Calorimetric Evaluation of Enzyme Kinetic Parameters

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The measurement of kinetic parameters (k_{cat}, K_m, K_i) for a wide range of proteolytic enzymes is vital to contemporary bioorganic and medicinal chemistry. Enzyme assays based on changes in optical properties of the system or changes in concentration of an ion detectable electrochemically are not viable for many enzyme-catalyzed reactions, including proteases and peptidases. Hydrolysis of an amide bond produces no change in the optical properties or pH of the reaction solution, and as a result no general direct method for the evaluation of protease kinetics exists using underivatized substrates. We report here a microcalorimetric assay which provides a general and straightforward technique for the measurement of kinetic parameters of hydrolysis of underivatized peptide substrates by proteases. Using this technique, k_{cat} values as high as 10^5 s^{-1} can be easily measured. We demonstrate the utility of the technique by measuring the kinetics of hydrolysis of several *N*-acylamino acids by the synthetically useful enzyme hog kidney acylase and the hydrolysis of tetrapeptide *p*-nitrophenyl anilides by subtilisin BPN′. Although we have used the technique to monitor amide bond hydrolysis, the methodology is applicable to any system with appropriate kinetic and thermodynamic properties.

Introduction

One of the most fundamental measurements in biological chemistry is of the kinetic parameters of enzymatic transformation. Determination of k_{cat} and K_m characterizes the ability of an enzyme to catalyze a chemical reaction. Today, much of medicinal and agricultural chemistry rests on the construction of inhibitors of various enzymes: measurement of the efficacy of enzyme inhibition is crucial to the development of an array of biologically active compounds.[1-3] The measurement of enzyme kinetics requires that an observable event accompany transformation. Typically, the events utilized to monitor enzyme-catalyzed reactions are changes in the optical properties, either absorption or emission, of the solution or changes in the concentration of an ion detectable electrochemically, most often H^+. Many enzyme-catalyzed reactions of interest to the medicinal or agricultural chemist produce no readily observable signal. Perhaps the most significant example of this situation is amide bond hydrolysis. The proteases and peptidases are an ubiquitous group of enzymes which in vivo control metabolic and regulatory events including viral assembly, hypertension, coagulation, complement production, fibrinolysis, cell migration, embryogenesis, hormone activation, growth factor activation, extracellular protein degradation, and mast cell function. The construction of inhibitors of these enzymes as therapeutic products requires accurate, efficient, and experimentally feasible assays for the enzymes involved. Hydrolysis of amides produces no change in pH, since the products are self-titrating. Because most peptides and proteins do not change absorption or fluorescence spectra on cleavage, optical assays for amide bond hydrolysis require the introduction of a UV active or fluorescent group at the site of hydrolysis, most frequently a *p*-nitrophenyl anilide. Assays of this nature are neither general nor desirable: hydrolysis of the native substrate cannot be observed, and for proteases which catalyze hydrolysis at an internal position, contributions to K_m by residues at the N-terminal side of the site of hydrolysis are excluded. Fluorescence assays of amide bond hydrolysis have been developed through incorporation of fluorescent side chains into target peptides.[4] Such assays are undesirable because of perturbations in enzyme–substrate binding caused by large hydrophobic fluorophores. A variety of discontinuous or semicontinuous assays have been developed, including HPLC methods,[5,6] derivatization,[7] and coupled enzyme assays,[8] despite the obvious problems with discontinuous assays. Thus, an accurate, convenient assay for amide bond hydrolysis remains a significant need in bioorganic chemistry. We report here a general, direct, and accurate calorimetric assay for amide bond hydrolysis.

Results and Discussion

The kinetic parameters of hydrolysis of a variety of substrates by both acylase I and subtilisin BPN′ were evaluated by measuring the power generated as a function of time following addition of enzyme to a solution of substrate in a calorimeter cell. The kinetic parameters for hydrolysis of nine natural and unnatural *N*-acetylamino acids by hog kidney acylase (EC 3.5.1.14) were measured. In all cases the reaction cell (1.3678 mL) was filled with a solution of substrate in 0.1 M phosphate buffer, pH 7.5 at 25 °C. Each reaction was initiated by addition of enzyme (70–1000 U) in a single 25-μL aliquot from a 250-μL syringe during 15 s. The cell was stirred at 400 rpm. The heat of dilution of the enzyme was large enough to interfere with the signal from the reaction: addition of 1 mg/mL of bovine serum albumin to the substrate suppressed this heat of dilution.[9] Typical plots of the time course evolution of both power (μcal s^{-1}) and integrated heat (μcal) are shown in Figure 1.

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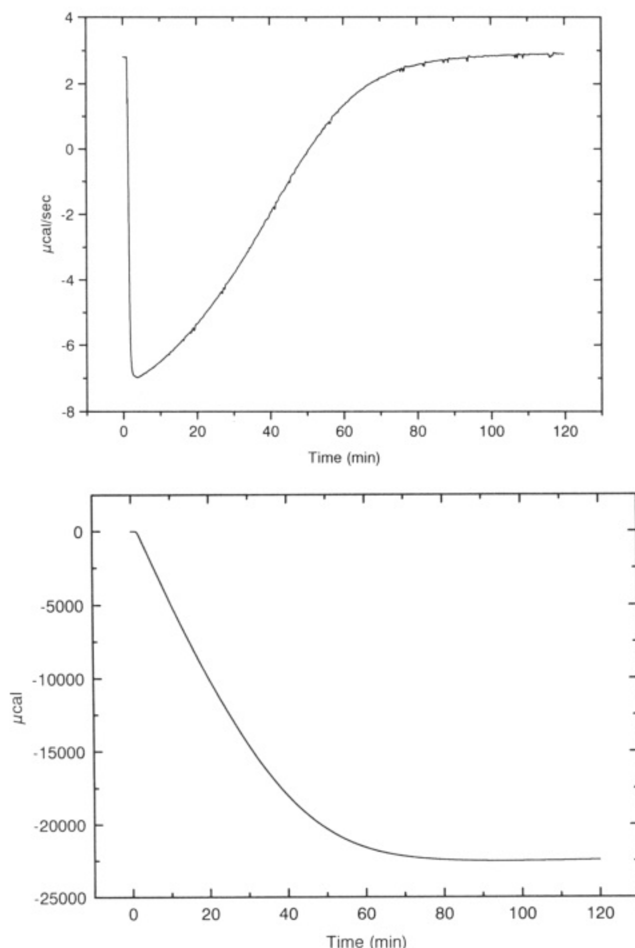

Figure 1. Power (top) and integrated heat (bottom) as a function of time for hydrolysis of *N*-acetyl-L-methionine (30.0 mM) by acylase 1 (70 U, 4.5×10^{-4} mM). Data points were collected at 2-s intervals.

Plots of the time course evolution of integrated heats were converted to product concentrations by dividing the curve by ΔH of hydrolysis (ΔH_R). Since amide bond hydrolysis is irreversible and all reactions go to completion, ΔH_R of each substrate was determined from the integrated heat of reaction at the highest substrate concentration (Table I). Kinetic runs were performed at 8-10 substrate concentrations ranging from 0.1 to 10 times K_m. Initial rates of reaction were determined from the slope of the product appearance curve: the portion of the curve from 40 to 100 s invariably gave straight lines with correlation coefficients >0.99. Values of k_{cat} and K_m were determined from standard reciprocal plots: in all cases correlation coefficients were >0.999. Measured values of k_{cat}, K_m, and ΔH_R are shown in Table I together with literature values of K_m.[10,11]

Kinetic parameters for hydrolysis of the peptides succinyl-Ala-Ala-Pro-Phe-(*p*-nitrophenyl)anilide and succinyl-Ala-Ala-Pro-Leu-(*p*-nitrophenyl)anilide by subtilisin BPN′ were measured in the same fashion as acylase I, in 100 mM Tris–HCl buffer, pH 8.6. Kinetic parameters and heats of reaction for these substrates together with values determined spectrophotometrically and literature constants are shown in Table II.

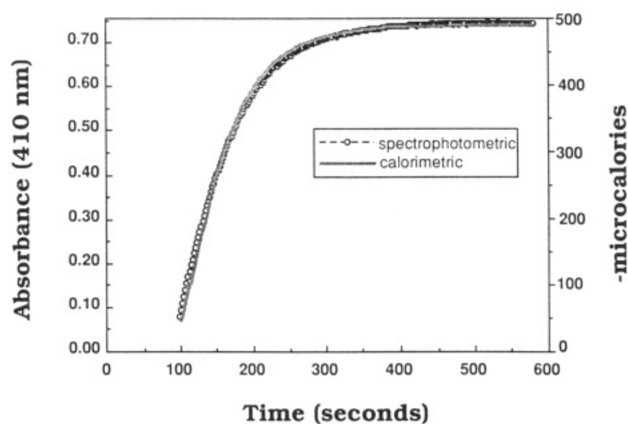

Figure 2. Time–course evolution of product for the hydrolysis of succinyl AAPF-(*p*-nitrophenyl)anilide measured calorimetrically and by UV assay. In both cases peptide concentration was 0.1 mM and subtilisin BPN′ concentration was 5.82×10^{-5} mM. Reactions were measured in 100 mM Tris–HCl buffer pH 8.6 at 22 °C. For both reactions, enzyme was added at 50 s.

Friere and co-workers recently addressed the issue of thermal lag in calorimetric kinetic studies.[12] Although instrument time constants for contemporary continuous power compensation calorimeters are significantly shorter than those of traditional instruments, thermal lag would have a substantial impact on measured reaction rates. Mathematical models have been developed to correct for heat lag effects: we chose to directly examine thermal lag in power compensation calorimetric kinetic experiments. Identical solutions of succinyl-AAPF-(*p*-nitrophenyl)-anilide were hydrolyzed with identical concentrations of subtilisin BPN′ at 22 °C in the calorimeter cell and in a UV assay. Figure 2 shows a time–course evolution of product from the two methods. The traces overlay exactly, demonstrating that for reactions with catalytic rate constants of the magnitude reported here, heat lag is negligable and rates measured calorimetrically are valid without compensation.

Results from calorimetric assays agree well with literature data. Chenault et al. reported initial rates of hydrolysis of a large number of natural and unnatural substrates by acylase I relative to *N*-acetylmethionine.[13] Entries **2**, **3**, **4**, **5**, and **7** were reported by these authors as "good" substrates, with initial relative rates of hydrolysis >10% of *N*-acetylmethionine (entry **1**). Entries **2–5** (Table I) show k_{cat} values such that initial rates of hydrolysis >10% the rate of *N*-acetylmethionine are expected under the Chenault assay conditions: *N*-acetylphenylalanine (**7**) is somewhat slower, with k_{cat} on the order of 5% of that of *N*-acetylmethionine. Calorimetrically determined values of K_m are in good agreement with values determined by discontinuous ninhydrin assay.[10,11]

Calorimetric evaluation of kinetic parameters for hydrolysis of tetrapeptide (*p*-nitrophenyl)anilides by subtilisin BPN′ yields k_{cat} and K_m values essentially identical

(9) The heat of dilution of any species is dependent on the initial and final concentration of the solute, i.e., $\Delta H_{dil} = (\Phi H)_{C2} - (\Phi H)_{C1}$, where H represents the heat content of the solute at infinite dilution and C1 and C2 represent the initial and final concentrations, respectively. Because the surfaces of all globular proteins are dominated by a relatively small group of hydrophilic amino acids, the addition of a second nonparticipating protein to the reaction mixture has the effect of more closely matching the initial and final "concentrations" of solute during addition of enzyme.

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Table I. Hydrolysis of *N*-Acetylamino Acids by Porcine Kidney Acylase I

Ia

entry	R_1	R_2	ΔH (cal mol^{-1})	K_m (mM)	K_m (lit.[10,11]) (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
1	$CH_3S(CH_2)_2$	H	−560	2.7 ± 0.3	3.0, 5	29 ± 2	1.1×10^4
2[a]	$CH_2{=}CH(CH_2)_3$	H	−880	0.49 ± 0.06		85 ± 2	1.7×10^5
3	$CH_3(CH_2)_3$	H	−830	3.2 ± 0.4	3.0	18 ± 1	5.6×10^3
4	CH_3	H	−1160	11 ± 1	10, 15	8.4 ± 0.9	7.3×10^2
5	$(CH_3)_2CH$	H	−730	17 ± 1	22	11 ± 1	6.7×10^2
6	$HSCH_2$	H	−770	5.4 ± 0.9		3.2 ± 0.3	5.9×10^2
7	$PhCH_2$	H	−660	6.9 ± 0.9	5	1.4 ± 0.1	2.0×10^2
8[a]	$HOCH_2$	CH_3	no reaction				
9	$H_2N(CH_2)_4$	H	no reaction				

[a] Racemic substrate; K_m and k_{cat} values are for the *S*-enantiomer only.

Table II. Hydrolysis of Peptides by Subtilisin BPN′

substrate	ΔH (kcal mol^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
succinyl-AAPF-*p*-NO$_2$φ				
calorimetry	−6.3 ± 0.1	0.32 ± 0.02	41 ± 3	1.3×10^5
UV		0.28 ± 0.01	48 ± 3	1.7×10^5
lit.[14]		0.14	50	3.6×10^5
succinyl-AAPL-*p*-NO$_2$φ				
calorimetry	−6.4 ± 0.1	0.40 ± 0.04	37 ± 3	9.2×10^4
UV		0.35 ± 0.1	30 ± 1	8.6×10^4
lit.[15]		0.27	28	1.1×10^5

to those obtained by UV assay and in good agreement with previously reported literature values.[14,15] Because experimental details for the literature values were not reported, direct comparisons of kinetic values are difficult to interpret. Enthalpies of reaction for (*p*-nitrophenyl)-anilides are roughly 10 times greater than those for hydrolysis of acetamides. Enthalpies of hydrolysis of amide bonds are typically near 1 kcal mol^{-1},[16] and the large values of ΔH for (*p*-nitrophenyl)anilide hydrolysis presumably reflects at least partial transfer of the resulting carboxylic acid proton to buffer instead of to (*p*-nitrophenyl)aniline: ΔH of protonation of Tris is −11.3 kcal mol^{-1}.

The potential for evaluation of kinetics through calorimetry was recognized several years ago; however, reports of the use of calorimetry for measurement of enzyme activity are scarce. Examples of the use of calorimetry for the detection of enzyme activity in various matrices have been reported.[17] Calorimetry has been used to measure Michaelis constants for urease,[18] fructose 1,6-diphosphate aldolase,[19] chymotrypsin,[20] acid phosphatase,[21] and lactate dehydrogenase.[22] More recently, Morin and Freire reported the use of calorimetry to examine the effect of salt concentration on the activity of cytochrome C oxidase, although neither k_{cat} nor K_m was measured.[12] Two major drawbacks of calorimeter design in the past have prevented the use of calorimetry as an analytical technique for the measurement of enzyme kinetic parameters: reaction cell size and instrument response time. Conventional calorimeters typically have reactions cells of 20 mL or more, requiring substantial amounts of both enzyme and substrate. Commercially available microcalorimeters now are available with cell volumes of ~1.5 mL, greatly reducing the amount of material required. More significantly, calorimeter instrument response times, defined as the time required for a signal to drop from a maximum value to $1/e$ of that value, have been greatly reduced through the use of power compensation instead of passive heat conductivity.[23] Titration calorimeters relying on passive heat transfer between two thermal sinks show time constants of 100–300 s, while currently available microcalorimeters utilizing continuous power compensation show instrument response times of approximately 16 s.[24]

The range of reaction rates which can be measured calorimetrically is dependent on a variety of technical features including solubility and availability of both enzyme and substrate. Fundamentally, the methodology is limited by the instrument time constant and the minimum and maximum power which can be accurately measured. Completion of the reaction should require at least ten times the instrument time constant.[12] The latter two constraints are both functions of ΔH, K_m, and k_{cat} for the transformation. The rms noise for state of the art microcalorimeters is no more than 10 ncal s^{-1}: power values of 1 μcal s^{-1} are readily measured. For creation of double reciprocal plots, the lowest reaction rate will be measured at a substrate concentration of $\sim 0.1K_m$. Thus, the expression

$$(k_{cat}/K_m[E][S])\Delta HV > 1\ \mu\text{cal s}^{-1}$$

where V is the cell volume in liters provides a lower limit on the k_{cat} values that can be measured for a given K_m and ΔH. The maximum accurately measurable power is approximately 150 μcal s^{-1}; thus,

$$V_{max}\Delta HV < 150\ \mu\text{cal s}^{-1}$$

provides an additional constraint on the upper limit of k_{cat} accessible for a given K_m and ΔH. Within these limits, for reactions with ΔH values between 0.5 and 5 kcal mol^{-1} and K_m values of 1–10 mM, k_{cat} values as high as 10^5 are measurable.

Conclusion

Calorimetry is a general and accurate method for evaluation of enzyme kinetics using underivatized sub-

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(24) Instrument response times are sometimes reported as the rise time to half maximal power output following generation of a heat pulse: this value for modern heat compensation calorimeters is roughly 10 s.

strates. The development of commercial calorimeters with rapid response times and small cell volumes makes calorimetry a feasible technique for nonspecialists. Currently available instrumentation allows measurement of systems with k_{cat} values greater than 10^5 s^{-1}. Although we have demonstrated the methodology for amide bond hydrolysis, the technique is generally applicable to any catalyzed reaction with appropriate kinetic and thermodynamic properties.

Experimental Section

Hog kidney acylase I (EC 3.5.1.14) and subtilisin BPN′ (EC 3.4.4.16, Sigma bacterial protease Type VII) were obtained from the Sigma Chemical Company. *N*-Acetylamino acids **2** and **8** were gifts from Professor G. M. Whitesides, Department of Chemistry, Harvard University. All other *N*-acetylamino acids were purchased from the Sigma Chemical Co. and used without further purification. Acylase I concentrations were measured spectrophotometrically by the method of Edelhoch.[25] Subtilisin BPN′ was determined using $E_{280}^{1\%}$ = 11.7 and a MW of 26 000.[26]

Calorimetry was performed with a Microcal Omega calorimeter (Northampton MA). The design and performance of this instrument has been described.[27]

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