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The Catalytic Mechanism of Arginine Specific Proteinases and the Localization of Arginine Specific Proteinases Present in Fibrosarcoma Cells Utilizing a Fluorescent Transition-State Analog Probe

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THE CATALYTIC MECHANISM OF ARGinine SPECIFIC PROTEINASES
AND THE LOCALIZATION OF ARGinine SPECIFIC PROTEINASES
PRESENT IN FIBROSARCOMA CELLS UTILIZING A FLUORESCENT
TRANSITION-STATE ANALOG PROBE

by

Karen A. Kozlowski

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of the Requirements for the Degree of
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This work is dedicated to my husband, Lou
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PUBLICATIONS


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Enzymes bring about large increases in the rates of physiological reactions which are often, though not always, found to occur at rates so slow as to be undetectable in their absence. Rate enhancements produced by enzymes are ascribed to a variety of factors:

1.) Increased effective concentration of reactants on the formation of the ES complex.
2.) Loss of entropy during the formation of the ES complex prior to the catalytic step.
3.) Nucleophilic catalysis.
4.) Acid - base catalysis.
5.) Stabilization of the transition-state by the enzyme active site.
6.) Destabilization of the ground state conformation on binding into the ES complex toward the transition-state.

However, it is difficult to quantitatively assess the
contributions made by each of these factors.

It is commonly held that when an enzyme converts a substrate to products, it must overcome an energy barrier, due to the formation of a high energy, metastable intermediate, known as the transition-state. The transition-state occurs at the peak in the reaction coordinate diagram (See Figure 1), in which the energy of the reactants is plotted as the reaction proceeds. In the transition-state, chemical bonds are in the process of being formed and broken, in contrast to the intermediates along the reaction pathway whose bonds are complete. These intermediates have longer half-lives and occupy the troughs in the energy profile of a reaction. The velocity of a reaction is proportional to the concentration of reactants that achieve the transition-state structure.

Enzymes exhibit a high degree of specificity as a result of strong attractive forces between the enzyme and the substrate. Early workers made an assumption that the enzyme and the substrate are structurally complementary as explained by Fischer's "lock and key" analogy (Fischer, 1894), that is to say that each binding group on the substrate is matched by a binding site on the enzyme. The enzyme-substrate complementarity hypothesis predicts a very tight interaction between the enzyme and
Figure 1: Difference free energy versus reaction coordinate diagram comparing an uncatalyzed reaction with an enzymatically catalyzed unimolecular reaction. S and P are the substrate and the product, respectively, and ES and EP are their respective complexes with the enzyme. For catalysis to occur in the enzymatic case, the difference free energy between the enzymatically catalyzed "activated state" (ES\textsuperscript{\textbullet}) and the activated state for the uncatalyzed reaction (S\textsuperscript{\textbullet}) (=G\textsubscript{T}) must be greater than the favorable free energy of S and E complexation (G\textsubscript{ES}).
the substrate with a very small $K_M$. However, if the full binding energy is realized in the formation of the ES complex, then there is little impetus for the reaction to proceed.

Pauling (1948) suggested a type of substrate distortion mechanism in which the enzyme has a configuration which is complementary to the structure of the substrate in its transition-state and not the ground state conformation. Accordingly, the initial binding forces between the enzyme and the substrate in its ground state within the Michaelis complex would be less that the forces between the enzyme and the substrate in its transition-state. Therefore, the initial ES complex would be strained toward its transition-state because the full binding energy would only be realized in the activated transition-state complex. Mechanisms of substrate enzyme activation in the Michaelis complex prior to the rate determining bond making or bond breaking step on the reaction pathway are referred to as "Association-Activation Mechanisms" (Schultz et al., 1977). Rate enhancements may then be due to (1) a conformational change in the enzyme upon binding of the substrate, i.e., induced fit (Koshland and Neet, 1969), (2) distortion of the ground state conformation of the substrate on binding
toward its transition-state (Eyring and Spikes, 1954), and (3) propinquity effects (Jencks, 1969). All of these mechanisms predict that specific substrates would have a high value for $K_M$, because of the utilization of the intrinsic binding energy to lower the energy of activation in the catalytic step (Fersht, 1977).

Expressed in terms of transition-state theory, as derived by Fersht (1977), the equilibrium constants between the transition-state, $ES^\ddagger$, and $E+S$ is proportional to the activation energy $\Delta G_T^\ddagger$. This activation energy is composed of two terms, an energetically unfavorable term, $\Delta G^\ddagger$, due to the activation energy of the chemical steps of bond making and bond breaking; and a compensating energetically favorable term $\Delta G_S$, due to the realization of the intrinsic binding energy. That is,

$$\Delta G_T^\ddagger = \Delta G^\ddagger + \Delta G_S$$ (1)

If the enzyme is complementary to the substrate in its ground state, then the formation of the transition-state will result in a loss of binding energy caused by the poor fit of the transition-state configuration in the enzyme. Therefore, the overall energy of activation for $E + S \rightarrow ES$ (second order rate constant $= k_{cat}/K_M$) then becomes

$$\Delta G_T^\ddagger = \Delta G^\ddagger + \Delta G_R + \Delta G_S$$ (2)
If, however, the enzyme is complementary to the structure of the substrate in its transition-state, then there will be an initial loss of energy in the formation of the ES complex due to the poor fit of the substrate which will result in a higher $K_M$ because the intrinsic binding energy is reduced by the amount $\Delta G_R$ due to the poorer fit of the substrate. However, when the transition-state is reached, $ES^\ddagger$ will be stabilized due to a better fit in the enzyme active site in the amount of $\Delta G_R$. Therefore, the $\Delta G_R$ terms cancel in the sum and the total energy of activation is

$$\Delta G_T^\ddagger = \Delta G^\ddagger + \Delta G_S$$

(3)

Thus, it is clear that the energy of activation for the second order rate constant $k_{cat}/K_M$ required for an enzyme which is complementary to the structure of the transition-state is less by the free energy term $\Delta G_R$ than the energy of activation for an enzyme complementary to the substrate in its ground state.

II. TRANSITION-STATE ANALOGS AS PROBES OF ENZYME MECHANISMS

From the previous discussion, one can predict that an enzyme inhibitor containing the structural features of
the configuration of the substrate in its transition-state would be an extremely potent and selective inhibitor. This prediction of high transition-state affinity is supported by an argument derived from the theory of absolute reaction rates (Wolfenden, 1976) as described by Figure 2. The top line of the equilibrium diagram shows the reaction of substrate (S) and its transition-state (S‡) to form product (P) in a non-enzymic reaction. The bottom line of the same figure depicts the same reaction catalyzed by enzyme (E). K_S is the equilibrium association constant for enzyme and substrate; KN and KE are the equilibrium association constants for the formation of the non-enzymic and enzymic transition-states respectively; and KT is the equilibrium association constant for the association of the substrate in its transition-state configuration to the enzyme. The equilibrium constants are related by equation 4.

\[ \frac{K_T}{K_S} = \frac{K_E}{K_N} \]  \hspace{1cm} (4)

Moreover, transition-state kinetic theory predicts that the rate of any reaction is proportional to its equilibrium constant for formation times a proportionality factor \((kT/h)\) (Eyring, 1935).

\[ k_r = \frac{kT}{h} (K^+) \]  \hspace{1cm} (5)

where \(k_r\) = the rate constant for a chemi-
Figure 2: Schematic representation of transition-state theory as applied to enzymatic catalysis.
\[
\text{cal reaction}
\]
\[
h = \text{Planck's constant}
\]
\[
k = \text{Boltzmann's constant}
\]
\[
T = \text{absolute temperature}
\]
\[
K^\ddagger = \text{the equilibrium constant for the formation of the transition-state}
\]

Equations 4 and 5 can be combined to give equation 6, where \( k_e \) and \( k_n \) are the rate constants for the enzymatic and non-enzymatic reactions, respectively, in which the reaction is carried out by an identical mechanism.

\[
\frac{K_T}{K_S} = \frac{K_E}{K_N} = \frac{k_e}{k_n} \quad (6)
\]

It is commonly believed that the ratio of \( k_e/k_n \) is greater than \( 10^8 \) for enzyme reactions (Lienhard, 1973). Accordingly, the equation predicts that the ratio \( K_T/K_S \) is greater than \( 10^8 \), or that a substrate in its transition-state will bind to an enzyme with an affinity at least eight orders of magnitude greater than that same substrate in its ground state.

While transition-state analogs should bind at least eight orders of magnitude more tightly than the corresponding substrates, the actual values for binding constants fall in the range from \( 10^2 - 10^5 \). These lower than anticipated values reflect the imperfect nature of
the analogs and emphasize the fact that the transition-state structures are the enzymologist's best guess as to what the transition-state may actually look like (Lindquist, 1975). It also assumes that the transition-state of the enzymatic and non-enzymatic reactions are identical. Other factors, such as solvation differences and protein conformation changes in the enzyme upon substrate association may also affect the predicted ratio of \( k_e/k_n \). Therefore, the ideal transition-state analog may be equivalent to a Platonic "form," possible to imitate but never to attain.

IO. GOALS OF THIS DISSERTATION

Aldehyde analogs of substrates of serine and cysteine proteases are known to form highly stable association complexes with their respective enzymes (Thompson, 1973). Their enhanced affinity for these enzymes over that for substrates has been attributed to possible transition-state like modes of association (Thompson, 1973). The experiments in this dissertation utilize a fluorescent peptidyl-aldehyde transition-state analog to further elucidate the mechanism of aldehyde association with the protein hydrolase enzyme, trypsin. In addition, the
properties of this transition-state analog, that is, its high affinity binding coupled to a fluorescent reporter group, will be exploited to localize and quantify arginine specific proteinases in normal and transformed cells.
CHAPTER II

REVIEW OF THE RELATED LITERATURE

IIA. TRYPsin - AN INTRODUCTORY REVIEW

The proteolytic enzyme, trypsin, and its inactive precursor, trypsinogen, were first obtained in crystalline form by Northrop and Kunitz in 1933 (Northrop et al., 1948). Trypsinogen is transformed into trypsin as the result of the cleavage of a single peptide bond (Lys6-Ile7) near the N-terminus of the zymogen (Davie and Neurath, 195), and the appearance of activity is accompanied by a conformational change (Neurath and Dixon, 1957). The activation process is catalyzed by a variety of enzymes including enterokinase and trypsin, itself (Kunitz, 1939). The latter autocatalytic process is accelerated by calcium ions which bind to the N-terminal region of the zymogen and promote the specific bond cleavage (Radhakrishan et al., 1969; Abita et al., 1969). A striking feature of the enzyme is the narrow specificity of its action, which is almost exclusively direc-
ted toward L-lysyl and L-argininyl bonds of polypeptides. Biologically, trypsin serves as the activator of all the other zymogens of pancreatic tissue. Thus, the control of the activation of trypsinogen has broad consequences in terms of the formation of the endopeptidase and exopeptidase components of pancreatic juice.

Trypsin belongs to a family of enzymes known as the serine proteases because it possesses a highly reactive serine residue in the enzyme active site. The major pancreatic enzymes, trypsin, chymotrypsin and elastase, are all serine proteases. They are kinetically very similar, hydrolyzing peptide bonds and synthetic esters and amides and are all active at neutral pH. The major difference between them is their specificity. The three dimensional structure of these three crystalline enzymes, obtained by x-ray crystallography, shows that the polypeptide backbone of all three are essentially superimposable. It is also known that there is a 50% sequence homology between their primary structures (Blow, 1971; Hartley and Shoten, 1971; Neurath, 1984). This means that kinetic information obtained on one of these enzymes may be applicable to other enzymes in this serine protease family. Chemical modification experiments show that there are two amino acid residues, serine-183 and
histidine-146, which are essential for catalytic activity (Kiel, 1971). X-ray crystallography indicates a third amino acid residue, aspartate-90, may also be catalytically essential as the aspartate residue is in hydrogen bonding distance of the histidine. These three amino acid residues compose a catalytic triad in the enzyme active site which is historically known as the "charge relay system" (Blow et al., 1969).

The mechanism of tryptic hydrolysis of peptide bonds is believed to involve a general base catalyzed nucleophillic attack on the carbonyl carbon of the substrate by the hydroxyl oxygen of the serine-183. Coincident with this attack, the hydroxyl proton of the serine is transferred to the histidine imidazole (Kraut, 1977). The unresolved mechanistic question has been whether the histidine-46 is the actual chemical base in the reaction or is it an intermediary through which the aspartate-90 functions as the general base. It has long been thought that the imidazole base of histidine increases the nucleophilicity of the serine hydroxyl by acting as a general base catalyst. This seemed to be consistent with the decrease in enzymatic activity observed at low pH with the ionization of a base with a pKa of 7, a characteristic value for a histidine residue. The other candi-
date for the general base is the aspartate-90, which
normally has a pKa of 4.5. Since the aspartate residue
is in a hydrophobic domain, shielded from the aqueous
media, the milieu may serve to elevate the pKa of the
aspartate. Therefore, Hunkapillar et al., (1973) sugges-
ted that the pKa of the histidine and aspartate may
actually be reversed. Data from nuclear magnetic reso-
ance experiments and infrared spectroscopy indicated that
the group ionizing with a pKa of 7 was actually the
aspartate residue (Hunkapillar et al., 1973; Koeppe and
Stroud, 1976). If this evidence is correct, then the
histidine remains unprotonated in the enzyme active site.
Therefore, during catalysis, a proton is transferred from
the histidine to the aspartate. However, more recent
evidence from proton inventory studies (Elrod et al.,
1980) and neutron diffraction (Kossiakoff and Spencer,
1980) indicate that histidine does have a pKa of 7.0 and
thus acts solely as the general base. The negatively
charged aspartate acts to stabilize the imidazolium ion
through electrostatic interactions, but not through the
delocalization of charge.

Trypsin is known to exist in three catalytically
active forms: alpha, beta and pseudo trypsin (Kiel,
1971). α-trypsin if formed from the proteolytic activa-
tion of trypsinogen with the removal of a hexapeptide from the amino terminus of the zymogen (Davie and Neurath, 1955). $\beta$-trypsin is formed from the cleavage of the lys-131 and ser-132 bond of $\alpha$-trypsin. At optimum pH with ester substrates, there are no significant differences between these two forms. Pseudotrypsin is the product of the cleavage of the lys-176 and asp-177 bond in $\beta$-trypsin and possesses esterolytic activity against uncharged substrates.

IIA-1. EARLY INVESTIGATIONS OF SUBSTRATE SPECIFICITY

Mares-Guia and Shaw (1965) early argued that the specificity site of trypsin contains an anionic site to which a substrate or inhibitor binds electrostatically through its the anionic binding site in trypsin is indicated by the fact that pseudotrypsin, which has lost the aspartate-177 residue, also has lost its characteristic lysine or arginine specificity while retaining its general esterase activity. X-ray crystallographic data confirm these features of the primary binding site (Stroud et al., 1974).

The effectiveness of benzamidine and phenylguanidine as competitive inhibitors of trypsin was attributed
to the binding of their aromatic rings in the hydrophobic slit. Mares-Guia et al. (1967) tested this interpretation of the orientation of bound benzamidine and phenylguanidine by preparing benzamidine and phenylguanidine derivatives containing ester groups in the para position and examining their behavior as substrates. Esters whose length approximated the extended form of normal arginine substrates were shown to be cleaved in a manner consistent with this picture. Mares-Guia and Shaw (1967) considered that ethyl p-guanidinobenzoate would be a model compound of rigid structure and a possible substrate which would help to further define the functional relationship between the specificity site and catalytic site of the active center of trypsin. Although the compound is closely related structurally to some aromatic esters split by trypsin, the unexpected observation was made that it inactivated the enzyme. Kinetic evidence indicated that a typical ES complex is formed which gives a stable acylenzyme which does not deacylate, even at a pH which is optimal for tryptic hydrolysis.

Difference spectra which appear during the inactivation indicate that the p-guanidinobenzoyl group is transferred from an aqueous to a hydrophobic environment, while an analogous shift of the tyrosine residues of
trypsin occurs (Mares-Guia and Shaw, 1967) These tyrosine residues may be involved in the hydrophobic binding site of the active center. The failure of this compound to deacylate was attributed to a distortion of the hydrolytic site of trypsin (Mares-Guia and Shaw, 1967).

Inagami and coworkers (1964, 1965 and 1969) are another early group who have made important investigations of the enzyme's active site. The specific substrates of trypsin, benzoyl-arginine ethyl ester (BAEE), and chymotrypsin, acetyl-tryptophan ethyl ester (ATEE), can be exchanged between the two enzymes and still undergo hydrolysis. Specificity with respect to side chain of an amino acid is thus of a quantitative nature rather than a qualitative nature. The factors that control specificity are determined by interactions between the side chain and part of the enzyme's active site. Inagami suggested that if the two enzymes react with a substrate without the amino acid side chain, such as acetyl-glycine ethyl ester (AGEE), the interaction between enzyme and substrate will involve the catalytic site alone. The catalyses of AGEE by the two enzymes were similar with respect to $K_M$, the maximal first order rate constant, $k_{cat}$, and its temperature dependence and the dependence of $K_M$ and $k_3$ on salt concentration. Inagami (1964)
concluded that for AGEE there was an absence of a specific interaction of the substrate with the specificity site of the enzyme; therefore catalyses was mediated by the active site alone. The kinetic similarity gave evidence that the catalytic sites of the two enzymes are structurally similar.

The rate of the trypsin catalyzed hydrolysis of AGEE is increased several fold by the addition of methyl-, ethyl-, or n-propylamine. The kinetic evidence indicated that the increased rate of hydrolysis is due to the binding of the alkylamine to the specificity determining site of the enzyme (Inagami et al., 1964).

Inagami (1965 and 1969) also studied this phenomenon using iodoacetamide, a molecule which is roughly comparable to AGEE in size and structure. He predicted that the iodoacetamide would have access to the enzyme active site when methylguanidine was present. It was observed that the presence of methylguanidine increased the rate of alkylation by iodoacetamide by a factor of 6 and increased the esterolytic rate of AGEE by a factor of 7, indicating that both the processes of non-specific substrate hydrolysis and alkylation of the histidine take place by the same stereo or conformationally facilitated mechanism. The pH dependence of the rate of
alkylation can be fitted by a single group titrating with a pKa of 6.66, suggesting that the unprotonated form of the histidine is alkylated.

An increased reactivity of the histidine imidazole group was suggested as the most likely explanation of the trypsin catalyzed hydrolysis of AGEE and the alkylation of trypsin by iodoacetamide under the influence of methylguanidine (Inagami, 1969). As the alkylation proceeds through the nucleophilic attack of iodoacetamide by the N-3 atom of the imidazole, which is specifically alkylated by the iodoacetamide, the nucleophilicity of the N-3 atom is presumably increased via a conformational change. An alkylguanidine can be considered to represent the basic side chain of an arginine substrate. The basic side chain of the substrate is bound to the cationic recognition site by coulombic and hydrophobic interactions and through a conformational or other changes in the protein increases the nucleophilicity of the N-3 atom of the histidine residue in its region of the catalytic site. This increased reactivity results in an increased catalytic activity.

Further work on the thermodynamics of binding of alkylammonium inhibitors strongly supported the existence of a hydrophobic binding site of limited size at the
trypsin active center (Heidberg et al., 1967). In addition, the authors found evidence for a secondary hydrophobic site which would become occupied when compounds of longer side chains (>C7) are used.

IIA-2. X-RAY CONFORMATION OF THE SPECIFICITY POCKET OF TRYPsin

Krieger, Kay and Stroud (1974) determined the x-ray structure of benzamidine-trypsin at pH 8.0 in order to study the specific side chain binding pocket of the enzyme. Benzamidine (BA) is a competitive, specific inhibitor of trypsin with a $K_I$ of $1 \times 10^{-5}$ M (Barman, 1969). The BA-trypsin structure provides a good model for the binding of the natural substrate side chains to trypsin during catalysis. Because the enzyme is inhibited, the structure could be determined at pH 8.0, where the enzyme is normally active, rather than at a pH where the enzyme is inactive.

The x-ray structure shows that the carboxyl group of asp-177, which is responsible for trypsin specificity, forms a salt bridge with the amidinium cation of benzamidine; a similar salt bridge can be constructed between the side chains of model substrates containing arginine.
and lysine. When benzamidine is bound in the enzyme active site, the binding pocket closes to sandwich in the phenyl ring of the molecule. In addition, binding of benzamidine induces alterations in the side chains of the amino acids present in the active site. If BA-trypsin is a good model for substrate binding, then the specific side chains of real substrates will produce similar rearrangements. Krieger speculates that a function of side-chain binding of specific substrates is to reposition parts of the enzyme structure. This hypothesis provides an explanation as to the mechanism by which small alkyl amines or guanidines binding increases the hydrolysis of non-specific substrates by trypsin. Methyl-guanidine is able to bind in the specificity pocket of trypsin and induce the necessary conformational changes within the enzyme to enhance the catalysis at the catalytic site.

IIA-3. SUBSTRATE ACTIVATION OF TRYPsin

Tosyl-L-arginine methyl ester (TAME) has been widely used as a model trypsin substrate for kinetic analyses (Scheraga et al., 1958; Ronwin, 1959; Martin et al., 1959). These early studies supported a simple Michaelis-Menten behavior for trypsin. However,
Trowbridge et al., (1963) studied the initial rates of hydrolysis of both the D and the L enantiomers of TAME over a $10^5$ concentration range and found that the hydrolysis of neither substrate fit simple Michaelis-Menten kinetics. Extension of the TAME concentration beyond the usual concentration limits utilized for simple kinetic analysis produced a curved Eadie plot showing enzyme activation by high concentrations of substrate. Similar results were obtained by Goto and Hess (1979) using the substrate dansyl-arginine. Goto and Hess reported two binding constants for dansyl-arginine of $6.7 \times 10^{-3} M$ and $4.8 \times 10^{-4} M$.

There are several possible explanations for this data including: (1) contamination of the trypsin preparation by another enzyme that also utilizes TAME as a substrate; (2) the presence of two catalytic sites per enzyme molecule; and (3) the presence of a secondary binding site on the enzyme to which a modifier can bind and activate the enzyme. By studying the kinetic behavior of three different trypsin preparations, Trowbridge et al., (1963) discounted the possibility of a contaminating factor in the enzyme or the presence of two catalytic sites. The data is best explained in terms of an allosteric effect. That is, a substrate molecule binds
not only to the active site of the enzyme, but also to a secondary site, similar to an allosteric site. On formation of a ternary complex (SES), the enzyme molecule changes conformation and catalyzes the conversion of substrate to product at a different rate. The existence of an auxiliary binding site is not unique to trypsin, but has also been suggested for chymotrypsin and subtilisin (McClure and Edelman, 1967; Smith and Hansch, 1973).

Trowbridge et al., (1963) suggested that the presence of two binding sites may play an important physiological role. The geometry of the sites and of the native protein substrate may be such that both substrate binding sites can be filled by the same protein molecule. Such an interaction can lead to a much faster hydrolysis for substrates filling both sites than if there were only a single interaction. This concept may well be of value in explaining the unusually high specificity of proteolytic enzymes in activating precursors of biologically active proteins. In such reactions, one or a few of the many susceptible bonds in the protein are hydrolyzed much more rapidly than the remaining chemically equivalent bonds.
III. MECHANISM OF SERINE PROTEASE ENZYMES

The general mechanism of serine proteases enzymes is depicted in Figure 3. The enzyme initially associates with the substrate to form the non-covalent Michaelis complex with a dissociation, $K_s$. This is followed by the formation of an acylenzyme through a putative tetrahedral intermediate. Deacylation of the enzyme proceeds by a similar pathway as acylation in the reverse direction. A molecule of water non-covalently associates with the acylenzyme, and then breaks the acylenzyme down liberating the second product and the free enzyme. The evidence for the sequence of steps comes from a variety of sources including x-ray crystallography (Steitz, Henderson and Blow, 1969), enzyme kinetics using chromophoric leaving groups (Hartley and Kilby, 1954), chromophoric acylating groups (Yu and Viswanatha, 1969), chemical modifying reagents (Kiel, 1971) and presteady state kinetic assays (Fastrez and Fersht, 1973). Because of its high energy and short half-life, the tetrahedral intermediate has never been directly observed, but its presence can be inferred from the kinetic results.
Figure 3: Reaction sequence for serine protease enzymes.
IIC. ALDEHYDE ASSOCIATION TO SERINE AND CYSTEINE PROTEASES

The inhibition of trypsin by the leupeptins Pr-LL and Ac-LL (i.e. propionyl- and acetyl-L-leucyl-L-leucyl-L-argininal) isolated from the culture filtrates of Actinomycetes was the first example of the inhibition of a serine protease by a peptide aldehyde (Aoyagi et al., 1969). It is now known that aldehyde analogs of peptide substrates form highly stable complexes with cysteine and serine proteases presumably due to a high transition-state affinity (Thompson, 1973). On binding to the active site of the enzyme, the carbonyl carbon of the aldehyde changes from a trigonal to a tetrahedral configuration to form a relatively stable hemiacetal or hemithioacetal adduct with the active site serine or cysteine as depicted in Figure 4 (Kennedy and Schultz, 1979; Frankfater and Kuppy, 1980). It is believed that substrates of serine and cysteine proteases undergo this same change in configuration in their transition-state during their catalytic transformation by enzymes. Hence aldehydes are known as transition-state analogs.

Controversy remains over whether or not aldehydes are true transition-state analogs. An additional feature
Figure 4: Scheme for the association of a peptide aldehyde with the active site serine of a serine protease enzyme. IA and IB are Michaelis complexes with the C1 carbonyl carbon of the aldehyde in sp² and sp³ configurations, respectively; IIA and IIB are hemiacetal adducts with the active site serine.
of the proposed transition-state of the serine protease enzymes includes an oxanion on the carbonyl carbon of the substrate. An oxanion is highly unstable, one with a pKa of approximately 13 (Gruen and McTigue, 1973), and would require approximately 12 kilocalories of stabilization energy from the enzyme at pH 8.0. X-ray crystallographers have identified a region in serine proteases known as the "oxanion hole" which is a hydrogen bond donating site proposed to stabilize the oxanion in the transition-state. Accordingly, for a peptidyl aldehyde transition-state analog, one would expect the formation of a charged hemiacetal adduct. In addition, by analogy to substrate catalysis, one would anticipate that formation of the hemiacetal adduct would require the basic form of the histidine imidazole side chain. By microscopic reversibility, the decomposition of the adduct will require the corresponding acid form of the general base-acid catalyst. Proper manipulation of terms describing the pH dependency of $k_2$ and $k_{-2}$ reveals a relationship which predicts that as the solution pH is lowered from the catalytically optimum pH 8.0 and below the apparent pKa of the histidine imidazole, then, $K_{I,al}$ should approach $K_s$ (Schultz and Cheerva, 1975) (note $k_{2,lim}$ and $k_{-2,lim}$ are the maximal pH independent rate
The data of Schultz and Cheerva (1975) on the binding of a non-specific aldehyde, hydrocinnamaldehyde, to chymotrypsin tended to confirm the predictions of equation 7, while the results of Schultz and Kennedy (1979) on the binding of a specific aldehyde to chymotrypsin tended to contradict equation 7. The above equation was derived on the assumption that the stable hemiacetal is anionic (Schultz and Cheerva, 1975). The lack of a significant pH dependence in \( K_{I,al} \) for benzoyl-phenylalaninal to chymotrypsin can be explained by assuming that two protons are transferred in steps \( k_2 \) and \( k_{-2} \) such that the stable hemiacetal formed is neutral and that step \( k_2 \) is general base - specific acid catalyzed, while step \( k_{-2} \) is specific base - general acid catalyzed. Since these two types of catalysis are kinetically indistinguishable, hydrogen ion induced alterations in the rate constant \( k_2 \) are mirrored in \( k_{-2} \) so that their pH dependency terms cancel in the ratio \( k_2/k_{-2} \). Conversely, Kuramochi et al. (1979) reported that the association of leupeptin shows a more significant pH dependence. Although these authors conclude that the trypsin-
leupeptin complex is neutral, this data suggests that trypsin may stabilize the anionic form of the hemiacetal to a greater extent than chymotrypsin. If aldehydes are truly transition-state analogs, they will provide specific information on the mechanism of enzymatic catalysis.

III. ROLE OF PROTEOLYTIC ENZYMES IN CELLULAR TRANSFORMATION

Transformation of cell cultures by oncogenic viruses or chemical carcinogens results in an increased level of proteolytic enzyme activity (Ossowski et al., 1974; Quigley, 1979). Therefore, it has been postulated that proteolytic enzymes may cause or facilitate changes in cells which will increase the probability that a normal cell will become malignant or that a tumor cell will have an increased capacity to metastasize.

Malignant cells exhibit three important phenotypic characteristics:

1.) Uncontrolled cell growth and division.
2.) Ability to degrade extracellular matrix.
3.) Ability to form metastases.
proteolytic enzymes have been implicated in each of these processes. For example, treatment of normal cells with proteolytic enzymes can lead to the appearance of phenotypic characteristics in the untransformed cells (Werb and Aggeler, 1978). These changes include loss of density-dependent inhibition of growth, induction of DNA synthesis and cell division, changes in cellular morphology, decreased cellular adhesion and loss of the cell surface glycoprotein fibronectin (Hynes, 1974). In addition, trypsin treatment of rabbit synovial fibroblasts induces the secretion of collagenases and plasminogen activator from these cells (Werb and Aggeler, 1978).

If limited proteolysis plays a role in the establishment or promotion of the malignant phenotype, then addition of protease inhibitors to cultures of transformed cells should bring about the restoration of a normal, non-malignant phenotype. Transformed cell cultures treated with inhibitors of proteolytic enzymes display a reduced growth rate, a normal flattened morphology, decreased agglutinability, decreased glucose transport and enhanced cellular adhesion (Roblin et al., 1975). It has also been demonstrated that protease inhibitors interfere with chemical transformation in C3H/10T1/2 cells (Kuroki et al., 1979) and suppress

The above studies indicate a role for proteases in the maintenance of the malignant phenotype, but not all reports support a dramatic role for protease inhibitors in the suppression of oncogenesis or metastases (Turner and Weiss, 1981).

A number of specific proteolytic enzymes have been implicated in the processes of tumorigenesis and metastasis. These enzymes include lysosomal hydrolases, plasminogen activator, trypsin-like neutral protease and collagenase. A significant amount of evidence exists for the secretion of these hydrolytic enzymes into the extracellular environment by the tumor cells (Reich et al., 1975; Strauli et al., 1980). In addition, recent reports also document the existence of protease activity associated with the cell membrane of transformed cells (Quigley, 1979). A high concentration of cell surface proteolytic activity may provide an intimate mechanism by which the transformed cell's membrane can both maintain malignant traits within the tumor and promote the degradation
of surrounding tissue during tumor cell invasion and metastasis.
CHAPTER III

MATERIALS AND METHODS

III.A. GENERAL MATERIALS AND SOURCES

The sources of uncommon chemical reagents and instrumentation utilized in this work are indicated throughout the text. All other chemicals and solvents were reagent grade or better as purchased through Scientific Products, Aldrich Chemical Company or Sigma.

Bovine trypsin (3x crystallized) was purchased from Sigma, and lot number 72F-8085 was used exclusively throughout this work. The concentration of this enzyme was calculated from a molecular weight of 23,800.

Bovine trypsinogen (1x crystallized) was purchased from Millipore Corporation and lot number 3649 TG 16P739 was used exclusively throughout this work. The concentration of the zymogen was calculated from a molecular
A specific staining procedure was routinely used to identify the spots corresponding to the aldehyde, the aldehyde hydrate or carbinolamine in thin layer chromatograms (Stahl, 1969). The developed and dried silica plate was initially sprayed with a solution containing 100 mg of 2,4-dinitrophenylhydrazine (DNP) (Aldrich) in 1 ml of 37% hydrochloric acid and 99 ml of 95% ethanol and dried at 100°C in an oven. The spots corresponding to the aldehyde were bright yellow in color.

An additional staining reagent was also used to identify the unprotected guanido group in the arginine compounds. The chromatogram was sprayed with a mixture of 16% urea solution and 0.2% ethanolic α-napthol solution (5:1). The chromatogram was dried in a 40°C oven and subsequently sprayed with a solution of 3.3 ml bromine in 500 ml 5% sodium hydroxide solution. Compounds containing an unprotected guanido group appeared as red spots on the chromatogram.

IIIB. SYNTHESIS OF DANSYL-L-ARGININAL

The synthetic methods utilized to prepare dansyl-L-argininal appear herein. Enantiomeric integrity was
maintained as documented previously (Ito et al., 1969).

Indicated TLC $R_f$ values (= distance traveled by a compound/distance traveled by the developing system) were obtained on Quantum UV$_{254}$ sensitized 10 cm silica gel plates. Developing systems are reported as volume/volume (V/V).

Infrared spectra (IR) were recorded on a perkin Elmer model 337 IR-spectrometer. When useful, characteristic absorbance maxima in reciprocal centimeters (cm$^{-1}$) are included.

$^1$H-nuclear magnetic resonance (NMR) spectra were recorded on a Varian EM360 60 megahertz spectrometer. Characteristic chemical shifts from tetramethylsilane in parts per million are included.

To 2.0 g, 7.4 mmole NO$_2$-arginine methyl ester (Sigma) in 100 ml of absolute ethanol, was added an equivalent of N-methylmorpholine. The mixture was then cooled to 0°C, and 2.7 g, 10 mmole dansyl-chloride (Sigma) were added as a solid. The mixture was stirred at 0°C for 30 minutes and then at room temperature for 3-4 hours. The solvent was removed by rotoevaporation (T<40°C), the residue dissolved in chloroform and the product extracted into 0.1 N HCl. The acidic layer was made basic with 5% Na$_2$CO$_3$ (pH 8.5) and the product was
extracted into chloroform. The chloroform layer was then washed with saturated saline, dried over sodium sulfate (anhydrous) and evaporated by roto-evaporation leaving an orange oily residue. The residue is triturated with ethyl ether resulting in a solid precipitate which is crystallized from benzene. M.P. 114-116°C. Yield 79.8%. IR: 1750 cm\(^{-1}\) (ester carbonyl). NMR: 7.4 ppm (aromatic protons), 2 ppm (OCH\(_3\)).

The dansyl-NO\(_2\)arginine methyl ester (300 mg, 0.6 mmoles) was dissolved in 40 ml of anhydrous tetrahydrofuran at -20°C to which 15 ml of diisobutylaluminum hydride (1 M solution in hexane) (Aldrich) was added dropwise over a period of 45 minutes, and stirred for an additional 20 minutes. The reduction was stopped by the addition of 50 ml of cold 2 M HCl and the temperature allowed to rise to 0°C. Ethyl acetate (50 ml) was then added and stirring was continued for an additional 30 minutes. The organic layer was separated from the aqueous layer and the acidic aqueous layer extracted with ethyl acetate several times. The ethyl acetate extracts were combined, washed with saturated saline, dried over sodium sulfate (anhydrous), and the solvent removed on the roto-evaporator. The impure dansyl-NO\(_2\)argininal was dissolved in a minimum amount of ethanol and an aqueous
Figure 5: Scheme for the synthesis of dansyl-L-argininal.
\[ \text{NO}_2\text{-arginine methyl ester} \]
\[ \downarrow \]
\[ \text{dansyl-chloride} \]
\[ \downarrow \]
\[ \text{dansyl-NO}_2\text{-arginine methyl ester} \]
\[ \downarrow \]
\[ \text{diisobutylaluminum hydride} \]
\[ \downarrow \]
\[ \text{dansyl-NO}_2\text{-argininal (impure)} \]
\[ \downarrow \]
\[ \text{sodium bisulfite} \]
\[ \downarrow \]
\[ \text{dansyl-NO}_2\text{-argininal as the bisulfite adduct} \]
\[ \downarrow \]
\[ \text{pH 8.5 (5\% Na}_2\text{CO}_3) \]
\[ \downarrow \]
\[ \text{dansyl-NO}_2\text{-argininal (pure)} \]
\[ \downarrow \]
\[ \text{semicarbazide HCl} \]
\[ \downarrow \]
\[ \text{dansyl-NO}_2\text{-argininal semicarbazone} \]
\[ \downarrow \]
\[ \text{catalytic hydrogenation} \]
\[ \downarrow \]
\[ \text{dansyl-argininal semicarbazone} \]
\[ \downarrow \]
\[ \text{formalin/HCl} \]
\[ \downarrow \]
\[ \text{dansyl-argininal} \]
solution of saturated sodium bisulfite was added, and the solution was stirred overnight at 4°C to form the bisulfite adduct. The aqueous solution was extracted with ethyl acetate until all of the dansyl-NO₂-arginine methyl ester and dansyl-NO₂-argininol were removed as verified using TLC. The pH of the bisulfite solution was then raised to 8.5 by the addition of 5% Na₂CO₃, and the solution was stirred for an hour at 4°C to decompose the bisulfite adduct, followed by extraction with ethyl acetate to remove the product. The organic extract was washed with saturated saline, dried over sodium sulfate (anhydrous) and the solvent was removed by roto-evaporation. TLC of the residue in chloroform/methanol (v/v) (9/1) gave two green fluorescent spots corresponding to the aldehyde hydrate and the carbinolamine forms of the aldehyde with Rf values of 0.55 and 0.53. Without further purification or crystallization the product from the previous reaction was dissolved in 5 ml of 70% ethanol. Semicarbazide hydrochloride (300 mg) and sodium acetate (150 mg) were added and the solution was stirred for 30 minutes at 80°C, before being allowed to stand at room temperature overnight. The desired product, dansyl-NO₂-argininal semicarbazone precipitated out of the solution. M.P. 175-178°C. Yield 34%. IR: 1650 and 1680 cm⁻¹
The dansyl-NO$_2$-argininal semicarbazone (100 mg, 0.2 mmoles) was subsequently dissolved in 40 ml methanol, 5 ml deionized water and 2.5 ml glacial acetic acid to which 25 mg palladium black was added and the mixture was placed in a Parr hydrogenator under 20 lbs/in$^2$ of hydrogen for 12 hours. TLC at the end of 12 hours gave a single green fluorescent spot with an $R_f$ of 0.31 in chloroform/methanol (v/v) (3/1). The solution was filtered through celite, the solvents evaporated and the dansyl-argininal semicarbazone crystallized from ethanol/ether. M.P. 143-145°C. The product gave a positive Sakaguchi reaction.

Dansyl-argininal semicarbazone (50 mg, 0.11 mmoles) was dissolved in 10 ml methanol, 1.5 ml of 2 N HCl and 2.0 ml of 37% formalin, stirred at room temperature for 2 hours prior to the addition of 10 ml of deionized water. The pH of the solution was brought to 7.0 with the addition of NaOH, extracted with methylene chloride and the methylene chloride dried over sodium sulfate (anhydrous). After evaporation of the solvent, dansyl-argininal was crystallized from hot ethanol. M.P. 180-185°C. $R_f$ of 0.58 and 0.54 in chloroform/methanol/acetic acid (15/5/1). The compound was both Sakaguchi and DNP posi-
Elemental Analysis (Galbraith Laboratories) (Calculated)
C 50.38% (50.64%), H 6.08% (5.90%), N 16.54% (16.40%).

$^1$H-NMR (D$_2$O) ppm: 1.55 (d) (CH$_3$-N-CH$_3$), 3.1 (CH$_2$-CH$_2$-CH$_2$), 3.6 (CH$_2$-CH$_2$-CH$_2$), 5.3 (carbinolamine anomeric CH), 6.2 (NH), 7.8 (aromatic).

IR spectra (KBr), cm$^{-1}$; 1740 (aldehyde).

III.C. GENERAL DISCUSSION OF THE DETERMINATION OF $K_I$

Two spectrophotometric methods have been utilized to quantitatively assess the dissociation constant for inhibition $K_I$ of dansyl-argininal to trypsin. The first method examines the inhibition of the hydrolysis of the trypsin substrate, benzoyl-arginine ethyl ester, while the second method utilizes the fluorescent properties of the inhibitor itself, to measure the binding of the inhibitor to the enzyme.

III.C-1. INHIBITION OF BENZOYL-ARGININE ETHYL ESTER HYDROLYSIS

The ester substrate, benzoyl-arginine ethyl ester (BAEE) has been shown to exhibit Michaelis-Menten type
kinetics of hydrolysis by trypsin over a fairly broad concentration range (Schwert and Takenaka, 1959) with a reported $K_M$ of 0.050 mM. BAEE was selected for these studies because it had a better $K_M$ for trypsin as compared to benzoyl-arginine p-nitroanilide and was not expected to cause product inhibition or enzyme activation within the concentration range to be used (Kiel, 1971; Schwert and Takenaka, 1959).

The course of the reaction can be followed spectrophotometrically to completion by observing the increase in absorbance due to the release of product, benzoyl-arginine, at 255 nm. Kinetic parameters were obtained from progress curves, rather than initial rate assays for several reasons:

1.) The change in absorbance upon product formation is relatively small with a molar absorptivity of 808 (Barman, 1969). Therefore, measuring this small change in absorbance could result in a high degree of error at the concentrations to be used in an initial rate assay.

2.) It would be possible to obtain more information on substrate activation or product inhibition through the use of progress curves.
3.) Numerous reports of irreversible or uncompetitive inhibition (Bajusz et al., 1979) of proteases by peptidyl aldehydes have appeared in the literature in recent years. The analysis of progress curves therefore, is needed to confirm competitive inhibition by dansyl-argininal in this work.

Kinetic parameters can be obtained from progress curves by integrating the Michaelis-Menten equation.

\[ v = \frac{dp}{dt} = \frac{V[S]}{K_M + [S]} \quad (8) \]

One of the three variables can be removed by means of the following relationship: \( S + P = S_0 \) \quad (9)

Then,

\[ \frac{dp}{dt} = \frac{V(S_0 - P)}{K_M + (S_0 - P)} \quad (10) \]

which may be integrated as follows:

\[ \int (K_M + S_0)dp/(S_0 - P) - \int Pdp/S_0 - P = Vdt \quad (11) \]

\[ \int -(K_M + S)\ln(S_0 - P) + P + S_0 \ln(S_0 - P) = Vt + \alpha \quad (12) \]

where \( \alpha \) is the constant of integration. The condition \( p=0 \) when \( t=0 \) gives \( \alpha = -K_M \ln S \), and so

\[ Vt = p + K_M \ln \left[ S_0 /(S_0 - P) \right] \quad (13) \]

or

\[ Vt = S_0 - S + K_M \ln \left( S_0 / S_9 \right) \quad (14) \]

The above equation can be transformed in order to permit plotting as a straight line:
\[
t/\ln(S_0/S) = 1/V[(S_0-S)/\ln(S_0/S)] + K_M/V
\] (15)

A plot of \(t/\ln(S_0/S)\) against \((S_0-S)/\ln(S_0/S)\) gives a straight line with an intercept of \(K_M/V\) and a slope of \(1/V_{\text{max}}\). Addition of a competitive inhibitor to the reaction will affect the substrate's \(K_M\) according to the following equation:

\[
K_{M,\text{apparent}} = K_{M,\text{true}} (1 + [I]/K_I)
\] (16)

In each rate assay determination, the enzyme concentration was 10 nm in 0.1 M phosphate or acetate buffer in a pH range from 5.0 to 7.8. BAEE was present at a concentration > \(K_M\) and several concentrations of substrate were utilized at each pH to rule out the possibility of product inhibition. The concentration of dansyl-argininal was > the concentration of enzyme.

IIIC-2. FLUORESCENCE BINDING ASSAY

Due to the increase in fluorescence of the dansyl group of dansyl-argininal upon formation of the dansyl-argininal-trypsin complex, it is possible to determine the dissociation constant for the complex. Dansyl-argininal exhibits a maximum excitation wavelength of 330 nm and maximum emission wavelength of 550 nm. However, the trypsin-dansyl-argininal complex has a maximum emis-
sion wavelength of 490 nm and the relative fluorescent yield is at least 40 fold greater than that of the fluorescent probe alone.

All fluorescence measurements were obtained on a Perkin Elmer MPF 44B spectrofluorimeter. Both the excitation and emission monochrometers were set at a slit width of 5 nm. The instrument was routinely standardized on the Raman band of water to ensure reproducibility of results. The temperature of the reaction cuvette was maintained at 25°C by circulating water from a circulating water bath. For each experiment, 100 ul of dansyl-argininal was added to 2.9 ml of 0.1 M phosphate buffer at pH 7.2 and allowed time for temperature equilibration. The emission spectrum of dansyl-argininal in aqueous solution was recorded between 400 and 600 nm. Then the reaction cuvette containing 10 nmoles of trypsin in 0.1 M phosphate buffer and dansyl-argininal was placed in the spectrofluorimeter and allowed 5 minutes for equilibration before the emission was recorded. To calculate the dissociation constant of the trypsin-dansyl-argininal complex, the fluorescent intensities, in arbitrary units, of the dansyl-argininal with trypsin (F), and without trypsin (F₀) were calculated by integrating the area under the emission peak. The fluorescent enhancement,
(\(\Delta F\)), was obtained as follows,

\[
\Delta F = F - F_0
\]  \hspace{1cm} (17)

The experiments were carried out at constant enzyme concentration and various dansyl-argininal concentrations under conditions such that \([\text{dansyl-argininal}] > [E_0]\). The relationship between fluorescent enhancement and dansyl-argininal concentration was plotted using as Eadie-Hofstee plot, \(\Delta F\) versus \(\Delta F/S_0\), where the equilibrium constant is equal to the slope of the line obtained.

Identical experiments with trypsinogen were also performed. Trypsinogen was assayed with BAEE under the conditions described previously, to demonstrate that the zymogen preparation did not contain active enzyme. No hydrolysis of BAEE was observed by the trypsinogen solution.

IIID. DETERMINATION OF THE CONCENTRATION OF THE EQUILIBRIUM FORMS OF ARGININAL

It has been shown by Umezawa and coworkers (1971) that the argininal moiety of leupeptin exists in three forms (Figure 6), the free aldehyde (III), the aldehyde hydrate (II) and the carbinolamine (I) formed by the covalent addition of the guanido \(\text{W}N\) to the aldehydic
Figure 6: Equilibrium forms of leupeptin, where (I) is the carbinolamine, (II) is the aldehyde hydrate and (III) is the free argininal.
(I)

RNH-ClI-CIIO

(CH) 1

NM

MN=C-NH 2

(II)

RNll- CI I-CIIO 2

(I12)

NII

HN=C-NH 2

(II)

(III)

R NH- CI I-CIIO

(CH)3

NH

WN=C- NH 2

k R

k D

R NH- CI I- CIIOH 2

(CH)3

NH

WN=C- NH 2

(II)

k N (SC or ESH)

R Argal-SC (IV)

or

R Argal- S- E (V)
carbon. Only the free aldehyde inhibits proteases (Kennedy and Schultz, 1979). In order to know the concentration of active inhibitor over the time course of protease inhibition studies, the equilibrium constants and rates of interconversion of the three forms must be determined. Kinetic experiments with the aldehyde trapping reagents, semicarbazide hydrochloride and hydroxylamine hydrochloride have been performed to determine these kinetic constants. The reaction scheme between the aldehyde and the trapping reagent is as follows (Bell and Evans, 1966):

1.) \[ RCH(OH)\textsubscript{2} \rightleftharpoons RCHO + H\textsubscript{2}O \]

or

1.) \[ \text{carbinolamine} \rightleftharpoons RCHO + H\textsubscript{2}O \]

2.) \[ RCHO + R'\text{NH}_\text{2} \rightleftharpoons RCOH-NHR' \]

3.) \[ RCOH-NHR' \rightleftharpoons RCHR=NR' + H\textsubscript{2}O \]

Since the reaction is followed by observing the absorption of the final product, it is necessary that reactions 2 and 3 should be much faster than reaction 1. This can be ascertained by measuring the rate of aldehyde trapping at several concentrations of trapping reagent to guarantee that the rate of the reaction is independent of the concentration of trapping reagent. These trapping reactions were followed by both UV spectroscopy and \(^1H-\)
IIIID-1. KINETICS OF ALDEHYDE TRAPPING UTILIZING UV SPECTROSCOPY

Reaction mixtures contained $1 \times 10^{-5} \text{M}$ leupeptin and semicarbazide hydrochloride ($1 \times 10^{-4} \text{M} - 5 \times 10^{-3} \text{M}$) in 0.2 M acetate buffer at pH 5.5. In a typical reaction, both the reaction and the reference cells were filled with a leupeptin solution, and a sufficient time allowed for temperature equilibration. The reaction was initiated by the addition of an aliquot of semicarbazide solution with mixing. The amount of time between the addition of the scavenger reagent and the beginning of the recording was measured. The reaction was followed continuously at 230 nm until an endpoint was reached or after the initial phase of the reaction, the reaction cuvettes were incubated at 25°C and readings were taken every 6-8 hours until an endpoint was obtained. Rate constants were determined from first order plots.

Trapping kinetics were also obtained on dansylargininal ($1 \times 10^{-5} \text{M}$) with semicarbazide hydrochloride, hydroxylamine hydrochloride and hydrazine sulfate ($1 \times 10^{-4} \text{M} - 5 \times 10^{-3} \text{M}$) in the pH range 5.0 to 7.8. Product
formation was observed at 230 nm for the semicarbazone, 225 nm for the hydroxime and 250 nm for the hydrazone (See Table 1).

IIID-2. KINETICS OF ALDEHYDE TRAPPING UTILIZING NMR

NMR spectra of leupeptin (10 mM) were obtained on a Nicolet 293 (200 MHz) NMR spectrometer at pH 5.0, 6.0 and 8.0 in 0.05 M acetate buffer at pH 5.0 and 0.05 M phosphate at pH 6.0 and 8.0. Both the buffer salts and leupeptin were lyophilized in deuterium oxide (Aldrich) prior to use.

NMR spectra of leupeptin (10 mM) were obtained on a Bruker SY200 NMR spectrometer at pH 7.0 in deuterium oxide. Leupeptin was lyophilized in D₂O prior to use.

Peak assignments were made based on the observed chemical shifts and peak splitting and also on an earlier identification made by Maeda et al., (1974). Determination of the relative amounts of each of the three equilibrium forms was based on measurement of peak heights.

Kinetic trapping experiments were carried out as follows. All of the reagents used in these experiments were dissolved in D₂O and lyophilized prior to use. Reaction solutions were made containing 20 mM semicarba-
Table 1

ALDEHYDE TRAPPING REAGENTS

<table>
<thead>
<tr>
<th>Reagent</th>
<th>pH Range</th>
<th>λ_{max}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semicarbazide</td>
<td>2.5-5.5</td>
<td>230</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>4.5-6.5</td>
<td>225</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>6.0-8.0</td>
<td>235</td>
</tr>
</tbody>
</table>

Experimental conditions as derived by Bell and Evans (1966).
zide HCl, 10 mM leupeptin and 0.05 N sodium acetate at pD 5.0 or 20 mM hydrazine sulfate, 10 mM leupeptin and 0.05 N phosphate buffer at pD 7.5. The rate of aldehyde trapping was determined on a Bruker SY 200 (200 megahertz) NMR spectrometer by measuring the rate of the disappearance of the anomeric carbinolamine proton at 5.4 ppm and the disappearance of the hydrate proton at 4.9 ppm (Maeda et al., 1974). The peak appearing at 1.8 ppm was used as an internal standard. Due to a dynamic range problem, which limits the detection of weak signals in the presence of strong signals, the data was calculated by measuring the peak heights in centimeters and comparing the peak height to that of an internal standard.

Additional kinetic experiments were performed on a Nicolet 293 NMR spectrometer under identical conditions. Spectra were obtained at 6.5 minute intervals for 130 minutes and then at 30 minute intervals for a total time of 7 hours. Data was calculated as above.

III. TUMOR CELL PROTEINASE VISUALIZATION

The properties of fluorogenic and chromogenic substrates for the assay of proteolytic enzymes has been exploited by enzyme kineticists in the study of enzymatic
reaction mechanisms. However, these substrate have only recently come into use by the histologists for the localization of enzymatic activity within a cell or tissue. Therefore, an attempt was made to utilize dansyl-argininal as a probe for proteolytic enzyme activity on the surface of or within cells.

IIIIE-1. CELL CULTURE

Cultures of HSDM_{1}C_{1} cells (American Type Culture Collection) were maintained in Ham's F-10 medium containing 15% horse serum with 2.5% heat inactivated fetal calf serum and antibiotics (penicillin and streptomycin). IMR-90 fibroblasts (American Type Culture Collection) were maintained in Eagle's minimal essential medium with non-essential amino acids in Earle's balanced salt solution containing 10% heat inactivated fetal calf serum with antibiotics. Cells were maintained at 37°C in 98% relative humidity, 5% CO_{2}/95% air.
Both HSDM₁C₁ and IMR-90 cells were maintained in serum-free medium for 24 hours prior to recovery by mechanical means. Harvested cells were tested for viability by the trypan blue dye exclusion test, counted using a hemocytometer, washed with phosphate buffered saline and aliquotted in phosphate buffered saline at cell concentrations of 50,000 cells per ml. Dansylargininal (0.11 mM in cuvette) was added to suspensions of either HSDM₁C₁ or IMR-90 cells in a mechanically stirred cuvetted (Instech, Fort Washington, Pa.). Fluorescence was measured using a Perkin Elmer MPF44B spectrofluorimeter. The suspensions were excited at 330 nm and emissions were measured between 400 and 600 nm. The emissions were standardized in each run against the Raman band of water and normalized against a spectrum of cells alone to correct for light scattering. The relative quantum yield was obtained by integrating the area under the emission peak. Other cell suspensions (50,000 cells per ml) of either HSDM₁C₁ or IMR-90 cells were preincubated with 1 mM diisopropylfluorophosphate (DFP) and/or 1 mM p-chloromercuribenzoate (pCMB) for 20 minutes at room temperature prior to the addition of
dansyl-argininal (0.11 mM). Additional preincubations were done adding the proteinase inhibitor, leupeptin, at concentrations ranging from $1 \times 10^{-4} \text{M}$ to $1 \times 10^{-7} \text{M}$ prior to the addition of dansyl-argininal. Solutions of dansyl-argininal alone and cells alone gave emissions that coincided with the buffer baseline at the sensitivity setting of the spectrofluorimeter. The spectrofluorimeter settings were those that gave a full scale recording of the dansyl-argininal-proteinase fluorescence.

**IIIE-3. ANALYSIS OF GLUTARALDEHYDE EFFECTS ON DANSYL-ARGININAL-TRYPsin COMPLEX**

The effect of glutaraldehyde on the fluorescence emission of dansyl-argininal-trypsin complexes was evaluated to facilitate the use of glutaraldehyde fixed cells for fluorescence microscopy. Trypsin (0.1 µM) and dansyl-argininal (0.11 mM) were stirred in 1.5 ml of phosphate buffered saline to which 1.5 ml of 0.1 M cacodylate buffered 2.5% glutaraldehyde was added, and fluorescence was measured between 400 and 600 nm.
Cells maintained in 75 cm² flasks were recovered by mild trypsinization and replated onto non-fluorescing Aklar plastic discs. Cells were grown in complete medium but were transferred to serum-free medium for 24 hours prior to the following procedure. Discs were examined by inverted phase contrast microscopy to determine that the cells were preconfluent. Prior to further treatment, the discs containing the cells in logarithmic phase growth were fixed in cold 0.1 M cacodylated buffered 2.5% glutaraldehyde (pH 7.4) and placed in phosphate buffered saline containing either 1 mM DFP or pCMB for 20 minutes. Other discs were not exposed to either DFP or pCMB prior to incubation in dansyl-argininal. The discs were then removed from the incubation solution and placed on glass slides with a drop of phosphate buffered saline. Cover-slips were placed on the slides and the cells were observed by dark-field fluorescence microscopy using a UG-1 exciter filter and barrier filter 50 (Zeiss). All fluorescent photomicrographs were acquired by standardizing the length of exposure so that semiquantification of the cell fluorescence would be attained.
CHAPTER IV

RESULTS

IVA. THE ASSOCIATION OF DANSYL-ARGININAL TO TRYSIN AND TRYSINOGEN

The equilibrium dissociation constants obtained for the binding of dansyl-argininal to trypsin and trypsinogen are given in Tables 2 and 3. A direct comparison of the equilibrium dissociation constant ($K_I$) of dansyl-arginine, shows that the $K_I$ observed for dansyl-argininal binding to trypsin is two orders of magnitude smaller than that for the structurally analogous group without the aldehydic function group.

The calculation of $K_I$ for dansyl-argininal in Tables 2 and 3 is based on the total concentration of dansyl-argininal (free aldehyde + hydrated aldehyde + carbinolamine). Double resonance NMR spectroscopy experiments on the binding of acetyl-phenylalaninal to chymotrypsin (Chen et al., 1979, Gorenstein and Shah, 1982) as well as presteady state kinetics (Kennedy and
TABLE 2

DISSOCIATION CONSTANTS FOR DANSYL-ARGININAL BINDING TO TRYPsin

<table>
<thead>
<tr>
<th>Dansyl-Arginine&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$6.7 \times 10^{-3}$</th>
<th>$4.8 \times 10^{-4}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dansyl-Argininal&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$6.9 \times 10^{-5}$</td>
<td>$5.1 \times 10^{-4}$</td>
</tr>
<tr>
<td>Dansyl-Argininal&lt;sup&gt;c&lt;/sup&gt;</td>
<td>$4.2 \times 10^{-5}$</td>
<td></td>
</tr>
</tbody>
</table>

Where $K_1$ is the equilibrium dissociation constant for binding to the active site and $K_2$ is the equilibrium dissociation constant for binding to the secondary site.

<sup>a</sup> Taken from Goto and Hess, 1979. Experimental conditions: pH 6.8 in 0.1 M phosphate buffer containing 0.1 M Na<sub>2</sub>SO<sub>4</sub>.

<sup>b</sup> As determined by fluorescence binding assay at pH 7.2 in 0.1 M phosphate buffer containing 0.1 M NaCl.

<sup>c</sup> As determined by the inhibition of BAEE hydrolysis utilizing progress curves. Experimental conditions: pH 7.2 in 0.1 M phosphate buffer containing 0.1 M NaCl and $1 \times 10^{-5}$ M dansyl-argininal.
### TABLE 3

**DISSOCIATION CONSTANTS FOR DANSYL-ARGININAL BINDING TO TRYSINOGEN**

<table>
<thead>
<tr>
<th></th>
<th>$K_1$, M</th>
<th>$K_2$, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dansyl-arginine&lt;sup&gt;a&lt;/sup&gt;</td>
<td>$6.9 \times 10^{-3}$</td>
<td></td>
</tr>
<tr>
<td>Dansyl-argininal&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$1.5 \times 10^{-4}$</td>
<td>$2.5 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

Where $K_1$ is the equilibrium dissociation constant for binding to the active site and $K_2$ is the equilibrium dissociation constant for binding to the secondary site.

<sup>a</sup> Taken from Goto and Hess, 1979. Experimental conditions: pH 6.8 in 0.1 M phosphate buffer containing 0.1 M Na<sub>2</sub>SO<sub>4</sub>.

<sup>b</sup> As determined by fluorescence binding assay at pH 7.2 in 0.1 M phosphate buffer containing 0.1 M NaCl.
Schultz, 1979) have shown that it is only the unhydrated form of the aldehyde that strongly associates with the enzyme. Based on these experiments, it is inferred that the free aldehydic form of the argininal is the species that strongly associates with trypsin.

NMR experiments have been carried out on leupeptin, which served as a model compound for argininal containing compounds due to the poor solubility of dansyl-argininal in aqueous solutions, to determine the percentage of argininal which is present as the free aldehyde (vide supra). These results show that approximately 1% of leupeptin is present as the free aldehyde (See Figure 7 and Table 4). It can be inferred from this data that the same is true for dansyl-argininal so that the binding constant of dansyl-argininal to trypsin is actually two orders of magnitude better than originally determined by the inhibition studies because the initial concentration of active inhibitor is only 1% of the total inhibitor present. Thus, dansyl-argininal has a 10,000 fold greater affinity than its corresponding substrates in agreement with the expectation of a greater affinity for a transition-state analog.
Figure 7: NMR spectrum of leupeptin (10 mM in D$_2$O at pD 7.0. Spectrum was obtained on a Bruker SY 200 NMR spectrometer.
9.4 ppm - aldehyde
5.4 ppm - carbinolamine
4.9 ppm - aldehyde hydrate
### Table 4

**Relative Amounts of Equilibrium Forms of Leupeptin**

<table>
<thead>
<tr>
<th>Equilibrium Form</th>
<th>Chemical Shift, ppm</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>free aldehyde</td>
<td>9.4</td>
<td>0.7-1.3</td>
</tr>
<tr>
<td>carbinolamine</td>
<td>5.4 (2d)</td>
<td>56</td>
</tr>
<tr>
<td>aldehyde hydrate</td>
<td>4.9 (d)</td>
<td>43</td>
</tr>
</tbody>
</table>

Above data obtained on a 10 mM leupeptin sample at pH 7.0 on a Bruker SY 200 NMR spectrometer. The percentage was calculated from a computer integration of the peaks due to the presence of the three forms of argininal.
IVA. DETERMINATION OF $K_I$ BY FLUORESCENCE BINDING ASSAY

Figure 8 shows the fluorescence emission spectrum of the trypsin-dansyl-argininal complex with an excitation maximum of 330 nm and an emission maximum of 490 nm. This is in contrast to an observed emission maximum of 530 nm for the dansyl-argininal alone in aqueous solution and an observed emission maximum of 530 nm reported for the trypsin-dansyl-arginine complex. The observed hyperchromic shift is similar to that observed for dansyl amino acids dissolved in apolar solvents. This indicates that hydrophobic interactions contribute significantly to the fluorescent properties of the bound inhibitor (Chen, 1967). The emission spectrum of the trypsinogen-dansyl-argininal complex is identical to that of trypsin-dansyl-argininal suggesting that the bound probe is associated with the zymogen and the active enzyme in a similar fashion.

In Figure 9, the values of the fluorescent enhancement, $\Delta F$, due to the formation of the enzyme-inhibitor complex are plotted against $\Delta F/S_0$, where $S_0$ is the initial concentration of dansyl-argininal. A linear relationship is not obtained in the Eadie-Hofstee plot suggesting that trypsin has more than one non-identical
Figure 8: Fluorescence emission spectra of dansyl-argininal alone and dansyl-argininal-trypsin complex with the excitation monochrometer set at 330 nm. Dansyl-argininal concentration was $4 \times 10^{-5}$ M and trypsin concentration was $1\mu$M in 0.1 M phosphate buffer, pH 7.2, containing 0.1 M NaCl.
Trypsin-Dansyl-argininal complex

Dansyl-argininal

Relative Fluorescence

Wavelength
Figure 9: Eadie-Hofstee Plot. Values of fluorescence enhancement, $\Delta F$, of the dansyl group due to the formation of the dansyl-argininal-trypsin complex plotted against the $AF/S_0$ where $S_0$ is the initial dansyl-argininal concentration. Fluorescence excitation wavelength is 330 nm and the emission was calculated by integrating the area under the emission peak scanned between 400-500 nm.
binding site for dansyl-argininal with dissociation constants of $6.9 \times 10^{-5} \text{M}$ and $5.4 \times 10^{-4} \text{M}$. These equilibrium dissociation constants were obtained directly from the slope of the lines in the Eadie-Hofstee plot. These results correlate well with kinetic results given in Table 5 and plotted in Figure 10 that demonstrate a decrease in the $K_{M, \text{apparent}}$ with dansyl-argininal concentrations greater than $0.2 \text{ mM}$. In addition, the dissociation equilibrium constant for dansyl-argininal to the secondary binding site is in good agreement with that observed for the binding of dansyl-arginine to the secondary site (See Table 2) (Goto and Hess, 1979).

Data from fluorescence titration experiments with trypsinogen also suggest the presence of two non-identical binding sites for dansyl-argininal with dissociation equilibrium constants of $1.5 \times 10^{-4} \text{M}$ and $2.5 \times 10^{-3} \text{M}$ (Figure 11). Although it is not possible to distinguish between binding to the active site of the enzyme and binding to the secondary site by this method, it is not unexpected that dansyl-argininal should be able to bind to the active site of the zymogen. Morgan et al., (1972) demonstrated that radiolabeled DFP can bind to trypsinogen and that the majority of the radioactivity is associated with the serine-183. In addition, Gertler
<table>
<thead>
<tr>
<th>KM,apparent</th>
<th>[DansylArgininal] x 10^5 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.050 ± .009</td>
<td>0</td>
</tr>
<tr>
<td>0.104 ± .007</td>
<td>4</td>
</tr>
<tr>
<td>0.138 ± .021</td>
<td>8</td>
</tr>
<tr>
<td>0.225 ± .025</td>
<td>15</td>
</tr>
<tr>
<td>0.168 ± .021</td>
<td>20</td>
</tr>
<tr>
<td>0.113 ± .014</td>
<td>30</td>
</tr>
<tr>
<td>0.041 ± .011</td>
<td>50</td>
</tr>
</tbody>
</table>

Kinetic rate assays were carried out in 0.1 M phosphate buffer containing 0.1 M NaCl. KM,apparent was calculated using the integrated rate equation. The substrate, benzoylarginine ethyl ester, was present at a concentration greater than KM.
Figure 10: Plot of the integrated rate equation.

Kinetic data was calculated from progress curves utilizing BAEE as the substrate present at a concentration $10 \times K_M$.

Solution contained 0.1 M phosphate buffer at pH 7.8 and 0.1 M NaCl. a) uninhibited reaction, b) $5 \times 10^5$ M dansyl-argininal, inhibited reaction, c) $1 \times 10^4$ M dansyl argininal, activated reaction.
Figure 11: Eadie-Hofstee Plot of Dansyl-Argininal-Trypsinogen Complex. Values of the fluorescence enhancement, $\Delta F$, of the dansyl group due to the formation of the dansyl-argininal-trypsinogen complex plotted against $\Delta F/S_0$ where $S_0$ is the initial dansyl-argininal concentration. The fluorescence excitation wavelength was 330 nm and the emission was calculated by integrating the area under the emission peak scanned between 400-600 nm.
et al., (1974) observed that p-aminobenzamidine could competitively inhibit the hydrolysis of p-nitrophenyl p'-guanidinobenzoate by trypsinogen. However, the apparent $K_I$ for p-aminobenzamidine is approximately four orders of magnitude larger for binding to the zymogen in comparison to the enzyme.

**IVA-2. pH DEPENDENCE OF DANSYL-ARGININAL BINDING TO TRYPsin**

As presented in Table 6, the $K_{I,obs}$ for the binding of dansyl-argininal to trypsin (as determined by the BAEE assay) was found to increase by only a factor of 3 over the pH range 7.8 to 5.0. These results are in close agreement with the pH dependency observed for the binding of benzoyl-phenylalaninal to chymotrypsin (Kennedy and Schultz, 1979) and for the binding of acetyl-phenylalaninal to chymotrypsin (Shah and Gorenstein, 1982).

A reasonable explanation for the pH independence of $K_{I,obs}$ for aldehyde binding is that two proton transfers occur on association of the aldehyde to the enzyme leading to the formation of a protonated tetrahedral complex (Figure 4, Structure IIB) as the major association species, rather than the anionic species (Figure 4,
TABLE 6

pH DEPENDENCY OF BINDING OF DANSYL-ARGININAL TO TRYPsin

<table>
<thead>
<tr>
<th>pH</th>
<th>$K_I$ (10$^{-5}$ M)</th>
<th>Buffer Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.8</td>
<td>4.22 ± 0.63</td>
<td>phosphate</td>
</tr>
<tr>
<td>7.2</td>
<td>3.78 ± 0.14</td>
<td>&quot;</td>
</tr>
<tr>
<td>6.8</td>
<td>8.56 ± 0.53</td>
<td>&quot;</td>
</tr>
<tr>
<td>6.2</td>
<td>7.34 ± 0.10</td>
<td>&quot;</td>
</tr>
<tr>
<td>5.5</td>
<td>7.71 ± 0.12</td>
<td>acetate</td>
</tr>
<tr>
<td>5.0</td>
<td>12.9 ± 2.2</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

$K_I$ values obtained against the substrate, BAEE, at 25°C under the following solution conditions: 0.01 M NaCl, 0.02 M buffer component at the appropriate pH. Values are not corrected for hydration and ring formation. 

$[S_0] >> [E]$. $K_I ± S.D.$
Structure IIA). Thus step $k_2$ may be depicted as a two step proton transfer process in which the histidine imidazole first accepts a proton from the $\gamma$-OH of the serine generating the $\gamma$0-nucleophile, and in a second step, either the protonated histidine or the solvent donates a proton to the oxanion of the C-1 of the aldehyde resulting in the formation of the stable tetrahedral adduct. Accordingly, in the latter mechanism, step $k_2$ is general base - specific acid catalyzed and step $k_2$ is specific base - general acid catalyzed. As specific base - general acid and general base catalysis are kinetically indistinguishable (Jencks, 1969), both $k_2$ and $k_2$ have the format $k_x = k_{lim}/(1 + [H^+] / K_a)$. The term $1 + [H^+] / K_a$ cancels out in the ratio $k_2 / k_2$ and thus $K_{I,obs}$ shows little variation with pH.

**IVB. HYDRATION AND CARBINOLAMINE FORMATION IN PEPTIDYL ARGININALS**

It is known that aldehydes covalently associate with water to form gem diols in aqueous solution. In addition to hydration, argininal also undergoes a spontaneous cyclization forming a carbinolamine (See Figure 6). Kennedy and Schultz (1979) have shown that it is only the
free aldehyde that strongly associates with serine protease enzymes. It is important to know the concentration of active inhibitor present in proteinase inhibitor studies with peptidyl argininal in order to determine true equilibrium dissociation constants.

In order to determine the amount of free aldehyde, proton NMR spectra were obtained on the peptidyl argininal, leupeptin (Figure 7), which served as a model for this class of inhibitors. Three peaks were observed at 9.41, 5.34 and 4.90 ppm for the aldehydic, the carbinolamine and the hydrate protons respectively based on prior assignments made by Maeda et al., (1971). The peak at 9.41 ppm which is due to the aldehydic proton comprises approximately 1% of the total leupeptin concentration with the value ranging from 0.7 to 1.3% for three spectra that were obtained at pD 7.0. The aldehyde hydrate generates a doublet at 4.90 ppm (J=4.7) and comprises approximately 43% of the total leupeptin concentration. The peak at 5.34 ppm is due to the anomeric CH of the carbinolamine and appears as two doublets (J=2.9, 2.2) due to the presence of both the R and the S configurations formed upon ring closure. Additional NMR experiments at pD's of 5.0, 6.0 and 8.0 were carried out to determine if this equilibrium was pH dependent. Results
of these experiments (See Table 7) show that the relative amounts of each of the equilibrium forms is invariant with pH.

IVB-1. KINETICS OF ALDEHYDE TRAPPING UTILIZING UV SPECTROSCOPY

Kinetic experiments were carried out to determine the rate of dehydration and ring opening using the aldehyde trapping reagents semicarbazide hydrochloride, hydroxylamine hydrochloride and hydrazine sulfate according to the method of Bell and Evans (1966). The trapping reactions were observed by measuring the increase in absorbance due to the formation of the semicarbazone, the hydroxime or the hydrazone. The experiments were difficult to follow due to the high molar absorptivity of the peptidyl argininals in comparison to the relatively small molar absorptivity of the complex. Leupeptin trapping experiments were performed only in the lowest pH range possible using semicarbazide hydrochloride as the other reactions could not be followed accurately due to the high background absorbance of leupeptin.

A first order plot of $\ln (A_\infty - A_t)$ (Figure 12) of the leupeptin trapping reaction resulted in a biphasic curve. Two rate constants were obtained corresponding to
TABLE 7

CONCENTRATION OF EQUILIBRIUM FORMS OF LEUPEPTIN WITH CHANGING pD

<table>
<thead>
<tr>
<th>Form</th>
<th>pD</th>
<th>Peak Height</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldehyde</td>
<td>5.0</td>
<td>27</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>47</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>30</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>49</td>
<td>2.4</td>
</tr>
<tr>
<td>aldehyde hydrate</td>
<td>5.0</td>
<td>598</td>
<td>49.9</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>973</td>
<td>51.7</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>960</td>
<td>49.0</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>968</td>
<td>47.8</td>
</tr>
<tr>
<td>carbinolamine</td>
<td>5.0</td>
<td>571</td>
<td>47.4</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>860</td>
<td>45.7</td>
</tr>
<tr>
<td></td>
<td>7.0</td>
<td>960</td>
<td>49.0</td>
</tr>
<tr>
<td></td>
<td>8.0</td>
<td>1007</td>
<td>49.7</td>
</tr>
</tbody>
</table>

---

a Calculation of relative concentration of each of the equilibrium forms was based on a measurement of the peak heights rather than integration due to a dynamic range problem. Comparisons must be made relative to peaks for other equilibrium forms at the same pD only.

b Percentage of equilibrium forms based on peak height data. Integrations, when made, gave approximately 1% free aldehyde (See text).
Figure 12: First order plot of the kinetics of aldehyde trapping utilizing UV spectroscopy. Reaction mixture contains $1 \times 10^{-5}$ M leupeptin and $1 \times 10^{-4}$ M semicarbazide hydrochloride in 0.2 M acetate buffer at pH 5.5. The change in absorbance due to the formation of the semicarbazone was monitored at 230 nm.
a half-life of 25 minutes for the fast reaction and 532 minutes for the slow reaction. Biphasic kinetics were expected with the more rapid rate assumed to be due to the dehydration reaction and the slow rate due to the opening of the carbinolamine ring. However, these rates were much slower than anticipated and although they correlate well with rates observed for dehydration and ring opening of galactose pentaacetates (Table 8), it was determined that additional experiments were needed to corroborate these results. Similar data was obtained for the trapping reactions carried out with dansyl-argininal (Table 9).

IVB-2. KINETICS OF ALDEHYDE TRAPPING UTILIZING NMR

Results obtained from NMR experiments provide additional information on the mechanism of aldehyde trapping and provide additional support for the much slower than anticipated rate of dehydration. However, kinetic constants obtained by NMR are not directly comparable to kinetic constants obtained by UV spectroscopy because of the high concentration of reagents required to obtain NMR spectra in a reasonable length of time. Bell and Evans (1966) reported that in order for the trapping reaction
### TABLE 8

**RATES FOR THE REACTION OF GALACTOSE ACETATES WITH HYDRAZINE AT pH 7.0**

<table>
<thead>
<tr>
<th></th>
<th>$k(\text{min})^{-1}$</th>
<th>Half-time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkyl Aldehyde Hydrate</td>
<td>0.058</td>
<td>12</td>
</tr>
<tr>
<td>Furanose</td>
<td>0.047</td>
<td>14.7</td>
</tr>
<tr>
<td>Pyranose</td>
<td>0.00093</td>
<td>745.6</td>
</tr>
</tbody>
</table>

---

*Taken from Compton and Wolford (1934).*
TABLE 9

RATES OF DANSYL-ARGININE ALDEHYDE TRAPPING REACTION

<table>
<thead>
<tr>
<th>pH</th>
<th>Half-Time (min)</th>
<th>Trapping Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>25</td>
<td>semicarbazide</td>
</tr>
<tr>
<td></td>
<td>693</td>
<td><em>w</em></td>
</tr>
<tr>
<td>5.5</td>
<td>33</td>
<td>semicarbazide</td>
</tr>
<tr>
<td></td>
<td>346</td>
<td><em>w</em></td>
</tr>
<tr>
<td>6.0</td>
<td>23</td>
<td>hydroxylamine</td>
</tr>
<tr>
<td></td>
<td>456</td>
<td><em>w</em></td>
</tr>
<tr>
<td>6.5</td>
<td>22</td>
<td>hydroxylamine</td>
</tr>
<tr>
<td></td>
<td>456</td>
<td><em>w</em></td>
</tr>
<tr>
<td>7.0</td>
<td>24</td>
<td>hydrazine</td>
</tr>
<tr>
<td></td>
<td>346</td>
<td><em>w</em></td>
</tr>
</tbody>
</table>

Reaction mixtures contained 0.01 M dansyl-argininal, 0.2 M buffer salts and a 10-100 fold excess of trapping reagent.
to occur instantaneously, the trapping reagent should be present in at least a 10 fold excess of the aldehyde and they also stated that at a concentration of \( > 1 \text{ mM} \), the semicarbazide may be capable of catalyzing the reaction so that the rates observed would be much faster than anticipated. In the NMR experiments performed in this work, the semicarbazide was present in only a two fold excess of the aldehyde and at a final concentration of 20 mM.

At \( \text{pD} \ 5.0 \), approximately 50% of the leupeptin reacted with the semicarbazide within the first ten minutes of the reaction. The peak at 9.4 ppm due to the presence of the free aldehyde disappeared immediately which was anticipated because of the assumed instantaneous reaction between the free aldehyde and the trapping reagent. The peaks due to the hydrate and the carbinolamine disappeared at similar rates, but due to the short reaction time, it was not possible to obtain enough data points to calculate a half time for the reaction (Table 10). The reaction rates observed in this experiment are much faster than rates of aldehyde trapping obtained by HPLC or UV spectroscopy (Table 11). Due to the very high concentration of trapping reagent present in the NMR reaction mixture, it may be assumed that the semicarbazide hydro-
TABLE 10

RATE OF ALDEHYDE TRAPPING AT pH 5.5 BY $^1$H-NMR

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Carbinolamine/Ref.</th>
<th>Hydrate/Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.234</td>
<td>0.186</td>
</tr>
<tr>
<td>6</td>
<td>0.181</td>
<td>0.162</td>
</tr>
<tr>
<td>12</td>
<td>0.172</td>
<td>0.139</td>
</tr>
<tr>
<td>60</td>
<td>0.173</td>
<td>0.137</td>
</tr>
<tr>
<td>240</td>
<td>0.174</td>
<td>0.137</td>
</tr>
<tr>
<td>420</td>
<td>0.173</td>
<td>0.136</td>
</tr>
</tbody>
</table>

---

Reaction mixture contained 10 mM leupeptin, 20 mM semicarbazide hydrochloride, and 0.02 N acetate buffer at pH 5.5. Experiment was carried out in a Nicolet NMR spectrometer. Peak heights of the carbinolamine and hydrate were compared to an internal standard. The reference peak chosen was found at 1.8 ppm and was due to the acetyl group of leupeptin.
TABLE 11

COMPARATIVE RATES OF ALDEHYDE TRAPPING WITH SEMICARBAZIDE AT pH 5.5

<table>
<thead>
<tr>
<th>Experimental Method</th>
<th>$t_{1/2}$</th>
<th>[Semicarbazide]</th>
<th>[Leupeptin]</th>
</tr>
</thead>
<tbody>
<tr>
<td>UV Spectroscopy</td>
<td>23-32 min.</td>
<td>0.1 - 1 mM</td>
<td>0.01 mM</td>
</tr>
<tr>
<td>HPLC</td>
<td>17-23 min.</td>
<td>0.5 - 10 mM</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>NMR</td>
<td>&lt; 5 min.</td>
<td>20 mM</td>
<td>10 mM</td>
</tr>
</tbody>
</table>

All of the above experiments were carried out at pH 5.5 in acetate buffer. Half-times were calculated from first order plots.
chloride catalyzed this reaction as was reported by Bell and Evans (1966). An NMR spectrum of the product, leupeptin semicarbazone (Figure 13), was obtained to determine if it was possible to observe the rate of formation of product in these reactions. The product gives a signal with a chemical shift of 7.2 ppm; however, this peak was too small to be seen during the kinetic assay. Alternatively, an additional peak was observed by NMR with a chemical shift of 4.89 ppm which appeared at the end of one hour of reaction and slowly increased in size over time. Due to its proximity to the hydrate peaks, it is not possible to determine if this new signal was a singlet or a multiplet. (See Figure 14). It is assumed that this peak is due to the accumulation of an intermediate in the reaction occurring between the aldehyde and the semicarbazone.

The results of the kinetic trapping reaction between leupeptin and hydrazine sulfate at pH 7.4 were significantly different than the results at pH 5.0. The peaks in the NMR due to the presence of the carbinolamine disappeared completely over the time course of the reaction with a half-time of 4.5 minutes (Table 12), while the hydrate peak disappeared at a much slower rate with a half-time of 27 minutes. (Table 12) The half-time of
Figure 13: NMR spectrum of leupeptin semicarbazone (10 mM) in D$_2$O containing 0.02 N sodium acetate at pD 5.5. The doublet appearing at 7.2 ppm is due to the semicarbazone.
Figure 14: Aldehyde trapping kinetics utilizing NMR.

The doublet appearing at 4.9 ppm is due to the presence of the aldehyde hydrate and the two doublets appearing in the range 5.3-5.4 ppm are due to the presence of the R and the S configurations of the carbinolamine. All spectra were obtained on a Nicolet NMR spectrometer in D$_2$O containing 0.02 M acetate, 20 mM semicarbazide hydrochloride and 10 mM leupeptin. All reagents were lyophilized in D$_2$O prior to use. (a) 10 minutes into the reaction, (b) 1 hour, (c) 2 hours, (d) 7 hours. Note the appearance of an additional peak at 4.89 ppm after 1 hour of reaction time.
### TABLE 12

**RATE OF ALDEHYDE TRAPPING AT pH 7.4 BY \textsuperscript{1}H-NMR**

<table>
<thead>
<tr>
<th>Time (min.)</th>
<th>Carbinolamine/Ref.</th>
<th>Hydrate/Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.192</td>
<td>0.119</td>
</tr>
<tr>
<td>3</td>
<td>0.161</td>
<td>0.101</td>
</tr>
<tr>
<td>6.5</td>
<td>0.073</td>
<td>0.090</td>
</tr>
<tr>
<td>9</td>
<td>0.036</td>
<td>0.085</td>
</tr>
<tr>
<td>27</td>
<td>0.0</td>
<td>0.067</td>
</tr>
</tbody>
</table>

Reaction mixtures contained 10 mM leupeptin, 20 mM hydrazine sulfate and 0.02 N phosphate buffer at pH 7.4. Experiment was carried out in a Bruker SY 200 NMR spectrometer. The carbinolamine and hydrate peak heights were compared to that of an internal reference peak. The reference peak was found at 1.8 ppm and was due to the acetyl group of leupeptin.
27 minutes for the dehydration of leupeptin is consistent with the half-times of 20 minutes and 25 minutes obtained by HPLC and UV spectroscopy, respectively, at pH 5.0 with semicarbazide hydrochloride as the trapping reagent (Table 11). The increased reactivity of the carbinolamine in this reaction may be due to the hydrazine sulfate acting as a general base catalyst in the ring opening reaction.

Aldehyde trapping reactions utilizing HPLC were carried out in tandem with this work. Chromatograms of leupeptin (Figure 15) showed three peaks assumed to be due to the presence of the three equilibrium forms of leupeptin, although it was not possible to identify each peak or to completely separate the three peaks by this method. The trapping reaction was carried out at various concentrations of semicarbazide hydrochloride and aliquots of the reaction mixture were injected over time. It was possible to measure the rate of appearance of product (Figure 15). Due to the significant overlap of all of the peaks, it was not possible to accurately measure the rate at which the equilibrium forms of leupeptin disappeared. However, product formation occurred with a half-time of approximately 20 minutes, and it appears that each of the two major peaks, putatively due
Figure 15: HPLC chromatogram of aldehyde trapping reaction. Leupeptin (0.1 mM) was injected initially on a C-18 reverse-phase column and eluted in acetonitrile/acetate buffer (85:15). Subsequent injections consisted of 0.1 mM leupeptin and 1 mM semicarbazide hydrochloride at various time intervals. The reaction was monitored for approximately 4 hours.
leupeptin

semicarbazone

leupeptin
to the hydrate and carbinolamine forms of the argininal also disappear with a half-time of 20 minutes. Due to the limitation in the accuracy of integration of peaks by HPLC it was not possible to determine the extent of the reaction with high accuracy, it appeared to go to completion. Assuming, however, that the HPLC reaction went to 90% completion, it is possible to calculate an equilibrium dissociation constant of 2.2M. In the NMR, the reaction appears to go to approximately 50% completion (Table 10), one calculates an equilibrium dissociation constant of 15 M which is increased by a factor of approximately 7 over that calculated in the HPLC analysis. One possible explanation for the large increase in the $K_{eq}$ in the NMR experiment is the effect of the deuterium solvent on both the aldehyde hydrate and the carbinolamine equilibriums with argininal. These forms should be stabilized to a greater extent in deuterium oxide than in water. Equilibrium deuterium solvent isotope effects for dehydration have been determined on alkyl aldehydes. An equilibrium deuterium isotope effect for hydration of aldehydes has been calculated to be on the order of 1.4 (Pocker, 1960) In addition to this, an equilibrium deuterium isotope effect for the addition of nitrogen nucleophiles to substituted benzaldehydes exhi-
bits an effect of approximately 2 for the overall reaction (Amaral et al., 1973). If one assumes an equilibrium deuterium isotope effect of 2-3 for hydration of the \(-amido argininal and an equilibrium deuterium isotope effect of 2 favoring the formation of the carbinolamine, one can easily expect to see an increase in the \(K_{eq}\) of a factor of 4-6. Because no models for deuterium isotope effects with argininal have previously been determined, one can only assume an estimated isotope effect, so that the actual effect on this reaction may be greater or lesser than the calculated effect. However, considering the relatively slow rate of dehydration and ring opening (half-time = 20 minutes) for the argininal containing compounds, it would appear that large equilibrium deuterium isotope effects are plausible and that a fivefold increase in the \(K_{eq}\) for this reaction is reasonable.

IVC. TUMOR CELL PROTEINASE VISUALIZATION AND QUANTIFICATION

In the following experiments with whole cell suspensions, bovine trypsin served as a model for a serine type proteinase and papain served as a model of a cysteine type proteinase. Figure 16 shows a typical
Figure 16: Emission spectra of trypsin-dansyl-argininal (above) complex and papain-dansyl-argininal complex (below). Trypsin (0.1 μM) in phosphate buffer (pH 7.4) containing 0.1 M NaCl and 0.11 mM dansyl-argininal (top). Papain (0.1 μM in 0.1 M sodium acetate buffer (pH 5.5) containing 0.16 M cysteine, 33 μM EDTA and 0.11 mM dansyl-argininal. Solutions of enzyme and dansyl-argininal were excited at 330 nm and emissions were scanned between 400-600 nm.
fluorescence emission spectrum between 400 and 600 nm on excitation at 330 nm from a solution of 0.1 µM trypsin or papain and 0.11 mM dansyl-argininal. Under these conditions, the dansyl-argininal is in excess of the equilibrium dissociation constants to the respective enzymes, and the enzyme active sites are >90% saturated. Neither dansyl-argininal nor the enzyme alone has an observable fluorescence under the conditions of the experiment. Thus, the inhibitor only exhibits an observable fluorescence upon binding to the enzyme.

Diisopropylfluorophosphosphate (DFP) is known to specifically modify the active site serine of the serine proteinases, and p-chloromercuribenzoate (pCMB) specifically modifies the active site cysteine of cysteine proteinases (Shaw, 1970). When trypsin and papain are preincubated with DFP and pCMB respectively prior to the addition of dansyl-argininal, no fluorescence was observed. These results support the mechanism that the argininal binds to the enzyme active site.

To ascertain whether glutaraldehyde interferes with this reaction, the effect of glutaraldehyde was studied under the same conditions used in the treatment of cells for microscopy. A decrease in the fluorescence intensity of 10% was observed in the presence of 2.5%
glutaraldehyde. Accordingly, the presence of the non-specific aldehyde, glutaraldehyde, does not significantly effect the binding of dansyl-argininal to either trypsin or papain, thus indicating that glutaraldehyde fixation of the cells for microscopy does not significantly alter the binding of dansyl-argininal to cell proteinases.

IVC-1. QUANTIFICATION OF PROTEINASE CONCENTRATION BY SPECTROFLUORIMETRY

Figure 17 shows the fluorescence emission from a solution of 0.11 mM dansyl-argininal containing $1 \times 10^5$ HSDM$_1$C$_1$ fibrosarcoma cells in 2.0 ml phosphate buffered saline and stirred within the cuvette of the spectrofluorimeter. The emission spectrum from the dansyl-argininal treated cells is observed to be identical to that found for trypsin and papain in solution. Furthermore, the emission spectrum appears immediately on addition of dansyl-argininal to the cells and remains constant for at least one hour. No significant fluorescence was observed from the cells alone in phosphate buffered saline or from the dansyl-argininal alone. A solution of IMR-90 fibroblasts (untransformed cells) in 2.0 ml of phosphate buffered saline with 0.11 mM dansyl-argininal
Figure 17: Emission spectrum of dansyl-argininal treated fibrosarcoma cells. To a suspension of HSDM₁₁C₁ (50,000 cells/ml) in phosphate buffered saline was added 0.11 mM dansyl-argininal. The cell suspension was excited at 330 nm and emissions were scanned between 400-600 nm. The cell suspension was preincubated with 1 mM DFP (----) or 1 mM pCMB (-----) for 30 minutes prior to the addition of dansyl-argininal.
gave 25% of the fluorescence observed for the fibrosarcoma cells. The four fold higher fluorescence yield observed in the fibrosarcoma cells is in agreement with the four fold higher proteolytic activity observed by Hatcher et al. (1976) in transformed cell membranes.

When the serine proteinase inhibitor, DFP, is incubated with the fibrosarcoma cells for 20 minutes prior to the addition of dansyl-argininal, the fluorescent yield is decreased by 67% (Figure 17). When the cysteine proteinase inhibitor pCMB is preincubated with the fibrosarcoma cells for 20 minutes prior to the addition of dansyl-argininal, the fluorescent yield is decreased by approximately 56% (Figure 17). When the cells are incubated with both inhibitors, no fluorescence is observed upon addition of dansyl-argininal. This data suggests that both serine and cysteine proteinases are present on or within the cells. Preincubation of the cells with the proteinase inhibitor leupeptin (1 x 10^{-5} M to 1 x 10^{-7} M) prior to the addition of dansyl-argininal also results in a complete inhibition of fluorescence (Table 12). Total inhibition of fluorescence upon addition of specific proteinase inhibitors suggests that the binding of the peptidyl aldehyde inhibitor is highly specific. The fluorescence yield form 0.1 \mu M trypsin-
### TABLE 13

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Relative Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5254</td>
</tr>
<tr>
<td>1 mM pCMB</td>
<td>2326</td>
</tr>
<tr>
<td>1 mM DFP</td>
<td>1726</td>
</tr>
<tr>
<td>1 mM pCMB + 1 mM DFP</td>
<td>&gt;500</td>
</tr>
<tr>
<td>.05 mM leupeptin</td>
<td>&gt;274</td>
</tr>
</tbody>
</table>

All experiments were carried out with a cell concentration of 50,000 cells per ml. and a dansyl-argininal concentration of $1.1 \times 10^{-4}$ M in phosphate buffered saline. The excitation wavelength was 330 nm and emissions were scanned and integrated between 400 and 600 nm. The cells were grown in serum free media for 48 hours prior to the experiment.
dansyl-argininal complex or papain-dansyl-argininal complex is approximately three times that of the fluorescence yield obtained from the fibrosarcoma cells at a concentration of 50,000 cells per ml. If one assumes that the fluorescent yield of the EI complex in the cell membrane is equivalent to the fluorescent yield of the EI complex in solution, then one can calculate the enzyme concentration of the cells.

\[
\frac{1}{3} \left( 1 \times 10^{-7} \text{ moles/L} \times \frac{1 \text{L}}{10^3 \text{ml}} \right) / 3 \text{ ml} = 0.5 \times 10^5 \text{ cells/ml}
\]

\[
= 6.66 \times 10^{-16} \text{ moles/cell}
\]

\[
= 4.01 \times 10^8 \text{ molecules/cell}
\]

Assuming a molecular radius of 15 Å for the enzyme molecule, one can calculate the cross-sectional area.

\[
A = \pi r^2 - 7.07 \times 10^{-16} \text{ u}^2/\text{molecule}
\]

\[
7.07 \times 10^{-16} \text{ u}^2/\text{molecule} \times 4.01 \times 10^8 \text{ molecules/cell}
\]

\[
= 2.83 \times 10^3 \text{ u}^2/\text{cell}
\]

Assuming a cell radius of 15 microns, the surface area of the cell can be calculated.

\[
\text{Surface Area} = 4\pi r^2 = 2.83 \times 10^3 \text{ u}^2
\]

If all of the above assumptions are correct and all of the proteinase activity is associated with the cell membrane, then it appears that the cell membrane is
completely covered with proteinases. However, if the assumptions are in error by an order of magnitude, then only 10% of the surface is occupied by enzyme.

A similar calculation can be carried out to determine the proteinase concentration, if all of the enzyme is present within the cell.

\[
\text{Volume of the cell} = \frac{4}{3} \pi r^3 = 1.4 \times 10^4 \text{ u}^3
\]

\[
\text{Volume of the protein} = 5.61 \text{ u}^3
\]

Therefore, 0.004% of the cell is occupied by proteinases. If there is \(6.6 \times 10^{-17}\) moles of proteinase/cell and the cell volume is \(1.4 \times 10^{-8}\) cm\(^3\) then the proteinase concentration is \(4.7 \times 10^{-6}\) M.

IVC-2. VISUALIZATION OF PROTEINASES BY FLUORESCENCE MICROSCOPY

Dark-field fluorescence microscopy was used to visualize and confirm the differences in dansyl-argininal binding to the HSDM\(_1\)C\(_1\) fibrosarcoma cells, the DFP and pCMB treated fibrosarcoma cells and the IMR-90 fibroblasts. A comparison of fluorescence in the different experiments can be made in a qualitative way from the photomicrographs obtained as all of the fluorescent photomicrographs were obtained under the same conditions.
with identical exposure times (Figures 17-19). The dansyl-argininal fluorescence appears to be widely distributed throughout the cell. The nuclei and other cellular organelles do not exhibit an apparent fluorescence. The photomicrographs confirm the inhibition of fluorescence when the cells are preincubated with the proteinase inhibitors DFP or pCMB and they also demonstrate the lower fluorescence yield obtained from the normal fibroblasts. These experiments demonstrate the usefulness of the dansyl-argininal probe in locating and quantifying cellular proteinases.
Figure 18: Fluorescent photomicrograph of HSDM$_1$C$_1$

Fibrosarcoma cells incubated with dansyl-argininal. Cells were grown to log-phase on non-fluorescing aklar plastic discs. The cells were fixed with glutaraldehyde and incubated with $1.1 \times 10^{-4}$ M at $37^\circ$ C for 1 hour. Discs were mounted on glass slides and observed by dark-field fluorescence microscopy using a UG-1 exciter filter and barrier filter 50 (Zeiss). X200.
Figure 19: Fluorescent photomicrograph of HSDM₁C₁ fibrosarcoma cells preincubated with DFP, followed by incubation in dansyl-argininal. Cells were preincubated with 1mM DFP for 30 minutes at room temperature prior to incubation in 1.1 x 10⁻⁴ M dansyl-argininal at 37°C for 1 hour. Dark field fluorescence microscopy. X200.
Figure 20: Fluorescent photomicrograph of normal fibroblasts incubated with dansyl-argininal. The cells were grown to log-phase, fixed with glutaraldehyde and incubated with $1.1 \times 10^{-4}$ M dansyl-argininal for 1 hour at $37^\circ$ C. Dark field fluorescence microscopy. X200.
CHAPTER V

DISCUSSION

The use of probe molecules which bind to the active center of enzymes with a concomitant spectral or fluorescence change has resulted in much mechanistic information. Proflavin was first reported as a spectral probe of serine proteases in 1965 and was used to study the transient kinetics of trypsin and chymotrypsin (Bernhard and Gutfreund, 1965). Kinetic studies utilizing proflavin have generated information on reaction mechanisms and conformational changes that occur during catalysis. Other probes of the trypsin active-site have been utilized since proflavin (Shaw, 1970). However, none of these spectral probes bound to the enzyme in a transition-state-like adduct. Therefore, the development of a specific transition-state analog probe molecule could serve to enhance the study of enzyme mechanisms and also the study of proteinase enzymes in situ.

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The work discussed in this dissertation involves the development of a specific enzyme probe which combines the high affinity binding of a transition-state analog with a fluorescent reporter group to give a highly sensitive and specific probe for trypsin-like and cathepsin B-like enzymes.

Dansyl-argininal is a competitive inhibitor of trypsin-like proteases and highly fluorescent when bound to the enzyme. The blue shift of its emission spectrum when bound to trypsin is similar to that observed when it is dissolved in non-polar solvents, indicating that hydrophobic interactions contribute significantly to the fluorescent properties of the bound probe (Chen, 1967).

Monitoring the increase in emission intensity associated with the binding of dansyl-argininal to trypsin permits a direct determination of the equilibrium constants for the dissociation of the complexes. The $K_{I,obs}$ of 0.069 mM for binding to trypsin as determined by the fluorescent binding assay is in good agreement with the $K_{I,obs}$ of 0.042 mM determined kinetically.

The fluorescent binding assay also confirmed the presence of a secondary binding site or possibly an allosteric site in trypsin with an equilibrium dissociation constant of 0.51 mM. This was confirmed kinetically
as one observes a decrease in the $K_{M,\text{apparent}}$ with concentra-
tions of dansyl-argininal in excess of 0.5 mM.

This is in agreement with earlier experiments (Trowbridge et al., 1963) which show substrate activation of trypsin by both the D and the L isomers of tosyl-L-arginine methyl ester designated D-TAME and L-TAME respectively. Data reported by Trowbridge show that at low concentra-
tions of D-TAME, the D isomer activates the hydrolysis of L-TAME. Trowbridge (1963) proposes a scheme based on the assumption that both binary and ternary complexes are formed. These ternary complexes deacylate at a rate much faster than the binary complexes.

In addition, it has been shown that trypsinogen is also capable of binding two molecules of dansyl-argininal with binding constants of 0.15 mM and 2.5 mM. The ability of trypsinogen to bind to dansyl-argininal is due to a number of factors. The structure of the zymogen is quite similar to that of the active enzyme except that the histidine and the oxanion hole are too far apart to cooperate in substrate catalysis (Kossiakoff et al., 1977). Since the aldehyde binds to the enzyme as the neutral hemiacetal, stabilization from the oxanion hole is not required. Furthermore, the histidine does not appear to play an intimate role in the binding of pepti-
dyl aldehydes as shown by the high affinity binding of benzoyl-phenylalaninal to methyl-chymotrypsin (Kennedy and Schultz, 1979). Therefore, one could expect that the active site histidine need not play an intimate role in dansyl-argininal binding to trypsin or trypsinogen.

**VA. PEPTIDYL ALDEHYDES AS TRANSITION-STATE ANALOGS**

The inhibition of trypsin by leupeptin was the first report of a serine protease enzyme by a peptide aldehyde (Aoyagi et al., 1969). The presence of the free aldehyde group and the guanidino group were shown to be essential for inhibition of trypsin. Thompson (1973) argued that peptidyl aldehydes were potent inhibitors because they formed tetrahedral adducts (hemiacetals) with the active site serine and that the enzyme stabilized this adduct because it resembled the transition-state. Due to the high affinity binding of peptidyl aldehydes and the formation of a tetrahedral adduct with the reactive serine, this class of inhibitors was called transition-state analog inhibitors.

An ideal transition-state analog for serine proteases should fulfill the following criteria:

1. Should be a competitive inhibitor with an
affinity for the enzyme at least eight orders of magnitude greater than its analogous substrate.

2. Should place an sp$^3$ configuration in the enzyme active site.

3. Should form an anionic hemiacetal that will be stabilized by the enzyme in the oxanion hole.

It is clearly demonstrated from the evidence reported in this work, that the peptidyl aldehydes do not meet all of the requirements for an ideal transition-state analog for serine protease enzymes. Dansyl-argininal is a competitive inhibitor of trypsin with an affinity for the enzyme which is four orders of magnitude greater than that of its corresponding substrate. Although this falls short of the affinity predicted by transition-state theory, it falls within the range of observed binding affinity for other putative transition-state analogs.

Peptidyl aldehydes are now known to place an sp$^3$ configuration in the enzyme active site. Evidence for hemiacetal formation comes from a variety of sources. Chen et al., (1979) used cross saturation NMR techniques to study the binding of benzoyl- and acetyl-phenylalaninals to chymotrypsin. Proton NMR signals for the hemiacetal were not directly observed, but were inferred from the selective cross-saturation experiments. The
first direct observation of hemiacetal structure was obtained by Shah and Gorenstein (1982) using fluorine nuclear magnetic resonance spectra of N-acetyl-DL-fluorophenylalaninal and chymotrypsin. Separate signals for the formation of the hemiacetal complex, the aldehyde hydrate, the Michaelis complex and the free aldehyde were observed. More recent evidence from Gorenstein and co-workers (1984) utilizing $^{13}$C NMR spectroscopy to characterize complex formation between N-acetyl-phenylalaninal to chymotrypsin resulted in the observation of two signals for hemiacetal formation at pH > 7.0. These two hemiacetal signals could be due to the presence of both the anionic and the neutral forms of the hemiacetal or the formation of two slowly interconverting conformational isomers. Similar results were obtained by Gamscik et al. (1983) utilizing $^{13}$C NMR spectroscopy under cryoenzymological conditions to study the binding of acetylphe-glycinal to papain. Gamscik proposed the formation of two diastereomeric hemithioacetals, one of which places an OH in the oxanion hole and is bound in a "productive" mode, while the other is bound in a "non-productive" mode (Frankfater and Kuppy, 1981). In addition, the x-ray crystallographic structure of a peptidyl aldehyde inhibitor with Streptomyces griseus protease infers
a tetrahedral adduct (Brayer et al., 1979).

Finally, the lack of a significant pH dependence and the ability of benzoyl-phenylalaninal to strongly associate with methyl-chymotrypsin (Kennedy and Schultz, 1979), demonstrates that the active site histidine does not play an intimate role in the binding of peptidyl aldehydes. The inherent stability of the hemiacetal may, in part, be a driving force in the reaction between the serine and the aldehyde without requiring the participation of the histidine.

Although aldehydes clearly do not fulfill all of the criteria for an ideal transition-state analog, they are still semantically placed in this class of inhibitors along with boronic acids and diisopropylfluorophosphate (Kraut, 1977) solely on the basis of their tetrahedral geometry.

VI. DANSYL-ARGININAL BINDING TO TRYPsinOGEN

The zymogens of several proteolytic enzymes react with site-specific reagents and catalyze reactions at rates much slower than their corresponding enzymes. Experiments carried out by Walsh and coworkers (1978) on the hydrolysis of aminoacyl esters by trypsinogen demon-
strated the ability of the zymogen to bind to peptide substrates, but with a $k_{cat}$ that is approximately 100 fold smaller than its corresponding enzyme for each of the substrates tested. Furthermore, trypsinogen showed improvement in catalysis when elongated peptide substrates were used. However, with trypsin the effect is expressed in an increase in $k_{cat}$, whereas with trypsinogen the effect is expressed in $K_M$. The authors conclude that the difference could be the result of an induced fit in trypsin which is precluded in trypsinogen or a result of the requirement for precise alignment of substrate by trypsin to elicit the cooperation of the oxanion hole with the active site serine and histidine. If the oxanion hole is unavailable, then the improved binding would not be reflected in $k_{cat}$ as is the case with trypsinogen. Based on these results, the ability of dansylargininal to bind to trypsinogen is not unexpected and may provide additional support for the lack of importance of the oxanion hole for the binding of peptidyl aldehydes to serine protease enzymes.

V.C. EQUILIBRIUM FORMS OF ARGININAL

Data reported in this dissertation demonstrate for
the very first time that only 1% of the argininal in leupeptin is readily available for enzyme inhibition. In addition, the data clearly shows that the hydrate and carbinolamine forms of the argininal are relatively stable and are converted to the free aldehyde at unexpectedly slow rates with half-times on the order of 20 minutes. The pH dependency for this equilibrium was also studied and it was demonstrated that the relative amounts of each of the equilibrium forms was invariant with pH. Inhibition studies utilizing peptidyl argininals, such as leupeptin, will have to take into account the equilibrium of Figure 6 which becomes kinetically limiting under experimental conditions of $K_{I,\text{true}} > [\text{active peptidyl argininal}] < [\text{enzyme}]$.

In a recent paper, Baici and Gyger-Marazzi (1982) reported that the binding of leupeptin to cathepsin B showed a significant lag phase lasting for approximately 8 minutes ($t_{1/2} = 1$ minute), indicating the existence of a transient leupeptin-proteinase intermediate preceding steady-state inhibition by leupeptin. These authors concluded that the slow approach to steady-state inhibition indicates a slow conformation change in the enzyme induced by leupeptin binding and that this slow change in leupeptin affinity fits into the hysteretic enzyme con-
cept of Frieden (1970). Evidence presented in this dissertation suggests an alternate explanation. The lag in inhibition by leupeptin may be due to the slow equilibrium which exists between the three conformational forms of the argininal. Although the half-life observed in the aldehyde trapping experiments is longer than that observed for the lag phase of inhibition, it is possible that the enzyme itself acts to catalyze the dehydration reaction. Work done in this laboratory demonstrates that increasing the total leupeptin concentration above the concentration of cathepsin B results in elimination of the lag phase for inhibition, supporting the hypothesis that cathepsin B is merely acting as a trapping reagent for the free aldehyde.

VD. DANSYL-ARGININAL AS A PROBE OF PROTEOLYTIC ENZYME ACTIVITY IN WHOLE CELLS

This work has demonstrated that due to its high specificity for arginine specific serine and cysteine proteinases, dansyl-argininal can be used to localize and semi-quantify the entire class of arginine specific proteinases associated with cells. The inhibitor was used at a high concentration so that the active sites of the
enzymes present on the cell surface would be saturated. The complete inhibition of observable fluorescence by the active-site modifying reagents DFP and pCMB together, the inhibition of fluorescence by leupeptin supports the argument that the inhibitor is active site directed and that non-specific binding interactions with the membrane are minimal.

It is not possible to determine from these studies whether the inhibitor is binding to membrane associated proteinases or to enzymes localized in the cytoplasm. In addition, due to the ability of dansyl-argininal to bind to trypsinogen, it is not possible to determine whether the cellular enzyme is present in an active conformation or as a zymogen. However, these studies indicate that a high concentration of proteolytic enzyme activity is available to the fibrosarcoma cells, which is not available to the normal control cells. This data shows that proteinase probes, like dansyl-argininal, can be used to look at changes in enzyme availability during malignant transformation and also during cell cycle events.
REFERENCES


1049-1059. "Acylation of the Enzymatic Sites of Chymotrypsin by Esters, Acid Anhydrides and Acid Chlorides."


Inhibitor Complexes of Papain by Direct NMR Observation."


Alterations in Cell Transformation: The Importance of Proteases and Surface Proteins.


logs."


Thompson, R.C. (1973) Biochem. 12, 47-51. "Use of Peptide Aldehydes to Generate Transition-State Analogs of Elastase."


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