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Direct observation of enzyme substrate complexes by stopped-flow fluorescence: mathematical analyses

R.R. Lobb and D.S. Auld*

Center for Biochemical and Biophysical Sciences and Medicine, Harvard Medical School, and the Division of Medical Biology, Brigham and Women's Hospital, Boston (Massachusetts 02115, USA), 17 June 1983

Summary. The fluorescence changes which occur upon the interaction of enzyme and substrate under stopped-flow conditions can provide a sensitive means to directly observe ES complexes. The interconversion of the intermediates during catalysis causes changes in fluorescence, signaling directly their existence, and allowing their quantitation. We have studied extensively an approach which measures radiationless energy transfer (RET) between enzyme tryptophanyl residues and a fluorescent peptide or ester substrate. Our studies of a number of proteolytic enzymes have validated the approach, which is sensitive and applicable to a variety of enzymes under a wide range of experimental conditions, including subzero temperatures. Direct excitation of fluorescent substrates can also be used to observe ES complex formation and breakdown and is complementary to the RET approach. Here we review both the RET and direct excitation kinetic approaches, with particular emphasis on the mathematical foundations we have developed which are critical to the successful interpretation of these or any other spectroscopic approach which yields a signal that is unique to the ES complex.
Key words. Enzyme-substrate complexes; stopped-flow fluorescence, proteolytic enzymes; radiationless energy transfer; enzyme kinetics.

1. Introduction

The interaction between enzyme and substrate is the characteristic feature of enzyme catalysis, and mechanistic studies are greatly facilitated by the direct visualization of reaction intermediates. This obviously desirable goal has proven elusive, because the inherently short lifetimes and low concentrations of ES complexes make particularly demanding requirements of available techniques. While stopped-flow equipment and methodology, now generally available, have done much to advance the field, visualization of ES complexes is not an inherent feature of this technique. Thus spectral systems that can directly signal the ES complex must be designed to be compatible with rapid mixing instrumentation.

Our studies of a number of proteolytic enzymes have demonstrated that measurement of radiationless energy transfer (RET) between enzyme tryptophanyl residues and a fluorescent dansyl-peptide or ester provides a sensitive means to observe ES complexes at both steady-state and pre-steady-state conditions[4, 6, 13, 22, 23]. The interconversion of ES complexes during catalysis causes changes in fluorescence, signaling directly their existence, and allowing their quantitation.

The direct observation of ES complexes using fluorescent substrates is also possible using an alternative approach in which the substrate fluorophore is excited directly, and is thus always fluorescent. On binding to the enzyme surface, the intrinsic fluorescence of the substrate is often greatly enhanced due to changes in the fluorophore environment. While less sensitive than the RET method, since there is always background fluorescence, this technique is complementary to the RET approach, and has been used effectively by Fruton and co-worders using N-mansyl peptide substrates for proteolytic enzymes[24, 25, 28].

The mathematical foundations critical to the successful interpretation of the observed fluorescence changes have received little attention, presumably because of the inherent difficulty in direct visualization of ES complexes. Here we review the RET kinetic approach and illustrate its applicability under a variety of experimental conditions, with particular emphasis on the theoretical framework we have developed for its interpretation. In addition, we extend the mathematics to the interpretation of stopped-flow fluorescence signals obtained upon direct excitation of fluorescent substrates. Moreover, we emphasize that this theoretical

framework can be applied to any other approach (e.g. electronic absorption, nuclear magnetic and electron parmagnetic resonance, circular dichroism, resonance Raman) which yields a change in spectral properties upon substrate binding to the enzyme that is characteristic of the ES complex.

2. Direct observation of the ES complex by radiationless energy transfer

Most enzymes contain tryptophanyl residues and their excitation with 285 nm radiation leads to light emission in the region of 340 nm (fig. 1A). Radiationless energy transfer, RET, between the intrinsic fluorescent enzyme tryptophanyl groups and an extrinsic acceptor in the substrate, e.g. a dansyl group, allows direct visualization of the enzyme substrate complex. Substrate binding permits the efficient transfer of energy from enzyme tryptophans to the substrate dansyl resulting in quenching of tryptophan fluorescence and simultaneous generation of dansyl fluorescence (fig. 1B). Since energy transfer occurs within nanoseconds changes in either dansyl or tryptophan fluorescence accurately monitor both substrate binding and catalysis. Changes in ES complex concentration, tryptophan or dansyl quantum yields, will result in changes in fluorescence, directly signaling the existence of the ES complex and allowing its quantitation.

The resultant fluorescence changes are readily monitored after mixing of enzyme and substrate in conventional stopped-flow equipment. Figure 2 shows the changes observed in enzyme and substrate fluorescence which occur on binding and hydrolysis of the ester Dns-Gly-Ala-Phe-OMe by α-chymotrypsin. Excitation of enzyme tryptophans at 285 nm results in a rapid decrease in enzyme tryptophan (fig. 2A) and enhancement of substrate dansyl (fig. 2B) fluorescence, signaling attainment of the steady state within 350 msec, followed by a slower change in fluorescence intensity as the substrate is hydrolyzed over a 50-sec time interval.

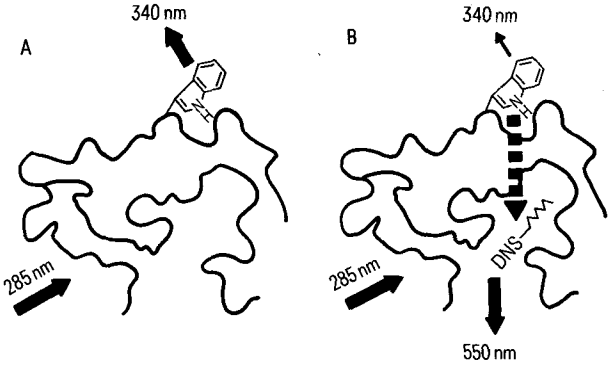

Figure 1. Diagramatic illustration of direct observation of the ES complex by radiationless energy transfer. For the purposes of simplicity only one tryptophan residue is shown in the enzyme and tryptophan and dansyl fluorescence are indicated by 340 and 550 nm emission. Radiationless energy transfer between enzyme tryptophan and the substrate dansyl group is denoted by the broken arrow.

3. Mathematical analysis of RET fluorescence changes

For the subsequent mathematical analyses we shall restrict ourselves to an irreversible one-substrate mechanism involving n intermediates.

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

Scheme A

where ES_n represents either non-covalent or covalent ES complexes. In this scheme all substrate is converted to product and substrate and product activation and/or inhibition is absent. If such inhibition or activation should occur during RET assays it will be readily detectable, manifesting in either non-linear Lineweaver-Burk plots or a substrate dependence of the kinetic parameters determined from a progress curve[23].

Relationship between fluorescence and ES complex concentration

If an absorbing species is also a fluorophore, then its fluorescence intensity is given by

$$I_F = I_0 Q_0 [1 - \exp(-\text{alc})]. \quad (1)$$

Where Q_0 is the quantum efficiency and I_0 is the incident light intensity on the sample at a concentration c with a molar absorptivity, a, in a cell of path length 1. The exponential may be expanded in a power series and, if alc is sufficiently small, i.e. if the fluorophore concentration is sufficiently low, then

$$I_F = I_0 Q_0 \text{ alc}. \quad (2)$$

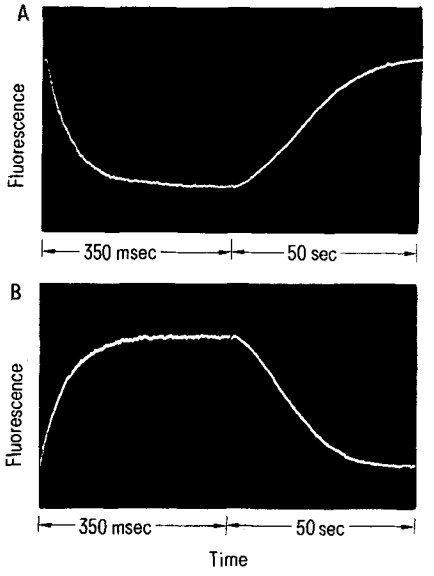

Figure 2. Stopped-flow fluorescence observation of the formation and breakdown of ES complexes of α-chymotrypsin (1 μM) and DNS-Gly-Ala-Phe-OMe (100 μM) under both pre-steady-state (350 msec) and steady-state (50 sec) time intervals. Excitation was at 285 nm and either (A) the enzyme tryptophan emission at 340 nm or (B) the substrate dansyl emission above 430 nm was observed. Assay conditions were 5 mM $CaCl_2$, 0.25 M KCl, 25 mM Mes, pH 6 and 20 °C.

Thus, fluorescence intensity is directly proportional to concentration under these conditions[30].

Enzyme tryptophan fluorescence during RET

Prior to an RET stopped-flow experiment the fluorescence cell, open to 285 nm light, contains fluorescing enzyme and unbound dansyl product from a prior experiment. The observed tryptophan fluorescence intensity at this time, measured at 340 nm, constitutes a baseline value, I_B, which has two components.

$$I_B = I_{E_T} + B_0. \tag{3}$$

Where I_{E_T} is the fluorescence due to the total enzyme E_T, and B_0 represents nonspecific background fluorescence, which remains constant with time. The fluorescence intensity at time t after mixing of enzyme and substrate is

$$I_t = I_E + \Sigma I_{ES_i} + B_0 \tag{4}$$

where I_E is the intensity due to free enzyme at time t, and ΣI_{ES_i}, the sum of the intensities of all intermediates, ES_i, at that time. (For simplicity, Σ refers to the sum over all i (i = 1, 2, ... n) unless otherwise stated.) The observed change in fluorescence at time t, F_t, is

$$F_t = (I_t - I_B) = \Sigma I_{ES_i} + (I_E - I_{E_T}). \tag{5}$$

If the total enzyme concentration is sufficiently low, then its fluorescence is proportional to its concentration. Hence, from equation (2)

$$I_E = C_T I_0 Q_0 a_0 [E] \tag{6}$$

where a_0 and Q_0 are the molar absorptivity and quantum yield of the enzyme respectively, l is 1 cm, [E] is the concentration of free enzyme at time t, and C_T is an instrumental factor relating the absolute tryptophan fluorescence intensity to the signal observed on the oscilloscope.
Similarly, for the ith ES complex

$$I_{ES_i} = C_T I_0 Q_{ES_i} a_{ES_i} [ES_i]. \tag{7}$$

Remembering that $E_T = (E + \Sigma ES_i)$, it follows from equations (5), (6) and (7) that the observed change in fluorescence is

$$F_t = C_T I_0 \Sigma (Q_{ES_i} a_{ES_i} - Q_0 a_0)\,[ES_i]. \tag{8}$$

Substrate dansyl fluorescence during RET

Energy transfer to the bound substrate dansyl group results in fluorescence in the region of 550 nm, and this may be monitored using a filter which eliminates radiation below 430 nm. The fluorescence intensity at zero time is

$$I_B = B_0 \tag{9}$$

where B_0 represents non-specific background and is again constant with time. The intensity at time t is

$$I_t = B_0 + \Sigma I_{ES_i} \tag{10}$$

where I_{ES_i} represents the intensity due to energy transfer from the enzyme to the dansyl group in the ith ES complex. Thus,

$$F_t = (I_t - I_B) = \Sigma I_{ES_i}. \tag{11}$$

If the fluorescence intensity of the enzyme is directly proportional to its concentration, then the intensity of the ES complex will also show such proportionality, i.e. the fluorescence intensity of the dansyl group is

$$I_{ES_i} = C_D I_0 a_{ES_i} Q_i T_i [ES_i] \tag{12}$$

where Q_i is the quantum yield of the dansyl group in ES_i, T_i the efficiency of energy transfer from enzyme to the ith ES complex, and C_D the appropriate instrumental factor related to dansyl fluorescence. Hence,

$$F_t = C_D I_0 \Sigma a_{ES_i} Q_i T_i [ES_i]. \tag{13}$$

Normalization

Compared to the enzyme, the dansyl group absorbs weakly at the excitation wavelength. Nevertheless, at high substrate concentrations this absorption may be significant, and small variations in the apparent light intensity, I_0, may occur for different initial substrate concentrations. Since I_0 is contained within equation (8) and (13), variation in this parameter at high substrate concentrations is undesirable. To obviate this difficulty, the fluorescence change in either tryptophan or dansyl signal may be normalized at any given substrate concentration by the fluorescence intensity of the enzyme I_{E_T}. That is F_t may be redefined as

$$F_t = (I_t - I_B)/I_{E_T}. \tag{14}$$

This normalization removes the variable I_0 appearing in equations (8) and (13). Thus, for changes in enzyme tryptophan fluorescence,

$$F_t = \frac{1}{[E_t]} \Sigma \left(\frac{a_{ES_i} Q_{ES_i}}{a_0 Q_0} - 1 \right) [ES_i] \tag{15}$$

and for changes in substrate dansyl fluorescence,

$$F_t = \Sigma \frac{C_D\, a_{ES_i}\, Q_i T_i}{C_T\, a_0\, Q_0} \frac{[ES_i]}{[E_T]}. \tag{16}$$

Importantly, equations (8) and (13), and if normalization is necessary, equations (15) and (16) are all of the form

$$F_t = \Sigma C_i [ES_i] \tag{17}$$

where C_i are constants for a given enzyme and substrate at fixed conditions of pH, temperature, and buffer, showing that the observed fluorescence changes are

dependent upon, and only upon, the concentrations of the individual ES complexes (or, by the mass conservation equation, the free enzyme concentration).

4. RET analysis at steady state

Equation (17) shows the relationship between the concentrations of fluorescent species and the observed oscilloscope signal. To relate such signals to the kinetic parameters k_{cat} and K_m requires a steady state, i.e. a time interval during which the conventional Michaelis-Menten equation is applicable.

$$V = \frac{-d[S]}{dt} = \frac{d[P]}{dt} = \frac{k_{cat}[E_T][S]}{K_m + [S]} \tag{18}$$

where k_{cat} and K_m are an assembly of rate and equilibrium constants and V and S represent the reaction velocity and free substrate concentration.
If $E_T \ll K_m$, it has been shown that this expression holds until hydrolysis is complete[29]. We have found these conditions to be readily achieved for a number of proteases acting on specific oligo- and depsipeptide substrates[22, 23].
Under such conditions k_{cat} and K_m may be obtained by multiple means from the observed fluorescence changes.

Definition of the RET parameters F_{max} and A_0

At the inception of the steady state, the fluorescence, F, is at a maximum, which we shall call F_{max}. This is illustrated schematically in figure 3. As hydrolysis proceeds, the concentration of ES complexes, and hence F, falls to zero, describing a total area A, defined by

$$A_0 = \int_0^\infty F_t \, dt. \tag{19}$$

It has been shown[26] that at steady state, for all i:

$$[ES_i] = c_i[ES_T] \tag{20}$$

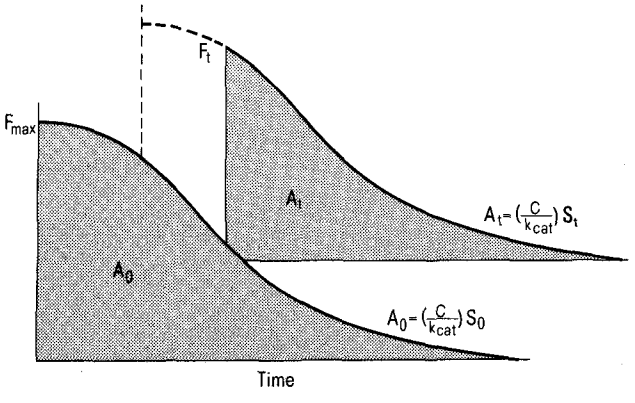

Figure 3. Schematic illustration of the steady-state portion of the oscilloscope trance defining the RET kinetic parameters, F_{max}, the maximal fluorescence; A_0, the total area; and F_t and A_t, the fluorescence and area at any time t.

where $[ES_T]$ is the total concentration of ES complexes, and c_i are complex constants comprising the individual rate and equlibrium constants that govern scheme A. It follows from equation (17) that

$$F_t = C[ES_T] \tag{21}$$

where $C = \Sigma(C_i c_i)$.
C is a constant for a given enzyme and substrate at fixed pH and temperature. This equation shows that under steady-state conditions the observed fluorescence at any time, F_t, is directly proportional to the total concentration of ES complexes $[ES_T]$ present at that time[23]. It should be noted that scheme A may contain partial product release prior to the breakdown of the nth intermediate, as occurs, for example, upon the formation of an acyl intermediate. However, even if the fluorescent probe leaves the enzyme surface prior to formation of the final intermediate, it is clear from equation (17) that the overall form of the equations are unchanged. 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.

Relationship between A_0 and S_0

Under steady-state conditions the rate of product formation is

$$\frac{d[P]}{dt} = V_t = k_{cat}[ES_T] \tag{22}$$

combining equations (21) and (22) leads to

$$F_t = \frac{C}{k_{cat}} \frac{d[P]}{dt}. \tag{23}$$

This equation shows that the observed change, F_t, at time t, is directly proportional to the reaction velocity at that time. The total area A_0, equation (19), may now be expressed in terms of k_{cat} and the reaction velocity.

$$A_0 = \int_0^\infty F_t dt = \frac{C}{k_{cat}} \int_0^\infty dp = \frac{C}{k_{cat}} [P_0]. \tag{24}$$

Since by definition all substrate is converted into product, then

$$A_0 = \frac{C}{k_{cat}} [S_0]. \tag{25}$$

The total area A_0 is directly proportional to the initial substrate concentration, S_0. Our RET studies of bovine and yeast carboxypeptidases and α-chymotrypsin have shown this relationship holds over nearly a thousand fold variation in substrate concentration[23].

Relationship between reaction velocity, V, and F_{max}

The combination of the appropriate equations[23] eliminates the constant, C, and gives

$$\frac{F_{max}[S_0]}{A_0} = \frac{k_{cat}[E_T][S]}{K_m + [S]} = V. \tag{26}$$

This equation shows the direct relationship between F_{max}/A_0 and the initial reaction velocity, V. It predicts the reaction velocity, i.e. $F_{max}[S_0]/A_0$, to be linearly dependent on the total enzyme concentration $[E_T]$ if the reaction is under steady-state conditions. If $[S_0] \gg [E_T]$, the concentration of unbound substrate, [S] in equation (26) is approximated by $[S_0]$. Measurement of F_{max} and A_0 values at different initial substrate concentrations, $[S_0]$ allows the determination of k_{cat} and K_m.

Determination of k_{cat} and K_m at a single substrate concentration

The complete reaction course at a single initial substrate concentration contains all the information needed to calculate K_{cat} and K_m. The velocity, V_t, at any time t can be expressed as

$$V_t = \frac{F_t [S]_t}{A_t} = \frac{k_{cat}[E_T][S]_t}{K_m + [S]_t}. \quad (27)$$

The variables F_t and A_t (fig. 3) are directly determined from the oscilloscope traces, while S_t is readily determined from the relationship between the area under the curve and the substrate concentration (equation 25). Thus a plot of $1/V_t$ vs $1/[S]_t$, or any other suitable manipulation of the Michaelis Menten equation, yields values of k_{cat} and K_m determined from a single substrate concentration. This method of analysis is of particular value for rapid screening of substrates (table 1) and facile evaluation of the effects of pH, temperature, ionic strength, solvents and inhibitors or activators of enzyme activity.

Table 1. Kinetic parameters from stopped-flow RET assays at a single substrate concentration

Enzyme	E_T μM	Substrate	S_o μM	k_{cat} sec^{-1}	K_m μM
Carboxypeptidase Y[a]	0.1	Dns-Gly-Gly-Gly-OPhe	20	16	4
	0.5	Dns-Phe-Ala	50	15	16
	0.5	Dns-Ala-Ala-Phe	100	420	15
Carboxypeptidase A[b]	0.5	Dns-Gly-Gly-Phe	50	200	330
	0.5	Dns-Ala-Gly-Phe	50	400	140
	0.5	Dns-Gly-Ala-Phe	50	170	42
	0.5	Dns-Ala-Ala-Phe	50	110	20
Carboxypeptidase B[b]	1.0	Dns-Gly-Gly-Arg	50	71	200
Thermolysin[a]	2.0	Dns-Gly-Ophe-Ala	100	7	50
	2.0	Dns-Gly-Gly-Phe-Ala	100	600	390
	0.5	Dns-Ala-Ala-Phe-Ala	100	180	20
S. griseus carboxypeptidase[b]	1.0	Dns-Gly-Gly-OLeu	50	200	75
	1.0	Dns-Gly-Gly-Phe	100	5	250
	0.5	Dns-Ala-Ala-Phe	100	4	27
	1.0	Dns-Gly-Gly-Lys	50	30	170
S. griseus neutral protease[a]	1.6	Dns-Gly-OPhe-Ala	50	65	150
Trypsin[a]	2.8	Dns-Gly-Gly-ArgOMe	50	12	30
α-Chymotrypsin[a]	0.6	Dns-Gly-Gly-PheOMe	30	24	45
	0.6	Dns-Gly-Ala-PheOMe	30	25	33
	0.6	Dns-Ala-Gly-PheOMe	30	11	28
Aeromonas aminopeptidase[b]	0.2	Leu-Ala-DED	10	38	4
	0.1	Ile-Ala-DED	10	2	0.3
B. cereus β-lactamase[c]	1.0	Dns-6-amino penicillanic acid	100	1600	440

[a] pH 6.2, 25 °C. [b] pH 7.5, 25 °C. [c] pH 6.8, 20 °C.

Determination of k_{cat}/K_m

When $[S]_t \ll K_m$, as occurs during the latter stage of catalysis, the rate of change in ES complex concentration (fig. 3) becomes first order with a constant, k_{obs}.

$$k_{obs} = [E_T]\, k_{cat}/K_m. \quad (28)$$

The determination of k_{cat}/K_m by this means is particularly useful for detecting the occurrence of product inhibition. Thus if product changes either k_{cat} or K_m it will be reflected in a dependence of this first order rate constant on substrate concentration.

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 (fig. 2, 3). This corresponds to the initial steady-state portion of the reaction, where the initial velocity is constant, and may be related to the initial free substrate concentration. It follows from equation (21) that

$$F_{max} = \frac{C[E_T][S]}{K_m + [S]} = \frac{F^*_{max}[S]}{K_m + [S]} \quad (29)$$

where F^*_{max} is the limiting value of F_{max} when the enzyme is saturated with substrate[23]. Using equation 29 K_m may be evaluated from the variation of F_{max} with [S]. Normalization of the observed signal to the background fluorescence (equations 15 and 16) may be needed if the fluorescent signal is observed in the presence of an absorbance greater than 0.05.

It is clear from the preceding sections that the generation of a steady-state under stopped-flow conditions allows the evaluation of the conventional kinetic parameters, k_{cat} and K_m, by multiple means, and is a particular advantage of the RET approach (table 2).

5. RET Analyses at pre-steady state

In general, the substrate concentration may be considered constant during the transient time interval prior to the attainment of the steady state, if enzyme and substrate are mixed under the conditions $E_T \ll S_0$. The pre-steady-state time interval has been extensively examined under these conditions, but generally in terms of the concentration of product produced[7, 19, 31] rather than in terms of ES complex concentrations, since it is the former that is generally monitored. However, for scheme A, the equations for ES_i are also soluble[7, 15].

Table 2. RET analysis at steady state[a]

Method	Equation	k_{cat}	K_m	k_{cat}/K_m
1/V vs 1/S	26[b]	+	+	+
$1/F_{max}$ vs 1/S	29[b]	−	+	−
$1/V_t$ vs $1/S_t$	27[c]	+	+	+
k_{obs}	28[c]	−	−	+

[a] The plus sign indicates which kinetic parameter can be determined. [b] Analysis requires different initial substrate concentrations. [c] Analysis performed at single initial substrate concentration.

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Under such conditions, scheme A may be rewritten as

$$X_0 \underset{k_{-1}}{\overset{k_1 S_0}{\rightleftharpoons}} X_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} X_2 \ldots X_n \xrightarrow{k_{(n+1)}} X_0 + P \qquad \text{Scheme B}$$

where X_0 represents free enzyme and X_i represent ES_i ($i = 1 \rightarrow n$). Because of the condition of mass conservation,

$$E_T = \sum_{i=0}^{n} X_i \tag{30}$$

we may write for the n ES complexes n linear differential equations which are soluble by standard methods of linear algebra. The solutions are of the general form

$$ES_i = \sum_{j=1}^{n} A_{ij} e^{-k_j t} \qquad (i = 1, 2 \ldots n). \tag{31}$$

Hence, from equations (17) and (31)

$$F_t = \sum_{j=1}^{n} C_j e^{-k_j t} \tag{32}$$

where

$$C_j = \left(\sum_{i=1}^{n} C_i A_{ij} \right).$$

Thus, the observed fluorescence changes may be expressed as a series of exponential terms whose coefficients, C_j and k_j, in principle are known functions of rate constants and concentrations. Hence, these changes may yield direct mechanistic information, both as to the number of intermediates and to their rates of interconversion.

While the overall form of equation (32) is complex, in general many of the reciprocal time constants k_j will be well separated. Under such conditions the mechanism may be studied under various time intervals, during each of which it can usually be considered as having a small number of intermediates, with the first and last 'intermediate' in rapid equilibrium. The complexity is thus greatly reduced and meaningful mechanistic information readily obtained[10, 22].

The examples of reaction mechanisms listed in table 3 illustrate how RET kinetics can aid in differentiating between mechanisms which all lead to Michaelis-Menten kinetics under steady-state conditions. If intermediates can be directly observed under pre-steady-state conditions an apparent first-order rate constant k can be derived from the exponential signal change for all the mechanisms. However for each mechanism the rate constant k will have a characteristic dependence on substrate concentration which can be diagnostic (table 3). Thus, mechanism I represents an isomerization in the free enzyme which must occur before substrate can bind. In this case, k will have a decreasing sigmoidal dependence on the increasing logarithmic substrate concentration (fig. 4A). Mechanism 2 represents a slow equilibration of substrate binding to the enzyme. The value of k will increase linearly with the substrate concentration (fig. 4B). The last two mechanisms, an isomerization of two ES complexes (No. 3) and formation of a covalent intermediate (No. 4) give rise to an increasing sigmoidal dependence of k on the value of log [S] (fig. 4C). The latter mechanisms can be resolved in three ways: 1) The calculated steady-state values of k_{cat} and K_m will be different for the two mechanisms. 2) The concentration of the first product, P_1, will increase k for mechanism 4 and not for 3, and 3) by performing trapping experiments with added nucleophiles, e.g. hydroxylamine or alcolhols[22]. RET kinetics can therefore readily differentiate between these four mechanisms which are often discussed in the biochemical literature. For example we have observed mechanism 3 for carboxypeptidase A at sub-zero temperatures[13] and mechanism 4 for α-chymotrypsin at ambient temperatures[22].

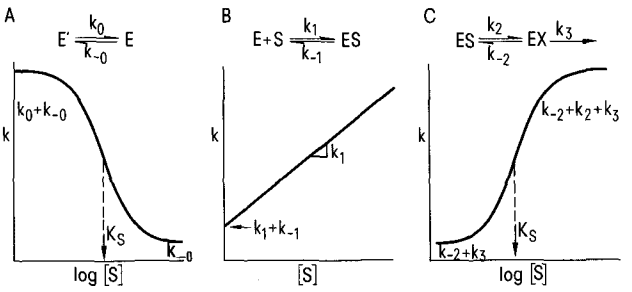

Figure 4. The substrate concentration dependence of a first order rate constant, k, evaluated from the pre-steady-state time interval. A represents the result expected for a conformation occurring in the free enzyme, B that for a direct substrate binding mechanism and C that for a slow equilibration of two ES complexes (see table 3).

Table 3. Reaction mechanisms distinquished by their pre-steady kinetics

No.	Mechanism	Rate expression for k
1.	$E' \underset{k_{-o}}{\overset{k_o}{\rightleftharpoons}} E \underset{\text{fast}}{\overset{SK_S}{\rightleftharpoons}} ES$	$k_o + k_{-o} \frac{K_S}{K_S + [S]}$
2.	$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2}$	$k_2 + k_{-1} + k_1 [S]$
3.	$E + S \underset{\text{fast}}{\overset{K_S}{\rightleftharpoons}} ES_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} ES_2 \xrightarrow{k_3}$	$k_{-2} + k_3 + k_2 \frac{[S]}{K_S + [S]}$
4.	$E + S \underset{\text{fast}}{\overset{K_S}{\rightleftharpoons}} ES \underset{k_{-2}[P_1]}{\overset{k_2}{\rightleftharpoons}} EA \xrightarrow{k_3}$	$k_{-2}[P_1] + k_3 + k_2 \frac{[S]}{K_S + [S]}$

6. *Direct observation of the ES complex by direct substrate excitation*

The direct observation of ES complexes using fluorescent substrates is also possible using an alternative approach, direct excitation of the substrate fluorophore. New intermediates have been directly observed and documented during peptide hydrolysis by both pepsin and papain[24, 25, 28].

In this approach the fluorophore of the substrate is excited directly, and is thus always fluorescent, be it in the form of substrate, product, or ES complex. However, on binding to the enzyme surface the intrinsic fluores-

cence of the substrate is often greatly enhanced due to changes in the rigidity and/or hydrophobicity of the fluorophore environment. Changes in concentrations and/or quantum yields, between ES complexes cause changes in fluorescence, signaling directly their existence, and allowing their quantitation. While less sensitive than the RET method, since there is always background fluorescence, this technique is particularly valuable under the condition $E_T \gg S_0$ and is complementary to the RET approach in many respects.

7. Mathematical analysis of fluorescence changes on direct substrate excitation

When the concentration of a fluorescent substrate is sufficiently low to be proportional to its fluorescence (equation 2) then the equations relating the observed fluorescence intensity to the fluorophore concentration may be readily derived in a manner analogous to that for the RET approach. These equations are similar to, yet have critical differences from, those governing the RET approach.

Prior to the stopped-flow experiment the fluorescence cell contains fluorescing unbound product. The signal intensity at that time is

$$I_B = I_{P_0} + B_0 \tag{33}$$

where I_{P_0} is the fluorescence due to the completely hydrolyzed unbound product and B_0 is non-specific background fluorescence which may be considered constant with time. The fluorescence intensity at time t after mixing of enzyme and substrate is

$$I_t = I_{P_t} + I_{S_t} + \Sigma I_{ES_i} + B_0 \tag{34}$$

where I_P, I_S, and I_{ES_i} are respectively the fluorescence intensities due to the free product, free substrate, and the ith ES complex, at that time. The observed fluorescence change F_t is

$$F_t = (I_t - I_B) = (I_{P_t} - I_{P_0}) + I_{S_t} + \Sigma I_{ES_i}. \tag{35}$$

If the quantum efficiencies and molar absorptivities of the fluorophore in the substrate, product and the ith ES complex are Q_s and a_s, Q_p and a_p and Q_i and a_i, respectively, then from equations (2) and (35),

$$F_t = CQ_pa_p(P_t - P_0) + CQ_sa_s S_t + \Sigma CQ_ia_i[ES_i] \tag{36}$$

where C is a constant. Substituting for P_0 and S_t from

$P_0 = S_0 = S_t + P_t + \Sigma ES_t$, then;

$$F_t = C(Q_sa_s - Q_pa_p)(S_0 - P_t) + C\Sigma(Q_ia_i - Q_sa_s)[ES_i]. \tag{37}$$

Equations (36) and (37) show that for this approach the fluorescence changes observed under stopped-flow conditions are related not only to the concentration of ES complexes but also to the substrate and product concentration. Only if the quantum yield and molar absorptivity of the substrate are identical to those for the product can equation (37) be simplified to an equation analogous to that obtained for RET analysis (equation 17), i.e.

$$F_t = C\Sigma(Q_ia_i - Q_sa_s)[ES_i]. \tag{38}$$

Direct excitation of the dansyl substrate at steady state

By virtue of its experimental limitations, the direct excitation process is not well suited to the conditions $E_T \ll S_0$. Thus if the concentration of the ES complex is 1% of the total substrate present the dansyl quantum yield for the substrate in the ES complex must increase considerably in order to observe it directly. In addition even a 1% difference in the fluorescent properties of substrate and product could cause a substantial interference at this level of ES complex detection. Fortunately, if the fluorophore is placed at a distance from the scissile bond, the quantum yield and molar absorptivity differences between substrate and product are frequently less than 1%. Moreover, the quantum yield of the dansyl group in water is very low, 0.06, and may increase to some ten times this value when bound to the enzyme surface. Thus if the concentration of S is approximately ten times that of E and the above conditions hold a considerable change in fluorescence will be observed upon binding substrate to the enzyme. When such conditions are achieved equation (38) is valid, and kinetic parameters can be calculated as in the case for RET analysis (table 2).

Direct excitation at pre-steady state

By definition, during the pre-steady-state interval the product formed is negligible. Thus, for the direct excitation approach, from equation (37)

$$F_t = C_0S_0 + \Sigma C_i[ES_i] \tag{39}$$

where

$$Co = C(Q_sa_s - Q_pa_p) \quad \text{and} \quad C_i = C(Q_ia_i - Q_sa_s)$$

and, from equation (31),

$$F_t = C_0S_0 + \sum_{j=1}^{n} C_j e^{-kjt}. \tag{40}$$

Equation (40) is analogous to equation (32), but contains an additional constant. C_0 represents the difference in fluorescence between substrate and product. Hence a rapid change in fluorescence, either positive or negative, may occur on mixing within the stopped-flow instrument. Moreover it will change with substrate concentration. In the RET approach such a change observed within the mixing time of the stopped-flow instrument signifies the rapid formation of an intermediate, but cannot be interpreted in the same way using the direct excitation approach without knowledge of C_0.

Constant enzyme time interval ($E_T \gg S_0$)

Because of its higher sensitivity, the RET approach is of most value when $E_T \ll S_0$, generating both pre-steady-

state and steady-state time intervals. In contrast, the direct excitation approach is more suited to the condition $E_T \gg S_0$. In this case, the free enzyme concentration may be considered constant and equal to the total enzyme concentration over the whole reaction course. Under such conditions, Scheme A may be rewritten as

$$x_0 \underset{k_{-1}}{\overset{k_1 E_T}{\rightleftharpoons}} x_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} x_2 \ldots x_n \xrightarrow{k_{(n+1)}} x_{(n+1)} \qquad \text{Scheme C}$$

where x_0 is free substrate, x_i is ES_i, and $x_{(n+1)}$ is free product. From the mass conservation equation

$$S_0 = \sum_{i=0}^{n+1} x_i . \tag{41}$$

Thus, we may write (n+1) linear equations whose solution is again of the general form

$$x_i = \sum_{j=1}^{(n+1)} A_{ij} e^{-k_j t} \qquad (i = 0, 1, 2 \ldots n+1). \tag{42}$$

Substitution of equation (42) into (37) gives the general solution for the direct excitation approach. Since the solutions for S, P, and ES_i all have the same general form, F_t is again represented as a series of exponentials, i.e.

$$F_t = \sum_{j=1}^{n+1} C_j e^{-k_j t} \tag{43}$$

where

$$C_j = \left(\sum_{i=0}^{n+1} C_0 A_{ij} + \sum_{i=1}^{n+1} C_i A_{ij} \right).$$

Thus, under the conditions $E_T \gg S_0$, the difference in fluorescence between substrate and product is of concern only when attempting to interpret any change in fluorescence which has occurred during the mixing time of the stopped-flow instrument.

Quasi-equilibrium assumption

If the breakdown of the final intermediate of scheme C is rate-limiting, we may assume that all the ES complexes are in thermodynamic equilibrium, and we may reformulate scheme C as

$$E + S \overset{K_m}{\rightleftharpoons} ES_T \xrightarrow{k_{cat}} E + P \qquad \text{Scheme D}$$

where ES_T is the total concentration of ES complexes and K_m a true equilibrium constant.
It is readily shown for scheme D, using equation analogous to those for RET analysis at steady state, that the ratio of the maximal fluorescence F_{max} to the total area A_0 is

$$\frac{F_{max}}{A_0} = \frac{k_{cat}[E_T]}{K_m + [E_T]}. \tag{44}$$

Thus, the observed fluorescence for scheme D under the conditions $E_T \gg S_0$ shows an exponential signal decay from its maximal value. A plot of the reciprocal of the enzyme concentration against A_0/F_{max}, or the reciprocal of the rate constant characterizing the signal decay, thus leads to the evaluation of K_m and k_{cat}.

8. Determination of transfer efficiencies and quantum yields of ES complexes

If the absorption properties of the fluorophore are unaffected by binding, the direct observation of fluorescence changes during catalysis may allow, under favorable conditions, the determination of quantum efficiencies of individual ES complexes.

Tryptophan transfer efficiency

The normalized intensity of enzyme tryptophan fluorescence at time t is given by equation (15)

$$F_t = \frac{1}{[E_T]} \sum \left(\frac{a_{ES_i} Q_{ES_i}}{a_0 Q_0} - 1 \right) [ES_i] \tag{15}$$

Since there is quenching of enzyme fluorescence, the quantum yield of the enzyme in the ith ES complex, Q_{ES_i}, is less than that, Q_0, of the native enzyme, and F_t is negative. Remembering that the efficiency of energy transfer between the ith ES complex and the substrate is $T_i = (Q_0 - Q_{ES_i})/Q_0$, then if the absorptivity a is constant,

$$F_t = -\frac{1}{[E_T]} \Sigma T_i [ES_i]. \tag{45}$$

Since, during the pre-steady-state time interval, the differential equations governing the variation of ES_i with time are soluble for all i, in principle the magnitude of the quenching of enzyme fluorescence during this time interval allows the determination of all T_i.

Dansyl quantum yield

The normalized intensity of substrate dansyl fluorescence is given by equation (16) which becomes, if a is constant,

$$F_t = \frac{C_D}{C_T [E_T] Q_0} \Sigma Q_i T_i [ES_i] \tag{46}$$

where Q_i is the quantum efficiency of the substrate fluorophore in the ith ES complex. Since T_i can be determined from the quenching of enzyme fluorescence, and C_D and C_T are experimental parameters which are determinable, at least in principle, then the quantum efficiencies of individual ES complexes may be determined. While such determinations would clearly be complex, if the dansyl quantum yield can be determined for any one substrate, e.g. a slowly turned over substrate, then relative quantum yields can be readily evaluated from F_t values for any substrate determined under the same stopped-flow conditions[21].
Quantum yields may be more easily evaluated on direct excitation of the substrate. If the observed fluorescence intensity is normalized, then from equation (37) the fractional increase in fluorescence observed is

$$\frac{F_t}{I_0 - B_0} = \frac{F_t}{CQ_pa_pS_0} = \left(\frac{Q_sa_s}{Q_pa_p} - 1\right)\left(1 - \frac{P_t}{S_0}\right) + \frac{1}{S_0}\sum\left(\frac{Q_ia_i - Q_sa_s}{Q_pa_p}\right)[ES_i]. \quad (47)$$

If the absorbance properties of the fluorophore are identical for substrate, product, and ES complexes then

$$\frac{F_t}{I_0 - B_0} = \left(\frac{Q_s}{Q_p} - 1\right)\left(1 - \frac{P_t}{S_0}\right) + \frac{1}{S_0}\sum\left(\frac{Q_i - Q_s}{Q_p}\right)[ES_i]. \quad (48)$$

Moreover, if the quantum yields of substrate and product are similar, as is generally the case if the fluorophore is remote from the scissile bond, then

$$\frac{F_t}{I_0 - B_0} = \frac{1}{S_0}\sum\left(\frac{Q_i}{Q_0} - 1\right)[ES_i] \quad (49)$$

where $Q_0 = Q_s \simeq Q_p$.

Hence a knowledge of the mechanism and Q_0 allows the ready evaluation of Q_i.

The evaluation of quantum yields for each ES complex should give information about the environment of the substrate during catalysis since the fluorescence efficiency of most organic molecules, reflected in their quantum yields, is very sensitive to orientation, hydrophobicity, and motional freedom[17,18]. Our investigations, using α-chymotrypsin and carboxypeptidase A, have both validated the appropriate equations and allowed the evaluation of quantum yields by this means (unpublished observations).

The variation of quantum yields betwen ES complexes may give both quantitative as well as qualitative information on substrate structure. We have already used RET to determine distances between the substrate fluorophore and an active-site metal ion during catalysis itself[20,21]. Accurate evaluation of such distance measurements requires accurate quantum yields, which were estimated by indirect means. Now we may estimate with greater accuracy the distance between metal ion and fluorophore for each ES complex, indicating geometry changes that occur as catalysis proceeds.

9. *Discussion*

The interaction between proteases and fluorescent peptide and ester substrates under stopped-flow conditions, monitored by radiationless energy transfer or by direct excitation of the substrate, allows the direct observation of ES complexes during catalysis[1-4,6,13,22-25,28]. The RET approach has been validated using the well-studied enzymes carboxypeptidase A[3,4,13,23] and α-chymotrypsin[22,23], and the method is now being applied to a wide variety of seryl- and metalloproteases[4,22] including aeromonas aminopeptidase[6] (table 1), *S. aureus* β-lactamase[1,2] and *B. cereus* β-lactamase (table 1, unpublished observations: King and Auld). In addition to the above enzymes, the direct excitation approach has been used with success to detect intermediates during catalysis by pepsin and papain[24,25,28]. Both the RET and direct excitation approaches are widely applicable to mechanistic studies.

Direct observation of ES complexes by means of radiationless energy transfer has a number of advantages over other electronic processes. The inherent sensitivity of fluorescence allows the use of micromolar or lower enzyme concentrations. Under these conditions, 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. Thus direct observation of the ES complex by RET under stopped-flow steady-state conditions provides excellent economy of enzyme and allows the use of rapidly turned over substrates while retaining the virtues of conventional initial rate analyses. 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 (table 2).

RET kinetic studies also overcome a major disadvantage of conventional initial rate assays, in that if a pre-steady-state exists, the direct observation of ES complexes under stopped-flow conditions can both determine the number and nature of intermediates directly and quantitate the individual binding and rate constants (table 3).

The basic feature of RET kinetics is that fluorescence is induced through the interaction of an enzyme donor group and a substrate acceptor group. We have used enzyme tryptophanyl residues as fluorescent donors and dansylated substrates as acceptors. In the absence of tryptophan, enzyme tyrosine residues could serve as the donor and a 7-nitrobenzo-2-oxa-1,3-diazole labeled substrate could be used as the acceptor. The fluorescent acceptor can be placed in the substrate at a position remote from the scissile bond. This permits considerable flexibility in the design of the substrates to be used for mechanistic studies. 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[3], ester-thiol ester, or phosphate-phosphonate pairs. 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. Our studies with fluorescent nucleotides suggest it can be applied to both phospodiesterases and nucleotidyl transferases[27]. This is an important class of enzymes for which there are no direct chromophoric assays for either convential steady-state or pre-steady-state kinetics. RET sudies of these enzymes should be extremely useful in defining their mechanisms of action.

The fluorescent approaches described here provide a means to overcome the problems of sensitivity caused by the low concentrations of ES complexes present during catalysis. However, the short lifetimes of ES complexes pose further difficulties. Thus, even when the stopped-flow technique is used, ES complexes may form and equilibrate during the instrumetnal mixing time, and hence only be collectively observable. The use of very low temperatures has proven particularly successful in circumventing this problem by slowing the time-scale of catalysis sufficiently to allow the use of conventional optical methods[5,8,9,11,12]. The combination of fluorescent substrates, which can directly monitor ES complexes, with low temperatures, to slow the time-scale of catalysis, would thus seem ideal for mechanistic studies. To this end we have constructed a low temperature stopped-flow apparatus capable of working effectively at $-55\,^{\circ}C$[5,16]. In our investigations of peptide and ester hydrolysis by both bovine and yeast carboxypeptidases we have been able to readily observe and characterize intermediates not detectable at ambient temperature[4,13,14]. The integration of approaches that allow direct visualization of ES complexes through the placement of probes within the enzyme and/or substrates with those that permit low temperature rapid mixing and observation conditions should allow detection of covalent intermediates as well as conformational changes which may accompany binding of substrate to an enzyme as well as the subsequent catalytic and product release steps. These methods should assist in discernment of previously unattainable details of mechanisms of enzyme action.

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