Kinetics of Hydrolysis of Dansyl Peptide Substrates by Thermolysin: Analysis of Fluorescence Changes and Determination of Steady-State Kinetic Parameters†

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ABSTRACT: The stopped-flow fluorescence technique has been used to study the hydrolysis of 10 dansyl peptides by thermolysin. The origin of the fluorescence changes observed during the reactions has been investigated in detail. Depending on the substrate and the excitation wavelength, the dansyl fluorescence changes observed arise either from energy transfer (maximal at λ_{ex} = 230 and 280 nm) between Trp residues of thermolysin and the dansyl group of the substrate in enzyme–substrate (ES) complexes or from direct excitation (maximal at λ_{ex} = 245 and 340 nm) of the free substrate and product, or from both sources. These two types of fluorescence signals reflect the concentrations of ES_i and free substrate, respectively. Both types of fluorescence changes have been used to monitor the reaction progress, and different mathematical formalisms have been used to determine the kinetic parameters for the reactions with results that are in good agreement. The efficiency of Trp quenching by a series of five dansyl tripeptides is shown to be related to the fractional saturation of enzyme and follows the K_M^{-1} values for the substrates. The quenching efficiency for a dansyl tetrapeptide is weaker due to the greater distance between the dansyl group and the Trp-115 donor in thermolysin. On the basis of these studies, substrates capable of supporting more detailed kinetic studies of thermolysin have been identified.

Thermolysin (EC 3.4.24.4) is a thermostable zinc proteinase from *Bacillus thermoproteolyticus* (Endo, 1962). The enzyme has been extensively studied and is believed to share many critical mechanistic features with mammalian metallopeptidases such as carboxypeptidase A, angiotensin converting enzyme, and the matrix metalloproteinases. The enzyme contains one zinc atom (Latt et al., 1969) located at the active site that participates in catalysis. It also contains four atoms of calcium that confer thermal stability by stabilizing its tertiary structure (Feder et al., 1971; Dahlquist et al., 1976; Voordrow et al., 1974). A refined three-dimensional structure of the enzyme is available (Holmes & Matthews, 1982), and the structures of complexes with a wide variety of inhibitors (Matthews, 1988) have been elucidated. Specific mechanistic roles have been postulated for several active-site residues, including Glu-143, His-231, and Tyr-157.

The substrate specificity of thermolysin has been examined in detail, where the enzyme hydrolyzes both peptides (Morihara & Tsuzuki, 1970) and esters (Holmquist & Vallee, 1976). A number of steady-state and pre-steady-state kinetic studies have also been carried out (Feder & Schuck, 1970; Holmquist & Vallee, 1976; Morgan & Fruton, 1978; Kunugi et al., 1982; Wayne & Fruton, 1983; Fukuda & Kunugi, 1984; Stein, 1988; Izquierdo & Stein, 1990). While a general picture for the mechanism of the enzyme has evolved, a number of questions remain. In particular, it appears that the choice of substrate markedly affects the outcome of certain studies. For example, some workers have presented evidence for multiple enzyme–substrate intermediates, ES_i[1] (Fukuda & Kunugi, 1984; Izquierdo & Stein, 1990), while others (Morgan & Fruton, 1978) have reported the resolution of the substrate-binding step leading to formation of a single ES. Depending on the substrate, the order of product release has been reported to be random (Wayne & Fruton, 1983) or ordered (Oyama et al., 1981). The effect of salts on the efficiency of the enzyme is also markedly dependent on the identity of the substrate (Holmquist & Vallee, 1976; Morgan & Fruton, 1978; Li, 1982; Inouye, 1992).

Mechanistic studies of thermolysin have been hampered by the lack of convenient methods for monitoring the reaction for a wide variety of substrates. As a result, very few systematic studies of closely related substrates have been carried out. Another problem has been that sensitive methods for directly observing catalytic intermediates in either steady-state or pre-steady-state studies have not been utilized. Lobb and Auld (1984) have pioneered the use of the stopped-flow fluorescence technique for the study of the steady-state and pre-steady-state kinetics of hydrolysis of dansyl peptide substrates. In principle, this technique provides a means to directly observe ES_i by taking advantage of energy transfer (ET) between the enzyme and the dansyl substrate and allows the rates of both pre-steady-state and steady-state events to be studied from the resulting stopped-flow fluorescence traces. The stopped-flow fluorescence technique can also be used to monitor the fluorescence changes associated with substrate-to-product (SP) conversion in steady-state studies (Lin et al., 1988a) or the

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[1] Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Dns or dansyl, 5-(dimethylamino)naphthalenyl-1-sulfonyl; Mns or mansyl, 6-(*N*-methylanilino)-2-naphthalenyl-1-sulfonyl; ET, energy transfer; DE, direct excitation; E, enzyme; S, substrate; P, product; ES, enzyme–substrate intermediate; HPLC, high-performance liquid chromatography; FAGLA, furanacryloyl-Gly-Leu-NH_2; TFA, trifluoroacetic acid; Cbz, benzyloxycarbonyl; Ac, acetyl; DMSO, dimethyl sulfoxide; S-Leu, L-α-mercaptoisocaproic acid.

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interconversion of ES_i by their direct excitation (DE) in pre-steady-state studies (Lin et al., 1988b).

To be able to abstract meaningful kinetic information from the fluorescence changes observed in stopped-flow fluorescence experiments, it is first necessary to establish the origin of the observed fluorescence changes, since the kinetic formalism used to analyze them depends upon their source (Lin & Van Wart, 1988). In this investigation, 10 dansyl substrates for thermolysin have been synthesized to provide a variety of candidates for more systematic studies. The experiments described here establish conditions under which both ET and SP experiments can be used to study the hydrolysis of individual dansyl substrates by thermolysin. In the accompanying paper (Yang et al., 1994), these studies are extended to examine the anion dependence of the steady-state kinetic parameters for selected substrates.

MATERIALS AND METHODS

Materials. Thermolysin (*Bacillus thermoproteolyticus rokko*) was purchased from Sigma and dialyzed extensively before use to remove salts. Dansyl chloride, Hepes, Phe-Ala, Phe-Gly, Leu-Ala, Leu-Gly, Phe-Phe, Leu-Phe, all amino acids, a dansyl amino acid kit, and Ac-Pro-Leu-Gly-(S-Leu)-Leu-Gly-OCH_3 were also obtained from Sigma.

Synthesis of Dansyl Substrates. The 10 dansyl substrates prepared in this study were synthesized by classical solution-phase methods and purified by high-performance liquid chromatography (HPLC) using an instrument that consisted of a Beckman 421 controller, two Beckman 110A pumps, an Altex 210 injector, a Varian UV 50 detector, and a Houston Instrument chart recorder. Analytical analyses were performed using a Beckman 5 μm C-18 (4- × 250-mm) reverse-phase column, while peptide purification was carried out on a Beckman Ultrasphere ODS 5 μm C-18 (10- × 250-mm) reverse-phase column. All HPLC steps were carried out either isocratically in 15% CH_3CN:H_2O/0.1% TFA or with a linear gradient from 15% CH_3CN:H_2O/0.1% TFA to 50% CH_3CN:H_2O/0.1% TFA in 30 min as described previously (Rivier et al., 1984). Peptides were recovered after preparative chromatography by lyophilization.

Peptides were sequenced before dansylation using an Applied Biosystem Model 477A automatic pulsed liquid protein sequenater using the standard protocol. The site of hydrolysis of all peptides was determined by dansylation of the reaction products followed by chromatographic analysis (Negro et al., 1987). Aliquots (20 μL) of the reaction mixtures were removed at 0 and 48 h and reacted with 20 μL of 0.5 M $NaHCO_3$ and 20 μL of 8 mM dansyl chloride in acetonitrile. After standing in the dark for 24 h, the samples were injected onto an analytical HPLC column and eluted using a linear gradient from 30% to 100% in 40 min, where the two solvents were 50 mM NaH_2PO_4/Na_2HPO_4, pH 6.4, and 80% methanol/H_2O. The site of hydrolysis was determined by comparing the retention times of the peptide products with those of dansylated peptides of known composition that corresponded to either the N- or C-terminal half of the substrate.

Optical and Fluorescence Spectra. The optical spectra of all samples were obtained with a Varian Model 219 spectrophotometer. Fluorescence studies were carried out using either the Perkin-Elmer Model LS-5 or LS-50 instrument using quartz cells. For most studies, an entrance slit width of 3 mm and an exit slit width of 10 mm were employed. A 420-nm long-pass filter was used during the detection of fluorescence at 540 nm to avoid interference from Rayleigh scattering. The concentrations of all dansyl substrates were determined spectrophotometrically using $\epsilon_{330} = 4.1 \times 10^3\ M^{-1}\ cm^{-1}$.

Spectrophotometric Steady-State Kinetic Assays. Some assays for thermolysin were carried out spectrophotometrically at 23 °C in assay buffer (50 mM Hepes, 1.0 M NaCl, 10 mM $CaCl_2$, pH 7.5) using furanacryloyl-Gly-Leu-NH_2 (FAGLA) as a substrate at a concentration of 0.3 mM by continuously monitoring the decrease in absorbance at 345 nm after the addition of enzyme (Holmquist & Vallee, 1974). The substrate and enzyme concentrations were determined spectrophotometrically using $\epsilon_{305} = 2.47 \times 10^4\ M^{-1}\ cm^{-1}$ and $\epsilon_{280} = 6.4 \times 10^4\ M^{-1}\ cm^{-1}$, respectively. In a typical assay, thermolysin (50 nM) was added to a solution of 0.3 mM FAGLA in assay buffer in a 1-mL cuvette and the change in absorbance at 345 nm was recorded. Initial velocities (v_o) were calculated from the slope of the absorbance change during the first 10% of hydrolysis using the relationship $v_o = (\Delta A/\Delta t)\Delta\epsilon_{345}$, where $\Delta\epsilon_{345} = 200\ M^{-1}\ cm^{-1}$. For assays carried out at a single substrate concentration, activity is expressed as $v_o/[E_o]$.

In other assays, the initial rate of hydrolysis of Ac-Pro-Leu-Gly-(S-Leu)-Leu-Gly-OCH_3 was measured in assay buffer at 23 °C (Weingarten & Feder, 1985). The reaction mixture contained 200 μM dithionitrobenzoic acid which allowed the hydrolysis of the Gly-(S-Leu) thiolester bond to be monitored continuously using a Varian Model 219 spectrometer by following the absorbance change at 412 nm ($\Delta\epsilon_{412} = 0.0142\ \mu M^{-1}\ cm^{-1}$). The assay was initiated by addition of 10 μL of substrate stock solution to give a final concentration of 20 μM.

Stopped-Flow Fluorescence Studies. A second-generation low-temperature stopped-flow instrument was constructed (Baek and Van Wart, manuscript in preparation) and used to carry out the stopped-flow fluorescence studies described here. This instrument has a stopped-flow module that consists of a 2-cm long observation cell made from a 2-mm diameter channel in a Kel-F block with quartz windows on each end, similar to a Gibson–Durrum-type design. The system uses unplasticized Kel-F sample syringes equipped with special ethylene–propylene O-rings (compound # E515-8; Parker Co.) on the plungers. The commercial 3-way fluid control valves were replaced by specially constructed self-tightening valves designed to be leak-free at low temperature. The dansyl or Trp emissions from the reactions in the stopped-flow cell were selected by use of appropriate filters. For monitoring changes in dansyl fluorescence, an Oriel 420-nm long-pass filter was used to cut off the Trp emission. For monitoring the Trp emission, a 340-nm band-pass filter was used.

THEORY

Origins of the Changes in Dansyl Fluorescence Observed during the Hydrolysis of Dansyl Substrates. In the reactions of interest in this study, the dansyl fluorescence signal observed at time t, F_t, is related to the instantaneous concentration of all of the dansyl species present, including the substrate (S), product (P), and all ES_i intermediates. The general expression for F_t is given by

$$F_t = F^{SP} + F^{ES} \quad (1)$$

where the F^{SP} term describes the contribution from changes in the concentrations of S and P and the F^{ES} term describes changes in F_t due to changes in the concentrations of ES_i

(Lobb & Auld, 1984; Lin & Van Wart, 1988a). The first term is given by

$$F^{SP} = C(Q_s a_s - Q_p a_p)([S_o] - [P_t]) = C(Q_s a_s - Q_p a_p)[S_t] \quad (2)$$

where Q_s and a_s are the quantum yields and the molar absorptivities, respectively, of S at the excitation wavelength, Q_p and a_p are the same quantities for P, C is a constant, $[S_o]$ is the total starting substrate concentration, $[P_t]$ is the product concentration at time t, and $[S_t]$ is the free substrate concentration at time t. The second term in eq 1 contains contributions from two distinct sources, denoted F^{DE} and F^{ET}, which represent the contributions to F_t from changes in $[ES_i]$ that arise from direct excitation of the dansyl group of S in the ES_i or from dansyl fluorescence that arises from energy transfer associated with excitation of enzyme donors, respectively. This term is given by (Lobb & Auld, 1984)

$$F^{ES} = F^{DE} + F^{ET} = c\sum(Q_i a_i - Q_s a_s)[ES_i] + I_o C_D \sum Q_i a_i T_i [ES_i] \quad (3)$$

where I_o is the incident light intensity, T_i is the efficiency of energy transfer in the ES_i, c is a constant, and C_D is an instrumental factor.

Dominance of Individual Terms in Equations 1–3. By adjusting the experimental conditions, it is possible to make one term in eqs 1–3 dominant, as described below, allowing the F_t values to be analyzed for the purpose of calculating kinetic constants for the reactions. The F^{SP} term will be nonzero when $Q_s a_s - Q_p a_p$ is nonzero at the excitation wavelength. The resulting F^{SP} term will outweigh the F^{ES} term in eq 1 when the excitation wavelength used is far from the absorbance of E so that the F^{ET} term is negligible and when $[S_o] \gg [E_o]$ so that the $[ES_i]$ will be very small compared to $[S_o]$, making the contribution of F^{DE} to F^{ES} negligible.

The contribution to F_t from one of the F^{ES} terms in eq 3 can dominate when there is a negligible contribution from F^{SP} due to a small $Q_s a_s - Q_p a_p$ term. In general, the magnitude of F^{ES} will be greatest at high $[E_o]$ where the $[ES_i]$ is highest. The selective excitation (λ_{ex} = 280 nm) of Trp residues on E can yield ET to the bound dansyl substrate in the ES_i. In this case, the changes in F_t reflect the changes in the concentration of ES_i and $F_t = F^{ET}$. Alternatively, if direct excitation of the bound dansyl substrate can be achieved selectively with no ET in the ES_i, F_t can be dominated by the F^{DE} term in eq 3.

Experimental Criteria for Establishment of the Origin of Dansyl Fluorescence Changes. Five criteria have been identified to establish the origin of F_t changes in stopped-flow fluorescence experiments (Lin & Van Wart, 1988). Three of these criteria will be used in the studies described below. The first criterion relates to whether there is a quenching in Trp fluorescence that parallels the change in dansyl fluorescence. For changes due to F^{ET} terms, this behavior is predicted. In contrast, F_t changes due to F^{SP} or F^{DE} terms do not exhibit parallel Trp-quenching patterns. The second criterion relates to the position of the peak in the "excitation profile" for the dansyl fluorescence change. For F^{ET} changes, this peak should coincide with the absorption band of the group on the enzyme responsible for the ET (normally near 285 nm for Trp). In contrast, this peak will occur for F^{SP} and F^{DE} terms at the wavelengths at which the differences in the quantities $Q_s a_s - Q_p a_p$ (eq 2) and $Q_i a_i - Q_s a_s$ (eq 3), respectively, are maximal. This often coincides with an absorbance maximum of the dansyl group (e.g., 245 or 330 nm). A third distinguishing feature of the dansyl fluorescence change is its dependence on $[E_o]$. F_o (the value of F_t at $t = 0$) should be directly proportional to $[E_o]$ for F^{ET} and F^{DE} terms as long as $[S_o] \gg [E_o]$, since both monitor $[ES_i]$. In contrast, for F^{SP} changes, F^o is determined only by the spectral properties of S and P and is independent of $[E_o]$.

Relationship between the Stopped-Flow Fluorescence Parameters and the Steady-State Kinetic Parameters. In F^{ET} experiments, the steady-state kinetic parameters k_{cat} and K_M can be evaluated in several ways from the kinetic traces. The important experimental parameters are F_t, F_o, A_t (the area under the curve from time t until completion of the reaction), and A_o (the total area under the curve). One means of evaluating k_{cat} and K_M from stopped-flow ET experiments is to measure F_o and A_o for a series of traces obtained at different substrate concentrations. Lobb and Auld (1984) have shown that

$$\frac{A_o[E_o]}{F_o[S_o]} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}[S_o]} \quad (4)$$

Thus, a plot of $(A_o[E_o])/(F_o[S_o])$ vs $1/[S_o]$ should be linear with x- and y-intercepts of $-1/K_M$ and $1/k_{cat}$, respectively. Equation 4 also holds for the changes in Trp fluorescence that are observed as a result of ET. The kinetic constants k_{cat} and K_M can also be determined from an F^{ET} trace obtained at a single substrate concentration, provided that there is no product inhibition and $[S]_o \gg K_M$, by employing the following form of eq 4

$$\frac{A_t[E_o]}{F_t[S_o]_t} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}[S_o]_t} \quad (5)$$

To evaluate k_{cat} and K_M, a similar plot of $(A_t[E_o])/(F_t[S_o]_t)$ vs $1/[S_o]_t$ is constructed. In F^{SP} experiments, F_t simply reflects the instantaneous substrate concentration and the instantaneous rate is reflected by $m_t = dF/dt$, the instantaneous slope of the fluorescence trace. Under these conditions, the following relationship holds

$$-\frac{F_o[E_o]}{[S_o]m_o} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}[S_o]} \quad (6)$$

Thus, one means of evaluating k_{cat} and K_M is from the m_o and F_o values obtained from a series of stopped-flow experiments carried out at variable $[S_o]$. A plot of $-(F_o[E_o])/([S_o]m_o)$ vs $1/[S_o]$ should be linear with x- and y-intercepts of $-1/K_M$ and $1/k_{cat}$, respectively. Alternatively, if there is no product inhibition, these parameters can be evaluated from a single stopped-flow trace acquired under $[S_o] \gg K_M$ conditions using

$$-\frac{F_o[E_o]}{[S_o]_t m_t} = \frac{1}{k_{cat}} + \frac{K_M}{k_{cat}[S_o]_t} \quad (7)$$

Thus, a plot of $-(F_o[E_o])/([S_o]_t m_t)$ vs $1/[S_o]_t$ will also allow evaluation of k_{cat} and K_M.

RESULTS

Description of Substrates. A series of substrates containing a dansyl group on either the N- or C-terminus has been synthesized. In all but one of the substrates (seven tripeptides and two tetrapeptides, all with unblocked C-termini), the dansyl group serves as an N-terminal blocking group (Table 1). In another substrate, the dansyl group has been attached to the C-terminus of a tripeptide with an unblocked N-terminus

Table 1: Properties of Dansyl Substrates

substrate	F^{SP} (arbitrary units)[a] λ_{ex} = 340 nm	F^{SP} (arbitrary units)[a] λ_{ex} = 245 nm	maximum solubility (mM)
Dns-Gly-Phe-Ala	7.0	27	5.4
Dns-Gly-Phe-Phe	5.5	20	0.73[b]
Dns-Gly-Leu-Phe	10	25	1.3[b]
Dns-Gly-Phe-Gly	5.5	23	5.8
Dns-Ala-Phe-Ala	6.2	39	4.5[c]
Dns-Ala-Leu-Ala	6.2	57	6.8
Dns-Gly-Leu-Gly	5.5	25	10
Dns-Gly-Gly-Leu-Gly	7.0	39	3.7
Dns-Ala-Ala-Phe-Ala	2.4	15	4.0[d]
Phe-Leu-Ala-NH$(CH_2)_2$NH-Dns	24	50	0.30[e]

[a] Total fluorescence change at 540 nm observed on full hydrolysis of each substrate at a concentration of 100 μM. [b] 6% DMSO. [c] 5% DMSO. [d] 7% DMSO. [e] 30% DMSO.

through an ethylenediamine spacer. This alternate placement of the fluorescent dansyl group at different ends of a variety of peptides with different sequences has been carried out to allow the choice of substrates with optimum recognition by thermolysin, as judged by the kinetic parameters for their hydrolysis, to identify substrates for which F^{ET} would be particularly efficient or to produce substrates with large values of F^{SP}. This would allow the greatest flexibility in the planned kinetic studies.

Some of the properties of these 10 substrates are summarized in Table 1. A property of significance is their solubility in aqueous solution. While thermolysin is inactivated by most organic cosolvents, it can tolerate up to 10% v/v DMSO to enhance substrate solubility. Phe-Leu-Ala-NH$(CH_2)_2$NH-Dns is poorly soluble in aqueous solution; even in 30% v/v DMSO, its solubility is only 0.30 mM. With the exception of Dns-Gly-Phe-Phe, all of the other substrates are soluble to a concentration of at least 1 mM in either aqueous solution or solutions containing 7% v/v or less DMSO.

Potential for F^{ET}-Based Fluorescence Studies. All of the substrates considered in this study have similar absorption and emission spectra that are dominated above 230 nm by the dansyl group. There are dansyl absorption bands near 247 and 330 nm. Excitation within either of these bands gives an emission band centered near 540 nm. The excitation and emission spectra of thermolysin exhibit bands at 280 and 340 nm, respectively, due primarily to its Trp residues. The emission band of thermolysin near 340 nm overlaps the 330-nm absorption band of these dansyl substrates. Thus, on excitation of enzyme Trp residues at 280 nm, there is the potential for resonance ET to the dansyl substrate in the ES_i formed during catalysis that should be manifested as dansyl fluorescence in the 540-nm region.

Potential for F^{SP}-Based Fluorescence Studies. Excitation (λ_{em} = 540 nm) and emission (λ_{ex} = 245 or 340 nm) spectra of all 10 dansyl substrates have been acquired (not shown), both before and after hydrolysis, and represent those of the substrate and the product, respectively. The shapes of the substrate and product spectra are very similar; however, both the emission and excitation bands of the product are less intense than those of the substrate. Thus, there is a decrease in dansyl fluorescence at 540 nm associated with hydrolysis of substrate to product due to a F^{SP} term that is largest with excitation wavelengths near 245 and 340 nm. These F^{SP} changes are listed in Table 1 (arbitrary units) for the hydrolysis of all 10 substrates at a concentration of 100 μM. Thus, the hydrolysis of these substrates can be studied at these wavelengths by using the F^{SP}-based formalism discussed above.

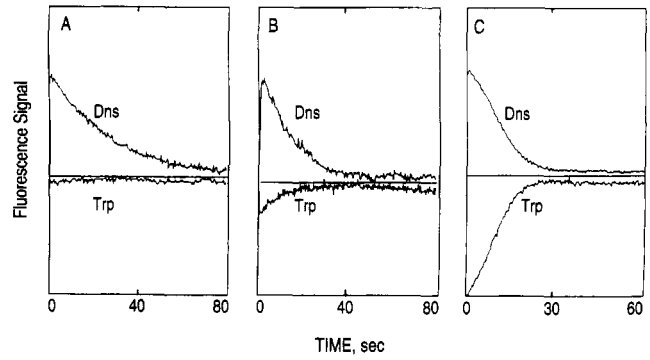

FIGURE 1: Stopped-flow fluorescence traces (λ_{ex} = 280 nm) showing the changes in Dns (top) and Trp (bottom) fluorescence during the hydrolysis of 0.10 mM (A) Dns-Ala-Leu-Ala, (B) Dns-Ala-Phe-Ala, and (C) Dns-Gly-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, 1 mM NaCl, pH 7.3, at 23 °C. The thermolysin concentrations were 7.5, 3.0, and 5.0 μM, respectively.

Stopped-Flow Fluorescence Traces for Hydrolysis of Dansyl Substrates. Stopped-flow fluorescence traces (λ_{ex} = 280 nm) for the hydrolysis of 0.10 mM Dns-Ala-Leu-Ala, Dns-Ala-Phe-Ala, and Dns-Gly-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, 1 M NaCl, pH 7.5, at 23 °C are shown in Figure 1. Due to the different susceptibilities of each peptide to hydrolysis, the time scale and the thermolysin concentrations used for each reaction differ somewhat. This excitation wavelength is one that should favor F^{ET}-based changes in fluorescence. For all three substrates, there is a significant decrease in dansyl fluorescence (λ_{em} = 540 nm) during the reaction. However, the change in Trp fluorescence (λ_{em} = 340 nm) is substrate dependent with a large signal for Dns-Gly-Phe-Ala, a much smaller signal for Dns-Ala-Phe-Ala, and essentially no signal for Dns-Ala-Leu-Ala. Thus, even when employing excitation at 280 nm to maximize F^{ET}, there is clearly a contribution from F^{SP} during the hydrolysis of some of these substrates. Therefore, it is necessary to establish what percentage of the fluorescence change for each reaction is due to each source at each excitation wavelength before performing any kinetic analyses.

Excitation Profiles for Dansyl and Trp Fluorescence Changes Observed on Substrate Hydrolysis. It is clear that both F^{SP}- and F^{ET}-based fluorescence changes are possible in these reactions. One means of assessing the origin of the dansyl or Trp fluorescence change observed at any particular wavelength on hydrolysis is to measure an "excitation profile" for each reaction. This is obtained by plotting the full fluorescence change for the reaction, F_o, vs the excitation wavelength. Such a profile will reveal which excitation wavelengths give rise to the observed changes in F_o. A series of dansyl (λ_{em} = 540 nm) and Trp (λ_{em} = 340 nm) excitation profiles for the hydrolysis of all 10 dansyl substrates by thermolysin has been measured. The dansyl excitation profiles for Dns-Ala-Leu-Ala, Dns-Ala-Phe-Ala, and Dns-Gly-Phe-Ala, respectively, are displayed in Figure 2. The profiles for the first two substrates exhibit clearly defined maxima only at 245 and 340 nm. These match the excitation bands of the dansyl group and are attributable to the F^{SP} changes listed in Table 1. Excitation at 245 nm produces a substantially larger dansyl fluorescence change than excitation at 340 nm.

The dansyl excitation profile for Dns-Gly-Phe-Ala, however, shows different behavior below 300 nm in that it has maxima at 230 and 260 nm. The corresponding Trp excitation profiles for this and several other substrates are shown in Figure 3A. The profile for Dns-Gly-Phe-Ala exhibits prominent maxima near 230 and 280 nm that are also associated with the

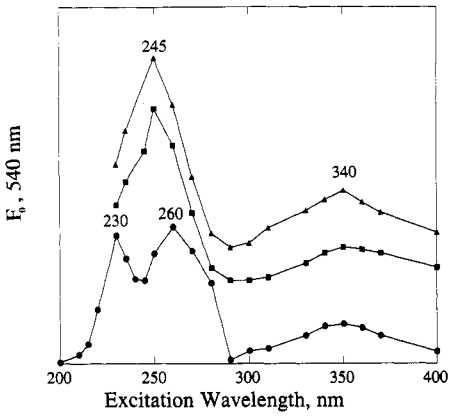

FIGURE 2: Stopped-flow fluorescence excitation profiles for the change in dansyl fluorescence (λ_{em} = 540 nm) observed on hydrolysis of 0.10 mM (▲) Dns-Ala-Leu-Ala, (■) Dns-Ala-Phe-Ala, and (●) Dns-Gly-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, 1 M NaCl, pH 7.5, at 23 °C. The thermolysin concentrations were 1.5, 1.5, and 2.5 μM, respectively.

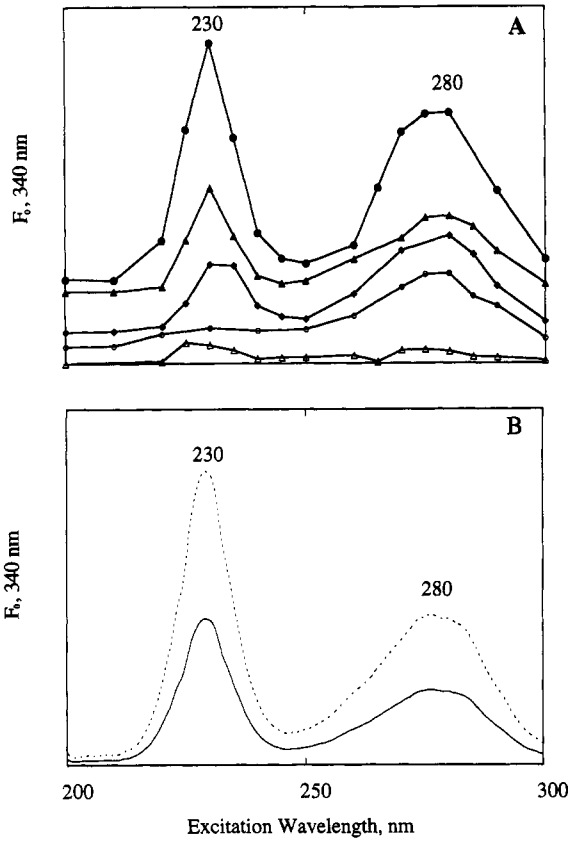

FIGURE 3: (A) Stopped-flow fluorescence excitation profiles for the change in Trp fluorescence (λ_{em} = 340 nm) observed on hydrolysis of 0.10 mM (●) Dns-Gly-Phe-Ala, (▲) Dns-Gly-Leu-Phe, (■) Dns-Gly-Phe-Phe, (□) Dns-Gly-Phe-Gly, and (△) Dns-Ala-Ala-Phe-Ala by 10 μM thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, 1 M NaCl, pH 7.5 at 23 °C. (B) Excitation spectra (λ_{em} = 340 nm) of (—) 1 μM thermolysin and (– – –) 3 μM L-Trp in 50 mM Hepes, 10 mM $CaCl_2$, 1 M NaCl, pH 7.5, at 23 °C.

quenching of Trp fluorescence (λ_{em} = 340 nm) in thermolysin during the hydrolysis of this substrate. A similar quenching is also seen for Dns-Gly-Phe-Phe, Dns-Gly-Leu-Phe, Dns-Gly-Phe-Gly, and Dns-Ala-Ala-Phe-Ala. These maxima also coincide with those in the excitation profiles (λ_{em} = 340 nm) of L-Trp and thermolysin (Figure 3B). Thus, the bands near 230 and 280 nm in Figures 2 and 3A arise from quenching of thermolysin Trp fluorescence by the dansyl substrate via a F^{ET} mechanism. For Dns-Ala-Leu-Ala and Dns-Gly-Leu-Gly, no changes in Trp fluorescence were detectable under the conditions studied. The markedly different extent of quenching for these substrates indicates that some are much better candidates for F^{ET} studies than others.

Dependence of F_o on Thermolysin Concentration. The results of the previous section indicate that, depending on the substrate and the excitation wavelengths, the contributions of F^{SP} and F^{ET} terms to F_o can vary widely. The magnitude of a F^{SP}-based signal at a given $[S_o]$ should be independent of $[E_o]$, while that for a F^{ET}-based signal should be directly proportional to $[E_o]$ as long as $[S_o] \gg [E_o]$. Therefore, the dependence of F_o on $[E_o]$ can be used at each excitation wavelength to assess what fraction of F_o arises from each source.

The dependence of dansyl F_o (λ_{em} = 540 nm) on $[E_o]$ observed on excitation at 340 and 245 nm has been examined for all 10 dansyl substrates (data not shown). The F_o values are independent of $[E_o]$ at both excitation wavelengths for all reactions, indicating pure F^{SP} behavior. The magnitude of F_o for each substrate reflects the magnitude of the $Q_s a_s - Q_p a_p$ term in eq 2 which is determined by the different spectral properties of each S and P. The magnitudes of these F_o values vary widely for the different substrates, as shown in Table 1. Phe-Leu-Ala-$NH(CH_2)_2NH$-Dns gives one of the largest fluorescence changes. However, its low solubility in aqueous solution limits its application for kinetic studies. Of the other substrates, Dns-Gly-Leu-Phe exhibits the largest F^{SP} value with 340-nm excitation and Dns-Ala-Leu-Ala exhibits the largest change with 245-nm excitation. Thus, these substrates are the best candidates for F^{SP} studies.

A plot of the dansyl F_o value (λ_{em} = 540 nm) vs $[E_o]$ observed on hydrolysis for seven dansyl substrates (0.10 mM) by thermolysin with 280-nm excitation is shown in Figure 4A. All of these substrates exhibit a linear increase in F_o with $[E_o]$, as expected for a F^{ET} signal, but with quite different slopes. The slopes follow the order Dns-Gly-Phe-Ala > Dns-Gly-Phe-Phe > Dns-Gly-Leu-Phe > Dns-Gly-Phe-Gly > Dns-Ala-Phe-Ala > Dns-Ala-Leu-Ala > Dns-Gly-Leu-Gly. Thus, substrates such as Dns-Gly-Phe-Ala and Dns-Gly-Phe-Phe are the best candidates for F^{ET} studies. None of the plots shown in Figure 4A have a zero intercept, however, indicating that there is always some contribution from F^{SP}. Dns-Gly-Leu-Phe and Dns-Gly-Phe-Phe have the largest y-intercept, while Dns-Gly-Leu-Gly and Dns-Ala-Phe-Ala have the smallest.

In contrast to the behavior for the dansyl F_o values, the variation in Trp F_o values (λ_{ex} = 280 nm, λ_{em} = 340 nm) for Dns-Gly-Phe-Ala, Dns-Gly-Phe-Phe, Dns-Gly-Leu-Phe, Dns-Gly-Phe-Gly, and Dns-Ala-Phe-Ala (Figure 4B) increases linearly with $[E_o]$ and intersects near the origin. Dns-Ala-Leu-Ala and Dns-Gly-Leu-Gly do not exhibit any detectable Trp quenching. The slopes of these F_o vs $[E_o]$ plots follow the same order observed above for the dansyl F_o values. Since these plots intersect very close to the origin, virtually all of the fluorescence changes are attributable to F^{ET}. Thus, F^{ET} studies are best carried out by monitoring the Trp signal.

Product Inhibition. Studies have been carried out with the hydrolysis products of the substrates examined here to quantitate any possible product inhibition. The effect of Leu-Ala, Phe-Ala, Leu-Gly, Phe-Gly, and Leu-Phe on the rate of hydrolysis of 1.0 mM FAGLA ($[S_o] \ll K_M$) by thermolysin has been examined in 50 mM Hepes, 10 mM $CaCl_2$, 1 M NaCl, pH 7.5. The IC_{50} values obtained from the resulting inhibition curves are listed in Table 2. The values for Leu-Ala, Phe-Ala, and Leu-Phe are the lowest but are still quite

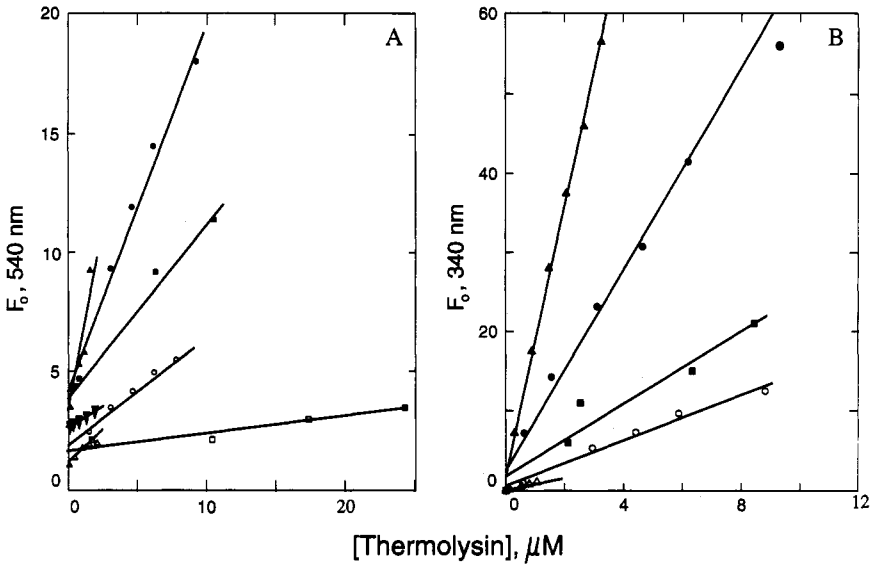

FIGURE 4: Dependence of (A) dansyl F_o (λ_{ex} = 280 nm, λ_{em} = 540 nm) and (B) Trp F_o (λ_{ex} = 280 nm, λ_{em} = 340 nm) on [E_o] for the hydrolysis of 0.10 mM (▲) Dns-Gly-Phe-Ala, (●) Dns-Gly-Phe-Phe, (■) Dns-Gly-Leu-Phe, (○) Dns-Gly-Phe-Gly, (△) Dns-Ala-Phe-Ala, (▼) Dns-Ala-Leu-Ala, and (□) Dns-Gly-Leu-Gly in 50 mM Hepes, 10 mM $CaCl_2$, 1 M NaCl, pH 7.5, at 23 °C.

Table 2: Inhibition of Thermolysin by Products of Hydrolysis of Dansyl Substrates

product	IC_{50} (mM)
Dns-Ala	15
Dns-Gly	>15
Leu-Ala	0.31
Phe-Ala	0.56
Leu-Gly	7.0
Phe-Gly	18
Leu-Phe	0.26

high. Thus, product inhibition should be appreciable only late in reactions for Dns-Ala-Leu-Ala, Dns-Gly-Phe-Ala, Dns-Ala-Phe-Ala, and Dns-Gly-Leu-Phe carried out at higher substrate concentration. Dns-Ala and Dns-Gly absorb strongly near 340 nm and interfere with the FAGLA assay. Therefore, inhibition by these species was measured using 20 μM Ac-Pro-Leu-Gly-(S-Leu)-Leu-Gly-OMe ([S_o] ≪ K_M). For both dansyl products, the IC_{50} value is greater than 15 mM, indicating that these products are very poor inhibitors.

Determination of Kinetic Parameters from F^{SP} Measurements. The kinetic parameters for the hydrolysis of dansyl substrates by thermolysin have been measured using a variety of approaches. The first entails measurement of dansyl fluorescence (λ_{em} = 540 nm) employing excitation wavelengths of 340 or 245 nm, conditions under which the fluorescence signal arises exclusively from the F^{SP} term. One approach is based on eq 6 and involves measuring initial rates under conditions where the concentration of products is not appreciable. Two different methods have been used to measure the initial rates m_o. Method I entails the use of [E_o] high enough to achieve full hydrolysis within a reasonable time period (<1 min) to get F_o but using only the first 10% of the reaction to obtain m_o from $\Delta F/\Delta t$ at t = 0. An example of a double-reciprocal plot obtained for this data (λ_{ex} = 340 nm) is shown in Figure 5A for the hydrolysis of Dns-Gly-Phe-Ala by thermolysin. Method II uses a lower [E_o] in order to greatly expand the first 10% of the reaction curve in order to more accurately evaluate m_o. A higher concentration of [E_o] is used in a separate experiment in order to measure F_o. The steady-state kinetic parameters for the hydrolysis of Dns-Gly-Phe-Ala determined with excitation at 340 nm using method I and with excitation at 245 and 340 nm using method

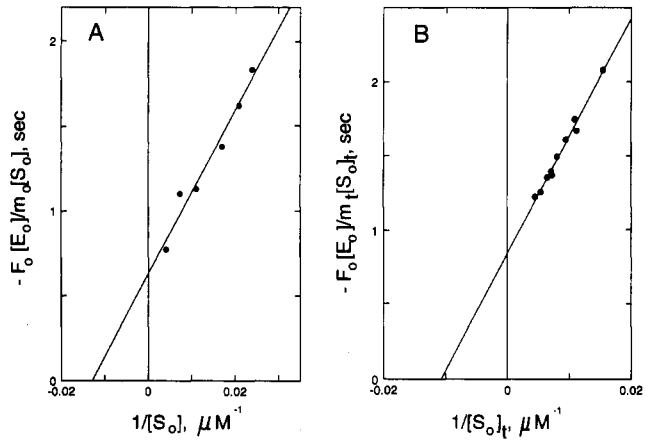

FIGURE 5: Plots of (A) $-F_o[E_o]/m_o[S_o]$ vs $1/[S_o]$ and (B) $-F_o[E_o]/m_t[S_o]_t$ vs $1/[S_o]_t$ for the hydrolysis of Dns-Gly-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, 1 M NaCl, pH 7.5, at 23 °C (λ_{ex} = 340 nm, λ_{em} = 540 nm).

Table 3: Steady-State Kinetic Parameters for the Hydrolysis of Dns-Gly-Phe-Ala by Thermolysin Measured by Different Stopped-Flow Fluorescence Analyses

method	λ_{ex} (nm)	k_{cat} (s^{-1})	K_m (mM)	$k_{cat}/K_M \times 10^{-4}$ (M^{-1} s^{-1})
I	340	1.6	0.080	2.0
II	340	1.6	0.081	2.0
II	245	1.6	0.080	2.0
III	340	1.4	0.090	1.6
IV	280	1.7	0.087	2.0
V	280	1.4	0.083	1.7

II are listed in Table 3. These three methods give essentially the same results with k_{cat} = 1.6 s^{-1} and K_M = 0.080 mM. The K_M value is 7-fold smaller than the IC_{50} value for either of the products, Phe-Ala and Dns-Gly (Table 2). Thus, there should be essentially no product inhibition during the first 10% of the reaction.

A second approach to the measurement of the kinetic parameters from F^{SP} studies is based on eq 7 which analyzes the m_t values from a single trace obtained at a high [S_o]. A plot of $-(F_o[E_o])/([S_o]_t m_t)$ vs $1/[S_o]_t$ obtained from the fluorescence trace of the hydrolysis of 0.24 mM Dns-Gly-Phe-Ala by thermolysin is shown in Figure 5B. The plot is

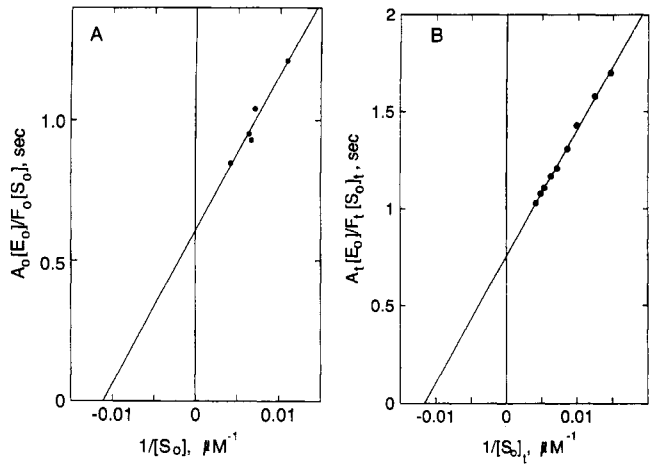

FIGURE 6: Plots of (A) $A_o[E_o]/F_o[S_o]$ vs $1/[S_o]$ and (B) $A_t[E_o]/F_t[S_o]_t$ vs $1/[S_o]_t$ for the hydrolysis of Dns-Gly-Phe-Ala by thermolysin in 50 mM Hepes, 10 mM $CaCl_2$, 1 M NaCl, pH 7.5, at 23 °C (λ_{ex} = 280, λ_{em} = 340 nm).

linear and gives k_{cat} = 1.4 s^{-1} and K_M = 0.090 mM (Table 3, method III), in reasonable agreement with methods I and II. The slightly smaller value of k_{cat}/K_M may be due to a small amount of product inhibition.

Determination of Kinetic Parameters from F^{ET} Measurements. Another approach to evaluating k_{cat} and K_M is from F^{ET} experiments in which F_o and A_o are measured from a series of traces obtained at different substrate concentrations using eq 4 to analyze the data (method IV). Thus, the hydrolysis of Dns-Gly-Phe-Ala by thermolysin was studied by monitoring the changes in Trp fluorescence at 340 nm on excitation at 280 nm. The dansyl fluorescence change was not studied since it contains a contribution from the F^{SP} term (Figure 4A). Values of A_o have been obtained by integration of the area between the trace and the base line. The data have been analyzed as a double-reciprocal plot of $(A_o[E_o])/(F_o[S_o])$ vs $1/[S_o]$ as shown in Figure 6A. The values of k_{cat} = 1.7 s^{-1} and K_M = 0.087 mM obtained from this plot agree well with those obtained from the F^{SP} experiments. Method V involves the evaluation of these kinetic parameters from the trace obtained at a substrate concentration of 0.20 mM by using eq 5. The plot of $(A_t[E_o])/(F_t[S_o]_t)$ vs $1/[S_o]_t$ shown in Figure 6B gives k_{cat} = 1.4 s^{-1} and K_M = 0.083 mM, in good agreement with the other methods.

Kinetic Parameters for All Substrates. The K_M and k_{cat} values for the hydrolysis of all 10 dansyl substrates have been determined from F^{SP} experiments by monitoring the dansyl fluorescence (λ_{ex} = 340 nm, λ_{em} = 540 nm) during the first 10% of hydrolysis and analyzing the traces by method II (Table 4). Because of the poor solubilities of Dns-Gly-Phe-Phe and Dns-Gly-Leu-Phe, 5% v/v DMSO was added to the buffer, while 30% v/v was used for Phe-Leu-Ala-$NH(CH_2)_2NH$-Dns. The total fluorescence change for the reaction (F_o) was measured at every substrate concentration to avoid errors due to self-absorption at high $[S_o]$. The kinetic parameters for these substrates vary widely. The k_{cat}/K_M values for the first six dansyl tripeptides listed in Table 4 vary only approximately 10-fold. Dns-Gly-Phe-Ala is notable for its low K_M value (0.080 mM), a feature that should greatly facilitate detailed kinetic studies. Dns-Gly-Leu-Gly is a much worse substrate than the other dansyl tripeptides. Dns-Ala-Ala-Phe-Ala is the best substrate studied with both a high k_{cat} (240 s^{-1}) and a low K_M (0.20 mM). In contrast, Dns-Gly-Gly-Leu-Gly is much worse, even though it is also a tetrapeptide. Phe-Leu-Ala-$NH(CH_2)_2NH$-Dns is a good substrate even in the presence of 30% v/v DMSO, but its poor solubility limits its utility for more detailed studies.

Table 4: Kinetic Parameters for the Hydrolysis of Dansyl Substrates by Thermolysin[a]

substrate	slope of F_o vs $[E_o]$[d]	K_M (mM)	k_{cat} (s^{-1})	$k_{cat}/K_M \times 10^{-3}$ (mM^{-1} s^{-1})
Dns-Gly-Phe-Ala	17	0.080	1.6	20
Dns-Gly-Phe-Phe[b]	5.6	0.30	1.8	6.0
Dns-Gly-Leu-Phe[b]	1.9	0.69	2.2	3.2
Dns-Gly-Phe-Gly	1.4	0.77	2.0	2.6
Dns-Ala-Phe-Ala	0.98	0.91	20	22
Dns-Ala-Leu-Ala	0	2.0	38	19
Dns-Gly-Leu-Gly	0	5.0	0.042	0.0084
Dns-Gly-Gly-Leu-Gly	0	13	1.3	0.10
Dns-Ala-Ala-Phe-Ala	0.67	0.20	240	1200
Phe-Leu-Ala-$NH(CH_2)_2NH$-Dns[c]	0	0.62	250	400

[a] All reactions were carried out in 50 mM Hepes, 10 mM $CaCl_2$, 1 M NaCl, pH 7.5, at 23 °C. [b] Assays carried out in 5% v/v DMSO. [c] Assay carried out in 30% v/v DMSO. [d] Taken from Figure 4B.

DISCUSSION

A number of recent kinetic studies with thermolysin have shown that the behavior of the enzyme is markedly dependent on the structure of the substrate as well as the conditions (pH and ionic strength, etc.) under which the reactions are examined. Morgan and Fruton (1978) studied the hydrolysis of a series of peptides with the structure X-Phe-Leu-Ala, where X = Cbz, Cbz-Gly, Z-Gly-Gly, Mns, Mns-Gly, or Mns-Gly-Gly. The specificity of thermolysin for the substrate series containing N-terminal Cbz at pH 7.5 was reported to differ from that for the Mns series at pH 8.5. These workers were also able to resolve a relaxation for Mns-Phe-Leu-Ala that they attributed to substrate binding. The rate-limiting step was proposed to be the decomposition of the first detectable ES complex. In contrast, Fukuda and Kunugi (1984) have studied the hydrolysis of FA-Gly-Phe-Ala and FA-Gly-Leu-Ala by thermolysin. Near pH 6, a pre-steady-state burst was observed that was interpreted as evidence for the existence of two ES_i.

Izquierdo and Stein (1990) have recently studied a variety of substrates including FA-Gly-X-NH_2, where X = Ala, Val, Phe, and Leu, and FA-Gly-Leu-Ala. Their analysis of the temperature dependence of the steady-state kinetic parameters supports a mechanism with consecutive, partially rate-limiting steps producing distinct ES_i. The two steps have differential temperature sensitivities that yield nonlinear Eyring plots for the overall reactions. Importantly, these authors also noted a marked substrate dependence on the relative rates of the two steps with the result that ES_2 accumulates for FA-Gly-Leu-Ala but ES_1 accumulates for FA-Gly-Leu-NH_2. The responses of the kinetic parameters for thermolysin to variations in salt concentration have also been reported to vary widely for different substrates (Holmquist & Vallee, 1976; Morgan & Fruton, 1978; Li, 1982; Inouye, 1992).

These observations emphasize the need to be able to study both the pre-steady-state and steady-state kinetics of a series of peptide substrates with variable lengths and sequences under a variety of conditions. Accordingly, this study has explored the utility of the stopped-flow fluorescence approach pioneered by Lobb and Auld (1984) to study the hydrolysis of a series of 10 dansyl substrates by thermolysin by monitoring both F^{SP}- and F^{ET}-based fluorescence changes. These studies reveal the utility of each substrate for different types of studies. All 10 substrates give F^{SP} changes that allow their study by this technique. The most sensitive are Dns-Gly-Leu-Phe and Phe-

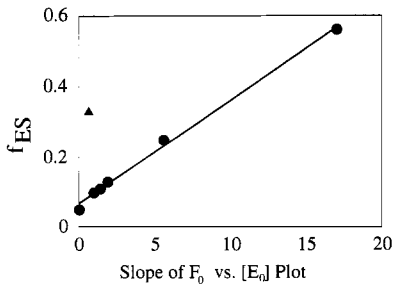

FIGURE 7: Plot of the fractional saturation of enzyme, f_{ES}, vs the slope of the F_o vs $[E_o]$ plots listed in Table 4. The ● symbols are for the dansyl-tripeptides, while ▲ is for Dns-Ala-Ala-Phe-Ala.

Leu-Ala-NH$(CH_2)_2$NH-Dns (λ_{ex} = 340 nm) because of the large F_o values produced on hydrolysis (Table 1). The poor solubility of Phe-Leu-Ala-NH$(CH_2)_2$NH-Dns, however, severely limits its utility for more detailed kinetic studies.

The feasibility of carrying out F^{ET} studies on these substrates is more limited. Only six of the 10 substrates produce a detectable quenching of Trp fluorescence in thermolysin during the reactions. The magnitude of this quenching is reflected by the slope of the F_o vs $[E_o]$ plot for each substrate (Figure 3B, Table 4). There are two possible reasons for the variation in the amount of quenching between substrates. The first is that different K_M values for the substrates relative to the $[S_o]$ used results in different fractions (f_{ES}) of E that are converted into ES. The second is that the ET efficiencies of ES are different for different substrates. A plot of f_{ES} calculated as $[S_o]/([S_o] + K_M)$ vs the slope of the F_o vs $[E_o]$ plots for these substrates is shown in Figure 7. The points for the five dansyl tripeptides fall on the same line, indicating that the extent of Trp quenching is accounted for satisfactorily by the fractional saturation of the enzyme with substrate. This implies that all of these substrates produce an ES in which the quenching efficiency is very similar. Molecular modeling studies show that the dansyl group of these substrates is very close to the Trp-115 residue in the active site of thermolysin (Yang et al., 1994), and there is little doubt that the emission from this residue is quenched in the complexes with these substrates.

In contrast, the amount of Trp quenching for the tetrapeptide Dns-Ala-Ala-Phe-Ala is much lower at the same degree of fractional saturation. Modeling studies confirm that the Ala residue in subsite P_2 pushes the dansyl group further away from Trp-115 than in the tripeptides, accounting for the lower quenching efficiency. The efficiencies of Trp quenching by dansyl substrates are known from other studies to be markedly dependent on the enzyme and the substrate. For example, the quenching of enzyme Trp emission during the hydrolysis of Dns-Gly-Tyr by carboxypeptidase A is very efficient, while there is virtually none observed during the hydrolysis of Leu-Gly-NHNH-Dns by leucine aminopeptidase (Lin & Van Wart, 1988). For thermolysin, this efficiency is markedly substrate dependent and can be rationalized by considering the magnitude of the K_M values for the reactions and the distance of the dansyl group from Trp-115. This underscores the importance of studying the origin of the observed fluorescence changes before attempting mathematical analyses.

In choosing substrates for more detailed studies of thermolysin, several criteria have been considered. These include the sensitivity of the fluorescence response for either F^{SP} or F^{ET} studies, the specificity of the substrate as reflected by its k_{cat}/K_M value, and the solubility of the substrate relative to its K_M value. In the studies described in the following paper on the effect of anions on thermolysin-catalyzed reactions (Yang et al., 1994), Dns-Gly-Phe-Ala has been chosen for study because of its low K_M value and favorable ET while Dns-Ala-Ala-Phe-Ala has been chosen because of its high k_{cat}/K_M.

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