Stopped-Flow Radiationless Energy Transfer Kinetics: Direct Observation of Enzyme–Substrate Complexes at Steady State†

Roy R. Lobb and David S. Auld*

ABSTRACT: Measurement of radiationless energy transfer (RET) between enzyme tryptophan residues and a fluorescent dansyl (5-dimethylaminonaphthalene-1-sulfonyl) substrate under stopped-flow conditions forms the basis of a rapid and sensitive kinetic approach to delineation of enzyme mechanisms. Both the pre steady state and steady state can be studied in one experiment. The ES complexes of even rapidly turned over dansyl substrates are observed directly at enzyme concentrations of 10^{-8}–10^{-6} M. If $[E_T] \ll K_m$, a steady state of ES complex formation and breakdown can be achieved and maintained even though the reaction is completed in a few seconds. RET kinetic analysis under stopped-flow steady-state conditions both simplifies and supplements conventional initial rate kinetic studies as illustrated here with bovine and yeast carboxypeptidases and α-chymotrypsin acting on Dns-(Gly)$_3$-L-OPhe and Dns-(Gly)$_2$-L-PheOMe, respectively. Since both the concentration and rate of breakdown of intermediates are observed, the kinetic parameters k_{cat} and K_m can be determined precisely by multiple means. The capability of observing both steady-state and pre-steady-state RET kinetics in the same experiment greatly reduces errors in quantitative analysis, allowing a more rigorous definition of enzyme mechanisms.

The measurement of initial rates under steady-state conditions is the conventional approach to enzyme kinetics. Low concentrations of enzyme, i.e., 10^{-6}–10^{-9} M, are frequently employed so that the overall rate of conversion of substrate to product is slow enough to be measured conveniently. Under such conditions all enzyme–substrate species, ES, are in the steady state and it is relatively easy to determine the kinetic parameters k_{cat} and K_m (Segal, 1975). These parameters can be related mathematically to the steady-state concentration of ES complexes and their rate of breakdown. Mechanistic information can then be obtained from the variation of k_{cat} and K_m with, e.g., pH, temperature, or isotopic substitution, and the mode of action of reversible inhibitors can be studied (Cleland, 1977).

It is a major advantage of this approach that the concentration of enzyme is orders of magnitude less than that of the substrate, thus facilitating the mathematical analysis, providing economy of enzyme, and allowing the use of rapidly turned over substrates.

Our previous studies have demonstrated that measurement of radiationless energy transfer (RET)[1] between enzyme tryptophan residues and a fluorescent dansyl substrate provides a sensitive means to observe ES complexes directly [Auld (1977) and references cited therein]. Direct analysis by RET of the rate of formation and breakdown of ES complexes serves as the basis of a novel kinetic approach to enzyme mechanisms (Auld, 1977; Lobb & Auld, 1979). Both pre-steady-state and steady-state kinetics can be observed in one experiment. Analyses of the pre steady state allows determination of the number of intermediates and individual binding and rate constants (Lobb & Auld, 1979), while, as demonstrated here, direct observation of ES complexes by RET under steady-state conditions both simplifies and supplements conventional initial rate kinetic studies.

Materials and Methods

α-Chymotrypsin, salt-free, 3 times crystallized, and lyophilized (Worthington Biochemical Corp., Lot CDI 36J835), was used without further purification. The concentration of active enzyme, determined by active-site titration with *N-trans*-cinnamoylimidazole (Schonbaum et al., 1961), was 89% of that determined spectrophotometrically at 280 nm by using a molar absorptivity of 5.0×10^4 M^{-1} cm^{-1} (Dixon & Neurath, 1957) and assuming a molecular weight of 25 000 (Wilcox et al., 1957). Yeast carboxypeptidase, purified to homogeneity and lyophilized in citrate buffer, pH 5.5, was the gift of Dr. J. T. Johansen of the Carlsberg Laboratory. Protein concentration was determined spectrophotometrically by using $A_{280}^{1\%} = 14.8$ and a molecular weight of 64 000 (Johansen et al., 1976). Bovine carboxypeptidase A prepared by the method of Cox et al. (1964) was obtained as a crystal suspension from Sigma Chemical Co. and purified by affinity chromatography (Peterson et al., 1976). Protein concentration was determined by using a molar absorptivity of 6.42×10^4 M^{-1} cm^{-1} at 278 nm (Simpson et al., 1963).

The substrates used were Dns-(Gly)$_2$-L-PheOMe (chymotrypsin) and Dns-(Gly)$_3$-L-OPhe (carboxypeptidase A, yeast carboxypeptidase). These substrates were selected from a series of dansylated depsipeptides and oligopeptides that have been synthesized in our laboratory for the investigation of proteolytic enzymes (Auld & Holmquist, 1974; Lobb & Auld, 1979).

Buffers were prepared from concentrated stock solutions of Tris and Mes (Sigma) and KCl, NaCl, and $CaCl_2$ (Fisher). Extraneous heavy metal ions were removed from buffers by extraction with 0.001% dithizone in carbon tetrachloride (Thiers, 1957). Calcium ion was included in the α-chymotrypsin assays to enhance enzyme stability (Delaage et al., 1968).

Esterase assays were performed by titration of protons released on substrate hydrolysis using 2 mM NaOH as titrant

† From the Biophysics Research Laboratory, Department of Biological Chemistry, Harvard Medical School, and the Division of Medical Biology, Peter Bent Brigham Hospital, Boston, Massachusetts 02115. *Received April 18, 1980.* This work was supported by Grant-in-Aid GM-24968 from the National Institutes of Health of the Department of Health, Education and Welfare to Harvard Medical School.

[1] Abbreviations used: RET, radiationless energy transfer; Dns or dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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Scheme I

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \ldots ES_i \ldots ES_n \xrightarrow{k_{n+1}} E + P$$

and a Radiometer titrator comprising an ABU 12 autoburet coupled to a TTTII autotitrator, TTA 31 titration assembly, and a PHM 28 pH meter.

Stopped-flow fluorescence measurements utilized either a Durrum-Gibson instrument equipped with the Durrum fluorescence accessory, No. 16400, a 75-W xenon lamp (Illumination Industries), and an EMI 9526B (S13) photomultiplier or a low-temperature stopped-flow instrument equipped with quartz fiber optics, a Schoeffel monochromator, a 200-W xenon lamp, and a low-noise–high-sensitivity detector system comprised of an EMI 9659QB trialkali (extended S-20) photomultiplier and a Sorenson high-voltage power supply (Auld, 1979; Hanahan & Auld, 1980). The signal to root mean square noise was typically in the range of 1000–8000. Fluorescence intensities were digitized and stored on floppy discs (BSAF) with a PDP 11/34 computer (DEC) equipped with an AR11 A/D converter and a VT-55 decscope. Heights, first-order rate constants, and integrated areas were calculated by using the computer.

One thousand time points were stored per experiment. If a pre steady state occurred within the time range of the instrument, the data could be collected at two different frequencies, 500 points being collected during the pre steady state and 500 during steady state.

A Fortran program was written to allow display of the data on the VT-55 screen for visual inspection and analysis. The fluorescent intensity F_i at any given time point was determined with a resolution of one part in a thousand. Areas were calculated by application of Simpson's rule (Thomas, 1956)

$$A = h/3\sum(F_1 + 4F_2 + 2F_3 + \ldots + 2F_{n-2} + 4F_{n-1} + F_n)$$

where h is the time interval between successive data points, F_i, and n may be up to 1000 points.

Turnover numbers, k, were computed from the measured values of F_{max} and A_0 (see Definition of RET Parameters) and the known values of initial substrate concentration $[S_0]$ and total enzyme concentration $[E_T]$. The kinetic parameters k_{cat} and K_m were determined by least-squares analyses of double-reciprocal plots of $1/k$ vs. $1/[S_0]$. Determination of k_{cat} and K_m from the progress curve at a single initial substrate concentration was accomplished by evaluating a series of intensities, F_t, and areas, A_t, at every tenth time interval, generating a series of rates, k_t, and substrate concentrations, $[S]_t$. Usually 100–500 data points were used in these calculations generating 10–50 k_t and $[S]_t$ values. The linear regression of $1/k_t$ vs. $1/[S]_t$ was then displayed on the VT-55 for visual inspection, and the calculated kinetic parameters were automatically recorded on a floppy disc. First-order rate constants were obtained by linear regression of $-\log(F_\infty - F_t)$ on time. The first-order plot, the rate constant, and associated error analysis were displayed on the VT-55 for visual inspection, and the analyses were automatically recorded on floppy discs.

Theory and Results

Mechanistic Assumptions. The analyses will be restricted to an irreversible one-substrate mechanism involving n intermediates where the total reaction scheme may be represented by Scheme I where ES_i represents either a noncovalent or a covalent ES complex. In this scheme all substrate is converted to product, and substrate and/or product activation and/or inhibition are absent. The following assumptions are made:

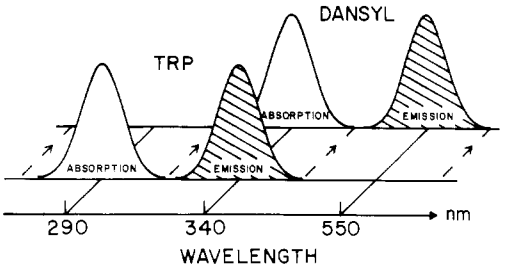

FIGURE 1: Schematic representation of the spectral overlap relationships between enzyme tryptophan and substrate dansyl groups, which consititute the energy donor–acceptor pair critical for observation of the ES complex by RET.

(1) under conditions where the total enzyme concentration $[E_T]$ is much less than the initial substrate concentration $[S_0]$, a steady state is rapidly attained, where the steady state is defined as that time interval during which conventional Michaelis–Menten kinetics are followed

$$V_t = \frac{k_{cat}[E_T][S]_t}{K_m + [S]_t} = k_{cat}[ES_T]_t \qquad (1)$$

where V_t, $[S]_t$, and $[ES_T]_t$ are the reaction velocity, free substrate concentration, and total concentration of ES complexes at any time t after the inception of the steady state, and k_{cat} and K_m are an assembly of rate and equilibrium constants (Peller & Alberty, 1959); (2) under the conditions $[E_T] \ll K_m$, Michaelis–Menten kinetics are followed until the reaction is complete.

Conventional initial rate assays have confirmed that assumption 1 is valid for the enzyme–substrate systems studied here, that the reactions are irreversible, and that product inhibition is negligible. Assumption 2 has been verified both in theoretical studies (Schauer & Heinrich, 1979; R. R. Lobb, J. L. Bethune, and D. S. Auld, unpublished experiments) and by experiment (see below).

Direct Observation of the ES Complex by RET. Radiationless energy transfer, RET, between fluorescent enzyme tryptophanyl residues as intrinsic donors and an extrinsically placed acceptor, e.g., a dansyl group in the substrate, allows direct visualization of the enzyme–substrate complex [Auld (1977) and references cited therein]. As illustrated schematically in Figure 1, excitation of enzyme tryptophans at 290 nm results in tryptophan fluorescence at longer wavelengths. The spectral overlap of the dansyl group absorption (λ_{max} ~330 nm) and tryptophan emission (λ_{max} ~340 nm) is excellent, and the dansyl emission spectrum (λ_{max} ~550 nm) is red shifted far enough not to overlap with its own absorption spectrum, properties which make these an exceptional donor–acceptor pair (Figure 1).

Binding of the substrate allows the transfer of energy from the enzyme tryptophans to the substrate dansyl group resulting in quenching of tryptophan fluorescence and generation of dansyl fluorescence. Since energy transfer occurs within nanoseconds, changes in either dansyl or tryptophan fluorescence accurately monitor both substrate binding and hydrolysis. Thus, changes in the concentrations of ES complexes or differences in their tryptophan to dansyl transfer efficiencies and/or dansyl quantum yields will result in changes in fluorescence, directly signaling their existence and allowing their quantitation. Since the method is sufficiently sensitive to allow the use of the kinetic conditions $[E_T] \ll [S_0]$ and $[E_T] \ll K_m$, a steady state is generated rapidly under stopped-flow conditions and maintained until the reaction is complete. For example, Figure 2A illustrates the changes observed in substrate dansyl fluorescence which occcur on the binding and

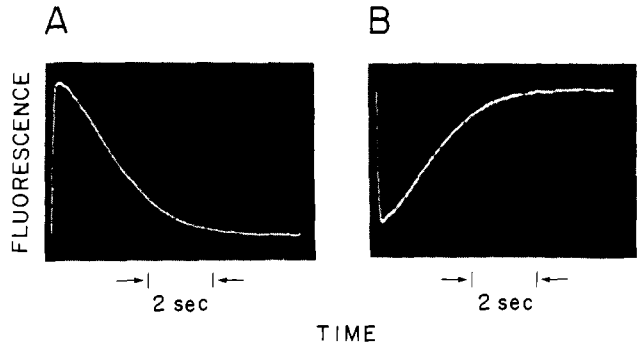

FIGURE 2: Stopped-flow fluorescence observation of the formation and breakdown of ES complexes of chymotrypsin (1.85 μM) and Dns-(Gly)$_2$-L-PheOMe (50 μM) under steady-state conditions. Excitation was at 285 nm and either (A) the substrate dansyl emission above 430 nm or (B) the enzyme tryptophan emission at 360 nm was observed. Assay conditions were 0.25 M KCl, 5 mM $CaCl_2$, and 25 mM Mes at pH 6.0 and 20 °C.

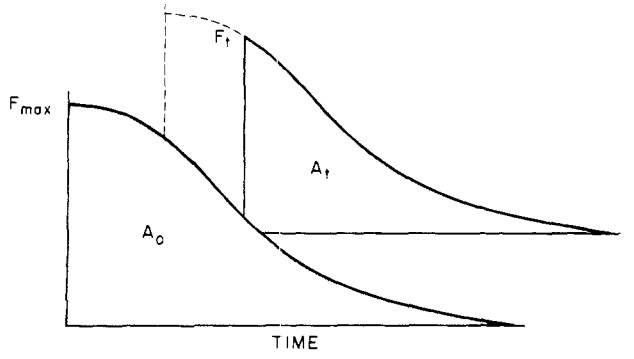

FIGURE 3: Schematic illustration of the steady-state portion of the oscilloscope trace defining the RET kinetic parameters F_{max}, the maximal fluorescence, A_0, the total area, and F_t and A_t, the fluorescence and area at any time t.

hydrolysis of Dns-(Gly)$_2$-L-PheOMe by chymotrypsin. A maximum in fluorescence is achieved rapidly, signaling attainment of the steady state, followed by a slower decay of fluorescence intensity as the substrate is hydrolyzed. Analogous signals are seen when the quenching of enzyme tryptophan fluorescence is monitored (Figure 2B).

Definition of RET Parameters. As illustrated schematically in Figure 3, the fluorescence intensity at any time t after the attainment of the steady state is F_t. As hydrolysis proceeds to completion the fluorescence intensity decreases, allowing description of an area A_t defined by eq 2. At the inception

$$A_t = \int_t^\infty F_t \, dt \tag{2}$$

of the steady state, operationally defined as time zero, the fluorescence is at a maximum, F_{max}, and the total area described by the stopped-flow trace is defined as A_0.

It has been shown that when the steady-state assumption is valid (Peller & Alberty, 1959) the ratio of the concentrations of any two intermediates is a constant, i.e.

$$[ES_j]/[ES_i] = b_{i,j} \; (i,j = 1, 2, ..., n) \tag{3}$$

Hence the ratio of any one intermediate, $[ES_i]$, to the total concentration of ES complexes, $[ES_T]$, also is a constant

$$[ES_i]/[ES_T] = c_i \; (i = 1, 2, ..., n) \tag{4}$$

where

$$[ES_T] = \sum_{j=1}^{n} [ES_j]$$

For the systems employed here, the fluorescent properties of the dansyl group are unchanged in substrate and product. Thus, both the quenching of tryptophan and the enhancement of dansyl fluorescence may be related directly to changes in

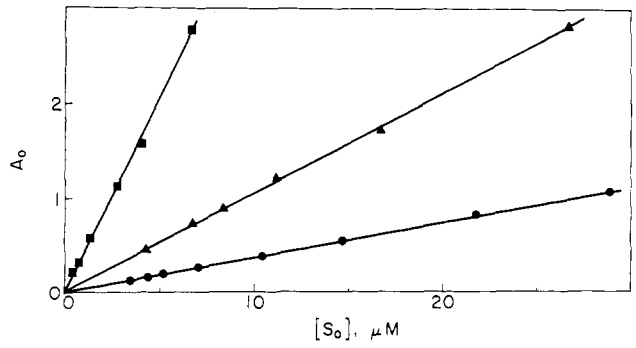

FIGURE 4: Dependence of A_0 on $[S_0]$ for the enzymes: 0.3 μM yeast carboxypeptidase (■) and Dns-(Gly)$_3$-L-OPhe; 1.85 μM chymotrypsin (▲) and Dns-(Gly)$_2$-L-PheOMe; 0.5 μM carboxypeptidase A (●) and Dns-(Gly)$_3$-L-OPhe. Assay conditions for yeast carboxypeptidase are 0.1 M NaCl and 25 mM Mes at pH 5.5 and 20 °C; for chymotrypsin are 0.25 M KCl, 5 mM $CaCl_2$, and 25 mM Mes at pH 6.0 and 20 °C; and for carboxypeptidase A are 1 M NaCl and 25 mM Tris at pH 7.5 and 20 °C.

the concentrations of ES complexes.[2] For the steady-state time interval of interest here, the fluorescence intensity at any time, t, is given by eq 5 where C_i represents constants for a

$$F_t = \sum C_i [ES_i]_t \tag{5}$$

given enzyme, substrate, pH, temperature, and ionic strength.[3]

From eq 4 and 5 it follows that

$$F_t = [ES_T]_t \sum c_i C_i \tag{6}$$

$$F_t = C[ES_T]_t \tag{7}$$

where C is a constant for a given enzyme and substrate at fixed pH, temperature, and ionic strength. This equation shows that under steady-state conditions the fluorescence observed at any time t, F_t, is directly proportional to the total concentration of ES complexes, $[ES_T]$, present at that time.[4]

Relationship between A_0 and S_0. The reaction velocity at time t may be stated (Peller & Alberty, 1959) as

$$\frac{d[P]}{dt} = k_{cat}[ES_T]_t$$

Substituting from eq 7 for $[ES_T]$ leads to eq 8. Equation 8

$$F_t = \frac{C}{k_{cat}} \frac{d[P]}{dt} \tag{8}$$

shows that the reaction velocity at time t is directly proportional to the observed fluorescence, F_t, at that time. The total area A_0, eq 2, may now be expressed in terms of k_{cat} and the reaction velocity.

$$A_0 = \int_0^\infty F_t \, dt = \frac{C}{k_{cat}} \int_0^\infty dP = \frac{C}{k_{cat}} [P_T] \tag{9}$$

[2] When the fluorescent properties of substrate and product differ, any weak direct excitation of the dansyl group at 285 nm may no longer be negligible, requiring different mathematical treatment (R. R. Lobb and D. S. Auld, unpublished experiments).

[3] For simplicity, $\sum$ refers to the sum over all i ($i = 1, 2, ..., n$) unless otherwise stated.

[4] Scheme I may contain partial product release prior to the breakdown of the nth intermediate, for example, the formation of an acyl intermediate. However, even if the fluorescent probe leaves the enzyme surface prior to formation of the final intermediate, the overall form of the equations is unchanged, only the constant being altered. In fact, the fluorophore need only be present in one intermediate to monitor the steady state completely. However, early release of the fluorophore must be taken into account during analysis under other conditions, for example, the pre-steady-state time interval.

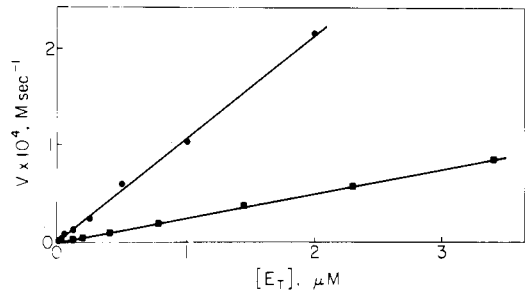

FIGURE 5: Dependence of initial rate on enzyme concentration. The rate, V, is obtained from the ratio of $F_{max}[S_0]/A_0$ for each concentration of carboxypeptidase A (●) acting on Dns-(Gly)$_3$-L-OPhe (30 μM) or chymotrypsin (■) acting on Dns-(Gly)$_2$-L-PheOMe (50 μM). See Figure 4 for other assay conditions.

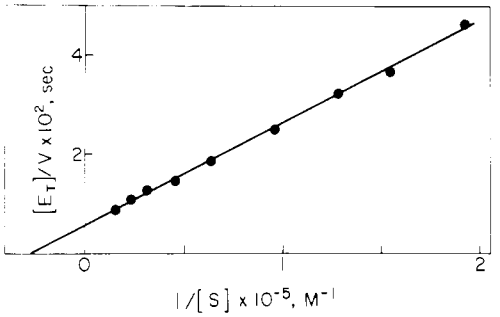

FIGURE 6: Stopped-flow fluorescence determination of the kinetic parameters k_{cat} and K_m for the hydrolysis of Dns-(Gly)$_3$-L-OPhe by carboxypeptidase A (0.5 μM) in 25 mM Tris and 1 M NaCl at pH 7.5 and 21 °C. Linear regression analysis of the reciprocal of eq 11 yields values of 220 s^{-1} for k_{cat} and 37 μM for K_m.

Since by definition all substrate is converted into product (Scheme I), then

$$\frac{A_0}{[S_0]} = \frac{C}{k_{cat}} = \frac{A_t}{[S]_t} \qquad (10)$$

Therefore, the total area, A_0, is directly proportional to the initial substrate concentration, $[S_0]$. Indeed, our RET studies of chymotrypsin, bovine carboxypeptidase A, and yeast carboxypeptidase show that this proportionality holds over as much as a 400-fold variation in substrate concentration (Figure 4).

Relationship between Initial Velocity, V, and F_{max}/A_0. At time $t = 0$ where $F_t = F_{max}$, eq 1, 8, and 10 may be combined to eliminate the constant C giving

$$\frac{F_{max}[S_0]}{A_0} = V = \frac{k_{cat}[E_T][S_0]}{K_m + [S_0]} \qquad (11)$$

These equations show the direct relationship between F_{max}/A_0 and the initial reaction velocity V. It predicts that under steady-state conditions the reaction velocity, expressed as $F_{max}[S_0]/A_0$, depends linearly on the total enzyme concentration, $[E_T]$.

Figure 5 illustrates this relationship for carboxypeptidase A and chymotrypsin. The reaction velocity was determined by measuring the stopped-flow parameters F_{max} and A_0 over an enzyme concentration range from 10^{-8} to 10^{-6} M. A linear dependence of velocity on enzyme concentration is found in both cases.

Determination of Kinetic Parameters. The direct observation of ES complexes at steady state under stopped-flow conditions allows the evaluation of k_{cat} and/or K_m in multiple ways. As illustrated in the following, they can be determined (a) from values of F_{max} and A_0, (b) from the initial maximal values of $[ES_T]$, or (c) from analysis of the progress curve.

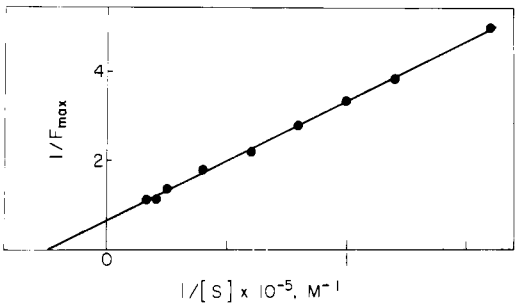

FIGURE 7: Stopped-flow fluorescence determination of the kinetic parameter K_m for the hydrolysis of Dns-(Gly)$_2$-L-PheOMe by chymotrypsin (1.85 μM) in 25 mM Mes and 5 mM $CaCl_2$ at pH 6.0 and 20 °C. Linear regression analysis of the reciprocal of eq 12 yields a value of 46 μM for K_m.

Table I: Comparison of Kinetic Parameters from Initial Rate and Stopped-Flow Assays:[a] Chymotrypsin-Catalyzed Hydrolysis of Dns-(Gly)$_2$-L-PhOMe

assay method	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\times 10^4$ $M\ s^{-1}$)
initial rate	24	45	53
stopped flow			
(1) $1/V$ vs. $1/[S]$	23	44	52
(2) $1/F_{max}$ vs. $1/[S]$		46	
(3)[b] $1/V_t$ vs. $1/[S]_t$	23	45	51
(4)[b] k_{obsd}			53

[a] Assays employed 0.25 M KCl, 0.025 M Mes, and 5 mM $CaCl_2$ at 20 °C and pH 6.0 (stopped flow) or 0.25 M KCl and 5 mM $CaCl_2$ at 20 °C and pH 6.0 (pH stat). [b] Concentration of substrate was 30 μM.

(a) Determination of K_m and k_{cat} from Dependence of F_{max}/A_0 on Substrate Concentration. When $[E_T] \ll [S_0]$, the concentration of unbound substrate $[S]$ approximates $[S_0]$. 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)$_3$-L-OPhe by 5×10^{-7} M carboxypeptidase A at pH 7.5 and 20 °C yield a K_m of 37 μM and a k_{cat} of 220 s^{-1} (Figure 6), in excellent agreement with conventional initial rate assays (Auld, 1977). We have used eq 11 to evaluate kinetic parameters for both endo- and exopeptidases acting on peptides as well as esters (Auld et al., 1972; Auld & Holmquist, 1974; Auld, 1977; Lobb & Auld, 1979).

(b) Determination of K_m from Dependence of F_{max} on Substrate Concentration. At the inception of the steady state the concentration of ES complexes, and hence F, is maximal (Figures 2 and 3). This corresponds to the initial steady-state portion of the reaction, where the velocity is constant, and may be related readily to $[S_0]$. From eq 1 and 8 at time $t = 0$

$$F_{max} = \frac{C[E_T][S_0]}{K_m + [S_0]} = \frac{F^*_{max}[S_0]}{K_m + [S_0]} \qquad (12)$$

where F^*_{max} is the limiting value of F_{max} when the enzyme is saturated with substrate. Clearly, a double-reciprocal plot of F_{max} values for each free substrate concentration provides another way of determining the steady-state parameter K_m. This is illustrated in Figure 7, with data obtained from the hydrolysis of Dns-(Gly)$_2$-L-PheOMe by chymotrypsin at pH 6.0 and 20 °C, under stopped-flow conditions. The resulting K_m is in excellent agreement with that determined from conventional pH-stat assays (Table I).

(c) Evaluation of k_{cat} and K_m from Dependence of F_t/A_t on Substrate Concentration. In principle, the complete re-

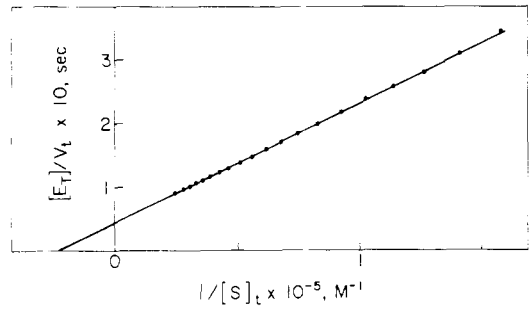

FIGURE 8: Determination of the kinetic parameters k_{cat} and K_m for the 1.85 μM chymotrypsin catalyzed hydrolysis of 30 μM Dns-$(Gly)_2$-L-PheOMe in 0.25 M KCl, 5 mM $CaCl_2$, and 25 mM Mes at pH 6.0 and 20 °C. Linear regression analysis of the reciprocal of eq 1 yields values of 23 s^{-1} for k_{cat} and 45 μM for K_m.

action course obtained at a single initial substrate concentration contains all the information needed to calculate k_{cat} and K_m. Thus, from eq 1, 8, and 10 the velocity, V_t, at any time t can be expressed as

$$V_t = \frac{F_t[S]_t}{A_t} \tag{13}$$

where $[S]_t$ is the free substrate cooncentration at time t and F_t and A_t are as previously defined (Figure 3).

The variables F_t and A_t are determined directly from the oscilloscope trace as described under Materials and Methods. The value of $[S]_t$ can be determined easily since, for a given enzyme–substrate system, there is a direct relationship between the area under the curve and the substrate concentration (eq 10). Thus, a plot of $1/V_t$ vs. $1/[S]_t$ may be obtained from the height and area variations over time from a single initial substrate concentration and hence constitutes an alternate means to determine K_m and k_{cat}.

Figure 8 illustrates such a plot for the binding and hydrolysis of Dns-$(Gly)_2$-L-PheOMe by chymotrypsin at pH 6.0 and 20 °C. The stopped-flow trace was stored digitally and analyzed as described under Materials and Methods. The resultant values of k_{cat} and K_m are in excellent agreement with those obtained by other methods (Table I).

Determination of k_{cat}/K_m. From eq 1 the concentration of ES complexes at any time t is

$$[ES_T]_t = \frac{[E_T][S]_t}{K_m + [S]_t}$$

When $[S]_t \ll K_m$, the rate of decrease in ES complex concentration becomes

$$\frac{d[ES_T]}{dt} = \frac{[E_T]}{K_m}\frac{d[S]}{dt} \tag{14}$$

Further substitution of $k_{cat}[ES_T]$ for $d[S]/dt$ shows that the decrease in the concentration of ES is first order under these conditions:

$$\frac{d[ES_T]}{[ES_T]} = \frac{k_{cat}[E_T]dt}{K_m} \tag{15}$$

The first-order rate constant, k_{obsd}, for the change in ES complex concentration during the latter stages of catalysis is thus $[E_T]k_{cat}/K_m$.

This is also seen readily from eq 11. When the initial substrate concentration is much less than K_m, the fluorescence trace is exponential from its initial maximal height through to completion of the reaction, and eq 11 becomes

$$\frac{F_{max}}{A_0} = \frac{k_{cat}[E_T]}{K_m} \tag{16}$$

Thus, since $[E_T]$ is known, the rate constant characterizing the exponential signal decay of the full progress curve allows an independent evaluation of k_{cat}/K_m. For example, the values of k_{cat}/K_m for chymotrypsin are in excellent agreement with those obtained by other means (Table I).

Discussion

Radiationless energy transfer between enzyme and substrate under stopped-flow conditions provides one of the most versatile approaches now available to study enzyme mechanisms. The formation and breakdown of ES complexes may be observed directly and with high sensitivity, allowing a more rigorous definition of enzyme mechanisms (Auld, 1977; Lobb & Auld, 1979). Further, the approach retains the virtues of conventional steady-state kinetic analyses. Since the ES complex is observed at enzyme concentrations of 10^{-8}–10^{-6} M, where $[E_T] \ll K_m$, a steady state in ES complex formation and breakdown can be achieved and maintained even though the entire reaction may be complete in a few seconds (Figures 2, 4, and 5). Thus direct observation of the ES complex by RET under stopped-flow steady-state conditions still provides excellent economy of enzyme and allows the use of rapidly turned over substrates. Moreover, since in each experiment independent measurements can be made of parameters reflecting the concentration of ES complexes, F_t, and their rate of breakdown, A_t, the kinetic parameters k_{cat} and K_m can be determined in multiple ways (Figures 6–8 and Table I). In all cases studied so far the values obtained by RET analyses are in excellent agreement with those derived from conventional initial rate assays.

The evaluation of k_{cat} and K_m from the progress curve at a single substrate concentration (Figure 8) should be of particular value for the screening of substrates and determination of the mechanism of modifiers of enzyme activity. The speed of the assay performed under stopped-flow conditions combined with computerized data storage and analysis allows both quantitative evaluation within seconds and, if desired, the continuous redesign of experimental strategy. Thus, this approach should allow a facile evaluation of the effects of pH, temperature, ionic strength, solvents, and inhibitors and activators of enzyme activity. Direct examination of ES complexes at a single substrate concentration during catalysis by carboxypeptidase A has already allowed the delineation of inhibitor modes (Auld et al., 1972) and of the consequences of chemical modification of the enzyme's activity (Auld & Holmquist, 1974; Van Wart & Vallee, 1978).

RET kinetic studies also overcome one of the disadvantages of conventional initial rate assays, in that if a pre steady state exists, the direct observation of ES complexes can both determine the number and nature of intermediates directly and quantitate the individual binding and rate constants (Auld, 1977; Lobb & Auld, 1979). For example, pre-steady-state fluorescence changes observed during the hydrolysis of Dns-$(Gly)_2$-L-PheOMe by chymotrypsin reveal the presence of two reaction intermediates (Lobb & Auld, 1979). Addition of the nucleophilic product methanol demonstrates that the second intermediate is the acyl enzyme. The quantitative analysis of the effects of substrate and product during the pre-steady-state time interval allows the evaluation of all kinetic parameters thereby defining the enzyme mechanism. The kinetic parameters derived from RET analyses of the steady-state region also complement mechanistic information from the pre-steady-state time interval, since a stringent test

of the mechanism lies in the equivalence of the overall kinetic parameters K_m and k_{cat} calculated from the individual binding and rate constants to those measured under steady-state conditions. The determination of K_m and k_{cat} from the same experiment as that in which the pre steady state is observed greatly reduces the errors encountered when measuring such parameters by different techniques. For the case of chymotrypsin, the calculated and measured values of k_{cat} and K_m are in excellent agreement (Lobb & Auld, 1979).

The absence of product inhibition is an important assumption for the derivation of the equations used in this study. However, if such inhibition occurs, it can be evaluated readily, for the accumulation of product will affect the apparent k_{cat} or K_m, or both. As a result, the values of the kinetic parameters determined from the progress curve at a single initial substrate concentration will no longer be independent of that concentration. In addition, since the exponential decay of the fluorescence signal for each substrate concentration is characterized by a pseudo-first-order rate constant, $k_{cat}[E_T]/K_m$ (eq 15), any product inhibition that changes k_{cat} or K_m will modify this constant. Further, although purely competitive product inhibition will not affect the total area, A_0 (Auld et al., 1972), other types of inhibition will result in nonlinearity of its variation with initial substrate concentration. Such checks provide a sensitive monitor of product inhibition. Importantly, they also form the basis for the facile evaluation of inhibition constants at a single substrate and inhibitor concentration (R. R. Lobb and D. S. Auld, unpublished experiments).

The use of RET to observe the ES complex permits considerable synthetic flexibility in the design of the substrate to be used for mechanistic studies. The introduction of chromophoric probes frequently results in substrates that are less than ideal. The chromophore is often inserted next to or is made part of the scissile bond in order to give measurable spectral changes. However, the active site of the enzyme may not readily accept such an unusual substrate structure, and catalytic activity may then be reduced drastically. In contrast, the use of the dansyl group for RET kinetics provides a sensitive and direct probe of catalytic events, while leaving those regions of the substrate critical for binding and catalysis unaltered. In fact, the placement of the dansyl group remote from the scissile bond results in weakly bound fluorescent products, obviating more complex mathematical analyses and, more importantly, enhances its versatility as a probe by increasing the variety of substrates into which it may be incorporated. For example, thus far the majority of transient-state mechanistic studies of proteolytic enzymes have relied on ester substrates because of the relative ease of synthesis of substrates containing chromophoric alcoholic leaving groups, e.g., nitrophenolate. The placement of the dansyl group remote from the scissile bond still permits the use of a chromophoric ester, if desired. More importantly, the hydrolysis of peptide substrates, which constitute more realistic substrates for proteolytic enzymes, can be observed with equal ease. Thus, the RET kinetic approach allows detailed mechanistic comparisons between substrates which are exact structural analogues, e.g., matched ester–peptide pairs (Auld & Holmquist, 1974) or ester–thiol ester or phosphate–phosphonate derivatives. Moreover, the RET kinetic approach is not restricted to proteolytic enzymes but can be applied to any enzyme in which a suitable fluorescent probe can be introduced into the substrate. Direct inspection of the reaction pathway in this manner permits rigorous definition of the enzyme mechanisms.

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References

Auld, D. S. (1977) in *Bioorganic Chemistry* (Van Tamelin, E. E., Ed.) Vol. 1, p 1, Academic Press, New York.

Auld, D. S. (1979) *Methods Enzymol. 61*, 318.

Auld, D. S., & Holmquist, B. (1974) *Biochemistry 13*, 4355.

Auld, D. S., Latt, S. A., & Vallee, B. L. (1972) *Biochemistry 11*, 4994.

Cleland, W. W. (1977) *Adv. Enzymol. Relat. Areas Mol. Biol. 45*, 273.

Cox, D. J., Bovard, F. C., Bargetzi, J. P., Walsh, K. A., & Neurath, H. (1964) *Biochemistry 3*, 44.

Delaage, M., Abita, J. P., & Lazdunski, M. (1968) *Eur. J. Biochem. 5*, 285.

Dixon, G. H., & Neurath, H. (1957) *J. Biol. Chem. 225*, 100.

Hanahan, D., & Auld, D. S. (1980) *Anal. Biochem.* (in press).

Johansen, J. T., Breddam, K., & Ottesen, M. (1976) *Carlsberg Res. Commun. 41*, 1.

Lobb, R. R., & Auld, D. S. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 2684.

Peller, L., & Alberty, R. A. (1959) *J. Am. Chem. Soc. 81*, 5907.

Peterson, L. M., Sokolovsky, M., & Vallee, B. L. (1976) *Biochemistry 15*, 2501.

Schauer, M., & Heinrich, R. (1979) *J. Theor. Biol. 79*, 425.

Schonbaum, G. R., Zerner, B., & Bender, M. L. (1961) *J. Biol. Chem. 236*, 2930.

Segal, I. H. (1975) in *Enzyme Kinetics*, Wiley-Interscience, New York.

Simpson, R. T., Riordan, J. F., & Vallee, B. L. (1963) *Biochemsitry 2*, 616.

Thiers, R. E. (1957) *Methods Biochem. Anal. 5*, 273.

Thomas, G. B. (1956) in *Calculus and Analytical Geometry*, 2nd ed., Addison-Wesley, Cambridge, MA.

Van Wart, H. E., & Vallee, B. L. (1978) *Biochemistry 17*, 3385.

Wilcox, P. E., Kraut, J., Wade, R. D., & Neurath, H. (1957) *Biochim. Biophys. Acta 24*, 72.