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Determination of enzyme mechanisms by radiationless energy transfer kinetics

(fluorescence/proteolytic enzymes/stopped-flow kinetics/metalloenzymes/transient enzyme–substrate complexes)

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ABSTRACT **Rigorous definition of the elementary steps of an enzymatic reaction requires visualization of transient enzyme–substrate (ES) complexes. Measurement of radiationless energy transfer (RET) between enzyme tryptophan residues and a fluorescent dansyl (5-dimethylaminonaphthalene-1-sulfonyl) substrate provides a sensitive means to observe ES complexes directly. Analysis of the rate of formation and breakdown of ES complexes by RET can serve as the basis of a rapid kinetic approach to enzyme mechanisms. Both pre-steady-state and steady-state kinetics can be performed in the same RET experiment. Analysis at steady state precisely determines k_{cat} and K_m values by multiple means. Analysis at pre-steady state determines the number of intermediates, the type of reaction mechanism, and all the individual binding and rate constants. Chymotrypsin was chosen as a standard of reference for RET kinetics because extensive investigations have established both the existence of transient intermediates in the course of its catalytic process and the range of values to be expected for pertinent kinetic constants. As predicted, RET kinetics readily detects the two known intermediates in the α-chymotrypsin-catalyzed hydrolysis of specific ester substrates. The results are both qualitatively and quantitatively in accord with data derived for this enzyme from classical kinetics. Hence, this experimental study both validates and demonstrates the theoretical advantages and potential of RET kinetics. The generality of the approach has been investigated by synthesizing a family of dansyl-labeled substrates designed to meet the specificity requirements of a number of metallo- and nonmetallo- exo- and endopeptidases. In all cases, the ES complex is observed readily at micromolar or lower concentrations of enzyme under stopped-flow conditions. The success of the RET kinetic approach on proteolytic enzymes shows its broad utility.**

Transient enzyme–substrate (ES) complexes are the characteristic feature of enzyme catalysis, and their direct observation simplifies kinetic analyses and provides a rigorous definition of enzyme mechanisms (1). However, their detection has proven difficult, both due to the extremely rapid rates of enzyme-catalyzed reactions and the lack of suitable means to visualize intermediates directly. The stopped-flow technique has greatly aided in the solution of these problems by allowing mixing of enzyme and substrate within a few milliseconds (2). However, even then it has often become necessary to infer the number and nature of ES complexes—e.g., from the kinetics of the release of chromophoric products (3–6) or from substrate displacement of absorbing or fluorescing inhibitor complexes (7, 8). Thus, direct detection of the ES complex by means of spectral systems that are compatible with stopped-flow instrumentation remains problematic.

Our previous studies on carboxypeptidase A (1, 9–12) show that measurement of radiationless energy transfer (RET) between enzyme tryptophanyl residues and a fluorescent dansyl (5-dimethylaminonaphthalene-1-sulfonyl) substrate provides a sensitive means to observe ES complexes directly. Thus, RET might serve as the basis of a rapid, kinetic approach to enzyme mechanisms. Since RET visualizes the ES complex directly, it should be possible to determine the minimal number of intermediates, the type of reaction mechanism, and the individual rate and binding constants. In order to test the potential of RET kinetics, an enzyme known to possess more than one intermediate was required. The serine protease α-chymotrypsin was chosen for this purpose because intensive investigations over the past 25 years have established the existence of intermediates in its catalysis and the kinetics of the individual steps (refs. 13–16 and refs. therein). The results of RET and of classical kinetics on chymotrypsin are both qualitatively and quantitatively in agreement. The generality of the approach has been validated by means of studies using a family of fluorescent dansyl substrates specific for a number of metallo- and nonmetallo- exo- and endopeptidases.

MATERIALS AND METHODS

α-Chymotrypsin, trypsin, and *Bacillus cereus* "Microprotease" were obtained from Worthington, carboxypeptidase B from Sigma, and thermolysin from Calbiochem. Yeast carboxypeptidase was the gift of J. Johansen. Pronase carboxypeptidase and Pronase neutral protease were gifts of T. Bazzone and K. Breddam. All other chemicals were of reagent grade. The *B. cereus* protease was purified by the method of Holmquist (17).

Substrates were synthesized by standard methods, as described elsewhere (10, 12, 18). Dns-Gly-L-OPhe-Ala and Dns-$(Gly)_2$-L-Arg were the gifts of B. Holmquist and S. Pastan. When necessary, the substrate was purified further by dry column chromatography or a Waters Associates high-pressure liquid chromatograph equipped with a 0.7 × 30 cm μ BONDAPAK C_{18} column. Thin-layer chromatography on silica gel, as described (19), or on micropolyamide (Schleicher & Schuell) with benzene/glacial acetic acid, 9:1 (vol/vol) or water/formic acid, 200:3 (vol/vol) showed substrates to be pure by this criterion and enzymatic cleavage to occur only at one bond.

The formation and breakdown of intermediates were measured by stopped-flow fluorescence on a Durrum–Gibson instrument and the data were analyzed as described (11, 12).

Trypsin was assayed in 5 mM $CaCl_2$/0.25 M KCl/25 mM acetate, at pH 5.5 and 20°C. Pronase carboxypeptidase, Pronase neutral protease, and carboxypeptidase B were assayed in 10 mM $CaCl_2$/0.1 M NaCl/25 mM Tris·HCl, pH 8.0, at 20°C.

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Abbreviations: ES complex, enzyme–substrate complex; RET, radiationless energy transfer; Dns or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

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Unless otherwise noted, thermolysin and *B. cereus* protease were assayed in 10 mM $CaCl_2$/0.1 M NaCl/25 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.5 at 20°C. Yeast carboxypeptidase was assayed in 0.1 M NaCl/25 mM Mes at pH 5.5 and 20°C.

RESULTS

Families of fluorescently labeled oligopeptide and depsipeptide substrates have been synthesized for RET kinetic studies of proteolytic enzymes. They have the general structure: Dns-$(Gly)_n$-X-Z. The fluorescent dansyl group, Dns, is always the NH_2-terminal blocking group, separated from the scissile bond, X-Z, by a glycine chain of n residues. Substitutions at positions X and Z satisfy enzyme specificity requirements. X is an amino acid, usually phenylalanine, glycine, or arginine. The choice of Z results in a wide variety of peptides and esters. For exopeptidases Z can be an amino acid such as Phe, or its direct ester analog, phenyllactate OPhe. For endopeptidases Z can be a peptide such as phenylalanylalanine, Phe-Ala, or its direct ester analog phenyllactylalanine, OPheAla. Substitution of NH_2 or methoxide, OMe, yields amide and methyl ester substrates.

The interaction between each enzyme and its respective substrate results in a characteristic oscilloscope trace reflecting the formation and breakdown of ES complexes, exemplified in Fig. 1*A* by the hydrolysis of 50 μM Dns-$(Gly)_2$-L-PheOMe by 1.85 μM α-chymotrypsin. The rapid increase in dansyl fluorescence to a maximum value signals the rapid attainment of the steady-state concentration of ES complexes. The slower decay of the signal reflects the reduction in enzyme-bound substrate as the concentration of substrate is reduced by hydrolysis. A complementary pattern is observed when the quenching of enzyme tryptophan fluorescence by bound substrate is measured. Fig. 1*B* schematically illustrates the general form of the oscilloscope trace which is characterized by two parameters, the maximal fluorescence intensity, F_{max}, and the total area, A_0, beneath the curve. Our previous studies on carboxypeptidase A (1, 11) have shown that, for a simple one-intermediate mechanism, these parameters are related to k_{cat} and K_m by

$$\frac{F_{max}[S_0]}{A_0[E_T]} = \frac{V}{[E_T]} = \frac{k_{cat}[S]}{K_m + [S]}, \qquad [1]$$

in which $[E_T]$ is the total enzyme concentration, V is the velocity at an initial substrate concentration, $[S_0]$, and $[S]$ is the concentration of free substrate. Under steady-state conditions, $[S_0] \gg [E_T]$, the concentration of unbound substrate, $[S]$, is approximated by $[S_0]$. We have found that under steady-state conditions, Eq. 1 may also be applied to enzymes with more than one intermediate, extending the usefulness and applicability of the RET approach. Thus, measurement of F_{max} and A_0 values at different initial substrate concentrations, $[S_0]$, allows the determination of k_{cat} and K_m. Stopped-flow fluorescence assays for the binding and hydrolysis of Dns-$(Gly)_2$-L-PheOMe by chymotrypsin at pH 6.0 and 20°C yield a K_m value of 44 μM and a k_{cat} value of 23 sec^{-1} (Fig. 2), in excellent agreement with those obtained by conventional initial rate assays (Table 1).

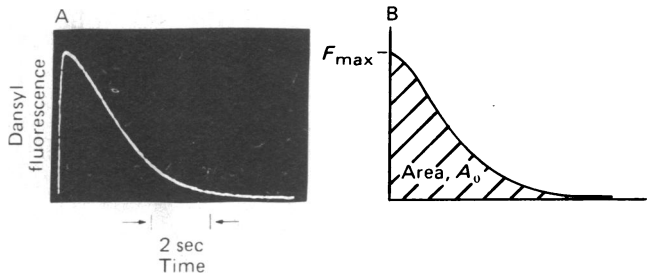

FIG. 1. (*A*) Binding and hydrolysis of 50 μM Dns-$(Gly)_2$-L-PheOMe by 1.85 μM chymotrypsin in 0.25 M KCl/5 mM $CaCl_2$/25 mM Mes, pH 6.0, at 20°C. (*B*) Schematic illustration of the oscilloscope trace defining the maximal fluorescence intensity, F_{max}, and the total area, A_0. $(F_{max}[S_0])/(A_0[E_T]) = V/[E_T] = (k_{cat}[S])/(K_m + [S])$.

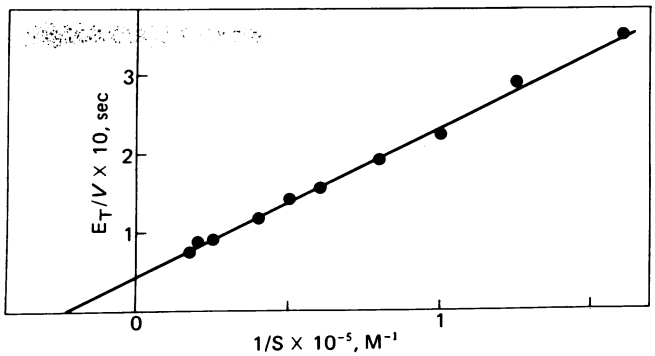

FIG. 2. Determination of the kinetic parameters k_{cat} and K_m for Dns-$(Gly)_2$-L-PheOMe hydrolysis by stopped-flow fluorescence. The rate, V, is determined from the ratio $F_{max}[S_0]/A_0$ for each substrate concentration. Conditions of assay: 1.85 μM α-chymotrypsin, 0.25 M KCl, 5 mM $CaCl_2$, pH 6.0, at 20°C.

RET kinetics also allows the evaluation of k_{cat} and K_m from a single oscilloscope trace because the observed trace (e.g., Fig. 1) contains all the information required to determine the rate of the reaction at any time t and the substrate concentration, S_t, at that time. The parameters k_{cat} and K_m can be determined from an equation analogous to Eq. 1:

$$\frac{F_t[S]_t}{A_t[E_T]} = \frac{k_{cat}[S]_t}{K_m + [S]_t}, \qquad [2]$$

in which F_t is the fluorescence intensity at time t and A_t is the area beneath the curve between times t and ∞. The detailed method of analysis will be reported. Importantly, the results are in excellent agreement with those from other methods (Table 1).

While the analysis of the reaction time course after attainment of the steady state allows the determination of k_{cat} and K_m by multiple means, the observation and analysis of the pre-steady-state time interval gives direct information on the number of reaction intermediates and their rates of formation and breakdown. Fig. 3*A* shows the interaction between chymotrypsin and the specific ester substrate Dns-$(Gly)_2$-L-PheOMe during the pre-steady-state time interval. As illustrated schematically in Fig. 3*B*, the steady-state level of F_{max} is attained in two steps, indicating the presence of two intermediates. During mixing an increase in signal intensity, F_1, has occurred, indicating the formation of the first intermediate in <3

Table 1. Chymotrypsin-catalyzed hydrolysis of Dns-$(Gly)_2$-L-PheOMe: Comparison of kinetic parameters from initial rate and stopped-flow assays

Assay	k_{cat}, sec^{-1}	K_m, μM
Initial rate	24	45
Stopped-flow		
Variation of substrate	23	44
Single substrate	23	45
Calculated from pre-steady state	22	46

Assays, at pH 6.0 and 20°C, contained 0.25 M KCl and 5 mM $CaCl_2$. The pH was kept constant with 25 mM Mes in the stopped-flow assay and by a pH stat for the initial rate assay.

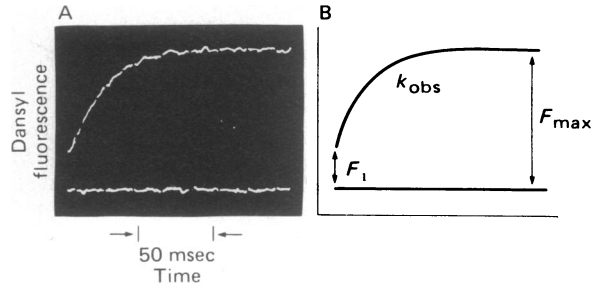

FIG. 3. (*A*) Interaction between 50 μM Dns-(Gly)$_2$-L-PheOMe and 1.85 μM chymotrypsin during the pre-steady-state time interval in 0.25 M KCl/5 mM $CaCl_2$/25 mM Mes, at pH 6.0 and 20°C. (*B*) Schematic illustration of the oscilloscope trace defining the initial fluorescence intensity, F_1, and the exponential increase in signal intensity to F_{max} from which the first order rate constant, k_{obs}, is derived.

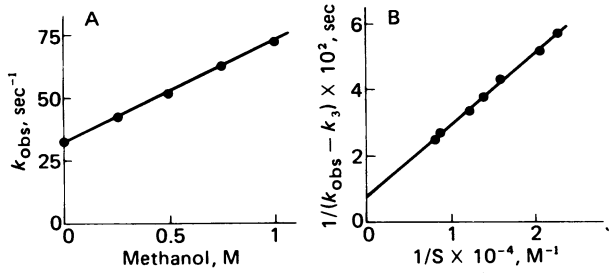

FIG. 4. (*A*) Dependence of the first-order rate constant, k_{obs}, on methanol concentration. (*B*) Determination of the kinetic parameters k_2 and K_s. Linear regression analysis of the plot of $1/(k_{obs} - k_3)$ against $1/[S]$ yields k_2 and K_s from the y and minus x intercept values (Eq. 5). See caption of Fig. 2 for conditions of assay.

msec. An exponential increase in signal intensity to F_{max}, characterized by the rate constant k_{obs}, reflects formation of a second intermediate.

These results are consistent with the accepted acyl intermediate mechanism for ester hydrolysis by α-chymotrypsin (refs. 13–16 and refs. therein).

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \underset{k_{-2}[P_1]}{\overset{k_2}{\rightleftharpoons}} EA \xrightarrow{k_3} E + P_2,$$

in which ES represents the reversible Michaelis complex and EA the acyl intermediate, formed concomitantly with loss of the first product P_1. Thus, the initial increase in intensity to F_1 reflects the formation of ES, while the exponential increase to F_{max} reflects the interconversion of ES and EA. For the above mechanism, this increase in intensity should be exponential, with a first-order rate constant, k_{obs}, described by the equation:

$$k_{obs} = k_3 + k_{-2}[P_1] + \frac{k_2[S]}{K_s + [S]}. \quad [3]$$

The concentration of the first product, P_1, produced during the pre-steady-state time interval is negligible under the conditions necessary for these studies. For this time interval the concentration of S is essentially constant and equal to S_0 since $[E_T] \ll [S_0]$. These restraints greatly simplify the analysis and the dissection of k_{obs} into its component parts. Thus, measurement of the rate of equilibration of ES and EA at varying concentrations of either $[S_0]$ or $[P_1]$, where $[P_1]$ refers to the concentration of added product, allows determination of the rate and equilibrium constants for the reaction mechanism.

At a fixed substrate concentration Eq. 3 can be simplified to:

$$k_{obs} = k' + k_{-2}[P_1], \quad [4]$$

in which k' is a constant. This equation predicts a linear dependence of k_{obs} on the concentration of P_1, methanol for the substrate Dns-(Gly)$_2$-L-PheOMe. Excellent first-order kinetics are observed both in the presence and absence of methanol, with an increased rate constant in the presence of the nucleophile. Further, as predicted from Eq. 4, a linear dependence of k_{obs} on methanol concentration is seen (Fig. 4*A*). The slope of the line determines k_{-2}, which is 40 sec^{-1}M^{-1}.

The acyl intermediate mechanism also predicts that addition of the product, P_1, will not affect the initial signal intensity, F_1, since F_1 reflects the initial concentration of the Michaelis complex ES, which depends only upon K_s and the concentration of E and S. Indeed, addition of methanol in concentrations up to $\approx$1 M (4%, vol/vol) negligibly affects F_1. In contrast, the maximal fluorescence, F_{max}, depends upon the steady-state concentrations of ES and EA, and because the presence of added product will affect these concentrations, the value of F_{max} should be affected by added methanol. As expected, increasing concentrations of methanol decrease values of F_{max}. These results are not due to a general solvent effect because the presence of 5% dimethyl sulfoxide or 5% acetonitrile negligibly affects F_1, F_{max}, or k_{obs}.

In the absence of added P_1, Eq. 3 can be simplified to:

$$k_{obs} = k_3 + \frac{k_2}{(1 + K_s)/[S]}. \quad [5]$$

This equation predicts that at concentrations of $S \ll K_s$, k_{obs} will approach a lower limit of k_3. Using a value for k_3 of 27 sec^{-1} estimated in this manner, k_2 and K_s may be determined from a linear transformation of Eq. 5, plotting $1/(k_{obs} - k_3)$ against $1/[S]$, as shown in Fig. 4*B*, which allows the evaluation of K_s (0.25 mM) and k_2 (120 sec^{-1}). Thus, all the individual rate and equilibrium constants for the chymotrypsin mechanism are determined readily, and may be summarized as follows:

$$E + S \underset{}{\overset{K_s = 0.25\ mM}{\rightleftharpoons}} ES \underset{40\ sec^{-1}M^{-1}}{\overset{120\ sec^{-1}}{\rightleftharpoons}} EA + P_1 \xrightarrow{27\ sec^{-1}} E + P_2.$$

The evaluation of the individual binding and rate constants permits an independent determination of the conventional kinetic parameters K_m and k_{cat}. This is particularly useful since a stringent test of a mechanism lies in the equivalence of the values of K_m and k_{cat} calculated from the binding and rate constants independently determined to those observed under steady-state conditions. The calculated values of $k_{cat} = k_2k_3/(k_2 + k_3)$ and $K_m = K_sk_2/(k_2 + k_3)$ are in excellent agreement with those evaluated under steady-state conditions both by initial rate and stopped-flow analysis (Table 1).

We have initiated investigations of the interaction between several other proteolytic enzymes and suitable *N*-dansylated specific ester and peptide substrates. The ease of RET analysis at a single substrate concentration (Eq. 2) allows the rapid screening of a variety of enzyme–substrate systems. The determination of the kinetic parameters k_{cat} and K_m at a single substrate concentration permits the evaluation of those systems suitable for further study. The results for a number of metallo- and nonmetallo- endo- and exopeptidases are listed in Table 2. In each case substrates are bound tightly and hydrolyzed rapidly and are thus suitable for further RET analysis under steady-state conditions. More importantly, a pre-steady-state time interval is readily observed for a number of these enzymes. Fig. 5 shows the pre-steady-state time interval for the yeast glycoenzyme, carboxypeptidase Y, and the bacterial zinc en-

Table 2. Kinetic parameters from stopped-flow assays at a single substrate concentration*

Enzyme	E_T, μM	Substrate	S_0, μM	k_{cat}, sec^{-1}	K_m, μM
Carboxypeptidase Y	0.1	Dns-$(Gly)_3$-L-OPhe	20	16	4
	0.5	Dns-Phe-L-Ala	50	15	16
Carboxypeptidase B	1.0	Dns-$(Gly)_2$-L-Arg	50	71	200
Thermolysin	2.0	Dns-Gly-L-OPhe-Ala	100	7	50
Pronase carboxypeptidase	1.0	Dns-$(Gly)_2$-L-OLeu	50	200	75
	1.0	Dns-$(Gly)_2$-L-OPhe	50	200	130
Pronase neutral protease	1.6	Dns-Gly-L-OPhe-Ala	50	65	150
Trypsin	2.8	Dns-$(Gly)_2$-L-ArgOMe	50	12	30
B. cereus protease	1.0	Dns-Gly-L-OPhe-Ala	100	18	87

* Conditions of assays are given in *Materials and Methods*.

zyme, thermolysin, acting on Dns-$(Gly)_3$-L-OPhe and Dns-Gly-L-OPheAla, respectively. For both enzymes, exponential changes in dansyl fluorescence are observed readily at micromolar enzyme concentrations or less.

DISCUSSION

Dynamic information defining the nature, number, and concentration of all reaction intermediates that occur during catalysis is a prerequisite for the description of the microscopic detail of enzyme action. At present, such information is generally obtained through two different kinetic approaches. One is the increasingly sophisticated analysis of factors affecting the rate of appearance of products or disappearance of reactants under steady-state conditions and, hence, the form of the overall rate equation. The other approach is the result of the development of increasingly complex techniques to monitor transient intermediates under pre-steady-state conditions.

It is a major advantage of the first approach that the concentration of enzyme is orders of magnitude less than that of substrate, facilitating mathematical analysis, providing economy of enzyme, and allowing the use of rapidly turned over substrates. It is a major disadvantage that the number and nature of intermediates can then only be inferred.

In principle, the second approach has the potential that the number and nature of intermediates can often be determined directly. Yet, in practice, several circumstances have greatly restricted the realization of these aims. Thus, an insensitive spectral readout may require milligram to gram quantities of enzyme, limiting the applicability of the approach to only a few enzymes. At enzyme concentrations approaching or exceeding those of substrate, mathematical analysis becomes more difficult. In addition, if the spectral systems are incompatible with rapid mixing techniques, slowly turned over substrates must be used and then reaction pathways may differ from those of substrates that are turned over rapidly.

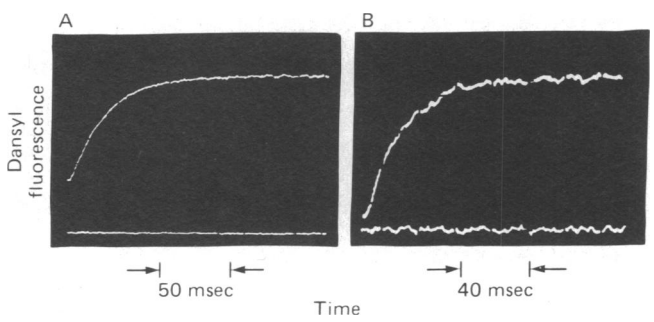

FIG. 5. Pre-steady-state variations in substrate dansyl fluorescence for: (*A*) 1.0 μM yeast carboxypeptidase and 20 μM Dns-$(Gly)_3$-L-OPhe in 0.1 M NaCl/25 mM acetate, pH 4.5 at 20°C; (*B*) 2 μM thermolysin and 45 μM Dns-Gly-L-OPhe-Ala in 0.1 M NaCl/10 mM $CaCl_2$/50 mM Mes, pH 6.0, at 15°C.

The direct observations of ES complexes by means of RET effectively combines the major advantages of these two approaches, allowing both the steady-state (Figs. 1 and 2) and the pre-steady-state phases (Figs. 3–5) to be observed readily under stopped-flow conditions. A number of reasons have led us to choose RET as the means to observe ES complexes. The most important are: (*i*) fluorescent events are inherently much faster than catalytic events; (*ii*) fluorescence can be induced through the interaction of a donor–acceptor pair; (*iii*) Forster's RET theory predicts optimal energy donor–acceptor pairs; (*iv*) tyrosyl and tryptophanyl residues of enzymes can serve as fluorescent donors; (*v*) fluorescent acceptors are incorporated readily into the substrate (e.g., as the NH_2-terminal blocking group for exo- and endopeptidases); and (*vi*) the inherent sensitivity of fluorescence allows the use of micromolar or lower enzyme concentrations.

The serine protease chymotrypsin was chosen for the first detailed RET kinetic study because of the intensive investigation of this enzyme over the past 25 years (refs. 13–16 and refs. therein). Hartly and Kilby in 1954 (3) first suggested that the hydrolysis by α-chymotrypsin of nonspecific ester substrates such as *p*-nitrophenyl acetate follows an acyl intermediate mechanism. Extensive kinetic and spectrophotometric studies using this and other chromophoric nonspecific esters confirmed this mechanism. Since then, a number of investigations of specific methyl and ethyl esters, both by steady-state kinetics [e.g., in the presence of added nucleophiles (20)] and pre-steady-state kinetics, such as proflavin displacement (21), have also confirmed this mechanism indirectly. Moreover, x-ray crystallographic studies using nonspecific substrates (22, 23) have demonstrated the existence of the acyl intermediate directly.

RET kinetics allow the direct observation of two reaction intermediates during the hydrolysis of the rapidly hydrolyzed specific methyl ester, Dns-$(Gly)_2$-L-PheOMe, by chymotrypsin. Addition of the product, methanol, affects the rate of interconversion of intermediates observed in the pre-steady-state time interval and identifies the second intermediate as the acyl enzyme. Detailed analysis of the changes in the stopped-flow trace in the presence of substrate and product allows the evaluation of all the individual binding and rate constants. The deacylation step is rate limiting, in agreement with numerous studies of ester hydrolysis by this enzyme (e.g., refs. 20 and 21). Further, the ratio of methanolysis to hydrolysis is 1.5, closely similar to the value of 1.6 obtained indirectly for the related substrate Ac-L-PheOMe (20). In short, the individual binding and rate constants obtained are both self-consistent and in the range of values expected for chymotryptic methyl ester hydrolysis.

While the use of chymotrypsin as a standard of reference validates the RET approach both qualitatively and quantita-

tively, our exploratory investigations of a number of other proteolytic enzymes indicate that RET kinetic analysis should be widely applicable. A number of metallo- and nonmetallo-exo- and endopeptidases acting on *N*-dansylated ester and peptide substrates designed to meet their specificity requirements were chosen for this survey. In most cases ES complex formation and breakdown can be observed readily at micromolar or lower concentrations of the enzyme. The capacity of RET kinetics to evaluate k_{cat} and K_m at a single substrate concentration permits rapid delineation of substrates optimal for detailed mechanistic studies. The results of these analyses indicate that a number of these peptide and ester substrates are suitable for further kinetic studies by RET (Table 2 and Fig. 5). Detailed investigation of the pre-steady-state time interval for these enzymes, in the manner here described for a α-chymotrypsin, should result in the identification of the number of reaction intermediates, the formulation of reaction mechanisms, and determination of the individual binding and rate constants.

The RET kinetic approach can be applied to any enyzme whose substrate can be labeled with a suitable fluorescent probe. Further, it is not restricted to a single enzymatic reaction but should be applicable to multienzyme chain reactions or to other systems such as those seen in transport, which contain intermediate species that are in a continuous state of flux.

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