N-benzoylimidazole is 241.5 nm (\( \varepsilon = 11400 \text{ M}^{-1} \text{ cm}^{-1} \)) in 1.7% DMSO pH 6.1 and 25°C. The \( \lambda_{\text{max}} \) of the products of the hydrolysis occurs at 234 nm (\( \varepsilon = 10900 \text{ M}^{-1} \text{ cm}^{-1} \)) (see Figure 1). The decrease in absorption is pseudo-first-order in substrate concentration and linear plots of \( \ln(-A_t - A_\infty) \) vs. \( t \) were obtained and used to determine the first-order rate constant \( k_{\text{obs}} \). The results listed in Table 4 are obtained from \( \log_{10} k_{\text{obs}} \) vs. pH profiles shown in Figure 2. The experimental points, in
Figure 1. Absorption spectrum of:

A. $1.06 \times 10^{-4}$ M N-benzoylimidazole in 0.1 M phosphate, 1.7% DMSO, pH 6.1, 25°C.

B. Same as in (A), after complete hydrolysis at 25°C (60 minutes later).
Figure 2. Hydrolysis of $1.06 \times 10^{-4}$ M N-benzoylimidazole in 0.1 M buffer, $I = 0.1$, in

(A) 1.7% DMSO, 25°C.
(B) 26.7% DMSO, 0°C.
each case, are found to be consistent with a curve described by Equation (31) and the constants listed Table 4.

Table 4

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Temp(°C)</th>
<th>$k_{H^+}(s^{-1})$</th>
<th>$k_{OH^-}(M^{-1}s^{-1})$</th>
<th>$k_{H_2O}(s^{-1})$</th>
<th>pK_a</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>25</td>
<td>3.93x10⁻²</td>
<td>2.92x10⁻²</td>
<td>9.12x10⁻⁵</td>
<td>3.34</td>
</tr>
<tr>
<td>1.7% DMSO</td>
<td>25</td>
<td>4.47x10⁻²</td>
<td>2.71x10⁻²</td>
<td>6.04x10⁻⁴</td>
<td>3.7</td>
</tr>
<tr>
<td>26.7% DMSO</td>
<td>0</td>
<td>4.79x10⁻³</td>
<td>3.24</td>
<td>2.08x10⁻⁵</td>
<td>4.0</td>
</tr>
</tbody>
</table>

*(results of Smith, 1976)*

The values of $k_{H^+}$ and $k_{OH^-}$ in 1.7% DMSO are in reasonable agreement with the results of Smith (1976). The largest differences are apparent in the $k_{H_2O}$ value which would be expected to be higher due to buffer catalysis which has been observed to increase the rate of hydrolysis in studies on N-benzoylimidazole (Smith, 1976), and N-acetylimidazole (Jencks and Carriuolo, 1959). Below pH 5 and above pH 8 non-enzymatic hydrolysis is rapid (see Figure 2). Above pH 8, the observed rate constant is dominated by the contribution from the $k_{OH^-}$ term. Below pH 5, rapid hydrolysis of protonated N-benzoylimidazole ($k_{H^+}$) is predominant.

The rates of hydrolysis in 26.7% DMSO, 0°C, were obtained and are significantly slower (Figure 2). The pK_a of the N-benzoylimidazole shows a slight increase although it might be expected to decrease in DMSO-water solutions. The decrease in temperature would account for an increase in the observed pK_a (Maurel et al., 1975), and the change in the buffer pK_a's may account for the slight differences. The rate constants plotted in Figure 2 are also consistent with a curve described by Equation (31).

The effect of cosolvent and temperature on the rate of deacylation of benzoyl-papain was investigated by absorption spectrophotometry to establish the conditions needed for a $^{13}$C-NMR investigation. The method of monitoring deacylation at 277 nm,
used by Zannis and Kirsch (1978), was unsuitable since the gel-filtration step needed to remove the excess N-benzoylimidazole and its hydrolysis products, dramatically reduces the concentration of the enzyme. For our purposes, it was necessary that the concentration remain as high as possible. Reconcentration of the acyl papain solution after gel-filtration is possible but would require additional time during which the concentration of the intermediate further decreases since the enzyme deacylates during the whole process. While removal of excess reactants or products would not be required for a $^{13}$C-NMR experiment, it was important to monitor the reaction by UV-spectrophotometry under identical conditions. A new technique was developed to monitor the extent of acylation and rate of deacylation of high concentrations of benzoyl-papain in the presence of an excess of N-benzoylimidazole and the products of the hydrolysis.

This technique utilizes the high reactivity of the titrant 2, 2'-dipyridyl disulphide (2-PDS) with the thiolate ion of papain at low pH. 2-PDS reacts rapidly ($k=15000 \text{ M}^{-1} \text{s}^{-1}$) with the thiolate ion of papain at pH 3.8 (Brocklehurst and Little, 1972). In a titration mixture containing 0.25 mM 2-PDS (thiolate concentration < 0.025 mM), the reaction with papain has a half-life of 0.27 seconds. Zannis and Kirsch (1978) report a limiting value of 150 seconds for the half-life of deacylation of benzoyl-papain, therefore deacylation is the rate limiting step. Addition of benzoyl-papain to a solution containing 2-PDS should produce a biphasic curve when the absorbance at 343 nm is measured as a function time. The initial phase ($t_{1/2} = 0.27 \text{ s}$), represents rapid titration of non-benzoylated papain with 2-PDS releasing 2-thiopyridone. This is followed by a slower phase which reflects the rate limiting deacylation since as the enzyme slowly turns over, more thiolate is then available for titration by 2-PDS. In practice, an initial burst of 2-thiopyridone is observed which is proportional to the concentration of non-benzoylated papain and is followed by a slower first-order
increase in absorbance. The final absorbance value \( (A_\infty) \) of the 2-thiopyridone in solution after the enzyme completely deacylates, is equal to the concentration of active enzyme present before acylation. A plot of \( \ln(A_\infty - A_t) \) vs. \( t \) produces a line with slope \( k_{\text{obs}} \) equal to the deacylation rate, and y-intercept equal to the initial burst of 2-thiopyridone (or free enzyme). Using this method, the deacylation rate in water at pH 3.8 and room temperature was obtained.

In a modification of this procedure, aliquots of the benzoylimidazole-papain solution were removed at various time intervals and added to the 2-PDS titration solutions. In this way, the amount of non-acylated papain present was obtained as a function of time by measuring the initial burst (\( = \) free thiol) of 2-thiopyridone for each addition. A plot of \( \ln(\text{fraction acylated}) \) vs time produces a straight line with a slope equal to the deacylation rate. The advantage of this method is that papain-benzoylimidazole reaction mixtures under various conditions of cosolvent concentration and temperature can all be titrated by the same method at room temperature. At 25°C, pH 3.8, the deacylation rate is sufficiently slow \( (t_{1/2} = 4.4 \text{ min.}) \), to allow accurate determination of the initial burst yet fast enough for complete deacylation to occur in a reasonably short time. The benzoyl-papain may therefore be successively titrated and the final \( A_\infty \) value for each titration should be consistent. If the final \( A_\infty \) value varies from one titration to the next, the results for that titration were discarded. Low \( A_\infty \) values may be caused by denaturation of the enzyme; this can be checked by titrating the denatured enzyme at pH 8 where it is most reactive (Brocklehurst and Little, 1973). The concentration of 2-PDS in the titration cell was varied between 0.1 to 0.75 mM and had no effect on \( k_{\text{obs}} \). Protein concentrations in the papain-N-benzoylimidazole mixtures varied between 0.1 to 1.7 mM and in the titration cell from 4 to 40 µM also without any effect on the observed rate. This indicates that deacylation is indeed the rate
limiting step in the titrations.

The two methods of determining the deacylation rate were directly compared. The half-life for deacylation, determined by measuring the bursts for a series of titrations, is 13.0 minutes. Following complete deacylation by removing a single aliquot of the papain-benzoylimidazole mixture and monitoring complete deacylation in a titration cell under similar conditions, has a \( t_{1/2} = 14.4 \) minutes. The results are in reasonable agreement and support the validity of this procedure for determining the rate of deacylation of benzoyl-papain.

Table 5 lists the results obtained for the deacylation of benzoyl-papain under different conditions of cosolvent and temperature. At 25°C, pH 3.2, the half-life for deacylation was 13.9 minutes. Raising the pH to 3.8 increases the deacylation rate 3.2-fold, which illustrates the importance of maintaining a constant, low pH. Also included in the table are the results of Zannis and Kirsch (1978) at the same pH values calculated from Equation (30) with \( k_{3}^{\lim} = 0.271 \text{ min}^{-1} \) and \( pK_a = 4.3 \). Their results are 2.5 times lower. The ratio of the rates at pH 3.2 and 3.8 however are similar. The differences in the results cannot be due to differences in buffer or ionic strength as both are similar in the two experiments. Included in the table are the half-lives for non-enzymatic hydrolysis under the same conditions. It is immediately apparent that the rate of non-enzymatic hydrolysis is much faster than the deacylation rate at acid pH.

The rate of deacylation of papain at low pH and 25°C was however too fast for a \(^{13}C\)-NMR experiment so it was necessary to reduce the rate of deacylation by lowering the temperature. At 0°C in 25% DMSO, the half-life for deacylation proved to be approximately one hour which in the range required for the \(^{13}C\)-NMR experiment. The freezing-point of a 25% DMSO cryosolvent is -15°C so that the lowest practical temperatures which could be used were in the -5 to -10°C range.
Table 5

<table>
<thead>
<tr>
<th>Solvent</th>
<th>pH (°C)</th>
<th>Temp. (°C)</th>
<th>Deacylation $t_{1/2}$ (± 1 s.d.)</th>
<th>Aq. Hydrolysis $t_{1/2}'$</th>
<th>Ratio $(t_{1/2}/t_{1/2}(3.2))$</th>
<th>No. of Determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7 to 3.8%</td>
<td>3.2</td>
<td>25</td>
<td>13.9 ± 2.0 min.</td>
<td>0.33 min.</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>DMSO</td>
<td>3.8</td>
<td>25</td>
<td>4.4 ± 0.7 min.</td>
<td>0.54 min.</td>
<td></td>
<td>18</td>
</tr>
<tr>
<td>100% H2O*</td>
<td>3.2</td>
<td>25</td>
<td>34.7 min.</td>
<td></td>
<td></td>
<td>3.3</td>
</tr>
<tr>
<td>100% H2O*</td>
<td>3.8</td>
<td>25</td>
<td>10.6 min.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25% DMSO</td>
<td>4.2</td>
<td>0</td>
<td>56.7 ± 2.5 min.</td>
<td>5.5 min.</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>25% DMSO</td>
<td>4.3</td>
<td>-6</td>
<td>72 min.</td>
<td>15.3 min.</td>
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<td>1</td>
</tr>
<tr>
<td>25% DMSO</td>
<td>4.1</td>
<td>-7</td>
<td>96 min.</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>25% DMSO</td>
<td>3.8</td>
<td>-7</td>
<td>156 min.</td>
<td></td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

* (values calculated from Zannis and Kirsch, 1978)

pH values at ≤ 0°C, 25% DMSO, corrected to 25°C assuming a pH change of 0.1 on lowering the temperature (Maurel et al., 1975).
A large excess of N-benzoylimidazole was used to ensure a high concentration of non-hydrolysed N-benzoylimidazole during acylation. For twenty determinations ranging from 1.7 to 25% DMSO, over a pH range 3.2 to 4.3, the average extent of acylation is 84 ± 12% (±1 s.d.) of the available active enzyme.

In the experiments listed in Table 5, 0.1M formate buffer was used to maintain a low pH. The increase in DMSO concentration causes an increase in the pK_a of the buffer (Douzou, 1973). Formate is the only suitable buffer available (at 0.1M concentration) with a pK_a low enough for these experiments. At higher pH, deacylation becomes rapid and lower temperatures would be required.

In the experiments at subzero temperatures, a lag phase was observed during which time little or no deacylation was observed. This is shown graphically in the results of a typical experiment (Figure 3). The figure shows a plot of ln(fraction acylated) vs. time for benzoyl-papain at -7°C. The benzoyl-papain concentration remained constant for the first two titrations over a 30 minute interval. The third titration at 60 minutes shows the first decrease in benzoyl-papain concentration and subsequent titrations indicate a first-order breakdown of acyl enzyme. The rate constant for deacylation is obtained from the slope of the linear portion. The lag phase may be due to the presence of unhydrolysed N-benzoylimidazole still in solution. At this low temperature, the N-benzoylimidazole hydrolysates slowly which may allow for reacylation of the enzyme. The rate constant for non-enzymatic hydrolysis of N-benzoylimidazole under similar conditions is 0.046 min⁻¹. At this rate, after 40 minutes [S]₀ = 23.8 mM, [E]₀ = 1.7 mM, 3.8 mM N-benzoylimidazole is still available for acylation. After 60 minutes, only 1.5 mM benzoylimidazole remains which is less than the enzyme concentration. At higher temperatures, where N-benzoylimidazole hydrolyses rapidly, and the enzyme has a higher rate of deacylation, a lag phase was not observed.

The purpose of the UV investigation was not to provide an
Figure 3. Lag phase observed in a typical deacylation at -7°C, pH 4.1. The deacylation is monitored by removing aliquots of the mixture at -7°C and adding this to a titration mixture at pH 3.8, 25°C. The burst ($A_0$) is observed, and the reaction is followed to completion ($A_\infty$). The fraction of enzyme acylated is calculated from ($1 - (A_0/A_\infty)$). The 2-PDS concentration in the titration cell is at least ten times greater than the active enzyme. Each determination has a half-life of approximately 5 minutes. The rate of deacylation is determined from the slope of the line.
extensive kinetic investigation of the deacylation of benzoyl-papain but rather to establish the experimental conditions required for successful application of $^{13}\text{C}$-NMR to the detection of a transient intermediate acyl enzyme. The spectrophotometric results indicated that low temperatures and DMSO cosolvent are needed to slow the deacylation rate and make detection and observation by $^{13}\text{C}$-NMR possible. The 2-PDS results suggested that N-benzoylimidazole acylates the active site of the enzyme but whether acylation takes place at a histidine, thiol, or other residue of the active-site can only be determined by other means.

Acylation of the active-site cysteine of papain by $[^{13}\text{C}=\text{O}]$-N-benzoylimidazole should produce a thioester which has a distinctive carbonyl resonance in the $^{13}\text{C}$-NMR spectrum. Hall and Wemple (1977) found that the chemical shift of the carbonyl carbon of thioesters generally occurs in the 193 to 203 ppm range which is 15 to 20 ppm downfield from the carbonyl carbons of all other carboxylic acid derivatives (see Table 6 for examples). They also predict that in biomolecules, a thioester formed for example with acetyl CoA, should have a resonance between 193 and 195 ppm. The resonance of a thioacyl papain should be within this region and detection of such a resonance would be unequivocal evidence for the identity of the intermediate. The only other compounds with such low-field carbonyl resonances are aldehydes and ketones which could not be formed from N-benzoylimidazole under the experimental conditions used.

The proton-decoupled $^{13}\text{C}$-NMR natural abundance spectrum of unenriched N-benzoylimidazole in DMSO-$d_6$ is shown in Figure 4. The carbonyl carbon is shown downfield of the other resonances at 166.0 ppm. The resonances between 118 and 138 ppm are due to the aromatic carbons of the imidazole and phenyl rings. The following spectrum (Figure 5), shows enriched $[^{13}\text{C}=\text{O}]$-benzoyl-
Table 6. Chemical shifts of the carbonyl carbon of carboxylic acid derivatives (ppm from TMS).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Solvent</th>
<th>δ</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ph-(\text{C}-\text{OH})</td>
<td>CDC(_3)</td>
<td>172.6</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Acetone-(\text{d}_6)</td>
<td>168.5</td>
<td>2</td>
</tr>
<tr>
<td>Ph-(\text{C}-\text{OCH}_3)</td>
<td>CDC(_3)</td>
<td>166.8</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Acetone-(\text{d}_6)</td>
<td>167.0</td>
<td>2</td>
</tr>
<tr>
<td>Ph-(\text{C}-\text{Cl})</td>
<td>CDC(_3)</td>
<td>168.0</td>
<td>1</td>
</tr>
<tr>
<td>Phthalic</td>
<td>DMSO-(\text{d}_6)</td>
<td>163.1</td>
<td>1</td>
</tr>
<tr>
<td>Anhydride</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ph-(\text{C}-\text{O})</td>
<td></td>
<td>162.8</td>
<td>3</td>
</tr>
<tr>
<td>CH(_3)C-N-(n-butyl)(_2)</td>
<td>Acetone-(\text{d}_6)</td>
<td>169.2</td>
<td>4</td>
</tr>
<tr>
<td>Ph-(\text{C}-\text{S-Et})</td>
<td>DMSO-(\text{d}_6)</td>
<td>191.2</td>
<td>5</td>
</tr>
<tr>
<td>Ph-(\text{C}-\text{S-CH}_3)</td>
<td>DMSO-(\text{d}_6)</td>
<td>195.4</td>
<td>5</td>
</tr>
<tr>
<td>Ph-(\text{C}-\text{S-Ph})</td>
<td>DMSO-(\text{d}_6)</td>
<td>189.1</td>
<td>This work</td>
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</table>

1-Johnson and Jankowski, 1972
2-Scott, 1972
3-Levy and Nelson, 1972 a
4-Levy and Nelson, 1972 b
5-Hall and Wemple, 1977
Figure 4. Proton-decoupled $^{13}$C-natural abundance spectrum of 0.1 M N-benzoyl-imidazole in DMSO-$d_6$. 
Figure 5. Proton-decoupled $^{13}\text{C}$-NMR spectrum of 0.01 M $[^{13}\text{C}=\text{O}]$-N-benzoylimidazole in DMSO-$d_6$. A five-fold vertical expansion of the natural abundance aromatic carbon resonances between 115 and 142 ppm is included.
imidazole recorded under the same conditions. The carbonyl carbon displays a single large resonance at 166.0 ppm along with a smaller resonance at 167.2 ppm. This smaller resonance has approximately the same intensity as the natural abundance resonances upfield and can be assigned to benzoic acid, the hydrolysis product of N-benzoylimidazole. A five-fold vertical expansion of the natural abundance signals between 115 and 142 ppm is also included. The ratio of the intensity of the carbonyl signal relative to the natural abundance resonances, compared with that determined for unenriched material under the same conditions, indicates that the N-benzoylimidazole is \( \geq 90\% \) enriched with \(^{13}\text{C}\).

Since the deacylation rate is sufficiently slow only at low pH and in formate buffer, the following spectra show the relative positions of the \([^{13}\text{C}=\text{O}]\)-N-benzoylimidazole, benzoic acid, and formate resonances. N-benzoylimidazole is hydrolysed rapidly in the effective buffering range of formate, so initially to obtain the \([^{13}\text{C}=\text{O}]\)-N-benzoylimidazole resonance, the spectrum is recorded in formate well above the \(pK_a\) of the buffer where hydrolysis of N-benzoylimidazole is relatively slow.

Figure 6 (a) shows the low-power noise-decoupled spectrum of 0.1 M formate, pH 7.1 in 15% DMSO-d\(_6\). A single resonance is observed at 172.2 ppm. At this pH, N-benzoylimidazole is relatively stable and addition of \([^{13}\text{C}=\text{O}]\)-N-benzoylimidazole to a concentration of 9 mM results in the appearance of an additional signal with a chemical shift of 169.8 ppm (Figure 6 (b)). To prove that the signal is indeed \([^{13}\text{C}=\text{O}]\)-N-benzoylimidazole and not the hydrolysis product, \([^{13}\text{C}=\text{O}]\)-benzoic acid was added with subsequent appearance of a new peak at 176.7 ppm (Figure 6 (c)).

The position of each resonance will change slightly with solvent composition (i.e., pH, DMSO concentration). The chemical shift and the multiplicity of the formate signal was significantly affected by the solvent composition. Under the same conditions
Figure 6.

a) Low-power noise-decoupled spectrum of 0.1 M formate in 15% DMSO, pH 7.1.

b) Same as in (a), with 9 mM $[^{13}\text{C}=\text{O}]$-N-benzyylimidazole added.

c) Same as in (b), with 4 mM $[^{13}\text{C}=\text{O}]$-benzoic acid added.
Figure 6

(a) 172.2

(b) 169.8

(c) 176.7

δ (ppm)
of low-power noise-decoupling, the formate signal appears as a doublet at low pH and a triplet at higher pH. The chemical shifts of the multiplets also changes with pH. Figure 7 shows a plot of chemical shift of the formate signals versus pH in 25% DMSO, 0°C. The pH of the solution can therefore be determined from the chemical shift of the formate resonances. The $pK_a$ determined from Figure 7 is approximately 4.0 which is a reasonable estimate for formic acid in 25% DMSO 0°C. The $pK_a$ in water is 3.7 and increases with increasing DMSO concentration and a decrease in temperature (Douzou, 1973). The change in multiplicity is caused by the relative efficiency of the decoupling power which is affected by changes in the solvent. Changes in pH and/or changes in DMSO affect the ionic strength and dielectric constant of the NMR solutions. The change in the solvent properties and therefore decoupler efficiency, means that at low pH the formate signal is fully coupled to the attached proton and hence a doublet is observed. At higher pH, the signal is partially decoupled and an apparent triplet, (singlet + doublet) is observed. Figure 8 illustrates the effect of varying decoupler power on the formate signal. Figure 8 (a) shows a fully coupled formate signal. Figure 8 (b) shows the same solution now accumulated under conditions of full decoupling (i.e. high-power decoupled). The bottom spectrum (Figure 8 (c)), shows a low-power partially decoupled spectrum of formate. This spectrum is a combination of the above spectra. The problem of varying multiplicity does not affect any other signals of interest since formate is the only carbonyl compound under study which has a directly attached proton.

The non-enzymatic hydrolysis of N-benzoylimidazole in 0.1 M formate was monitored by a time-course $^{13}$C-NMR experiment as a control for the enzyme experiment. Figure 9 shows the time-course for the hydrolysis of $[^{13}\text{C}=\text{O}]$-N-benzoylimidazole with the first spectrum of the sequence on the bottom. The spectra on the left side of the page show the carbonyl region of interest between
Figure 7. The dependence of the chemical shift $\delta$ (ppm) and the multiplicity of the formate resonances on pH under conditions of low-power noise-decoupling. 25% DMSO-$d_6$, 0°C, 0.2 M formate.
Figure 8. The effect of varying the decoupler power on the $^{13}$C-NMR spectrum of 0.2 M formate, 25% DMSO, p$^*$H 4.2, 20°C.

(a) Non-decoupled

(b) Decoupler power (DP) = 3 H (6.4 W), fully decoupled spectrum.

(c) Low-power noise-decoupled spectrum.

DP = 15 H (0.4 W).
164 and 176 ppm. In order to observe the weaker resonances, the spectra on the right side of the page show a 12-fold vertical expansion of the same time course spectra from 164 to 172 ppm. The spectra shown if Figure 9 were recorded sequentially 4 minutes after mixing $^{13}$C=N-benzoylimidazole in the formate buffer. After 45 minutes, accumulation of the first spectrum (Figure 9 (a)) is complete and it shows that most of the N-benzoylimidazole at 169.1 ppm has been hydrolysed to benzoic acid resulting in a signal at 173.7 ppm. The relative peak heights provide a measure of relative concentration, and it can be estimated that approximately 12% ($\sim$2.3 mM) $[^{13}\text{C}=\text{O}]$-N-benzoylimidazole remains after 49 minutes. Figures 9 (b) to (e) show the rapid decrease in the N-benzoylimidazole signal at 169.1 ppm slightly upfield from the formate triplet centered at 170.7 ppm. Also appearing in the spectra is one additional peak at 165.5 ppm. This peak also eventually disappears and since no additional resonances appear in the spectra, it must be assumed that the hydrolysis product(s) of this peak is also benzoic acid. This resonance must therefore represent some acid labile derivative of benzoic acid. Possible derivatives could be a benzoic acid ester, amide, or anhydride which all have carbonyl resonances in that region of the $^{13}$C-NMR spectrum (see Table 6). Since no additional carbonyl resonances appeared in the spectrum of $[^{13}\text{C}=\text{O}]$-N-benzoylimidazole in 100% DMSO (Figure 5), the additional resonance must have been produced during hydrolysis. A similar resonance is present in the hydrolysis of N-benzoylimidazole at acid pH in the absence of formate buffer. A possibility is benzoic anhydride formed during the reaction by nucleophilic attack of the product benzoate on benzoylimidazole:

$$
\begin{array}{c}
\text{Ph-C-O}^- \\
\text{Ph-C=O} \\
\text{N} \\
\text{H}
\end{array}
\rightarrow
\begin{array}{c}
\text{Ph-C=O} \\
\text{Ph-C-O} + \text{H-N-H}
\end{array}
$$
Figure 9. Hydrolysis of 19.2 mM $[^{13}\text{C}=\text{O}]$-N-benzoylimidazole in 0.1 M formate, 1 mM EDTA, 25% DMSO, -6°C, pH 4.4. Low-power noise-decoupled spectra. 12-fold vertical expansion of the region from 164-172 ppm shown on the right.

(a) Spectrum accumulated 4 minutes after mixing $[^{13}\text{C}=\text{O}]$-N-benzoylimidazole in buffer. Each spectrum required 45 minutes accumulation time (i.e. NS $= 10000$

(b)-(e) Recorded sequentially after (a) during the times indicated on the spectra.
The spectrum of non-enriched benzoic anhydride in DMSO is shown in Figure 10 (a). The resonance at 163.2 is the carbonyl signal of benzoic anhydride and the resonance at 167.3 ppm is benzoic acid which was confirmed by adding additional benzoic acid to the solution (Figure 10 (b)).

The spectrum of formate, pH 3.8, in 25% DMSO, is shown in Figure 11 (a), below which is the spectrum after addition of \(^{13}\text{C}=\text{O}\)-benzoic anhydride (Figure 11 (b)). The peak at 172.5 ppm is benzoic acid and the anhydride resonance in 25% DMSO, formate buffer, has a chemical shift of 165.4 ppm. After complete hydrolysis of the anhydride, the spectrum in Figure 11 (c) was recorded showing only the formate and benzoic acid resonances. The additional peak at 165.5 ppm present in the control hydrolysis in Figure 9 is therefore assigned to benzoic acid anhydride.

In the enzyme experiments, the expected chemical shift of a thioester intermediate between 190 and 200 ppm should be well downfield of all the resonances observed in the control experiments. The natural abundance spectrum of papain is shown in Figure 12 (h) and shows the broad resonances due to protein carbonyls between 165 and 180 ppm. The quaternary carbons of arginine C-6 and tyrosine C-7 are also evident at 158 and 156 ppm respectively. Figure 12 (g) shows the same sample after addition of formate. The triplet due to partially decoupled formate is clearly seen. The \(^{13}\text{C}=\text{O}\)-N-benzoylimidazole was added and the spectrum in Figure 12 (a) was recorded starting at 6 minutes from the addition. Each spectrum requires 45 minutes accumulation time for adequate S/N so the spectra were recorded sequentially starting at 6, 51, 96, 141, 186, and 231 minutes after addition of the N-benzoylimidazole. A sample of the solution from the \(^{13}\text{C}\)-NMR experiment was removed two minutes after the addition and kept at -7°C. Titration of aliquots of this solution showed that 5 minutes after the addition of N-benzoylimidazole, the enzyme was 96% acylated. Subsequent titrations
Figure 10. a) Low-power noise decoupled spectrum of 0.2 M benzoic anhydride in DMSO-d$_6$.

b) Same as in (a) plus 0.09 M benzoic acid.
Figure 11. Low-power noise-decoupled spectra of benzoic anhydride hydrolysis.

a) 0.1 M formate, 25% DMSO-d$_6$, pH* 3.8 -6°C.

b) Added [13C=O]-benzoic anhydride to (a) to a concentration of 0.5 mM.

c) After complete hydrolysis of [13C=O]-benzoic anhydride to [13C=O]-benzoic acid.
Figure 12. The reaction of papain and $^{13}\text{C}=\text{O}]^{-}\text{N-}$benzoylimidazole.

a) to f) 1.7 mM papain (1.2 mM active SH), 5.4 mM KCl, 25% DMSO, 0.1 M formate, pH $^*4.1$, 23.6 mM $^{13}\text{C}=\text{O}]^{-}\text{N-}$benzoylimidazole. The N-benzoylimidazole was added at 0°C, stirred for 1.5 min., and cooled to -7°C. NMR accumulations started 6 minutes after mixing. Spectra (a) to (f) represent 10000 accumulations recorded sequentially under conditions of low-power noise-decoupling at the times indicated on the spectra. The insert from 192-200 ppm is a two-fold expansion of the spectral range.

g) 2.03 mM papain (1.46 mM active SH), 6.5 mM KCl, 25% DMSO, 0.1 M formate buffer, pH $^*3.8$.

h) 2.3 mM papain (1.66 mM active SH), 7.5 mM KCl, 25% DMSO, pH $^*6.4$. 
indicated that the half-life for deacylation is 96 minutes. The
spectra in Figure 12 can be directly compared to the control
experiment in Figure 9. The position of the formate triplet shifts
slightly with a change in pH from 169.3 (pH 3.8) in Figure 12 (g)
to 170.1 ppm (pH 4.1) in Figures 12 (a) to (f). The resonance
due to benzoic anhydride is also present at 165.2 ppm in Figures
12 (a,b), but then disappears. The rate of hydrolysis of the anhydride
appears to be much faster in the enzyme experiments (compare
Figure 9 to Figure 12) which may be due to groups on the protein
aiding in hydrolysis. The resonance due to $^{13}$C=O]-N-benzoyl-
imidazole is present in Figures 12 (a,b) at 168.7 ppm but rapidly
hydrolyses. The most significant resonance observable in these
spectra and not found in any of the control experiments is the rather
broad resonance at 196.0 ppm. A two-fold vertical expansion of
this signal is shown above each spectrum. The time-course shows
the disappearance of this broad (25 ± 5 Hz) resonance.

The resonance at 196.0 ppm is characteristic of thioesters
(see Table 6) and is assigned to the formation of benzoylpapain
by the following scheme:

\[
\text{Papain-S}^- + \text{Ph-C-N'}^+\cdot\text{NH} \xrightarrow{\text{H}^+} \text{Papain-S-Ph} \xrightarrow{\text{H}_2\text{O}} \text{Papain-S}^- + \text{PhC-OH}
\]

\[
168.7 \text{ ppm} \quad 196.0 \text{ ppm} \quad 173.1 \text{ ppm}
\]

- $^{13}$C-label

This provides the first unequivocable evidence that a transient
thioacyl papain is formed as an intermediate.

The signal-to-noise ratio of the thiobenzoyl signal at 196.0
ppm is not large enough to accurately determine its half-life. The
signal-to-noise ratio in Figure 12 (a) is approximately 4 : 1. At
this ratio, the signal should not be visible after two half-lives, i.e.
after 192 minutes (from the thiolate assays determined by UV, \( t_{1/2} = 96 \) minutes). This is in good agreement with the time-course spectra shown in Figure 12 where there is little or no signal observable after 186 minutes. These results show the correspondence between the signal due to benzoyl-papain in the \(^{13}\)C-NMR experiment and the amount of acylated enzyme calculated from the thiolate titration of the same mixture.

The broad (25 ± 5 Hz) thioester resonance can be compared to the resonance of benzoic acid in the same spectrum which has a linewidth of 5 Hz. Oldfield et al. (1975a) calculated the linewidths (LW) for the non-protonated carbons of horse heart cytochrome c (MW=12300, LW=0.49 Hz), hen egg-white lysozyme (MW=14300, LW=0.55 Hz), and human adult haemoglobin (MW=64500, LW=1.1 Hz), from the relaxation times for these resonances. These determinations were made at a field strength of 15.2 MHz in water at 36°C. The relaxation times and linewidths are related to the size of the protein molecule because of the increased rotational correlation times (\( \tau_R \)) of larger molecules. Non-protonated carbons in papain (MW=23406) therefore should have a linewidth between 0.5 and 1 Hz if measured under these same conditions.

Many factors can influence the width of spectral lines. Instrumental broadening places a lower limit on the linewidth obtainable for a certain spectrometer. As a comparison to the results above, at 25°C in 100% water, the linewidth of the benzoic acid carbonyl resonance was measured as 1.1 Hz in a spinning sample and 2.5 Hz in a non-spinning sample at 75.47 MHz. Spinning the sample reduces instrumental broadening due to magnetic-field inhomogeneity. Placing the sample in aqueous-DMSO solvent and lowering the temperature increased the benzoic acid linewidth from 2.5 to 5 Hz in non-spinning samples. Low molecular weight compounds such as benzoic acid would be expected to have a much narrower linewidth than the enzyme-bound benzoyl group. The linewidth of the thioester should then be > 5 Hz.
According to the theoretical calculations of Norton et al. (1977), the linewidth of non-protonated carbons in a high magnetic field should be dominated by the contribution from CSA. This contribution is again dependent on the rotational correlation time which for papain should be between 19 and 49 nsec. (Oldfield, 1975a; see also discussion in Ac-Phe-Glycinal section). For a rotational correlation time in this range, the linewidth should be around 10 Hz at a field strength of 75.47 MHz. Add to this, the effect of lowering the temperature in aqueous-DMSO solutions and not spinning the samples, and a 25 Hz linewidth does not seem unusually broad. Line-broadening to this extent can be expected for non-protonated carbons of bound substrates and inhibitors at low temperature.

After the time-course experiment was completed (∼18 h at -7°C), the sample was diluted four times, the low molecular weight material was removed by gel-filtration at room temperature, and the papain solution was reconcentrated. Thiol titration showed no loss of the original reactive thiolate (72%). DMSO was added to the sample and the $^{13}$C-NMR spectrum was accumulated for a long period of 200000 scans (Figure 13 (a)). This improves the sensitivity of this spectrum approximately five-fold over the time-course spectra shown in Figure 12. Formate was not added to this sample in order to get an unobscured view of the carbonyl region. Two additional resonances at 168.7 and 169.1 ppm are immediately apparent. The spectrum of papain itself (Figure 13 (b)) was subtracted to give the difference spectrum shown in Figure 13 (c). The difference spectrum clearly shows the two resonances which appear in the region characteristic of amide and ester carbonyl signals. By analogy with the acylation of trypsin with N-acetylimidazole (Houston and Walsh, 1970), these resonances may be assigned to non-specific benzylation of the O-€ of tyrosine and N€ of lysine residues.

The papain used in this experiment was determined to have 0.72 thiol/mole of protein. The preparation used was subjected to covalent gel chromatography (Brocklehurst et al., 1973) but difficulties
Figure 13. a) 0.852 mM papain (0.614 mM SH), 25% DMSO-d₆, 7.5 mM KCl, pH 5.9 at 20°C. Low power noise decoupled spectrum. NS = 200000.

b) 2.3 mM papain (1.66 mM SH), 25% DMSO-d₆, 7.5 mM KCl, pH 6.6 at 0°C. Low power noise decoupled spectrum. NS = 180000.

c) Difference spectrum (a)-(b).
with the procedure did not increase the purity of the enzyme over that obtained from the salt-precipitation procedure (Baines and Brocklehurst, 1979). No contaminating thiol containing proteins were present since the 2-PDS titration at pH 4 and 8 both showed 0.72 thiol/mole.

These results provide unambiguous evidence for detection of a thioacyl intermediate in the reaction of papain with N-benzoyl-imidazole. Normally, this transient intermediate would be undetectable by $^{13}$C-NMR at room temperature and appropriate techniques had to be developed in order to successfully observe this resonance. $^{13}$C-NMR was also shown to be particularly well suited to observing non-specific acylation. This series of experiments demonstrated the feasibility of using low-temperature techniques combined with $^{13}$C-NMR to provide important structural information on enzyme-bound intermediates.
Papain and N-acetyl-L-phenylalaninylglycinal

There is considerable indirect evidence for formation of a tetrahedral intermediate in the reactions catalysed by the proteolytic enzymes (see introduction). Direct evidence for such an intermediate is not abundant and the interpretation of the cryoenzymological results of Fink and co-workers with the serine proteases (Fink and Meehan, 1979; Compton and Fink;1980) and possibly with papain (Angelides and Fink, 1979 a, b) must be viewed with some caution in light of the results of Markley et al. (1981). Asbóth and Polgár (1983) suggested that stabilization of an oxyanion THI is important with the serine proteases but is absent or is not significant with the thiol proteases. Whether a distinct THI exists at high concentration or is present only at very low steady-state concentrations also has not been established.

By synthesizing substrate analogues which form transition-state-like structures with enzymes, Wolfenden and co-workers have obtained valuable information on the possible structures of enzyme-bound intermediates (Wolfenden, 1972). Wolfenden proposes that if papain catalysis proceeds through the stabilization of tetrahedral intermediates, then the stability of analogues resembling these intermediates should be enhanced (Westerik and Wolfenden, 1972). The reversible inhibition of papain by aldehydes is thought to occur through stabilization of a hemithioacetal formed between the thiol and the aldehydic carbonyl (Westerik and Wolfenden, 1972; Lewis and Wolfenden, 1977 b; Mattis et al., 1977; Bendall et al., 1977; Frankfater and Kuppy, 1981).

\[
\begin{align*}
R-C-H & \overset{E-S^{-}}{\xrightarrow{\text{H}_2O}} \overset{E-S^{-}}{\xrightarrow{\text{H}^+}} \overset{\text{OH}}{\xrightarrow{E-S}} R-C-H \\
\text{OH} & \quad \text{Hemithioacetal} \\
\text{OH} & \quad \text{Hydrate}
\end{align*}
\]}
This hemithioacetal can be envisaged as resembling a tetrahedral intermediate formed between papain and an ester or amide substrate during acylation and deacylation:

\[
\begin{align*}
\text{O} & \quad \text{E-S}^- \\
\text{R-C-X-R} & \quad \text{R-C-X-R} \\
\text{E-S} & \quad \text{R-C} \\
\text{H}_2\text{O} & \quad \text{R-C-OH} \\
\text{X=-NH-} & \quad \text{THI} \\
-\text{O-} & \quad \text{THI'} \\
\end{align*}
\]

The tight binding of aldehyde inhibitors by formation of a hemithioacetal argues in support of a tetrahedral intermediate in substrate hydrolysis since it illustrates the capability of the enzyme to accommodate and stabilize the intermediate.

The previous investigations into aldehyde inhibition of papain relied upon observing changes in the fluorescence spectra, (Mattis et al., 1977; Frankfater and Kuppy, 1981) or UV-visible spectrophotometry of substrate inhibition (Lewis and Wolfenden, 1977 b; Westerik and Wolfenden, 1972; Frankfater and Kuppy, 1981), to study the papain-inhibitor complexes. \(^1\)H-NMR has also been used to study papain-aldehyde interactions (Bendall et al., 1977; Clark et al., 1977). Evidence in favour of hemithioacetal formation has been provided from deuterium isotope effects on inhibition (Lewis and Wolfenden, 1977 b) and cross-saturation of the hemithioacetal resonance by \(^1\)H-NMR (Bendall et al., 1977).

From these studies it was also concluded that the aldehyde itself and not its hydrate is the effective inhibitor, although in aqueous solution the aldehyde exists primarily as its hydrate (Bendall et al., 1977; Lewis and Wolfenden, 1977 a, b).

One of the most effective inhibitors of papain was determined to be N-acetyl-L-phenylalanylglycinal (Westerik and Wolfenden, 1972; Mattis et al., 1977; Frankfater and Kuppy, 1981). This substrate analogue inhibits papain in a 1 : 1 molar ratio with an extremely low \(K_i\) (see Table 7). The value for \(K_i\) may be corrected for the
fraction of aldehyde (and not hydrate) in solution. Because of the low inhibition constant for N-acetyl-L-phenylalanylglycinal, the methods of Henderson (1972) and Morrison (1969) must be used to estimate $K_i$ and the stoichiometry of inhibition. These methods are used with tightly bound inhibitors where Michaelis-Menten kinetics may not be valid.

The stoichiometry and inhibition constant were obtained and compared to the results of previous investigations (Table 7). These results agree well with earlier findings and the 1:1 stoichiometry of the inhibition was also confirmed.

Table 7.

<table>
<thead>
<tr>
<th>Reference</th>
<th>$K_i$ (M)</th>
<th>pH</th>
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</thead>
<tbody>
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<td>Westerik and Wolfenden (1972)</td>
<td>$4.6 \times 10^{-8}$</td>
<td>5.5</td>
</tr>
<tr>
<td>Lewis and Wolfenden (1977 b)</td>
<td>$5.2 \times 10^{-9}$</td>
<td>5.5</td>
</tr>
<tr>
<td>Mattis et al. (1977)</td>
<td>$1.0 \times 10^{-8}$</td>
<td>6.5</td>
</tr>
<tr>
<td>Frankfater and Kuppy (1981)</td>
<td>$8.3 \times 10^{-9}$</td>
<td>6.5</td>
</tr>
<tr>
<td>This work</td>
<td>$1.0 \times 10^{-8}$</td>
<td>7.0</td>
</tr>
</tbody>
</table>

$^{13}C$-NMR spectroscopy should enable direct observation of the inhibitor-enzyme complex and provide information on the mechanism of inhibition. Formation of a tetrahedrally substituted carbon should be readily observed by $^{13}C$-NMR. The results of Rich et al. (1982), and Malthouse et al. (1983) illustrate the potential of $^{13}C$-NMR when studying the inhibition of serine proteases with ketone inhibitors. Covalent attachment of the sulphur of Cys-25 to the $^{13}C$-enriched aldehyde carbonyl to form a hemithioacetal should directly confirm the nature of the inhibition. If the aldehyde or hydrate inhibits papain by non-covalent binding in the active-site,
only a small shift in the resonances would be expected since bonds directly attached to the enriched carbon remain intact.

The reaction of N-acetyl-L-cysteine with N-acetyl-L-phenylalanyl-[1-\(^{13}\)C]-glycinal was first studied as a model system by \(^{13}\)C-NMR spectroscopy. From the equilibrium constants of thiol-aldehyde reactions studied by UV (K=4-40 M\(^{-1}\)), an excess of aldehyde or thiol is needed for significant amounts of hemithioacetal to be formed (Lienhard and Jencks, 1966; Kanchuger and Byers, 1979).

The \(^{13}\)C-labelled aldehyde was provided as its more stable dimethyl acetal since free aldehydes are susceptible to polymerization and/or condensation when at high concentration. The dimethyl acetal itself is acid labile and was hydrolysed to the aldehyde in 0.2 M HCl and the reaction was followed by \(^{13}\)C-NMR. Figure 14 (a) shows the resonances of the dimethyl acetal at 102.4 ppm and the aldehyde hydrate at 88.2 ppm. After hydrolysis is complete (\(\sim 5\) h), a single large resonance due to the hydrate was detected (Figure 14(c)). Below each spectrum are the proton coupled spectrum of the acetal (Figure 14 (b)), and the hydrate (Figure 14(d)). The \(^{13}\)C-\(^1\)H coupling constant for the acetal is 167 Hz, and for the hydrate, \(J_{^{13}\text{C}-^{1}\text{H}}=164\) Hz.

The natural-abundance proton-decoupled spectrum of N-acetyl-L-cysteine is shown in Figure 15(f). Figure 15(a) shows the hydrate resonance at pH 2 as in Figure 14(c). Five resonances are clearly visible and assignable to each carbon atom on the basis of analysis of the proton-coupled spectrum (not shown). Adding approximately equimolar N-acetyl-L-cysteine to the aldehyde solution at low pH did not change the position of the hydrate resonance nor was any new signal detectable (Figure 15(b)). Adjusting the pH to 7 did not change the spectrum (Figure 15(c)). Addition of a large excess of N-acetyl-L-cysteine resulted in the appearance of a broad resonance at 75.7 ppm along with the natural-abundance resonances of N-acetyl-L-cysteine at 56.6, 26.3, and 21.9 ppm.
Figure 14. Hydrolysis of N-acetyl-L-phenylalanyl-[1-$^{13}$C]-glycinal dimethyl acetal to the aldehyde.

a) 4.49 mM N-acetyl-L-phenylalanyl-[1-$^{13}$C]-glycinal dimethyl acetal in 0.2 M HCl, 95% D$_2$O. Decoupled spectrum. 

b) Same as in (a), non-decoupled spectrum, 

\[ J_{CH} = 166.7 \text{ Hz.} \]

c) After 5 hours, dimethyl acetal in (a) is > 90% hydrolysed to N-acetyl-L-phenylalanyl-[1-$^{13}$C]-glycinal hydrate. Decoupled spectrum.

d) Same as in (c), non-decoupled spectrum, 

\[ J_{CH} = 164.3 \text{ Hz.} \]
The pH was then lowered and two sharp resonances at 75.8 and 76.9 ppm were observed (Figure 15(e)). These are in the region predicted for a hemithioacetal carbon. The hemithioacetal resonances should be upfield from the hydrate resonance in view of the smaller $\alpha$-effect of sulphur as compared to oxygen. Since sulphur is less electronegative than oxygen, there is less deshielding of the attached carbon and therefore the resonance of a sulphur substituted tetrahedral carbon is at higher field than a similar oxygen containing compound (Stothers, 1972; Breitmaier and Voelter, 1978).

The fact that two hemithioacetal resonances are observed in this experiment is predictable because of the two configurations which are possible when an aldehyde, with two pro-chiral faces, undergoes nucleophilic addition:

![Diagram of hemithioacetal configurations](image)

The sulphur atom of the thiol is able to add either to the "re" or "si" face (Hanson, 1966) of the aldehyde producing either and R- or S-hemithioacetal respectively. When the thiol is an achiral molecule, two enantiomers are formed. The enantiomers are indistinguishable by $^{13}$C-NMR (in achiral solvents) since the tetrahedral carbon is in the same environment in both (Raban and Mislow, 1967). This is illustrated by the single chemical shift observed for the hemithioacetal formed between N-acetyl-L-phenylalanyl-[1-$^{13}$C]-glycin al and the achiral thiol 2-mercaptoethanol ($\delta = 73.3$ ppm; Primrose et al., 1983).

With a chiral group such as N-acetyl-L-(S)-cysteine, 2 diastereoisomers...
Figure 15. Model experiment; reaction of N-acetyl-L-cysteine with N-acetyl-L-phenylalanyl-[1-^{13}C]-glycinal. Decoupled spectra.

a) Same as in Figure 14 (c).

b) 3.74 mM N-acetyl-L-phenylalanyl-[1-^{13}C]-glycinal as in (a) with 3.0 mM N-acetyl-L-cysteine, pH 2, 80% D_2O.

c) 3.21 mM ^{13}C-labelled aldehyde with 2.6 mM N-acetyl-L-cysteine, 7 mM phosphate, pH 7.1, 70% D_2O.

d) 2.81 mM ^{13}C-labelled aldehyde with 27 mM N-acetyl-L-cysteine, 6 mM phosphate, pH 7.3, 60% D_2O.

e) Same as in (d), adjusted to pH 3.7.

f) 32 mM N-acetyl-L-cysteine, 90% D_2O, 7 mM phosphate, pH 3.7.
are formed; R-hemithioacetal-S-cysteine, and S-hemithioacetal-
S-cysteine. In this case, the carbons are non-equivalent and two
resonances are observable. This approach has been used to detect
mixtures of enantiomers by NMR (Raban and Mislow, 1967).

The effect of pH on the intensity of the hemithioacetal signals
(Figure 15(d) and (e)), can be explained by the effect of pH on the
formation and breakdown of hemithioacetals. Jencks and co-workers
have shown that the equilibrium constants \( K_{\text{hemi}} \) for formation of
a hemithioacetal are pH independent (Lienhard and Jencks, 1966;
Gilbert and Jencks, 1977).

\[
K_{\text{hemi}} = \frac{[\text{Hemithioacetal}]}{[\text{Thiol}][\text{Unhydrated aldehyde}]} \tag{32}
\]

For thiols such as N-acetyl-L-cysteine (\( pK_a = 9.52 \), Friedman
et al., 1965), the addition of thiolate to aldehyde may be represented
by the following equilibria (Gilbert and Jencks, 1977).

\[
\begin{align*}
R' & \overset{k_1[R S^-]}{\rightleftharpoons} R-S-\overset{\cdot O^-}{\text{C}}-\overset{\cdot}{H} & \overset{k_h[H_2O]}{\rightleftharpoons} R-S-\overset{\cdot O}{\text{H}} \tag{R'}
\end{align*}
\]

For the wide range of thiols studied by Jencks and co-workers,
the pH independent rate constants \( k_1 \) and \( k_h \) are large and are not
the rate limiting steps in formation and breakdown of the hemithio-
acetals at the pH values used. The rate constants \( k_1 \) and \( k_h \) are
also pH independent but both steps rely upon reactants with pH
dependent concentrations. These steps are the rate determining
steps in formation and breakdown of the hemithioacetal. Since data
is not available for the hemithioacetal formed between N-acetyl-L-
cysteine and N-acetyl-L-phenylalanylglycinal, a reasonable estimate
of rate constants for this system can be made from the data for
the hemithioacetal formed between methoxyethanethiol (pK \textsubscript{a} = 9.5)
and acetaldehyde (Gilbert and Jencks, 1977).

For formation of the hemithioacetal, the observed rate
limiting step will be dependent on the concentration of the thiolate
ion:

\[ k_{1(\text{obs})} = k_1[RS^-] \]  \hspace{1cm} (33)

Since [RS\textsuperscript{-}] is dependent on pH the observed rate constant
will be pH dependent:

\[ k_1(\text{obs}) = \frac{k_1}{1 + \frac{[H^+]}{K_a}} \]  \hspace{1cm} (34)

Where K\textsubscript{a} is the dissociation constant for RSH. The
observed rate limiting breakdown step is also pH dependent:

\[ k_{-h(\text{obs})} = k_{-h}[\text{OH}^-] \]  \hspace{1cm} (35)

From the data of Gilbert and Jencks (1977), \( k_{-h} = 1 \times 10^{10} \)
M\textsuperscript{-1}s\textsuperscript{-1}, and \( k_1 = 4.7 \times 10^5 \text{ M}^{-1}\text{s}^{-1} \). The table below presents
results for the rate constants at different pH values.

<table>
<thead>
<tr>
<th>pH</th>
<th>9</th>
<th>8</th>
<th>7</th>
<th>4</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_1(\text{obs}) ) (s\textsuperscript{-1})</td>
<td>1.13 \times 10^5</td>
<td>1.44 \times 10^4</td>
<td>1.48 \times 10^3</td>
<td>1.49</td>
<td>1.49 \times 10^{-2}</td>
</tr>
<tr>
<td>( k_{-h(\text{obs})} ) (s\textsuperscript{-1})</td>
<td>1 \times 10^5</td>
<td>1 \times 10^4</td>
<td>1 \times 10^3</td>
<td>1</td>
<td>1 \times 10^{-2}</td>
</tr>
</tbody>
</table>

(The values for \( k_{-1} = 2.3 \times 10^7 \text{ s}^{-1} \), and \( k_h = 5 \times 10^8 \text{ s}^{-1} \)
from Gilbert and Jencks, 1977 are much greater than the values
in the table)
In order to distinguish two distinct resonances in an NMR spectrum the difference in chemical shift (in Hz) between the two species must be much greater than the rate constant for exchange between the two (Breitmaier and Voelter, 1978)

\[
\text{\textsuperscript{\textit{v}}} \text{aldehyde} - \text{\textsuperscript{\textit{v}}} \text{hemithioacetal} \gg k_{\text{exchange}} 
\]  

(36)

For the aldehyde (\(\delta = 200.9\) ppm = 15150 Hz), and the hemithioacetals (\(\delta \sim 74\) ppm = 5580 Hz) and the difference is \(\sim 9500\) Hz. Between pH 7 and 8 the observed rate constants for formation and breakdown are of the order 1000 to 14000 s\(^{-1}\). In this pH region, the rates of exchange are too fast for detection of sharp resonances and instead a broadening of the resonances results (Figure 15(d)). On lowering the pH to less than 4, the rate constants are well below the limit for \(\textsuperscript{13}\)C-NMR observation of sharp resonances (Figure 15(e)). It must be emphasized that the equilibrium constant (\(K_{\text{hemi}}\)) remains the same at pH 7 and 4, but the rate of exchange is beyond the detectable range for \(\textsuperscript{13}\)C-NMR at the higher pH. The rate constants here are also estimates and may be slightly different for N-acetyl-L-cysteine and N-acetyl-L-phenylalanyl-glycinal.

The proton-decoupled spectrum of papain is shown in Figure 16 (b) below the decoupled spectrum of N-acetyl-L-phenylalanyl-[1-\(\textsuperscript{13}\)C]-glycinal (Figure 16 (a)). A small amount of the aldehyde was added to the papain solution to give a 3-fold excess of protein. The \(\textsuperscript{13}\)C-NMR spectrum (Figure 16 (c)) shows a weak, broad resonance near 74 ppm and the notable absence of the aldehyde hydrate resonance at 88.2 ppm. Addition of more N-acetyl-L-phenylalanyl-[1-\(\textsuperscript{13}\)C]-glycinal to bring its concentration approximately equimolar with papain resulted in the appearance of a strong, broad resonance at 74.7 ppm (Figure 16 (d)). Also observable is a small peak at 102.4 ppm corresponding to the quaternary carbon of the unhydrolysed dimethyl acetal. A third addition of labelled inhibitor
Figure 16. Inhibition of Papain with N-Acetyl-L-Phenylalanyl-[1-$^{13}$C]-glycinal
Decoupled spectra.

a) 3.41 mM N-acetyl-L-phenylalanyl-[1-$^{13}$C]-glycinal in 10 mM phosphate
   80% D$_2$O, NS = 1000, pH 7.1.

b) 0.936 mM Papain (1.04 mM protein), 10 mM phosphate, 10% D$_2$O
   pH 7.1, NS = 5000 (Spectra (c) to (e) have the same NS)

c) First addition of aldehyde. 0.851 mM papain (0.945 mM protein), 0.280 mM
   N-acetyl-L-phenylalanyl-[1-$^{13}$C]-glycinal, 10 mM phosphate, 16% D$_2$O, pH 7.1

d) Second addition of aldehyde. 0.720 mM papain (0.800 mM protein), 0.710 mM
   $^{13}$C-labelled aldehyde, 10 mM phosphate, 26% D$_2$O, pH 7.1

e) Third addition of aldehyde. 0.624 mM papain, 1.76 mM $^{13}$C-labelled aldehyde,
   10 mM phosphate, 33% D$_2$O, pH 7.1.
brought the aldehyde to a 3-fold excess. The hemithioacetal signal at 74.7 ppm increases only slightly and the free hydrate is also observable at 88.2 ppm (Figure 16 (e)). Figure 17 shows some of these solutions after longer accumulation times. The aldehyde hydrate is shown in Figure 17 (a), papain in Figure 17 (b), papain with equimolar aldehyde in Figure 17 (c), and papain with excess aldehyde in Figure 17 (d). These clearly show the strong resonances for each of the enriched species. The observation of the hemithioacetal resonance provides the first direct evidence for formation of this tetrahedral adduct during the inhibition of papain with N-acetyl-L-phenylalanylglycin. The low \( K_i \) value for this inhibitor enables a strong signal to be observed with 1:1 stoichiometric amounts of aldehyde to papain. The large difference between the chemical shift of the hydrate at 88.2 ppm and hemithioacetal at 74.7 ppm rules out non-covalent binding of the hydrate. Non-covalent binding would shift the resonance only slightly (0.1 to 2 ppm) due to the different environment of the active-site. The appearance of both the hydrate and hemithioacetal resonances (Figure 17 (d)) with different line-widths rules out fast exchange for these two species. The line-widths from each enriched resonance in Figure 17 (d) are shown in the table.

<table>
<thead>
<tr>
<th>Species</th>
<th>( \delta ) (ppm)</th>
<th>LW (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemithioacetal</td>
<td>74.7</td>
<td>88</td>
</tr>
<tr>
<td>Hydrate</td>
<td>88.2</td>
<td>11</td>
</tr>
<tr>
<td>Dimethyl acetal</td>
<td>102.4</td>
<td>12</td>
</tr>
</tbody>
</table>

The broad hemithioacetal resonance is due in part to attachment of the low molecular weight inhibitor to the large protein molecule. Covalent bonding to the protein shortens the relaxation time and broadens the resonance lines.

The thiol titrant 2-PDS (natural-abundance spectrum in
Figure 17. Enzyme aldehyde experiment, longer accumulations, improved signal-to-noise in each spectrum.

a) Same as in Figure 16 (a)

b) Same sample as in Figure 16 (b), NS = 29000

c) Same sample as in Figure 16 (c), NS = 29000

d) Same sample as in Figure 16 (d), NS = 32000
Figure 18(b)) was then added to the papain-inhibitor mixture (shown again in Figure 18 (a)). This titrant rapidly reacts with free thiol and prevents formation of the hemithioacetal by the reversible aldehyde inhibitor. The addition at pH 7 results in a dramatic increase in the free hydrate resonance at 88.2 ppm. There is also a significant amount of the hemithioacetal present but the linewidth of the resonance decreased to 52 Hz. (Figure 18 (c)). By comparison, the linewidths of the hydrate and dimethyl acetal resonances in Figure 18 (a) and (c) remain approximately the same. After 48 hours, the spectrum remains the same and only upon changing to pH 4 is most of the hemithioacetal signal eliminated (Figure 18(d)). The intensity of the free hydrate signal in Figure 18 (d) shows the effect of line broadening on the resonances. Also apparent in the spectrum is the free unhydrated aldehyde at 200.9 ppm. This resonance is not observed in the spectra with the hemithioacetal present which is consistent with this species being the effective inhibitor and/or $K_{\text{hemi}}$ being very large ($K_{\text{hemi}} \gg K_{\text{hyd}}$). The ratio of the hydrate to aldehyde ($K_{\text{hyd}}$) is approximately 10 : 1 (estimated from the peak intensities), which agrees with values obtained earlier for hydration of aldehydes (Lewis and Wolfenden, 1977 a; Bendall et al., 1977). Each species has now been detected in the experiments and assignments can be made as follows:

<table>
<thead>
<tr>
<th>Species</th>
<th>Chemical Shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OMe dil HCl</td>
<td>R-C-H</td>
</tr>
<tr>
<td>R-C-H</td>
<td>E-S</td>
</tr>
<tr>
<td>OMe</td>
<td>OH</td>
</tr>
<tr>
<td>102.4 ppm</td>
<td>200.9 ppm</td>
</tr>
<tr>
<td></td>
<td>74.7 ppm</td>
</tr>
<tr>
<td></td>
<td>88.2 ppm</td>
</tr>
</tbody>
</table>

(Chemical structures are not rendered in this text format)
Figure 18. Addition of 2-PDS to papain-aldehyde mixture

a) Same as in Figure 17 (d)

b) Natural abundance spectrum, 1.37 mM 2, 2'-dipyridyl disulphide, 33% D₂O, pH 7.2, NS = 44000.

c) Addition of 2-PDS. As in (a) plus 10 mg 2-PDS, stirred 1.5 hours, NS = 42000.

d) After 24 hours, adjusted (c) to pH 4.1, NS = 42000.
On closer inspection, the broad hemithioacetal resonance ($LW = 88$ Hz) is seen to be split into two resonances at 75.1 and 74.7 ppm, separated by 28 Hz (Figure 19 (a)). The proton-coupled spectra also show similar splitting of the two resonances (Figure 19 (b)). After addition of 2-PDS at pH 7, the upfield resonance remains at 74.7 ppm while the resonance at 75.1 ppm disappears (Figure 19 (c)). The remaining resonance at 74.7 ppm has a linewidth of 52 Hz. This resonance is shown in the proton-coupled spectrum (Figure 19 (d)), and the coupling constant obtained is 160 Hz. This is equivalent to the coupling constant obtained in Figure 19 (b) for the upfield resonance at 74.7 ppm. The downfield resonance at 75.1 ppm in Figure 19 (a) shows a slightly different coupling constant of 155 Hz in Figure 19 (b). These results offer convincing evidence that two hemithioacetal resonances are present. The broad resonance of 88 Hz is composed of two resonances of ~50 Hz each. Since both resonances are of equal intensity, and the aldehyde inhibition has been shown to be reversible, then the equilibrium constants for the two forms of the hemithioacetal must be similar.

From the model studies, the two forms may result from the two modes of addition of the papain thiol to the aldehyde. The sulphur atom is able to add either to the "re" of "si" face of the inhibitor showing no preference for either conformation. This suggests that binding of the inhibitor aldehyde group is not stereospecific and that a proposed oxygen binding site for a substrate carbonyl oxygen (Drenth et al., 1976) does not influence binding of the inhibitor carbonyl. These results are consistent with the conclusion of Asbóth and Polgár (1983) in that a specific oxygen binding site does not play an important part in papain-catalysed hydrolyses. Different stereochemical considerations may apply to the binding of specific amide and ester substrates since it has been determined that the $S_{1}^{1}$ subsite is specific for non-polar leaving groups of the substrates (Alecio et al., 1974). The less
Figure 19. Proton coupled and decoupled spectra of the hemithioacetal resonances.

a) Same spectrum as in Figure 17 (d) showing only the region from 70 to 80 ppm.

b) Same sample as in (a), non-decoupled, NS = 128000

c) After addition of 2-PDS at pH 7, as in Figure 18 (c)

d) Non-decoupled spectrum of the same sample as in (c). NS = 168000.
sterically demanding aldehyde is free to assume two conformations since a proton is present in the position usually occupied by the leaving group. The results are not consistent with one conformation of hemithioacetal in two ionic states, i.e. the anion and the neutral hemithioacetal. The two resonances are separated by only 28 Hz which would require that the rate constants for proton transfer between the two species to be less than 28 s\(^{-1}\). For two hemithioacetals in the same conformation, this would be a very slow rate for proton exchange.

The equal intensity of the two hemithioacetal resonances in the \(^{13}\)C-NMR spectrum suggests that the equilibrium constants \(K_{\text{hemi}}\) for the two conformers must be similar. The separate titration of the two resonances with 2-PDS however suggests that the rate constants for breakdown of the hemithioacetals differ markedly. One possible explanation is that one of the conformations has an enhanced rate of formation and breakdown compared to the other so the equilibrium constants remain similar. For example, two binding conformations of the aldehyde in the active-site can be visualized (Figure 20).

One conformer corresponds to the non-productive binding mode suggested by Frankfater and Kuppy (1981). In this mode, (A) in Figure 20, the carbonyl oxygen is not bound in an oxygen binding site as may occur with specific substrates, but rather is in close proximity to the imidazole group of His-159. This is the position usually thought to be occupied by the leaving group heteroatom of specific substrates. This conformation allows facile proton transfer between the imidazole and heteroatom assisting in leaving group expulsion. In this case, the proton is in position for transfer to the hemithioacetal anion formed (B), and assist in removal of the proton as a general-base catalyst during breakdown.

Assuming that the phenylalanine side-chain of the inhibitor is bound tightly in \(S_2\), the other conformation allowing for nucleophilic
Figure 20. Possible mechanism of papain inhibition with aldehydes.

\[ \text{Figure 20. Possible mechanism of papain inhibition with aldehydes.} \]
attack on the opposite face of the aldehyde would place the aldehydic proton near the imidazole (D), and require protonation of the hemithioacetal from the water solvent, (E) to (F) in Figure 20. This conformer may have a rate constant for formation much lower than with (C) but breakdown would also be slower. The two species may therefore be present in solution in equal amounts \(K_{\text{hemi}}(C) = K_{\text{hemi}}(F)\) but the rate constants are unequal \(k_1 \gg k_2, k_{-1} \gg k_{-2}\). The 2-PDS may rapidly titrate the conformer with a fast exchange rate and slowly titrate the other conformer. At low pH (i.e. \(\leq 4\)), the inhibition constants for papain and N-acetyl-L-phenylalanylglycinal increases (Frankfurter and Kuppy, 1981) and both conformers dissociate rapidly.

The results are also consistent with another less likely mechanism. If both conformations are equally stable and in equilibrium, the imidazole may be able to adopt two conformations to stabilize both modes of inhibition. This would resemble the UP-DOWN transitions suggested by Angelides and Fink (1978) for substrate binding. The 2-PDS results could then be rationalized by binding of 2-PDS at the active site which enhances the stability of one conformer.

The two hemithioacetal signals do not result from the presence of denatured enzyme in the solution. At the completion of the \(^{13}\)C-NMR experiments, the 2-PDS blocked papain-aldehyde solution was subjected to gel-filtration to remove the aldehyde and excess 2-PDS present. The protein concentration was determined at pH 8.2 and excess cysteine was added to reactivate the enzyme. The reactivation unblocks the enzyme and liberates 2-thiopyridone. From this experiment it was determined that \(\geq 90\%\) of the active enzyme was blocked with 2-PDS. The reactivated enzyme showed no loss of activity to Na-CBZ-glycine-p-nitrophenyl ester at pH 5.

The \(^{13}\)C-NMR results may be compared to the \(^1\)H-NMR results of Bendall et al. (1977). In particular, the coupling constants,
obtained by these authors between the aldehydic proton
and a \(^{13}\)C-enriched aldehyde should be directly comparable.
The aldehyde signal in Figure 17 (d) has a coupling constant of
183 Hz as compared to the aldehyde coupling determined by Bendall
et al. (1977) where it was measured as 182 Hz. The aldehyde hydrate
signal is split by 164 Hz compared to the hydrate in the previous
study of 165 Hz. Bendall et al. (1977) reported the splitting of the
hemithioacetal signal as 173 Hz. The substitution of a sulphur
for an oxygen atom should however decrease the splitting because
of the lower electronegativity of the sulphur (Stothers, 1972;
Breitmaier and Voelter, 1978). The hemithioacetal signal should therefore
have a smaller splitting than that of the hydrate which is consistent
with the result obtained, \(J_{\text{13} \text{C}-\text{1H}}^{\text{hemi}}\) (hemithioacetals) = 160, 155 Hz.
The results of Bendall et al. (1977) may be rationalized by the
fact that they did not directly observe the hemithioacetal splitting,
but estimated it from cross-saturation experiment. By applying
a second radio-frequency in the region expected for a hemithioacetal
proton (which they could not observe), they cross-saturated the
resonance of the unbound aldehyde which they could observe. In
this way they obtained a reasonable, but not accurate, estimate of
the hemithioacetal coupling. More accurate results are obtained
in directly measuring this splitting by \(^{13}\)C-NMR. The \(^{1}\)H-NMR
technique also suffers from resolution problems. The 28 Hz (0.4 ppm)
difference between the hemithioacetal signals observed in the \(^{13}\)C-
NMR spectrum would be more difficult to resolve in the \(^{1}\)H-NMR
spectrum where this same difference corresponds to a separation of
0.1 ppm. This resolution problem would also contribute to errors
in the estimate of the hemithioacetal coupling since two peaks would
appear as one. The \(^{13}\)C-NMR spectrum of the hemithioacetal offers
a far more accurate picture of hemithioacetal formation and provides
much more information about the conformation and molecular
events occurring during inhibition.

The broad hemithioacetal resonances at 74.7 and 75.1 ppm
have been shown to have a linewidth of \(~50\) Hz. This can be compared to the hemiketal formed with trypsin and a chloromethyl ketone which has a linewidth of \(~9\) Hz (Malthouse et al. 1983). Protonated carbons are expected to have broader resonance lines due to the decrease in the relaxation time caused by the attached proton. The \(^{13}\)C-\(^1\)H dipole-dipole relaxation mechanism is dominant for the protonated carbon and the linewidth is related to the contribution from this interaction. For a carbon with a single proton attached, an estimate of the contributions of dipolar interactions to the linewidth is shown by the following equation (assuming no internal rotations) (Oldfield et al. 1975a).

\[
\text{LW} = \frac{1}{20\pi} \frac{\hbar^2}{\gamma_C^2} \gamma_H^2 r_{\text{CH}}^6 \phi_H
\]

Where \(\gamma_C\) and \(\gamma_H\) are the gyromagnetic ratios for \(^{13}\)C and \(^1\)H respectively, \(r_{\text{CH}}\) is the interatomic distance (\(~1.09\) Å), and \(\phi_H\) is a function of the rotational correlation time \((\tau_R)\) and Larmor frequencies of the carbon \((\omega_C)\) and proton \((\omega_H)\) nuclei in the magnetic field used.

\[
\phi_H = \frac{\tau_R}{1 + (\omega_H - \omega_C)^2 \tau_R^2} + \frac{3\tau_R}{1 + \omega_C^2 \tau_R^2} + \frac{6\tau_R}{1 + (\omega_H + \omega_C)^2 \tau_R^2} + \frac{4\tau_R}{1 + \omega_H^2 \tau_R^2}
\]

The correlation time for overall rotation of the papain-inhibitor complex may be calculated for a 50 Hz linewidth as \(\tau_R = 36\) nsec. This is a reasonable estimate for a carbon covalently attached to a large protein macromolecule. This value compares
well with the results for protonated carbons of proteins calculated by Oldfield et al. (1975a). They found that for myoglobin ($MW = 17000$), $\tau_R = 19 \text{ nsec}$, and for haemoglobin ($MW = 65000$), $\tau_R = 47 \text{ nsec}$. Since the rotational correlation time is inversely related to the size of the molecule, papain ($MW = 23406$) would be expected to have a $\tau_R$ value in between these two. The linewidth of a non-protonated carbon with the same correlation time would be expected to have a linewidth near 10 Hz (Oldfield et al. 1975a). These results further confirm that the hemithioacetal is covalently bound to the enzyme. The hemithioacetal must also be rigidly held since free rotation would result in narrower linewidths.

The results of this experiment confirm the fact that a hemithioacetal is formed during the inhibition of papain with N-acetyl-L-phenylalanylglycinal. For the first time, evidence is presented for non-stereospecific hemithioacetal formation. This application of $^{13}C$-NMR to the study of the stereospecificity of enzyme reactions may have further implications for the results of previous investigations. The single hemiketal resonances observed by Rich et al. (1982), and Malthouse et al. (1983), means that either for these complexes binding is essentially stereospecific, or that two resonances are not resolved.
Papain and Na-CBZ-L-lysine-chloromethyl ketone

The inhibition of serine proteases with chloromethyl ketone (CMK) substrate analogues occurs by alkylation of the active-site histidine and formation of a tetrahedral hemiketal by nucleophilic attack of the active-site serine hydroxyl on the ketone carbonyl (Poulos et al., 1976; Malthouse et al., 1983). With papain, alkylation occurs at the thiol of Cys-25 (Bender and Brubacher, 1966; Husain and Lowe, 1965; Whitaker and Perez-Villaseñor, 1968), without reaction at the ketone carbonyl. Drenth et al. (1976) studied the interaction of a number of peptide-CMK inhibitors with papain and presented a detailed study of papain inhibition by x-ray crystallography. Since CMK inhibitors covalently bond irreversibly at the active-site of papain, a $^{13}$C-NMR study was initiated to observe the effects of the protein environment on the carbonyl resonance of a $^{13}$C-enriched CMK.

Na-CBZ-L-lysine-CMK has been shown to irreversibly inhibit trypsin (Coggins et al., 1974) and was chosen for the papain study on the basis of preliminary inhibition experiments. The rate of hydrolysis of Na-CBZ-glycine-p-nitrophenyl ester was determined for papain solutions preincubated with various concentrations of CMK. An equimolar amount of CMK to papain thiol inhibited > 90% of the activity (see Table 10) but an excess of CMK was required for full inhibition.

Table 10. The inhibition of the papain-catalysed hydrolysis of Na-CBZ-glycine-pNP with Na-CBZ-L-lysine-CMK.

<table>
<thead>
<tr>
<th>Papain-SH : CMK</th>
<th>$k_{cat}(s^{-1})$</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 : 0</td>
<td>5.1</td>
<td>100%</td>
</tr>
<tr>
<td>1 : 1</td>
<td>0.56</td>
<td>11%</td>
</tr>
<tr>
<td>1 : 1.5</td>
<td>0.10</td>
<td>2%</td>
</tr>
<tr>
<td>1 : 2</td>
<td>0.012</td>
<td>0.23%</td>
</tr>
<tr>
<td>1 : 2</td>
<td>0.014</td>
<td>0.27% **</td>
</tr>
</tbody>
</table>

**(After gel-filtration)**
2-PDS titration of the protein solution after gel-filtration to remove unreacted CMK showed no reactive thiol which indicated that the active-site cysteine residue is irreversibly blocked.

As a model system for the enzyme reaction, [2-\textsuperscript{13}C]-Na-CBZ-L-lysine-CMK was reacted with N-acetyl-L-cysteine. The spectrum of [2-\textsuperscript{13}C]-Na-CBZ-L-lysine-CMK in DMSO-d\textsubscript{6} is shown in Figure 21 (a). A single resonance is observable at 200.6 ppm. This resonance is at the high-field end of the ketone carbonyl region of the \textsuperscript{13}C-NMR spectrum (\textasciitilde200-220 ppm), due to the deshielding effect by the chlorine atom on the adjacent carbon (see entries 1-3 in Table 11). In 1 mM HCl, the \textsuperscript{13}C-NMR spectrum shows two enriched resonances at 204.7 and 95.4 ppm which can be assigned to the ketone and its hydrate respectively (Malthouse et al., 1983) (Figure 21 (b)). The linewidth of the ketone resonance is 4.0 Hz. The spectrum of the ketone and hydrate was unchanged between pH 3 and 7. The intensity of the hydrate signal decreased between pH 7 and 9 as expected for a hydrated carbonyl group where hydration is acid catalysed (Hellström and Almqvist, 1970). Above pH 9 the signals at 204.7 and 95.4 are not detected due to breakdown of the CMK (Malthouse et al., 1983). Addition of CMK to a solution of N-acetyl-L-cysteine at pH 7 resulted in the appearance of a new resonance at 207.8 ppm with the disappearance of the signals at 95.4 and 204.7 ppm (Figure 21 (c)). The resonance was unchanged between pH 7 and 10. This downfield shift is consistent with substitution of the chlorine atom and is characteristic of aliphatic ketones (see Table 11). The resonances can be assigned to the species in the following reaction scheme:
When equimolar [2-^{13}C]-Nα-CBZ-L-lysine-chloromethyl ketone is added to a papain solution at pH 6.8, a single, strong resonance at 217.1 ppm is observable (Figure 22 (a)), and the activity of the enzyme is reduced to 10% of the original activity. Adding a second aliquot of CMK resulted in little or no activity to Nα-CBZ-glycine-p-nitrophenyl ester and a slight increase in the 217.1 ppm resonance is detected along with a weak resonance due to unreacted ketone at 204.7 ppm (Figure 22 (b)). Gel-filtration of the sample resulted in observation of a single strong resonance at 217.1 ppm (Figure 22 (c)). The linewidth of the enriched resonance at 217.1 ppm has increased from 4.0 Hz in the free inhibitor to 23.3 Hz in Figure 22 (c). The covalent binding of the ketone to the large protein molecule causes the broadening. Changing the pH between 3.6 and 9.2 had a slight effect on the position of the resonance (< 0.5 ppm shift over this range). At pH 9.2 the intensity of the signal decreases and above this pH, was unobservable. Returning the pH to 6.4 resulted in recovery of the resonance. The signal at 217.1 ppm
Figure 21. The reaction of Na-CBZ-L-lysine-CMK with N-acetyl-L-cysteine.

a) 11.42 mM $[2-^{13}\text{C}]$-Na-CBZ-L-lysine-CMK in DMSO-$d_6$, NS = 6000 (5 mm sample tube).

b) 47.6 mM $[2-^{13}\text{C}]$-Na-CBZ-L-lysine-CMK in 1 mM HCl, 12% D$_2$O, NS = 1500, (5 mm tube).

c) $^{13}\text{C}$-labelled CMK added to solution of N-acetyl-L-cysteine. Resulting solution: 5 mM N-Ac-L-cysteine 2.5 mM $^{13}\text{C}$-labelled CMK, 10 mM phosphate, 12.5% D$_2$O, pH 6.6, NS = 5000. Spectrum was unchanged over 3 hours. Adjusting the pH between 4 and 10 also did not change the spectrum (10 mm sample tube).
Figure 21
Table 11: Chemical Shifts (from TMS) of Ketone Carboxyls

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemical Shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>206.7</td>
</tr>
<tr>
<td>1-Chloroacetone</td>
<td>200.7</td>
</tr>
<tr>
<td>1,1,1-Trichloroacetone</td>
<td>186.3</td>
</tr>
<tr>
<td>2-Butanone</td>
<td>206.3</td>
</tr>
<tr>
<td>3-Pentanone</td>
<td>208.7</td>
</tr>
<tr>
<td>2-Hexanone</td>
<td>206.8</td>
</tr>
<tr>
<td>2-Octanone</td>
<td>206.5</td>
</tr>
<tr>
<td>2-Nonanone</td>
<td>206.4</td>
</tr>
<tr>
<td>5-Nonanone</td>
<td>208.3</td>
</tr>
<tr>
<td>2-Octanone</td>
<td>206.5</td>
</tr>
<tr>
<td>2-Nonanone</td>
<td>206.4</td>
</tr>
<tr>
<td>5-Nonanone</td>
<td>208.3</td>
</tr>
</tbody>
</table>

References:
1. Breitmaier and Voelter, 1978
2. Levy and Nelson, 1972a
3. Hirsch and Jarmas, 1978
Figure 22. Papain inhibition with Na-CBZ-L-lysine-CMK

a) 0.280 mM papain (0.250 mM SH), 0.291 mM [2-\(^{13}\)C]-Na-CBZ-L-lysine-CMK, 10% D\(_2\)O, 10 mM phosphate, pH 7.2. After 40 minute NMR accumulation (10000 scans), 0.05 ml removed for rate assay with CBZ-gly-pNP.

b) 0.274 mM papain (0.247 mM SH), 0.570 mM \(^{13}\)C-labelled CMK, 10% D\(_2\)O, pH 7.2. After accumulation (NS = 10000) second aliquot removed for rate assay.

c) Excess papain added to NMR sample to react with excess CMK, sample on Sephadex G-25 and eluted with 10 mM KCl. Sample reconcentrated to 0.318 mM papain (0.284 mM SH), 10 mM phosphate, 10% D\(_2\)O, 8 mM KCl, pH 7.2, NS = 200000.

d) Denatured papain. To the sample in (c) was added SDS to a concentration of 0.6% by weight. Addition of SDS caused precipitation so the pH of the solution was adjusted from 7.2 to 2 with 0.025 ml 10M HCl. Precipitate redissolved. NS = 205000.
Figure 22
is significantly different from that of the model compound N-acetyl-L-cysteine-CMK. Resonances in this region are characteristic of highly branched ketones (see entries 10-12 in Table 11), or cyclic ketones (entries 13-21 in Table 11). Since substitution at the adjacent carbons is the same as in the model compound, the shift in resonance due to polysubstitution at the C-1 carbon is unlikely.

To confirm that the CMK does indeed react at the active-site sulphur, papain blocked with an (-S-CH$_3$) group on the Cys-25 sulphur was prepared by the method of Smith et al. (1975). This blocked papain (papain-S-S-CH$_3$), was unreactive toward Na-CBZ-glycine-pNP and 2-PDS. Addition of $^{13}$C-CMK to papain-S-S-CH$_3$, pH 7.2, showed no change in the CMK resonances at 204.5 and 95.3 ppm (Figure 23 (a)). The linewidth of the ketone resonance at 204.5 ppm shows only a slight increase to 6.4 Hz indicating that covalent binding of inhibitor to protein has not occurred. (Linewidths are accurate to ±4 Hz depending on sample homogeneity, i.e. a change of 2 Hz is not significant) Even with a four-fold excess of CMK after 18 hours, the resonance positions were unchanged. The additional sharp resonance at 174.1 ppm superimposed on the protein carbonyl resonances, can be assigned to one of the products of the breakdown of CMK in aqueous solution. The resonance may be due to an enriched carboxylic acid formed by a Favorskii-type rearrangement (Kende, 1960):

\[
R-\overset{\bigcirc}{\text{C}}-\text{CH}_2-\text{Cl} \rightarrow R-\text{CH}_2-\overset{\bigcirc}{\text{C}}-\text{OH}
\]

The lysine carbonyl of Na-CBZ-L-lysine has a chemical shift of 174.5 ppm under similar conditions.

After gel-filtration the $^{13}$C-NMR spectrum of the enzyme showed no evidence of enriched resonances (Figure 23 (b)). Re-activation of the enzyme with cysteine resulted in recovery of full
Figure 23. Papain-S-S-CH₃ and Na-CBZ-L-lysine-CMK.

a) 0.261 mM papain-S-S-CH₃ (0.96 thiol/mol), 10 mM phosphate, 10 % D₂O, 0.510 mM [2-¹³C]-Na-CBZ-L-lysine-CMK, pH 7.2, NS = 10000 (part of 25 ml sample)

b) Sample from (a) after gel-filtration, reconcentration. 0.800 mM papain-S-S-CH₃, 10 mM phosphate, 10 % D₂O, pH 7.2, NS = 40000.
activity to Na-CBZ-glycine-pNP. These results suggest that the inhibitor is reactive at the Cys-25 sulphur and that non-specific alkylation does not occur—particularly at the active site His-159 in an analogous manner to the serine proteases with papain-S-S-CH$_3$.

Two possibilities may account for the observation of the low-field resonance of the papain-ketone. The alkylation of the active-site cysteine and binding of the Na-CBZ-L-lysyl group in the active-site region acts to hold the inhibitor in a particular conformation with the carbonyl group fixed in a position similar to the cyclic ketones. A possible conformation was suggested in the dithioacyl-papain studies by Ozaki et al. (1982). In that case, the dithioacyl papain was suggested to be held in a conformation where interaction of the dithioacyl group and N-acyl group takes place (see introduction). Lowe and Yuthavong (1971 a) suggest a mutual distortion of substrate and enzyme in their study of the papain-catalysed hydrolysis of specific substrates. Drenth et al. (1976) observed such a distortion of the enzyme along the active-site cleft after inhibition of the enzyme in the x-ray crystallographic study of papain-CMK complexes.

Another possibility is that upon reaction with the enzyme, the ketone is able to cyclize. This cyclization of the ketone may occur by the following mechanism where non-specific binding of the Na-CBZ-L-lysyl moiety brings the CBZ carbonyl group into the catalytic site:
Since only one resonance is observed, this would require complete binding of the inhibitor in this non-specific mode. The $^{13}$C-NMR results cannot distinguish between these two possibilities. Addition of trypsin to digest the papain did not produce a change in the 217.1 ppm resonance after 18 hours. SDS was added to a papain-CMK mixture to denature the enzyme and the pH lowered to 2 (concentrated, denatured papain was soluble at pH 2). The $^{13}$C-NMR spectrum shows a resonance at 206.7 ppm (Figure 22 (d)). This resonance is very similar to the model compound. Unfortunately, lowering the pH to solubilize the denatured papain means that the results cannot definitely distinguish between the two possibilities. The experiment suggests that denaturation of the enzyme resulted in relief of the conformational restraints on the inhibitor complex and therefore the chemical shift is similar to the model compound. An alternative explanation is that the cyclic ketone is acid labile and hydrolyses to the 1-hydroxy-2-ketone which has a similar chemical shift. From entries 22 and 23 in Table 11, an oxygen attached to the C-1 should have a resonance slightly downfield of the sulphur ketone. The differences are too small to distinguish between these two possibilities. Further $^{13}$C-NMR investigation would be required using similar CMK inhibitors which could not form cyclic species or which were doubly labelled at both carbonyl positions. The results do show that a unique carbonyl resonance is produced upon inhibition of papain with a CMK inhibitor. The resonance may be due to a unique binding mode with cyclization, or to the formation of a conformationally distorted enzyme-inhibitor complex.
The analysis of spectrophotometric data from cryoenzymological experiments is complicated by the fact that the UV-visible spectrum of a chromophore will change as the solvent composition and temperature changes. The next section describes the kinetic analysis of papain-catalysed hydrolyses of specific p-nitrophenyl esters substrates. In each case, the reaction was monitored by the spectrophotometric observation of the p-nitrophenol chromophore produced during the acylation reaction. Prior to these experiments, the effect of co-solvent and temperature on the substrate and product UV-visible spectra was studied. The data from this study provides the difference extinctions ($\Delta \varepsilon$) needed to analyse the progress curves and initial rate data.

Hargreaves et al. (1965) have determined that the $pK_a$ of p-nitrophenol increases from 7.28 in water to 7.67 in 44% acetone in water, to 8.13 in 51% dioxane-water, and 8.04 in 49% DMF-water (all at 20°C). The $pK_a$ of p-nitrophenol in 40% DMSO has been determined to be 7.65 (Malthouse, 1983; unpublished result). p-nitrophenol is commonly used as a spectrophotometric acid-base indicator with the appearance of the p-nitrophenol chromophore as a single peak at 317.5 nm at low pH, which decreases in intensity as the pH is raised with concomitant increase in the p-nitrophenolate absorption which has a $\lambda_{\text{max}} = 402$ nm in highly basic solutions (Doub and Vandenbilt, 1947). Figure 24 illustrates the effect of added DMSO on the absorption spectrum of p-nitrophenol. Curve A shows the absorption spectrum in water at pH 6.1. A large absorption at 318 nm due to p-nitrophenol is present with a small amount of p-nitrophenolate seen as a shoulder on the main peak. Curve B shows the spectrum of the same concentration of p-nitrophenol in 60% DMSO, pH 6.1. The shoulder due to p-nitrophenolate is much smaller since the $pK_a$ increases in DMSO and less p-nitrophenolate is present at the same pH. The position of the $\lambda_{\text{max}}$
Figure 24. The effect of DMSO on the absorption spectrum of p-nitrophenol, 25°C.

A-6.55 \times 10^{-5} \text{ M p-nitrophenol in 0.1 M phosphate, pH 6.1.}

B-6.55 \times 10^{-5} \text{ M p-nitrophenol in 0.1 M acetate, 60% DMSO, pH 6.1.}
shifts slightly to 320 nm with a molar extinction coefficient of \( \sim 9400 \text{ M}^{-1}\text{cm}^{-1} \). A slight change in the pH can cause a change in the value of \( \epsilon \) at each wavelength.

Keilen and Hartree (1949) have examined the effects of low temperatures on the absorption spectrum and noted a sharpening of peaks. This same effect was observed with p-nitrophenol as shown in Figure 25. As the temperature of the solution of p-nitrophenol in DMSO-water is lowered, the absorption spectrum sharpens with the \( \epsilon \) value increasing from 10400 at \( 18^\circ\text{C} \) to 12300 \( \text{M}^{-1}\text{cm}^{-1} \) at \(-65^\circ\text{C}\). The sharpening of the absorption spectrum causes increases in the molar extinction coefficient near \( \lambda_{\text{max}} \) and decreases at wavelengths further from \( \lambda_{\text{max}} \). Similar behaviour occurred in 40 and 50\% DMSO solutions. Figures 26 to 28 show the variation in p-nitrophenol molar extinction coefficients with temperature at pH 6.1. The figures show the increase in \( \epsilon \) at wavelengths near 340 nm with a decrease in temperature, and the decrease in \( \epsilon \) with temperature at longer wavelengths. The extinction values obtained from Figures 26 to 28 were used to calculate the difference extinction by subtraction of the contribution of the substrate molar extinction coefficient under the same conditions.

The absorption spectra of the p-nitrophenyl ester substrates used were all very similar and the effect of temperature on the spectrum of one of these, CBZ-glycine p-nitrophenyl ester, is shown in Figure 30. With decreasing temperature, the substrate spectrum also sharpens and the \( \epsilon_{\text{max}} \) increases. The spectrum at 340 nm and above is not greatly affected by temperature and the molar extinction coefficients obtained are about 10\% of the p-nitrophenol value under the same conditions. The substrate spectra did not change significantly in 40 or 50\% DMSO and the values used for all of the substrates described were obtained from Figure 29. The \( \epsilon \) values in Figure 29 show little change with temperature.
Figure 25. Effect of temperature on the absorption spectrum of $6.55 \times 10^{-5}$ M p-nitrophenol in 0.1 M acetate, 10 mM KCl, 60% DMSO pH* 6.2 (at $1^\circ$C).

(A) $+18^\circ$C  (B) $-24^\circ$C  (C) $-65^\circ$C
Figure 26. Molar extinction coefficients for p-nitrophenol in 0.1 M acetate, 1 mM EDTA, 60% DMSO, pH 6.1. The values of $\varepsilon_x$ are calculated from the concentration of p-nitrophenol used and the absorbance at wavelength ($x$).

Figure 27. Molar extinction coefficients for p-nitrophenol in 0.1 M acetate, 1 mM EDTA, 50% DMSO pH 6.1.
Figure 26
Figure 27
Figure 28. Molar extinction coefficients for p-nitrophenol in 0.1 M acetate, 1 mM EDTA, 40% DMSO, pH 6.1

Figure 29. Molar extinction coefficients calculated from the spectrum of Na-CBZ-glycine p-nitrophenyl ester in 60% DMSO and used for all p-nitrophenyl ester substrates since the spectra were virtually identical. DMSO concentration down to 40% had no significant effect on the molar extinction coefficients at the wavelengths shown.
Figure 28.

Figure 29.
Figure 30. The effect of temperature on the absorption spectrum of 92.6 μM Nα-CBZ-glycine p-nitrophenyl ester in 0.1 M acetate, 60% DMSO, pH 6.2.

(A) +2.1°C    (B) -63°C
The $\Delta \varepsilon$ values, which can be calculated from the temperature dependence of the product p-nitrophenol and substrate molar extinction coefficients, were used in the analysis of the spectrophotometric data obtained during the enzyme catalysed reaction. The contribution of the papain absorption spectrum at $> 340$ nm is very small at the concentrations used and can be ignored. The values of $\Delta \varepsilon$ were also calculated in each experiment where $A_0$ and $A_\infty$ were obtained and these values provided a cross-check on the concentration of substrate used. After the completion of the hydrolysis, a small amount of p-nitrophenol was often added to the cell to check the p-nitrophenol extinction at the pH of the experiment.
Papain and Na-CBZ-L-lysine-p-nitrophenyl ester

The unambiguous detection of a thioacyl enzyme formed in the reaction of papain and the non-specific substrate N-benzoyl-imidazole demonstrated the unique capabilities of $^{13}$C-NMR when applied to enzymology. The success of these experiments encouraged the application of the techniques to the detection of enzyme-bound intermediates in the papain-catalysed hydrolysis of specific substrates. The spectrophotometric observation of a stoichiometric burst of p-nitrophenol liberated in the hydrolysis of the p-nitrophenyl esters of N-acyl-amino acids has been accepted as good indirect evidence for acyl papain intermediates with specific substrates (Bender, et al., 1966). The p-nitrophenyl ester of Na-CBZ-L-lysine is a good substrate (i.e. $K_m$ is low, solubility is high), and has been previously studied at 25°C by a number investigators (Bender and Brubacher, 1966; Holloway and Hardman, 1973; Hinkle and Kirsch, 1970). Of particular interest is the low temperature study by Fink and Angelides, (1976). According to their results, it is possible to fully acylate papain at low temperatures and to trap the CBZ-lysyl-papain with little or no turnover taking place over a period of hours. This system therefore appeared to be particularly amenable to a $^{13}$C-NMR-cryoenzymological investigation. An extensive UV investigation was first required in order to establish the necessary conditions of temperature, co-solvent concentration, and pH for a $^{13}$C-NMR experiment.

At 25°C, the kinetic parameters were determined from complete hydrolysis curves, and the values obtained are in good agreement with previous results (see Table 12).

Table 12. Papain-catalysed hydrolysis of CBZ-L-lysine-pNP, pH 6.1

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ ($\times 10^6$ M)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6% CH$_3$CN*</td>
<td>44.5</td>
<td>1.7</td>
<td>2.62 x 10$^7$</td>
</tr>
<tr>
<td>1.7% DMSO</td>
<td>43.2±2.8</td>
<td>2.44±0.9</td>
<td>1.77(±1.21) x 10$^7$</td>
</tr>
</tbody>
</table>

*(Bender and Brubacher, 1966) (± Standard error)
Buffers containing 60% (v/v) DMSO were used by Fink and Angelides (1976) to study the low temperature hydrolysis of Na-CBZ-L-lysine-p-nitrophenyl ester by papain. They found that papain is stable and active in this cryosolvent below +10°C. Above this temperature, papain in 60% DMSO was reversibly denatured. High concentrations of DMSO also improves the solubility of papain at low temperatures which is essential for the detection of an acyl intermediate by $^{13}$C-NMR. The effect of DMSO on the kinetic parameters was investigated and the results are listed in Table 13. The reaction was studied at +3°C in order to avoid freezing problems with solutions at low DMSO concentrations, and to prevent denaturation of papain in solutions of higher co-solvent concentration at warmer temperatures. Also listed in Table 13 are the results of Fink and Angelides (1976) for a similar investigation at 0°C.

The results in Table 13 clearly show that $k_{\text{cat}}$ increases with increasing DMSO concentration in marked contrast to the trend reported by Fink and Angelides (1976). Other investigators have observed similar increases in the rates of papain-catalysed reactions in the presence of organic solvents. Hinkle and Kirsch (1970) observed large increases in the deacylation rate of furylacryloyl-papain upon addition of organic co-solvents including DMSO. In the same study, $k_{\text{cat}}$ for the papain-catalysed hydrolysis of Na-CBZ-glycine-p-nitrophenyl ester and Na-CBZ-L-lysine-p-nitrophenyl ester was increased 3.3 and 1.6-fold respectively in 27.6% dioxane as compared to water. The increase in rates is attributed to a mechanism involving co-solvent molecules binding at the active-site. This binding improves the orientation of the acyl-group with respect to the catalytic groups on the enzyme thereby increasing the rate of acyl-transfer to water. Henry and Kirsch (1967) also observed a rate increase with an increase in acetonitrile concentration in the papain-catalysed hydrolysis of p-nitrophenyl hippurate. The rate of the papain-catalysed hydrolysis
Table 13. The effect of DMSO on the kinetic parameters, +3°C, pH 6.1 (± 1 standard deviation)

<table>
<thead>
<tr>
<th>% DMSO</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (x 10$^6$ M)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
<th>% DMSO*</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (x 10$^6$ M)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
<td>13.5 ± 2.8</td>
<td>5 ± 2</td>
<td>3.06 (± 1.45) x 10$^6$</td>
<td>0</td>
<td>5.0</td>
<td>10</td>
<td>5 x 10$^5$</td>
</tr>
<tr>
<td>10</td>
<td>16.3 ± 6.5</td>
<td>7 ± 1</td>
<td>2.48 (± 0.83) x 10$^6$</td>
<td>15</td>
<td>4.8</td>
<td>25</td>
<td>1.92 x 10$^5$</td>
</tr>
<tr>
<td>20</td>
<td>22.6 ± 4.6</td>
<td>14 ± 3</td>
<td>1.59 (± 0.76) x 10$^6$</td>
<td>30</td>
<td>3.2</td>
<td>560</td>
<td>5.71 x 10$^3$</td>
</tr>
<tr>
<td>30</td>
<td>34.5 ± 7.3</td>
<td>58 ± 10</td>
<td>4.79 (± 2.38) x 10$^5$</td>
<td>40</td>
<td>2.94</td>
<td>1100</td>
<td>2.67 x 10$^3$</td>
</tr>
<tr>
<td>40</td>
<td>44.5 ± 4.4</td>
<td>98 ± 25</td>
<td>3.15 (± 1.86) x 10$^5$</td>
<td>50</td>
<td>2.54</td>
<td>3200</td>
<td>7.94 x 10$^2$</td>
</tr>
<tr>
<td>50</td>
<td>65.3 ± 12.6</td>
<td>681 ± 260</td>
<td>1.18 (± 0.52) x 10$^5$</td>
<td>60</td>
<td>2.08</td>
<td>10000</td>
<td>2.08 x 10$^2$</td>
</tr>
<tr>
<td>60</td>
<td>(see text)</td>
<td></td>
<td>3 x 10$^4$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*(Fink and Angelides (1976) equate 7.65 M DMSO with 60% v/v)*
of ethyl hippurate increased in solutions containing 20% DMSO (Sluyterman, 1967). Fink and co-workers however have reported the same uniform decrease in $k_{cat}$ with trypsin (Fink, 1974 b), chymotrypsin (Fink, 1973 a, b; Fink and Wildi, 1974), and papain (Fink and Angelides, 1976; Angelides and Fink, 1978), with a wide variety of N-acyl amino acid esters. In each case, the decrease in $k_{cat}$ is claimed to be proportional to the decrease in the water concentration. Maurel (1978) studied the effects of various co-solvents, including DMSO, on several enzymes and found that the effect of cosolvent varies with the enzyme. For example, in the chymotrypsin-catalysed hydrolysis of N-acetyl-L-tryptophan ethyl ester, $k_{cat}$ decreases with increasing DMSO, whereas $k_{cat}$ for the trypsin-catalysed hydrolysis of L-BAEE is not affected by an increase in the co-solvent concentration.

The $k_{cat}$ value in 50% DMSO is approximately 25-times greater than found by Fink and Angelides (1976). Since the $k_{cat}$ values are larger in the present study, this could suggest that this preparation contained an activator. This explanation is unlikely as the kinetic parameters ($k_{cat}$, $K_m$) for the hydrolysis of Na-CBZ-L-lysine-p-nitrophenyl ester, Na-CBZ-glycine-p-nitrophenyl ester (see Table 20 in next section), and L-BAEE (Table 2 in enzyme purity section) at 25°C in water agree well with those reported by other workers. It is more likely that the enzyme preparations of Fink and Angelides (1976) contains an inhibitor, denatured or not fully active papain, chymopapain contaminants, or that they over-estimated the enzyme concentration. The purity of the enzyme preparation used in their study was not as extensively investigated as in this study (see enzyme purity section). Enzyme activity was determined by a kinetic and burst assays (Fink and Angelides, 1976) with p-nitrophenyl ester substrates which would not easily detect contaminating enzymes and would give a less accurate estimate of active enzyme.

The $k_{cat}$ values in Table 13 are plotted versus % DMSO in
Figure 31. Up to 50% DMSO, $k_{cat}$ increases exponentially. A plot of $\ln(k_{cat})$ vs % DMSO (Figure 32) produces a straight line. Determination of the kinetic parameters in 60% DMSO at $+30^\circ C$ was impossible since the products of the hydrolysis are insoluble at this temperature and a precipitate forms as the reaction proceeds (lowering the temperature improves the solubility and the precipitate redissolves). Kinetic determinations in 60% DMSO at $+30^\circ C$ were limited to trials with low substrate concentrations where only an estimate of $k_{cat}/K_m$ could be obtained. This value is included in Table 13. $k_{cat}$ values in Table 13 were obtained from complete hydrolysis curves, and in solutions containing $\leq 40\%$ DMSO, $k_{cat}$ values were also obtained from initial rate studies. The data for $k_{cat}/K_m$ vs % DMSO and $\ln(k_{cat}/K_m)$ vs % DMSO are presented in Figures 33 and 34. The values of $k_{cat}/K_m$ were determined from first-order curves ($[S] < K_m$), and from the individual kinetic parameters $k_{cat}$ and $K_m$ determined from complete progress curves ($[S] > K_m$).

The values of $K_m$ (and $k_{cat}$ in 50 and 60% DMSO solutions) listed in Table 13 and in the low temperature experiments, were calculated from the complete progress curves and are the average of at least five determinations. The determination of $K_m$ values from complete curves is prone to errors due to the possible effects of substrate or product inhibition, or denaturation-inactivation of the enzyme during hydrolysis (Atkins and Nimmo, 1980). The values of $k_{cat}/K_m$ determined with $[S] > K_m$ are in agreement with the same values determined from first-order curves ($[S] < K_m$) (see examples in Tables 15 and 17). In all of the determinations with $[S] < K_m$, the log-plots of the first-order curves were linear over $\geq 90\%$ of the reaction indicating no significant denaturation of the enzyme occurred during the hydrolysis. The correspondence between $k_{cat}/K_m$ values at from first-order curves and complete progress curves was found over a wide temperature range. This is illustrated in Tables 15 and 17 and Figure 38. The similar values for $k_{cat}/K_m$
Figure 31. The dependence of $k_{\text{cat}}$ on DMSO concentration for the papain-catalysed hydrolysis of Na-CBZ-L-lysine-p-nitrophenyl ester. The values are from Table 13. The error bars represent ± one standard deviation and each point is the average of at least five determinations. For solutions containing ≤ 40% DMSO, $k_{\text{cat}}$ values were obtained from both initial rates and complete progress curves.

Figure 32. The linear relationship of $\ln(k_{\text{cat}})$ to DMSO concentration for the data in Table 13 and plotted in Figure 31.
Figure 31

Figure 32
Figure 33. The dependence of $k_{\text{cat}}/K_{m}$ on DMSO concentration for the papain-catalysed hydrolysis of Nα-CBZ-L-lysine-p-nitrophenyl ester from the data listed in Table 13. The points represent the average of at least five determinations obtained from complete progress curves and first-order curves. The error bars are ± one standard deviation.

Figure 34. The dependence of $\ln (k_{\text{cat}}/K_{m})$ on DMSO concentration for the data in Table 13.
Figure 33

$k_{cat}/K_m \times 10^{-6} \text{ M}^{-1} \text{s}^{-1}$

% DMSO

Figure 34

$\ln(k_{cat}/K_m)$

% DMSO
obtained with $[S]_0 > K_m$ show that no significant denaturation occurs at higher substrate concentrations. In 40% DMSO solution, the $k_{cat}$ and $K_m$ values determined from a complete progress curve are also in good agreement with those determined by initial rates (Table 14). This confirms that complete curves do provide reliable estimates of $k_{cat}$ and $K_m$, and also shows that there is no detectable enzyme denaturation or product inhibition under these conditions.

Table 14. Papain-catalysed hydrolysis of Na-CBZ-L-lysine-p-nitrophenyl ester in 40% DMSO, pH 6.1, 0°C.

<table>
<thead>
<tr>
<th></th>
<th>$[E]_0$ ($x 10^6$ M)</th>
<th>$[S]_0$ ($x 10^3$ M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial rates</td>
<td>0.013</td>
<td>0.2 to 3.5</td>
<td>37.5 (\pm) 1.6</td>
<td>111 (\pm) 8</td>
</tr>
<tr>
<td>Complete curve</td>
<td>0.049</td>
<td>0.191</td>
<td>37.2 (\pm) 1.2</td>
<td>122 (\pm) 23</td>
</tr>
</tbody>
</table>

(± Standard Error)

In 50 and 60% DMSO at higher substrate concentration (e.g. $[S]_0 > 5$ mM) $k_{cat}$ and $K_m$ appear to increase and saturation of the enzyme with substrate was not possible (see last two entries in Table 15). There are several possible explanations for the increases in $k_{cat}$ and $K_m$ at high substrate concentrations. Transacylation by the -amino group of the substrate or product lysine side-chain could occur by nucleophilic attack on the acyl enzyme and would cause an increase in the apparent $k_{cat}$ and $K_m$ values (Hinkle and Kirsch, 1971). Competitive product inhibition would also have the effect of raising the apparent $K_m$ obtained from complete progress curves (Orsi and Tipton, 1979). However, due to the high extinction of p-nitrophenol and the difficulty of maintaining a constant pH during the reaction at high substrate concentrations (the extinction of p-nitrophenol changes with pH), these effects were not examined in detail. Since the complete curve method is sensitive to enzyme denaturation or inactivation (an effect which
would be most pronounced over the long periods required for complete substrate hydrolysis when $[S]_0$ is large), this method was not suitable for investigating these effects further. Due to the experimental difficulties, an initial rate study at low temperature was not undertaken, and at higher temperatures, the decreased product solubility ruled out initial rate studies with high substrate concentrations.

The data in Table 13 for $K_m$ vs % DMSO is shown in Figure 35 and $\ln (K_m)$ vs % DMSO in Figure 36.

In 60% DMSO, with $[S]_0 < 5$ mM, reproducible results for $k_{cat}$ and $K_m$ were obtained but for determinations where $[S] < K_m$, the values obtained must be regarded as minimum estimates for $K_m$. Table 15 lists the results for 60% DMSO, pH 6.1 where values of $k_{cat}$ and $K_m$ can be estimated from the complete progress curves.

Table 15. Papain-catalysed hydrolysis of Na-CBZ-L-lysine-p-nitrophenyl ester in 60% DMSO pH 6.1.

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>$[E]_0$ (µM)</th>
<th>$[S]_0$ (mM)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (mM)</th>
<th>$k_{cat}/K_m$ (M⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3</td>
<td>0.28</td>
<td>0.06</td>
<td>(First-order curve)</td>
<td>12500</td>
<td></td>
</tr>
<tr>
<td>-3</td>
<td>1.12</td>
<td>2.8</td>
<td>62.0±20.1</td>
<td>5.6±2.9</td>
<td>11100</td>
</tr>
<tr>
<td>-11</td>
<td>1.09</td>
<td>2.0</td>
<td>26.3±9.9</td>
<td>3.44±1.3</td>
<td>7650</td>
</tr>
<tr>
<td>-41</td>
<td>22.6</td>
<td>1.14</td>
<td>0.56±0.24</td>
<td>6.76±2.8</td>
<td>82.8</td>
</tr>
<tr>
<td>-41</td>
<td>11.3</td>
<td>7.46</td>
<td>0.42±0.16</td>
<td>53.1±13.4</td>
<td>7.91</td>
</tr>
<tr>
<td>-40**</td>
<td>10.1</td>
<td>39.0</td>
<td>467±171</td>
<td>139±45</td>
<td>3360</td>
</tr>
</tbody>
</table>

** (0.2 M Buffer) (+ Standard Error)

The results for $K_m$ are fairly consistent when $[S]_0 < 5$ mM. The temperature dependence observations were therefore limited to determinations of $k_{cat}/K_m$ with low substrate concentrations ($[S]_0 << 2$ mM) from first-order curves and initial rates. A plot of
Figure 35. The dependence of $K_m$ on DMSO concentration for the papain-catalysed hydrolysis of $\text{Na-CBZ-L-lysine-p-nitrophenyl ester}$ at $+3^\circ\text{C}$. (From the data in Table 13.

Figure 36. The dependence of $\ln(K_m)$ on DMSO concentration.
Figure 35

% DMSO

Figure 36

% DMSO
ln (k_{cat} /K_m) vs (1/T) is shown in Figure 37. For a comparison, the results of Fink and Angelides (1976) are also included in this figure. Although specific values of k_{cat} /K_m at different temperatures were not given in their work, these values can be calculated from the activation energy of 14.6 kcal/mole for k_{cat}, a K_m value of 10 mM, which is reported to remain unchanged between 0 and -20°C, and the kinetic parameters given at 0°C. As mentioned in the introduction, the activation energy was obtained from a plot of \log_{10}(k_{cat}) vs (1/T) for 60% DMSO between 0 and -45°C with \[E]_o = 3 \mu M, and \[S]_o = 1 mM. This means that for 60% DMSO (K_m = 10 mM), "k_{cat}" values were obtained with \[S] \ll K_m, at least down to -20°C. Assuming that there is not a typographical error in the paper by Fink and Angelides (1976), then the reported temperature dependence of "k_{cat}" is really the temperature dependence of k_{cat} /K_m. Since they report that K_m is constant down to -20°C, they may have assumed that the temperature dependence of k_{cat} /K_m is the same as that of k_{cat} and may have multiplied their k_{cat} /K_m values by K_m to give k_{cat}^{'} . It is not explained how these k_{cat} values were estimated, but for comparison their data will be used as presented.

Below -20°C, the K_m values are not reported by Fink and Angelides (1976). However, a typical acylation at -70°C is reported to result in 86% acylated papain with \[E]_o = 14 \mu M and \[S]_o = 1 mM with little or no deacylation observed. In order to acylate papain to that extent with 1 mM substrate, K_m must be much less than 1 mM. For example, using the equation for the magnitude of the burst during acylation:

\[
\pi = \left[ \frac{k_2}{k_2 + k_3} \right]^2 \frac{K_m}{1 + \frac{[S]_o}{K_m}}
\]

and substituting \( \pi = 0.86, [S]_o = 1 \text{ mM}, \) and
Figure 37. The temperature dependence of $\ln \left( \frac{k_{\text{cat}}}{K_m} \right)$ for the papain-catalysed hydrolysis of Na-CBZ-L-lysine-p-nitrophenyl ester, 60% DMSO, pH 6.1.

- Initial rates, $[S]_0 \ll K_m$
- Complete first-order curves
- Data of Fink and Angelides (1976) calculated from an activation energy of 14.6 kcal/mol, $K_m = 10$ mM.

From the slope of the line, $E_a = 64.6 \pm 2.7$ kJ/mol
\[ 15.4 \pm 0.6 \text{ kcal/mol} \]
since \( k_2 \gg k_3 \), \( k_2/(k_2 + k_3) \approx 1 \), \( K_m \) may be calculated as 80 \( \mu \text{M} \). This is a 100-fold decrease in \( K_m \) between -20 and -70\( ^\circ \text{C} \). This dramatic decrease was not discussed by Fink and Angelides (1976) and no evidence for such a decrease was observed in this study.

The value of the activation energy \( (E_a) \) calculated from the temperature dependence of \( k_{\text{cat}}/K_m \) (Figure 37) is 64.6 \( \pm 2.7 \) kJ/mole (15.4 \( \pm 0.6 \) kcal/mole) which is similar to the value of 14.6 kcal/mole obtained by Fink and Angelides (1976) for "\( k_{\text{cat}} \"." Below -45\( ^\circ \text{C} \), 60% DMSO solutions were extremely viscous, and complete mixing of substrate and enzyme was difficult and introduction of air bubbles produced fluctuations in the hydrolysis curves. Therefore data was only obtained at temperatures greater than -45\( ^\circ \text{C} \).

From the temperature dependence of \( k_{\text{cat}}/K_m \), the temperature dependence of \( k_{\text{cat}} \) can be estimated by assuming \( K_m \) remains unchanged and has a value of approximately 3 mM (this estimate is based on the results in Table 15 and extrapolation of the line in Figure 36 to 60% DMSO). Using this assumption, the following values of \( k_{\text{cat}} \) were calculated and compared to values calculated from the results of Fink and Angelides (1976). In both cases, the temperature dependence was determined down to -45\( ^\circ \text{C} \) but this should be linear below that temperature provided there is no change in the rate determining step.

Table 16. \( k_{\text{cat}} \) values calculated from the temperature dependence in 60% DMSO.

<table>
<thead>
<tr>
<th>Temp (( ^\circ \text{C} ))</th>
<th>If ( K_m = 3 \text{ mM} )</th>
<th>Fink and Angelides (1976)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( -40 )</td>
<td>0.35</td>
<td>0.021</td>
</tr>
<tr>
<td>( -50 )</td>
<td>0.080</td>
<td>0.0050</td>
</tr>
<tr>
<td>( -60 )</td>
<td>0.016</td>
<td>0.0011</td>
</tr>
<tr>
<td>( -70 )</td>
<td>0.0025</td>
<td>0.00019</td>
</tr>
</tbody>
</table>
The \( k_{\text{cat}} \) values listed in Table 16 are approximately 15-times greater than the values calculated from Fink and Angelides (1976). For an acyl-enzyme to be observed by \( ^{13} \text{C-NMR} \), the enzyme must be saturated with substrate over long periods of time (\( \geq 1 \) hour); this requires \( [S]_0 \gg K_m \). The \( ^{13} \text{C-NMR} \) experiments require high concentrations of enzyme (\( \sim 1 \) mM) in order to detect an enzyme intermediate in a reasonably short period of time (\( \sim 1 \) hr). At \(-50^\circ \text{C}\), 1 mM enzyme would hydrolyse 0.08 mM \( \text{s}^{-1} \) of substrate under saturating conditions. In one hour, the enzyme would hydrolyse 288 mM substrate. At \(-60^\circ \text{C}\), 58 mM substrate would be hydrolysed over the same time period. Although this is much less than at \(-50^\circ \text{C}\), a large amount of substrate is still required. At \(-70^\circ \text{C}\), the enzyme hydrolyses a relatively small amount of substrate (9 mM in 1 hr). A \( ^{13} \text{C-NMR} \) experiment would then require a temperature of \(-70^\circ \text{C}\) or below. A UV investigation at this temperature was not possible due to the extremely viscous solutions obtained under these conditions.

In order to alleviate some of the viscosity problems, 50% DMSO solutions were used. These solutions have the advantage that they are slightly less viscous, the \( K_m \) is lower, and the results at \(+3^\circ \text{C}\) suggest that \( k_{\text{cat}} \) may be lower (assuming that the plot of \( \ln (k_{\text{cat}}) \) vs % DMSO, Figure 32, is linear to 60% DMSO). In a similar study to the one in 60% DMSO solution, the papain-catalysed hydrolysis was studied over a temperature range from \(-42\) to \(+3^\circ \text{C}\). The results are shown in Table 17.

The data in Table 17 shows no significant change in \( K_m \) over a 45\(^\circ\) temperature range and the similarity between the \( k_{\text{cat}}/K_m \) values obtained from first-order curves and complete progress curves is illustrated. In 50% DMSO, \( k_{\text{cat}} \) and \( K_m \) can be obtained over a wide temperature range. The temperature dependence of \( k_{\text{cat}}/K_m \) and \( k_{\text{cat}} \) are shown in Figures 38 and 39 respectively. Included in Figure 38 are values of \( k_{\text{cat}}/K_m \) from complete progress curves, first-order curves, and initial rates.
Table 17. Papain-catalysed hydrolysis of Na-CBZ-L-lysine-p-nitrophenyl ester in 50% DMSO, pH 6.1.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>[E]₀ (µM)</th>
<th>[S]₀ (mM)</th>
<th>kₓ (s⁻¹)</th>
<th>Kₘ (mM)</th>
<th>kₓ/Kₘ (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+3</td>
<td>0.1</td>
<td>0.905</td>
<td>76.4 ± 4.2</td>
<td>0.500 ± 0.048</td>
<td>153000</td>
</tr>
<tr>
<td>+3</td>
<td>0.1</td>
<td>0.044</td>
<td></td>
<td></td>
<td>149000**</td>
</tr>
<tr>
<td>0</td>
<td>0.03</td>
<td>0.423</td>
<td>94.9 ± 23.3</td>
<td>1.16 ± 0.39</td>
<td>81800</td>
</tr>
<tr>
<td>-15</td>
<td>0.23</td>
<td>2.77</td>
<td>21.3 ± 1.3</td>
<td>0.974 ± 0.119</td>
<td>21900</td>
</tr>
<tr>
<td>-21</td>
<td>0.21</td>
<td>1.00</td>
<td>14.8 ± 1.2</td>
<td>0.968 ± 0.112</td>
<td>13200</td>
</tr>
<tr>
<td>-21</td>
<td>0.20</td>
<td>0.07</td>
<td></td>
<td></td>
<td>15000**</td>
</tr>
<tr>
<td>-31</td>
<td>1.02</td>
<td>1.33</td>
<td>8.78 ± 1.15</td>
<td>1.07 ± 0.20</td>
<td>8210</td>
</tr>
<tr>
<td>-42</td>
<td>1.16</td>
<td>1.41</td>
<td>1.21 ± 0.12</td>
<td>0.520 ± 0.092</td>
<td>2330</td>
</tr>
<tr>
<td>-42</td>
<td>2.33</td>
<td>0.028</td>
<td></td>
<td></td>
<td>1990**</td>
</tr>
</tbody>
</table>

** (First-order curves) (± Standard Error)

In 50% DMSO, Buffer concentrations from 0.01 to 0.2 had no effect on the kinetic parameters.
Figure 38. The temperature dependence of $\ln \left( \frac{k_{\text{cat}}}{K_m} \right)$ for the papain-catalysed hydrolysis of $\text{N}^\alpha$-CBZ-$\text{L}$-lysine-$p$-nitrophenyl ester in 50% DMSO solutions pH* 6.1.

- Initial rates, $[S]_0 \ll K_m$
- Complete first-order curves
- Complete progress curves, $[S]_0 \geq K_m$

From the slope of the line $E_a = 52.6 \pm 3.6 \text{ kJ/mol}$

$12.6 \pm 0.9 \text{ kcal/mol}$
Figure 39. The temperature dependence of $k_{cat}$ for the papain-catalysed hydrolysis of Na-CBZ-L-lysine-p-nitrophenyl ester in 50% DMSO solutions pH 6.1. All data was obtained from complete progress curves and corresponds to the data included in Figure 38.

From the slope of the line $E_a = 46.2 \pm 2.0 \text{ kJ/mol}$ $\frac{1}{T} (x 10^3 \text{ K}^{-1})$

Since there is no indication for a change in $K$ with temperature, the $E_a$ values for $k_{cat}$ and $k_{cat}/K_m$ are similar.
$k_{\text{cat}}$ values were obtained from complete curves with $K_m \approx S < 4K_m$ and must be regarded as minimum values. Below $-50^\circ \text{C}$, mixing problems produced unreliable data. Markely et al (1981) observed mixing artefacts in their attempts to reproduce the earlier cryoenzymological results of Fink and co-workers.

The lower activation energy in 50% DMSO solutions (52.6 kJ/mol calculated from the slope of Figure 38) indicates that at low temperature the $k_{\text{cat}}$ values in 50% DMSO solutions would be greater than those predicted for 60% DMSO solutions. The lower activation energy in 50% DMSO solutions is consistent with the results of Maurel et al. (1975) who observed an increase in the activation energy with an increase in co-solvent concentration for the trypsin-catalysed hydrolysis of BAEE. The slowest reactions are therefore observed in 60% DMSO, and from the previous discussion, this would require a temperature of $\leq -70^\circ \text{C}$ for detection of an acyl-enzyme by $^{13}\text{C}$-NMR.

The effects of temperature on a carbonyl resonance in the $^{13}\text{C}$-NMR spectrum was studied in 50% DMSO solution. The $N$-benzoylimidazole results are useful for providing an estimate of the intensity and resolution that can be expected for an enriched acyl-enzyme. In that experiment, the low molecular weight benzoic acid carbonyl signal was much narrower (5 Hz) than the enzyme-bound benzoyl resonance (25 Hz). The linewidth of the benzoic acid signal doubled in changing from 100% water at $25^\circ \text{C}$ to 25% DMSO at $-7^\circ \text{C}$. Similarly, the linewidth of $[1-^{13}\text{C}]$-DL-lysine changed from 2.95 Hz in water at $25^\circ \text{C}$ to 5.7 Hz in 50% DMSO at $-5^\circ \text{C}$. The following table lists the linewidth of $[1-^{13}\text{C}]$-DL-lysine in 50% DMSO containing 0.8 mM enzyme to produce a system similar to that required for detection of an acyl enzyme. The NMR acquisition parameters are exactly the same as described for the $N$-benzoylimidazole experiments; i.e. low-power noise-decoupling, 10000 scans over a 45 minute period.
Table 18. The variation in the linewidth of $[1^{-13}\text{C}]-\text{DL-lysine}$ with temperature in 50% DMSO, pH*.

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Linewidth (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-5</td>
<td>5.7</td>
</tr>
<tr>
<td>-27</td>
<td>6.9</td>
</tr>
<tr>
<td>-50</td>
<td>19.8</td>
</tr>
</tbody>
</table>

The linewidth increases over 3-fold down at -50°C; the same temperature at which the UV experiments indicated that the solutions became extremely viscous. This 3-fold increase in the linewidth would also be expected for other resonances in the sample. That means that an acyl-enzyme resonance similar to the benzoyl-papain resonance would decrease in intensity 3-fold due to line broadening.

The signal-to-noise ratio of the transient thioester resonance in the N-benzoylimidazole experiments was approximately 4 : 1. From the results of the $[1^{-13}\text{C}]-\text{DL-lysine}$ experiments, -50°C would be the lowest temperature possible for detecting a thioester resonance in 50% DMSO. The natural-abundance protein spectrum in the same sample was undetectable below -5°C after 10000 scans. Remembering that a thioacyl resonance will most likely be of a similar intensity as the natural-abundance protein spectrum (see Figure 12 in the N-benzoylimidazole section), the lower limit of -50°C in 50% DMSO may be optimistic for observation of an acyl-enzyme by $^{13}\text{C}$-NMR.

The results show that the reaction in 60% DMSO, pH* 6.1 is too fast at -50°C and that temperatures below this are unsuitable for NMR detection.

The reaction in 50% DMSO was studied at low pH in an attempt to reduce the turnover rate. At pH* 3.3, substrate concentrations in the range 1 to 2 mM produced first-order curves showing $K^m >> 2$ mM. The $K^m$ in water at 25°C has been previously shown to increase seven-fold on lowering the pH from 6.2 to 3.2 (Bender and Brubacher, 1966),
therefore any favourable decrease in $k_{\text{cat}}$ is offset by the increase in $K_m$.

The temperature dependence of the reaction in 40% DMSO is shown in Figures 40 and 41. The activation energy is further reduced and the reaction is limited to temperatures above -41°C, the freezing point of these solutions.

Several alternative ternary cryosolvents were tested to reduce viscosity and the effects on $K_m$. The most promising cryosolvent was 40% DMSO, 20% methanol, 40% water which was found to be fluid down to the -70 to -80°C range. Preliminary trials at -60°C showed a high $K_m$ (~5-10 mM) and $k_{\text{cat}}$ values higher than predicted for the 50 and 60% DMSO cryosolvents at -60°C. DMSO:acetone:water (4:2:4) cryosolvent had a similar viscosity to the methanol ternary solvent but was not a good solvent for the substrate.

Douzou and Balny (1977) found that addition of water soluble polyelectrolytes to cryosolvents reduced the co-solvent effects on the kinetic parameters. They observed a ten-fold decrease in $K_m$ for the trypsin-catalysed hydrolysis of BAEE in 50% DMSO cryosolvent by the addition of highly polymerized RNA. It was proposed that the polyelectrolyte creates a microenvironment around the enzyme which excludes the co-solvent. This preferential solvation for the enzyme acts to protect it from co-solvent effects and reduces the perturbation of the kinetic parameters. A mole ratio of 1:1 polyelectrolyte to enzyme was found to be suitable in reducing co-solvent effects in 50% DMSO.

The effect of RNA on the $k_{\text{cat}}$ and $K_m$ of the papain-catalysed hydrolysis was studied in 50% DMSO solution. The results of a preliminary investigation are shown in Table 19.
Figure 40. The temperature dependence of $\ln \left( \frac{k_{\text{cat}}}{K_m} \right)$ for the papain-catalysed hydrolysis of Na-CBZ-L-lysine-p-nitrophenyl ester in 40% DMSO, pH 6.1. The data was obtained from complete hydrolysis curves.

From the slope of the line, $E_a = 46.2 \pm 2.0$ kJ/mol

$= 11.0 \pm 0.5$ kcal/mol

Figure 41. The temperature dependence of $k_{\text{cat}}$ in 40% DMSO, pH 6.1. The data was obtained from complete progress curves.

$E_a = 35.0 \pm 3.5$ kJ/mol

$= 9.36 \pm 0.84$ kcal/mol
\[ \ln\left(\frac{k_{\text{cat}}}{K_m}\right) \]

\[ \frac{1}{T} \times 10^3 \frac{1}{\text{K}} \]

Figure 40

\[ \ln(k_{\text{cat}}) \]

\[ \frac{1}{T} \times 10^3 \frac{1}{\text{K}} \]

Figure 41
Table 19. The effect of RNA on $k_{\text{cat}}$, $K_m$ in 50% DMSO, pH 5.9 at 0°C; $[E]_O = 0.11 \mu M$, $[S]_O = 1.53 \text{ mM}$. (± Standard Error)

<table>
<thead>
<tr>
<th>RNA (µg)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}} / K_m$ (M$^{-1}$s$^{-1}$)</th>
<th>RNA:Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>48.4 ± 3.8</td>
<td>0.818 ± 0.110</td>
<td>59200</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>22.6 ± 0.9</td>
<td>0.215 ± 0.029</td>
<td>105000</td>
<td>1:1</td>
</tr>
<tr>
<td>48</td>
<td>23.4 ± 0.5</td>
<td>0.187 ± 0.017</td>
<td>125000</td>
<td>3:1</td>
</tr>
<tr>
<td>95</td>
<td>25.0 ± 0.4</td>
<td>0.190 ± 0.012</td>
<td>132000</td>
<td>6:1</td>
</tr>
<tr>
<td>190</td>
<td>23.2 ± 0.8</td>
<td>0.182 ± 0.023</td>
<td>127000</td>
<td>10:1</td>
</tr>
</tbody>
</table>

While there is a five-fold decrease in $K_m$, there is only a two-fold decrease in $k_{\text{cat}}$. An RNA to protein ratio $> 1:1$ is the most effective range for the reduction of co-solvent effects on $K_m$. Changing the substrate concentration from 0.05 to 1.53 mM with a constant RNA concentration (12 µg) and enzyme still resulted in $K_m$ values near 0.18 mM and $k_{\text{cat}}$ of 23 s$^{-1}$. The RNA results, although very interesting, were not studied in detail since $k_{\text{cat}}$ was not reduced enough to make a $^{13}$C-NMR study feasible.

The aim of this study was to investigate the suitability of this system for adaptation to a cryoenzymological-$^{13}$C-NMR investigation. The results show that the reaction is far too rapid even at temperatures as low as -50°C, and detection of an acyl-enzyme by $^{13}$C-NMR with this substrate in DMSO is not possible using state-of-the-art instrumentation.
Other Ester Substrates

The failure of the Na-CBZ-L-lysine-p-nitrophenyl ester investigation to detect an acyl-enzyme by UV-spectrophotometry demonstrated the need for alternative specific substrates with lower turnover rates in aqueous-organic cryosolvents. Other p-nitrophenyl ester substrates were studied to determine their suitability for a cryoenzymological-¹³C-NMR investigation. Na-CBZ-glycine-p-nitrophenyl ester has previously been studied in water at 25°C (Kirsch and Igelstrom, 1966; Williams and Whitaker, 1967) and was used earlier in the work described in this thesis as a substrate for determination of relative enzyme activities by rate assay. In water at 25°C, the kinetic parameters $k_{cat}$ and $K_m$ were obtained from complete hydrolysis curves. $k_{cat}$ was also obtained from initial rate studies. The values listed in Table 20 agree with previous results.

Table 20. The papain-catalysed hydrolysis of Na-CBZ-glycine-p-nitrophenyl ester at 25°C, pH 5.

<table>
<thead>
<tr>
<th>Initial rates</th>
<th>Complete Curves</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ (s⁻¹)</td>
<td>$k_{cat}$ (s⁻¹)</td>
</tr>
<tr>
<td>4.81 ± 0.25</td>
<td>4.62 ± 0.11</td>
</tr>
<tr>
<td>4.96 ± 0.33**</td>
<td>5.61 ± 1.05**</td>
</tr>
</tbody>
</table>

ᵃ(Results of Williams and Whitaker, 1967)

**(Average of five determinations, ± 1 standard deviation)

The activation energy in 60% DMSO at pH 6.3 was obtained from $k_{cat}/K_m$ from initial rate studies ($S_o << K_m$) and complete first-order curves. The values obtained are comparable, and an activation energy of 74.0 ± 7.8 kJ/mol was obtained (Figure 42). $K_m$ for the reaction could not be determined due to the low solubility
Figure 42. The temperature dependence of $k_{cat}/K_m$ for the papain-catalysed hydrolysis of Na-CBZ-glycine-pNP in 60% DMSO pH 6.1

- Complete first-order curves
- Initial rates

The activation energy was calculated from the slope of the line $E_a = 74.0 \pm 7.8 \text{ kJ/mol}$

$17.1 \pm 1.6 \text{ kcal/mol}$
of \( \text{Na-CBZ-glycine-p-nitrophenyl ester} \) in 60% DMSO solutions (~600 to 800 \( \mu \text{M} \)). The value of \( k_{\text{cat}} / K_m \) extrapolated to \(-50^\circ\text{C}\) (3.7 M\(^{-1}\) s\(^{-1}\)) is approximately one-seventh the value predicted for \( \text{Na-CBZ-L-lysine-p-nitrophenyl ester} \) at that temperature (26 M\(^{-1}\) s\(^{-1}\)). The limited solubility of this substrate in the cryo-solvent precluded further investigation.

Similarly, \( \text{Na-CBZ-L-tyrosine-p-nitrophenyl ester} \) and \( \text{Na-CBZ-L-alanine-p-nitrophenyl ester} \) were not investigated due to preliminary studies which showed limited solubility (approximately 200 to 500 \( \mu \text{M} \) in 60% DMSO).

\( \text{N-acetyl-phenylalanylglycine-p-nitrophenyl ester} \) was studied by Lowe and Yuthavong (1971a) and was shown to have a low \( K_m \) (0.39 \( \mu \text{M} \)) in water at 25°\text{C}. The \( k_{\text{cat}} \) value determined by Lowe and Yuthavong (1971a) is approximately one-eighth that of \( \text{Na-CBZ-L-lysine-p-nitrophenyl ester} \) (Bender and Brubacher, 1966). Initial rate studies in water show a good comparison with the previous results.

Table 21. The papain-catalysed hydrolysis of \( \text{N-acetyl-L-phenylalanylglycine-p-nitrophenyl ester} \), pH 6.

<table>
<thead>
<tr>
<th></th>
<th>( k_{\text{cat}} (\text{s}^{-1}) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2% DMF, 35°C</td>
<td>6.6</td>
</tr>
<tr>
<td>1.7% DMSO, 25°C</td>
<td>7.5 ± 0.6</td>
</tr>
</tbody>
</table>

** (Average of 3 determinations, ±1 standard deviation)

In 50% DMSO at -40°C, \( K_m \) was determined to be 1.9 mM. This would mean a greater co-solvent effect on \( K_m \) for this substrate than for the \( \text{Na-CBZ-L-lysine-p-nitrophenyl ester} \) substrate. In the latter case, \( K_m \) increased from 1.7 \( \mu \text{M} \) in water to 650 \( \mu \text{M} \) in 50% DMSO. This greater increase may be due to the increased
contribution of hydrophobic interactions to the binding of the dipeptide substrate. The effect of temperature on \( \frac{k_{\text{cat}}}{K_m} \) is shown in Figure 43. An activation energy of 64.0 ± 2.1 kJ/mol was calculated from the data in Figure 43 and the value of \( \frac{k_{\text{cat}}}{K_m} \) predicted at -50°C (63 M⁻¹s⁻¹) is approximately one-sixth the value of \( \frac{k_{\text{cat}}}{K_m} \) predicted from the temperature dependence of \( \frac{k_{\text{cat}}}{K_m} \) for Na-CBZ-L-lysine-p-nitrophenyl ester under these same conditions. Assuming a \( K_m \) of 1.9 mM at -50°C, the \( k_{\text{cat}} \) value at this temperature would be only a third of that for the lysine substrate. The decrease in the turnover rate was not great enough to merit further investigation and this substrate was concluded to be unsuitable for a cryoenzymological-\(^{13}\)C-NMR investigation.

None of the ester substrates investigated was found to be adaptable to a \(^{13}\)C-NMR-cryoenzymological investigation designed to detect an acyl-enzyme. The results illustrate the specific requirements needed in a substrate, namely, high solubility, low \( k_{\text{cat}} \) and \( K_m \), and a high activation energy.
Figure 43. The temperature dependence of $k_{cat}/K_m$ for the papain-catalysed hydrolysis of N-acetyl-L-phenylalanylglycine-p-nitrophenyl ester in 50% DMSO, pH 6.1.

- From complete first-order curves
- Initial rates

The activation energy calculated from the slope of the line $E_a = 64.0 \pm 2.1 \text{ kJ/mol}$

$15.3 \pm 0.5 \text{ kcal/mol}$
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PUBLICATION
Cryoenzymology of Proteases: NMR Detection of a Productive Thioacyl Derivative of Papain at Subzero Temperature


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Received August 11, 1982

It is generally accepted that the hydrolysis of peptides and amides catalyzed by the thiol protease papain can be represented by a minimal three-step pathway as in Scheme I. The reactions are controlled by a thiolate ion (cys-25) at the active site of papain in a sequence involving binding, acylation, and deacylation. Structural evidence for the thioacyl intermediate is limited to electronic absorption data in which acylation of papain by N-\textit{trans}-cinnamoylimidazole gave rise to a UV spectrum red shifted by 0 nm relative to the model, (S)-\textit{trans}-cinnamoylcysteine. More direct evidence bearing on this point comes from the observation of a species assigned to a dithioester structure with \( \lambda_{\text{max}} 313 \text{ nm} \) (cf. dithioacetate, \( \lambda_{\text{max}} 305 \text{ nm} \)) in the papain-catalyzed hydrolysis of methyl thionohippurate. As a result of the development in our laboratory of reliable protocols for the observation of covalently bound intermediates of enzymes and their substrates by \(^{13}\text{C} \) NMR spectroscopy at subzero temperatures, we can now report on the direct observation of a productive thioacyl intermediate prepared from papain and \([^{13}\text{C}==\text{O}]\)-N-benzoylimidazole by adapting the techniques of cryoenzymology to a \(^{13}\text{C} \) NMR experiment. To monitor the extent of benzoylation of papain and the rate of deacylation, we used the high reactivity of 2,2'-dipyridyl disulfide toward the thiolate ion of cys-25 in papain at pH 3.8 to titrate free thiolate in aliquots of incubation mixtures corresponding to the time course NMR experiment, using 1-2 mM solutions of papain and a large excess (~20 mM) of substrate in formate buffer. After many trials the following conditions gave completely reproducible results in which a suitable concentration (~1 mM) and \( t_{1/2} > 30 \text{ min} \) of the intermediate were achieved. Papain (1.7 mM) in formate buffer (0.1 M, pH 4.1) was mixed with 90% enriched \([^{13}\text{C}==\text{O}]\)-N-benzoylimidazole \((23.6 \text{ mM})\) in 25% MeSO-\( d_6 \) at 0 °C then rapidly cooled to -6 °C. An aliquot of this solution was kept at -6 °C and active site thiol concentration measured throughout the NMR time course.

At 0 °C papain was 96% acylated (thiolate assay) while at -6 °C the half-life of deacylation is 96 min. The time course of the CMR experiment is shown in Figure 1 a–f. 

The broad (25 ± 5 Hz) resonance at 196.0 ppm is assigned to the thiobenzoate (2, Scheme II) of papain labeled at \(^{13}\text{C} \) (cf. phenylthiobenzoate, \( \delta 189.1 \); \(^{13}\text{C} \) n-butyl thioacetate, 194.1). The rate of disappearance of the signal at 196.0 ppm (allowing for experimental error due

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*Dedicated to the memory of the late Professor F. Sorm.*

*To whom correspondence should be addressed at the Center for Biological NMR, Texas A&M University.*

1 University of Edinburgh.
2 Texas A&M University.
4 Presumed tetrahedral intermediates have not been included in the scheme.
11 Schiller, R.; Otto, R. *Chem. Ber.* 1876, 9, 1635. \(^{13}\text{C} \) NMR spectrum recorded in MeSO-\( d_4 \) (present work).
to line broadening) corresponds well with 1,12, measured independently by titration with 2,2'-dipyridyl disulfide. Moreover, the thiobenzoate signal at 196.0 ppm was not observed in the control hydrolysis experiment in the absence of papain at pH 4.1. Apart from the resonances due to papain at natural abundance, in which Arg C-6 (158 ppm) and Tyr C-7 (156 ppm) are clearly discerned, together with broad carbonyl resonances between 170 and 180 ppm, a signal at 165.2 ppm (A in Figure 1a,b) was also detected in the absence of both formate and papain and is ascribed to the carbonyl resonance of benzoic anhydride, which has been shown to hydrolyze to benzoic acid at pH 4.1. Apart from the resonances due to papain at natural abundance, in which Arg C-6 (158 ppm) and Tyr C-7 (156 ppm) are clearly discerned, together with broad carbonyl resonances between 170 and 180 ppm, a signal at 165.2 ppm (A in Figure 1a,b) was also detected in the absence of both formate and papain and is ascribed to the carbonyl resonance of benzoic anhydride, which has been shown to hydrolyze to benzoic acid at pH 4.1. It should be noted that the remaining triplet centered at 170.1 ppm (pH 4.1) (Figure 1a-f) is due to partially decoupled formate and is shifted to 169.3 ppm at pH 3.8 (Figure 1g).

After 17 h at -6 °C, the reaction mixture used in the above experiments was diluted (×4), low molecular weight material was removed by gel filtration, and the sample was concentrated by ultrafiltration, whereupon titration with 2,2'-dipyridyl disulfide showed no loss of fast-reacting thiol at pH 3.8. The 13C NMR spectrum of this sample exhibited a broad resonance at 168-170 ppm, which resolved clearly into two peaks at 168.7 and 169.1 ppm by subtraction of the 13C NMR spectrum of papain. By analogy with model compounds and studies on trypsin,14 the main sites of this nonspecific benzoylation are assigned to the amino and phenolic side chains of the 10 lysine and 19 tyrosine residues of papain.

From the above data we conclude that (a) papain reacts with benzoylimidazole to form a thioacyl intermediate unambiguously detected by 13C NMR spectroscopy at -6 °C; (b) the rate of decay of this intermediate is equal to the rate of regeneration of the active center thiolate ion of papain as measured by titration with 2,2'-dipyridyl disulfide; and (c) nonspecific benzoylation of lysine and tyrosine residues of papain can be observed by virtue of the appearance of resonances at 168-170 ppm.

These results show that it is possible to characterize a labile covalent enzyme–substrate intermediate under well-defined crenzymological conditions by 13C NMR spectroscopy and to observe its transformation to product. Previous studies15 have provided 13C NMR evidence for acetyl chymotrypsin stabilized at pH 5.1 at room temperature. The experiments described herein show that it is now possible to observe at subzero temperatures enzyme-substrate intermediates that would escape detection above 0 °C or at higher ratios of substrate to enzyme. Further refinement of the technique to evolve parameters for the observation of acyl and tetrahedral intermediates16,17 of thiol and serine proteases is in progress.

Figure 1. (a–f) 1.7 mM papain (72% active enzyme), 5.4 mM potassium chloride, 25% v/v Me2SO, 0.1 M sodium formate buffer (pH 4.1), 23.6 mM benzoylimidazole. [13C=C=O]Benzoylimidazole was added at 0 °C after 1.5 min, the reaction mixture cooled to -6 °C, and the NMR data acquisition commenced 6 min after the reaction was initiated. Spectra a–f represent 10 000 accumulations recorded sequentially starting at 6, 51, 96, 141, 186, and 231 min after adding benzoylimidazole. The insert at 200-192 ppm is a 3-fold vertical expansion of this spectral range. (g) 2.03 mM papain (72% active enzyme), 6.5 mM potassium chloride, 25% v/v Me2SO, 0.1 M sodium formate buffer (pH 3.8). (h) 2.3 mM papain (72% active enzyme), 7.5 mM potassium chloride, 25% v/v Me2SO (pH = 6.4). Papain was purified by salt precipitation and covalent chromatography18,19 to 95% activity. Concentration and treatment with aqueous Me2SO reduced activity to 72%.

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Acknowledgment. We thank the Science and Engineering Research Council (U.K.) for generous support of this work and for the provision of a Bruker WB 300-MHz spectrometer.

Registry No. Papain, 9001-73-4; N-benzoylimidazole, 10364-94-0.

I have attended the following lectures and seminars:

Biosynthesis - Dr. T. J. Simpson (5 lectures)
Natural Products - Professor A. I. Scott (5 lectures)
β-Lactam Antibiotics - Glaxo Research (5 lectures)
Pulse sequences in NMR - Dr. G. A. Morris (5 lectures)
Bio-organic chemistry - series of 5 lectures
Current topics in organic chemistry - organic staff (10 lectures)

Professor Scott's research group seminars over a period of one year.
Various departmental colloquia and seminars and Monday evening organic seminars over three years.