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Enzyme Kinetics Determined Using Calorimetry: A General Assay for Enzyme Activity?[1]

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Two techniques for determining enzyme kinetic constants using isothermal titration microcalorimetry are presented. The methods are based on the proportionality between the rate of a reaction and the thermal power (heat/time) generated. (i) An enzyme can be titrated with increasing amounts of substrate, while pseudo-first-order conditions are maintained. (ii) Following a single injection, the change in thermal power as substrate is depleted can be continuously monitored. Both methods allow highly precise kinetic characterization in a single experiment and can be used to measure enzyme inhibition. Applicability is demonstrated using a representative enzyme from each EC classification, including (i) oxidation–reduction activity of DHFR (EC 1.5.1.3); (ii) transferase activity of creatine phosphokinase (EC 2.7.3.2) and hexokinase (EC 2.7.1.1); (iii) hydrolytic activity of *Heliobacter pylori* urease (EC 3.5.1.5), trypsin (EC 3.4.21.4), and the HIV-1 protease (EC 3.4.21.16); (iv) lyase activity of heparinase (EC 4.1.1.7); and (v) ligase activity of pyruvate carboxylate (EC 6.4.1.1). This nondestructive method is completely general, enabling precise analysis of reactions in spectroscopically opaque solutions, using physiological substrates. Such a universal assay may have wide applicability in functional genomics.

One commonly used method of comparative biochemistry is the analysis of the rate of enzymatic conversion of substrate to product. The ease with which rate information can be obtained varies from simple (e.g., a continuous spectrophotometer assay) to complex (e.g., fixed time points followed by chromatography or electrophoresis and quantitation). For certain enzymes (especially those producing small changes in macromolecules) there exists no straightforward method for obtaining rate information. Alternatively, nonphysiological chromogenic derivatives or coupling enzymes may be exploited to measure activity indirectly. With the expansion of genetic information (and a desire to understand functional genomics) and the simplification of methods for creating site directed mutations, an increase in comparative enzyme analysis is anticipated. A generally applicable assay that is rapid, simple, and precise should help facilitate these studies.

The use of calorimetry to measure the heat generated during enzymatic activity has long been recognized (1–4). These earlier studies, however, did not utilize current features of modern power-compensated titration calorimeters, including (i) the observable, thermal power is directly proportional to the rate (4, 5) and need not be corrected for the time constant of the calorimeter, simplifying data analysis; (ii) multiple additions of substrate allow multiple rate determinations in a single experiment (under pseudo-first-order conditions); and (iii) low amounts of enzyme are required. We demonstrate two methods that exploit the high sensitivity and rapid response time of modern power-compensated isothermal titration calorimeters (6, 7) and present kinetic analysis of the activity of biologically relevant enzymes from every EC classification.

EXPERIMENTAL METHODS

Enzymes. Recombinant urease from *Helicobacter pylori* was synthesized in *Escherichia coli* SE5000, containing the plasmids pHP808/pUEF204 (8), and purified by subjecting the supernate of broken cells to three chromatographic steps. Crude extract was applied to a Q-Sr column (SP, Pharmacia) equilibrated with 20 mM

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potassium phosphate, pH 7.5, 1 mM EDTA, 1 mM 2-ME[4] and eluted with 0–1 M KCl in the same buffer. Fractions containing urease activity (~100 mM KCl) were pooled, concentrated via ultrafiltration, diluted twofold, and reapplied to the Q-Sr column equilibrate with 50 mM borate pH 9.0, 1 mM EDTA, 1 mM 2-ME. The column was developed with a 0–1 M NaCl gradient. Active fractions were pooled, concentrated, and applied to a Superose-12 column equilibrated with 20 mM potassium phosphate, pH 7.0, 100 mM NaCl, 0.1 mM EDTA, 1 mM 2-ME. The purity of fractions containing urease activity was assessed using SDS–PAGE.

GroEL was purified as previously described (9), ensuring that no tryptophan contaminants were present. HIV protease (Q7K/L33I/L63I (10)) was purified as previously described (11). Acetolactate synthetase was a gift from Dr. S. Gutteridge (DuPont). Other enzymes were purchased from Sigma and used without further purification.

Calorimetry. Calorimetric assays were done on Omega-ITC and MCS-ITC instruments (MicroCal Inc.). Reaction cells (1.36 ml) were filled with degassed solutions and equilibrated at the indicated temperatures. Stirring speed was 400 rpm, thermal power was recorded every 2 s, and instrumental feedback was 10–50%.

Enzyme assays. The isothermal power-compensation calorimeter consists of two cells (sample and reference) maintained at constant temperature through the continuous addition of thermal power (6). A user-defined level of power is supplied to the reference cell, usually filled with buffer alone. The temperature difference between the reference cell and the sample cell is measured, and a variable level of thermal power is supplied to the sample cell in order to drive this temperature difference to zero. The raw signal, therefore, is the thermal power supplied to the sample cell. Considering all sources of heat, the total thermal power in the sample cell is a sum of that supplied by the instrument *plus* any chemical thermal power generated within the cell and remains constant during the experiment.

Enzyme reaction rates are determined by measuring the change in instrumental thermal power supplied to the sample cell after addition of the substrate or enzyme through a stirred injection syringe. If the reaction in the cell generates heat (exothermic), the calorimeter must supply less power to maintain constant temperature, and a negative deflection in the observed thermal power is observed. Conversely, if a reaction consumes heat (endothermic), the calorimeter must supply additional power to maintain isothermal conditions, and a positive deflection in the observed thermal power is observed.

In practice, once thermal equilibrium was reached, 5–10 min of baseline instrumental thermal power was collected before the start of the reaction. To determine reaction rates, the power change associated with the ith addition of substrate was averaged over the 10–60 s immediately preceding the i + 1st injection and subtracted from the original baseline. In this manner, the noise associated with the dilution event was ignored and the precision of the rate data increased. The power change, dQ_i/dt was divided by ΔH_{app}, the enzyme concentration (which decreased with each injection), and the cell volume to obtain enzyme turnover rates.

Data were fit to kinetic equations using Kaleidagraph.

RESULTS

Pseudo-First-Order Assays

A thermodynamically favorable chemical reaction is driven by a decrease in free energy, which is the sum of an enthalpic and an entropic term ($\Delta G = \Delta H - T\Delta S$); the enthalpic component is observed as heat. Thus an assay method based on measuring the rate of heat generation accompanying conversion of substrate to product should give a measure of the enzyme reaction rate. Heat (Q) measured as a function of time (dt) is defined as the thermal power:

$$\text{Power} = \frac{dQ}{dt}. \qquad [1]$$

The amount of heat associated with converting n moles of substrate to product is given by

$$Q = n \cdot \Delta H_{app} = [P]_{Total} \cdot V \cdot \Delta H_{app}, \qquad [2]$$

where V is the volume of the solution in the reaction cell, P is the molar concentration of product generated, and ΔH_{app} is an experimentally determined molar enthalpy for the reaction. Thus a measure of thermal power generated by an enzyme immediately gives knowledge of the reaction rate:

$$\text{Power} = \frac{dQ}{dt} = \frac{d[P]}{dt} \cdot V \cdot \Delta H_{app}. \qquad [3]$$

[4] Abbreviations used: BAEE, *N*-α-benzoyl-L-arginine ethyl ester; DHFR, dihydrofolate reductase; DHF, dihydrofolate; 2-ME, 2-mercaptoethanol; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); TEA, triethanolamine.

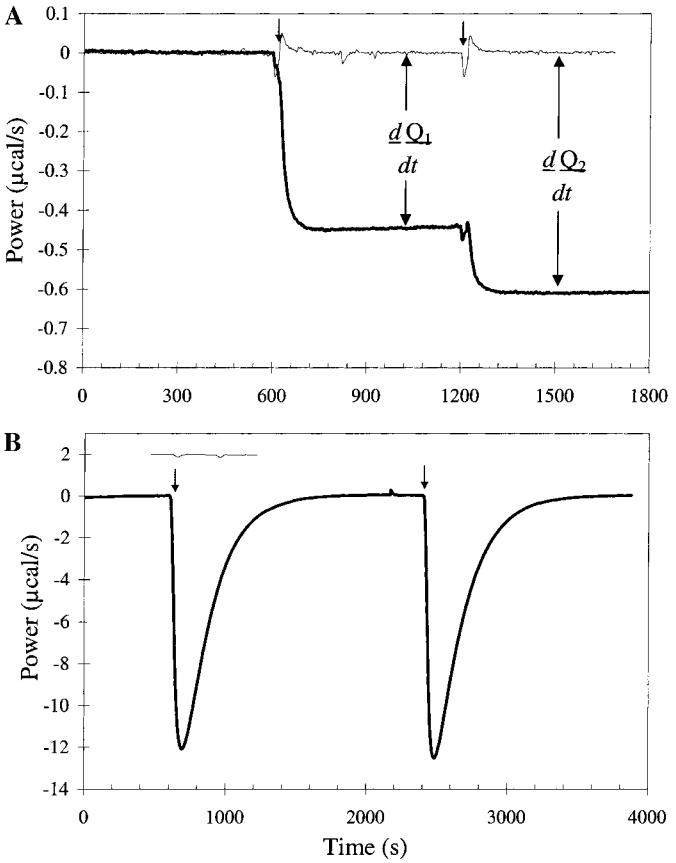

FIG. 1. Calorimetric rate measurements. (A) Thermal power required to maintain constant temperature after addition of substrate to enzyme. Two milliliters of 0 pM (thin line) or 50 pM (thick line) *H. pylori* urease in 20 mM Hepes pH 7.8, 0.1 mM EDTA was loaded into the 1.365-ml reaction cell, and 40 mM urea in the same buffer was loaded into a 250-μl syringe. After thermal equilibrium was reached at 37°C, 3 μl urea was injected (arrow), giving a final urea concentration of 88 μM. A second injection was made 10 min later. (B) Complete conversion of substrate to product gives ΔH_{app} of reaction. Two milliliters of 0 nM (thin line) or 4 nM (thick line) urease in 50 mM Hepes, pH 7.8, 0.1 mM EDTA was loaded into the sample cell and equilibrated at 37°C. Twenty-eight microliters of 25 mM urea was injected (700 nmol, final concentration = 513 μM), and hydrolysis was monitored until the baseline returned to the original level. Numerical integration of the area under the peak gave the total heat for urea hydrolysis, thus ΔH_{app}. A second injection was made 30 min later; the similar peak size/shape confirmed no product inhibition. Identical values for ΔH_{app} were obtained (-10.4 ± 0.1 kcal/mol).

Rearranging, we have

$$\text{Rate} = \frac{d[P]}{dt} = \frac{1}{V \cdot \Delta H_{app}} \cdot \frac{dQ}{dt}. \qquad [4]$$

In Fig. 1A, a calorimeter reaction cell containing buffer (thin line) or buffer plus enzyme (thick line) was allowed to reach thermal equilibrium (0–600 s), and substrate was injected (first arrow). In the absence of enzyme, a small change in power occurred due to the heat of dilution, followed by a return to the original baseline value. In the presence of enzyme, the dilution event was followed by a decrease in instrumental thermal power required to maintain isothermal conditions. The remaining power arose from the enzymatic reaction such that the total thermal power remained constant. The rate of heat generated by the enzyme was equivalent to the decrease in instrumental thermal power (dQ_1/dt). The negative deflection in Fig. 1A indicates that this reaction is exothermic. As negligible substrate depletion occurred, the reaction proceeded at a steady state and the instrumental thermal power remained constant. A second injection (arrow at 1200 s) increased the substrate concentration further, and the enzyme generated thermal power increased (dQ_2/dt).

Reaction rates (in units of power) can be converted to enzyme turnover if the apparent enthalpy (ΔH_{app}) is known (Eq. [4]). The ΔH_{app} can be determined by allowing the reaction to proceed to completion and then integrating the signal to obtain the total heat evolved. In Fig. 1B, the substrate was injected into a reaction cell containing much higher concentration of enzyme. As expected, the chemical thermal power generated by the enzyme was greater; thus the magnitude of the offsetting change in instrumental thermal power was larger. However, as the substrate was completely consumed, the power returned to the original baseline. Integration of the area described by the curve (minus the heat associated with the dilution event) gave an experimental ΔH_{app}:

$$\Delta H_{app} = \frac{1}{[S]_{Total} * V} \int_{t=0}^{t=\infty} \frac{dQ(t)}{dt}\, dt. \qquad [5]$$

After reequilibration, a second injection of substrate into the sample cell (now containing product) gave a curve with identical shape and area, confirming ΔH_{app} and showing that product inhibition was negligible. This experimental determination of ΔH_{app} assumes the reaction proceeds to completion. For reactions that do not proceed to completion, some independent measure of the extent of reaction is necessary to obtain ΔH_{app} from calorimetric experiments.

Urea Hydrolysis by Helicobacter pylori Urease

H. pylori is a human pathogen that can thrive in the mucosal lining of the stomach and is associated with peptic ulcer formation (12). An active urease enzyme contributes to the viability of the organism in the acidic environment of the stomach by rapidly hydrolyzing blood urea nitrogen to ammonia (13). Figure 2 demonstrates a complete calorimetric assay using 70 fmol of *H. pylori* urease. After thermal equilibrium, injections of urea were made every 3 min: a decrease in instru-

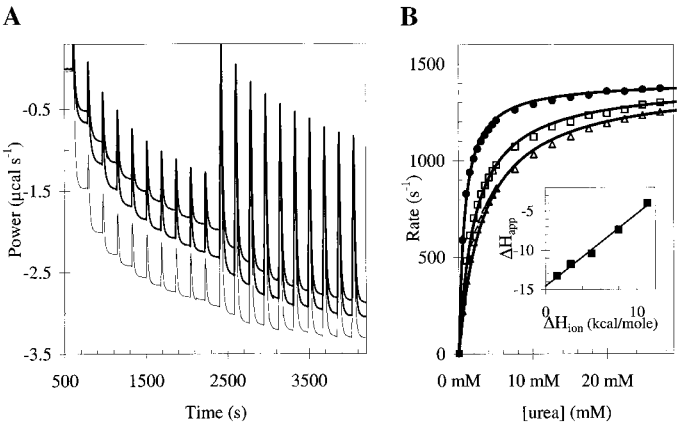

FIG. 2. Urea hydrolysis by *H. pylori* urease. (A) 88 pM *H. pylori* urease in 100 mM Hepes, pH 7.75, 1 mM EDTA plus 0 (bottom trace, ●), 5 mM (middle trace, □) or 10 mM (upper trace, △) 2-ME was allowed to reach thermal equilibrium at 37°C. After a 10-min wait, 10 successive injections of 2 μl of 350 mM urea were made every 3 min and then 10 injections of 10 μl 350 mM urea every 3 min. Injections 11–20, where larger quantities of substrate are introduced, produce correspondingly larger dilution heat, but the instrumental thermal power returns to a steady-state level within a few minutes. The change in thermal power was obtained from the baseline shift, converted to rate using Eq. [4], and corrected for enzyme concentration (including consideration of enzyme dilution). (B) Rate versus urea concentration was fit to Eq. [6], to give kinetic parameters listed in Table 1. (Inset) ΔH_{app} in different buffers. ΔH_{app} for the complete hydrolysis of urea was determined as described in the legend to Fig. 1B, using buffers with differing heats of ionization. All reactions were at 25°C, in 50 mM buffer, pH 7.75, 0.1 mM EDTA, and used 1 nM *K. aerogenes* urease. Buffers include phosphate (ΔH_{ion} = 1.22 kcal/mol), Pipes (ΔH_{ion} = 2.74 kcal/mol), Hepes (ΔH_{ion} = 5.02 kcal/mol), TEA (ΔH_{ion} = 8.0 kcal/mol), and Tris (ΔH_{ion} = 11.2 kcal/mol). Linear regression analysis of the data gave an intercept of $\Delta H_{int} = -14.6$ kcal/mol and a slope of 0.92 protons taken up during urea turnover.

mental thermal power was observed following each injection (urea hydrolysis is exothermic). The change in instrumental thermal power after an injection was complete after several minutes (Fig. 1A); thus data collection at each substrate concentration was truncated after 3 min, and another injection was made.[5] Enzyme reaction rates were obtained from the data in Fig. 2A by measuring the difference between the original baseline and the new baseline after substrate addition. The thermal power obtained was averaged for the 30 s prior to the subsequent injection, to obtain the most accurate power measurements. These rates were corrected for ΔH_{app} (Eq. [4]; Fig. 1B) and are shown as enzyme turnover in Fig. 2B). Data were fit to the Michaelis–Menten (14) equation (Eq. [6]) using nonlinear least-squares regression analysis to give the kinetic constants for urea hydrolysis (Table 1):

$$\text{Rate}_{(s)} = \frac{k_{cat} \cdot [E_{Total}] \cdot [S]}{K_m + [S]}. \qquad [6]$$

Also shown in Fig. 2 are two assays of *H. pylori* urease in the presence of 2-mercaptoethanol. Competitive inhibition is apparent even in the raw data, since an equivalent decrease in instrumental thermal power requires more substrate. The maximum thermal power, however, was similar regardless of inhibitor concentration. Enzyme turnover rates (Fig. 2B) were consistent with 2-ME acting as a competitive inhibitor of *H. pylori* urease (K_i = 4.1 ± 0.3 mM).

The experimental ΔH_{app} (used above to convert power measurements to rate) not only is reaction-dependent but also can depend on the reaction conditions. For example, if a reaction includes the uptake or release of a proton, ΔH_{app} will depend on the buffer heat of ionization (15). The experimentally determined ΔH_{app} for urea hydrolysis, measured in buffers with different heats of protonation, is shown in Fig. 2B, inset. The apparent enthalpy, ΔH_{app} increased linearly with increasing buffer heat of ionization because the complete reaction for urea hydrolysis under neutral, aqueous conditions includes the uptake of one proton, as shown in the scheme

$$\begin{aligned} H_2N\text{-}CO\text{-}NH_2 + H_2O &\rightarrow CO_2 + 2\ NH_3 \\ 2\ NH_3 + 2\ H^+ &\rightarrow 2\ NH_4^+ \\ CO_2 + H_2O \rightarrow H_2CO_3 &\rightarrow H^+ + HCO_3^- \\ \hline H_2N\text{-}CO\text{-}NH_2 + 2\ H_2O + H^+ &\rightarrow 2\ NH_4^+ + HCO_3^- \\ Buffer \cdot H^+ &\rightarrow Buffer + H^+ \end{aligned}$$

The slope of the line in the Fig. 2B inset (0.92 ± 0.05) gives the experimentally determined number of protons associated with urea hydrolysis at pH 7.75. The slope is slightly less than 1 because the product, ammonia (pK_a 9.25), is predicted to be only 97% ionized at pH 7.75, leaving an expected uptake of 0.94 proton ($0.97 \times 2 - 1$). The intrinsic enthalpy ($\Delta H_{int} = -14.5$ kcal/mol) is given by extrapolating the line in the Fig. 2B inset to zero heat of buffer ionization.

Continuous Assay of Trypsin

The experimental design described above gives a direct measure of reaction rates under steady-state reaction conditions. For well-characterized reactions, this initial limitation can be relieved. Continuous rate measurements following a single injection of substrate (typically at concentrations much greater than the K_m) can be made by monitoring the chemical thermal power as substrate is completely consumed. Figure 3

[5] The time required between injections was kept to a minimum in order to maintain pseudo-first-order reaction conditions. In general, one only needs to collect thermal power measurements long enough to prevent the dilution heats from affecting the measurements. With some enzymes, injections could be made as often as every 90 s.

TABLE 1

Kinetics of Enzymes Assayed Calorimetrically versus Published Values

Enzyme	Calorimetric		Literature values	
	K_m	k_{cat}	K_m	k_{cat}
EC 1.5.1.3 (DHFR)[a]				
Substrate = DHF	1.2 μM	6 s^{-1}	6 μM	3 s^{-1} (35)
EC 2.7.1.1 (yeast hexokinase)[b]				
Substrate = glucose	72 μM	270 s^{-1}	100 μM	450 s^{-1} (36)
EC 3.3.2.6 *B. cereus* penicillinase I[c]	120 μM	3600 s^{-1}	50 μM	2800 s^{-1} (37)
EC 3.4.21.4 (trypsin)[d]	4 μM	15 s^{-1}	5 μM	22 s^{-1} (38)
EC 3.4.21.16 (HIV protease)[e]	5–300 μM [NaCl] dependent	10 s^{-1}	15 μM	45 s^{-1} (33)
Substrate = KARVnLF(NO_2)EAnL				
Substrate = VSQNYPIVQ				
EC 3.5.1.5 (*H. pylori* urease)[f]	0.79 mM	1400 s^{-1}	0.17 mM	2700 s^{-1} (39)
EC 4.1.1.7 (*F. heparinum* heparinase)[g]	1.8 μM	0.059 s^{-1}	10.2 μM	92 s^{-1} (40)
EC 4.1.1.39 (rubisco)[h]				
Substrate = ribulose bisphosphate	0.15 mM	1.95 s^{-1}	0.053 mM	1.76 s^{-1} (41)
EC 4.1.3.18 (acetolactate synthase)[i]	4.8 mM	11 s^{-1}	5.5 mM	5.3 s^{-1} (42)
EC 5.99 (GroEL)[j]	3 μM n = 2.9	0.052 s^{-1}	5 μM n = 2.5	0.08 s^{-1} (22)
EC 6.4.1.1 (pyruvate carboxylase)[k]				
Substrate = ATP	85 μM		58 μM	
Substrate = pyruvate	105 μM		440 μM	(43)

[a] Bovine DHFR (EC 1.5.1.3, Sigma) was assayed at 25°C in 100 mM potassium phosphate, pH 7.5, 100 mM KCl, 77 μM NADPH, using the experimental design in Fig. 3. Thirty microliters of 34 μM dihydrofolate (DHF) was injected into the calorimeter reaction cell containing 28 nM DHFR, and the reaction was monitored until the baseline returned to original levels. Enzyme turnover rates were determined as in Fig. 3.

[b] Yeast hexokinase (EC 2.7.1.1, Sigma) in 50 mM Hepes, pH 7.5, 100 mM KCl, 20 mM $MgCl_2$, 1 mM 2-ME, and 9 mM ATP was assayed at 25°C using the experimental design in Fig. 2. The syringe contained 5.6 mM glucose in the same buffer. After equilibration, twenty 5-μl injections were made every 3 min, and enzyme turnover rates were determined as in Fig. 2.

[c] *B. cereus* penicillinase I (EC 3.3.2.6, Type I, Sigma) in 50 mM KP_i, pH 7.0, 150 mM KCl was assayed at 30°C using the experimental design in Fig. 2. After equilibration, twenty 5-μl injections of 36 mM benzylpenicillin (Sigma) were injected every 3 min into the reaction cell containing 32 pM penicillinase-I.

[d] Kinetic parameters for trypsin are from Fig. 3.

[e] HIV-1 protease (Q7K/L33I/L63I) was purified as described (11) and assayed at 25°C in 100 mM Na acetate, pH 5.1, 1 mM EDTA, 100 mM NaCl using the experimental design in Fig. 2. After equilibration, fifteen 5-μl injections of 4.3 mM KARVnLFEAnL or VSQNYPIVQ were made every 90 s into the reaction cell containing 56 nM protease dimer.

[f] Kinetic parameters for *H. pylori* urease are from Fig. 2 and are reported assuming one active site per 96 kDa heterodimeric unit.

[g] Kinetic parameters for *F. heparinum* heparinase-I are from Fig. 4B.

[h] *R. rubrum* rubisco (a kind gift of Dr. G. Lorimer) in 50 mM Tris–HCl, pH 7.0, 5 mM $MgCl_2$, 5 mM $NaHCO_3$ was assayed using the experimental design in Fig. 2. After equilibration at 25°C, five 2.8-μl injections of 25 mM ribulose bisphosphate were made every 2 min, followed by four 14-μl injections every 3 min into the reaction cell containing 50 nM rubisco dimer.

[i] Acetolactate synthase (a kind gift of C. Herman and Dr. S. Gutteridge) in 50 mM KP_i, pH 7.0, 20 mM $MgCl_2$, 0.5 mM thiamine pyrophosphate, 20 μM FAD was assayed using the experimental design in Fig. 2. After equilibration at 25°C, eight 2-μl injections and then eight 8 μl injections of 350 mM pyruvate were made every 5 min into the reaction cell containing 1.4 nM protein.

[j] Kinetic parameters for *E. coli* GroEL are from Fig. 4A.

[k] Bovine liver pyruvate carboxylase (Sigma) in 100 mM Tris–HCl, pH 7.7, 5 mM $MgCl_2$, 20 mM $NaHCO_3$, 10 mM pyruvate, 0.25 mM acetyl-CoA was assayed (at 200 ng/ml) using the experimental design in Fig. 2. After equilibration at 30°C, thirty 5-μl injections of 7 mM ATP were made every 3 min. To measure the K_m for pyruvate, the cell contained the same solution minus pyruvate, but including 1 mM ATP. After equilibration, ten 5-μl injections and then ten 20-μl injections of 14 mM pyruvate were made every 3 min. Literature values are for enzyme from chicken liver.

illustrates the calorimetric response to trypsin hydrolysis of benzoylarginine ethyl ester (BAEE). After an initial baseline was collected, substrate was injected (arrow). In the presence of enzyme (thin line), a rapid decrease in instrumental thermal power occurred because BAEE hydrolysis is exothermic. The thermal power remained constant for several minutes (400–1200 s) because the reaction was proceeding at maximal rate (V_{max}). Substrate depletion caused a decrease in rate and the power returned to the initial baseline (1200–1500 s). A second injection of substrate (data not shown) generated an identically shaped curve, demonstrating no product inhibition using this substrate.

The raw data in Fig. 3A were corrected for the instrument response time[6] (6, 16) and integrated to give

[6] In practice, the raw data need not be corrected for the instrument response time, before integrating to give the ΔH_{obs} for the reaction. Correcting the rate data using the instrument response time had a small effect on rate values, since the time course over which the

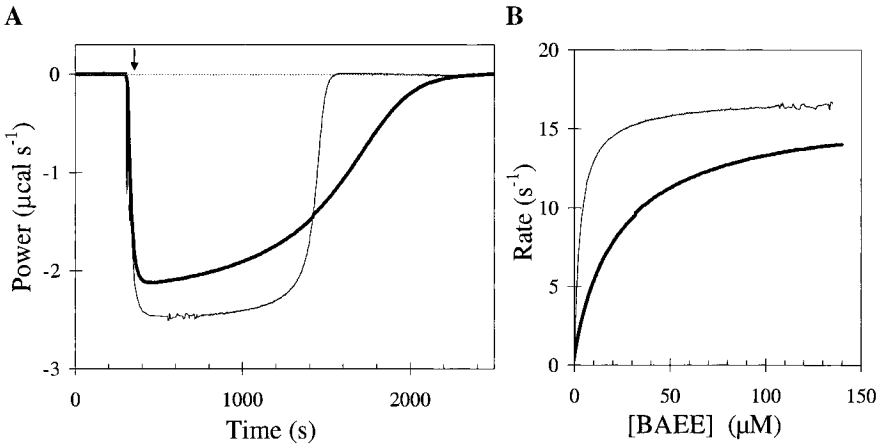

FIG. 3. Trypsin catalyzed hydrolysis of BAEE. (A) 9.6 nM Trypsin in 200 mM Tris–HCl, pH 8.0, 50 mM $CaCl_2$, and 0.2% PEG-8000 was equilibrated at 25°C with 0 (thin line) or 94 μM (thick line) benzamidine. After thermal equilibration (300 s), BAEE was injected (to 171 μM). The change in instrumental thermal power was monitored until substrate hydrolysis was complete, when it returned to the original baseline. (B) Thermal power was converted to enzyme turnover ($\Delta H_{app} = -11.45$ kcal/mol) and fit to Eq. [6], giving $K_m = 4$ μM and $k_{cat} = 16$ s^{-1}. K_i for benzamidine under these conditions was 16 μM.

ΔH_{app} for the reaction (Eq. [5]; −11.45 kcal/mol). At any given time, the rate could be determined from the power (using Eq. [4]) and the remaining substrate concentration from the integral of heat evolved:

$$S_{(t)} = S_{Total} - P_{(t)} = S_{Total} - \frac{\int_{t=0}^{t} Q_{(t)}}{\Delta H_{app} \cdot V}. \qquad [7]$$

The rate plotted as a function of the substrate concentration generated a *continuous* kinetic curve (Fig. 3B).

Figure 3 also shows the hydrolysis of BAEE in the presence of benzamidine (thick line), a known competitive trypsin inhibitor (17). Again, the power immediately approached V_{max} and slowly returned to original levels as substrate was completely hydrolyzed. As expected, the areas described by the two curves (ΔH_{app}) were identical. Enzyme turnover vs concentration (Fig. 3B) confirmed competitive inhibition, and a fit to standard equations yielded a K_i of 16 μM, in close agreement with a reported value of 18 μM (17).

Cooperative ATP Hydrolysis by the E. coli GroEL Chaperonin

The chaperonin proteins are a ubiquitous class of large, ring-shaped proteins (18, 19) that interact with nonnative forms of many cellular proteins (20). *In vivo,* they function to assist other proteins in achieving the correct conformation (21). Chaperonin-catalyzed protein folding (22–24) requires complex interactions with a smaller cochaperonin protein and is driven by a cooperative, K^+-dependent hydrolysis of $Mg^{2+} \cdot$ ATP (25). This cooperative ATP hydrolysis, measured calorimetrically, is shown in Fig. 4A. Changes in thermal power (left panel) were converted to enzyme turnover (right panel) using Eq. [5] and an experimentally determined ΔH_{app} (−16 kcal/mol). Rate data for the cooperative hydrolysis of ATP were fit to the Hill equation (see Fig. 4 legend), and the kinetic constants obtained (Table 1) agree favorable with literature values.

Heparin Degradation by Flavobacterium heparinum Heparinase I

Metastasizing tumor cells secrete heparin-degrading enzymes that digest the basement membrane of capillaries, in order to invade host tissue and initiate secondary tumors (26). Thus, inhibitors of these enzymes have potential clinical value (27). Although the biosynthesis of heparin and heparin sulfate is fairly well understood (28), the enzymatic degradation is less well-defined, partly due to a lack of a convenient assay. The steady-state rate of heparin lysis by *Flavobacterium heparinum* heparinase-I (29, 30) is shown in Fig. 4B (left). The raw data give an example of a reaction in which a large dilution heat (the concentrated heparin in the syringe becomes significantly more hydrated upon dilution) is followed by an *increase* in the instrumental thermal power; thus the lysis of heparin, under the described conditions, is endothermic. The change in thermal power was converted to enzyme turnover (Eq. [4]) using an experimentally determined ΔH_{app} (+55 ± 3 kcal/mol), and the rate as a function of heparin concentration is shown in Fig. 4B (right). Kinetic con-

enzyme reaction rate decays to zero is much larger than the instrument time constant (typically 10–20 s for power-compensated calorimeters).

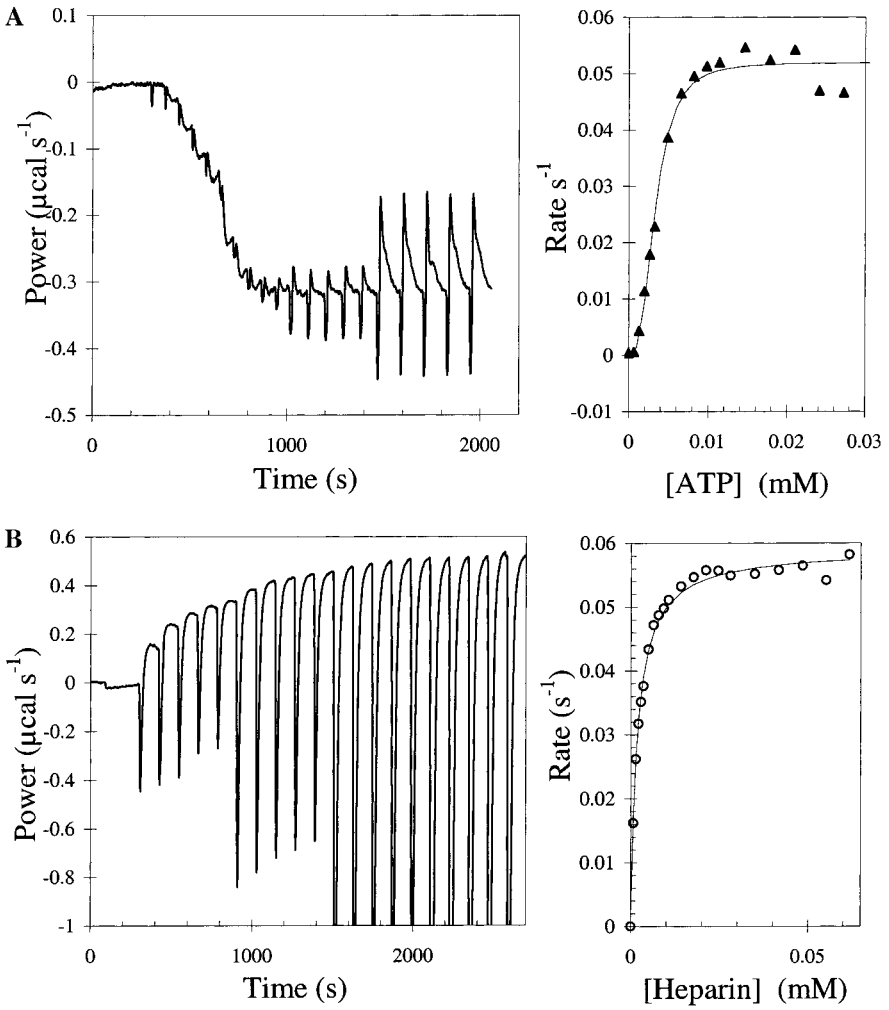

FIG. 4. Calorimetric assays of other enzymes. (A) *E. coli* chaperonin GroEL has cooperative ATP hydrolysis. Two milliliters of 28 nM $GroEL_{14}$ in 50 mM Tris, 5 mM $MgCl_2$, 100 mM KCl, pH 7.7, was allowed to reach thermal equilibrium at 37°C. After 5 min, 0.45 mM ATP was injected according to the following schedule: five injections of 2 μl every 70 s, five injections of 5 μl every 73 s, and five injections of 10 μl every 120 s. Enzyme turnover rates were obtained by dividing the measured baseline shift by ΔH_{app} (-16 ± 0.6 kcal/mol). Rate versus ATP concentration was fit to the Hill equation $[v_{(S)} = [S]^n/(K^{*n}_s + [S]^n)]$, with results given in Table 1. (B) Heparin lyase is an endothermic reaction. 115 nM *F. heparinum* heparinase-I was equilibrated in 40 mM Pipes, pH 7.1, 5 mM $CaCl_2$. After thermal equilibrium was reached and a baseline established (5 min, 37°C), five successive injections of 1 μl 0.96 mM heparin were made every 120 s, then five injections of 2 μl, five injections of 5 μl, and five injections of 10 μl, each time waiting 120 s to reestablish equilibrium conditions before the next injection. Enzyme turnover rates were obtained by dividing the measured baseline shift, by an experimentally determined ΔH_{app} ($+55 \pm 3$ kcal/mol using high-molecular-weight heparin). Rate versus heparin concentration was fit to the Michaelis–Menten equation (Eq. [6]), with results reported in Table 1.

stants (Table 1) were obtained by fitting the data to Eq. [6].

Compared with published values, a much slower V_{max} is observed using the calorimetric assay. This apparent discrepancy occurs because the experimentally determined ΔH_{app} is the enthalpy for the *complete* lysis of heparin. Heparin is a highly heterogeneous polysaccharide with an average molecular weight of ~15,000 and varying degrees of sulfation. Complete lysis includes numerous cleavage events, resulting in a heterogeneous collection of di- and tetrasaccharides. Thus ΔH for an individual cleavage event is significantly smaller (10- to 100-fold) and V_{max} for a single cleavage event is proportionally smaller. Consistent with this interpretation, ΔH_{app} for complete lysis of low-molecular-weight heparin (average M_w ~3000 g/mol) was significantly less (9.5 ± 0.9 kcal/mol).

Peptide Hydrolysis Activity of the HIV-1 Protease

Maturation of the human immunodeficiency virus, the causative agent of AIDS, requires an active virus-encoded protease to cleave the gag, and gag-pol polyproteins (31). The HIV protease is the target of

numerous clinical inhibitors that function by binding at the active site and preventing peptide hydrolysis (32). The simplest assay for proteolytic activity involves monitoring absorbance (or fluorescence) changes upon hydrolysis of substrates specifically designed to contain chromophores (33) or fluorophores (34). The hydrolysis of a commonly used chromogenic substrate analogue and a peptide corresponding to a natural cleavage site was measured calorimetrically, with results reported in Table 1. As expected, the thermal power generated by cleaving the peptide bond was identical regardless of the sequence of the peptide being cleaved ($\Delta H_{app} = -1.1$ kcal/mol).

DISCUSSION

Benefits of Calorimetric Assays over Conventional Methods

Universality of calorimetric assays. Calorimetric determination of enzyme activity has been applied to enzymes from every E C. classification, with simple (i.e., hyperbolic) and complex (i.e., cooperative) kinetics and to enzymes for which there is no direct assay currently available. The kinetic parameters agree well with published values in most cases (Table 1). When differences occur, the calorimetrically measured parameters often had different meanings (see heparinase-I, above) or revealed partial reactions not detected by conventional assays (M. Todd, unpublished). In all cases, the thermal power gave a nondestructive, direct, highly precise measurement of the reaction rate.

High-quality kinetic data are obtained using calorimetry for several reasons: First, the rate is determined directly, not calculated as a derivative of a measured quantity. Second, the noise level is constant (<0.01 μcal/s), regardless of the thermal power produced chemically. Thus an enzymatic reaction generating 0.1 μcal/s will have a signal-to-noise ratio of 10, whereas one developing 10 μcal/s (perhaps using greater amounts of enzyme) will have a signal-to-noise ratio of 1000. Third, an average change in thermal power (over several seconds preceding the subsequent dilution event) is determined, increasing the precision compared to that of an individual measurement. Fourth, numerous mechanized additions of substrate (or inhibitor) are made in the same reaction cell; thus the variability associated with multiple, independent determinations is reduced.

Assays in spectroscopically opaque solutions. A major advantage of using calorimetry to assay enzymes is the independence from optical methods. Thus, natural substrates should generate the same thermal power as their chromogenic/fluorogenic analogues, allowing experimentalists to use the most physiologically relevant compound. In addition, the presence of other components (high product concentration, inhibitors, etc.) does not mask the signal. For example, the reaction kinetics of *H. pylori* urease were measured in the presence of 0.5 M ammonium ion. The kinetic constants obtained were similar to those determined in the absence of product ($K_m = 1.9$ mM, $V_{max} = 1650$ s^{-1}), demonstrating that product has little affinity for the enzyme. During the hour-long assay, ~0.5 mM additional substrate was hydrolyzed (<0.1% change in product concentration). Such experiments emphasize the sensitivity of using thermal power to examine chemical reactions.

In a calorimeter, the need to "couple" a desired reaction to a second, optically detectable reaction using additional enzymes is not necessary. Alternatively, the activities of multiple enzymes or whole enzyme pathways could be assayed simultaneously. In some experiments, activity *in vivo* was compared to activity *in vitro,* simply by loading the calorimeter cell with whole bacterial cells (M. Todd, unpublished). The optically opaque mixture produced identical thermal power when titrated with substrate, revealing (i) substrate diffusion across the membrane was not rate limiting, and (ii) the enzyme exhibited kinetic parameters *in vivo* similar to those measured *in vitro.*

Applicability—measurable kinetic constants. A complete rate profile (such as those presented in Figs. 2–4) can easily be generated under conditions where an enzyme generates ~0.5 μcal/s in a 1.4-ml reaction at V_{max}. This represents 100-fold signal over the noise, and rate measurements at 10% V_{max} would have ~10% error. Assuming an "average" ΔH for a reaction of ± 5 kcal/mol, a reaction must generate >0.007 μM/s of product at 10% V_{max}. Each measurement is made following a delay, the length of which depends on instrument response time and the size of the dilution event. Therefore, calorimetric assays under pseudo-first-order conditions may be applicable only when the K_m is larger than ~10 μM. In addition, E_{total} should be $\ll [S]_{total}$, so calorimetric assays are only applicable to enzymes with $k_{cat} > 1$ min^{-1}. Lower K_m values have been determined using steady-state techniques when the reaction is more energetic or if a substrate-regenerating system is included. Alternatively, the continuous calorimetric assays described in Fig. 3 can be used when K_m is lower than 10 μM.

Some reactions may not release sufficient heat, either because they are entropically driven or because the free energy difference between substrate(s) and product(s) is small (e.g., racemases, isomerases). A portion of these reactions may be amenable to calorimetric techniques simply by altering the reaction conditions (i.e., changing buffer, pH, temperature; e.g., Fig. 2, inset). Other enzymes may require coupling the de-

sired reaction to one that has a larger enthalpic component.

Proteomics and mutational analysis. As the results of numerous genome sequencing projects become available, the number of potential new drug targets has increased dramatically. Often, homology within regions of translated sequence will give clues to the relevant biochemical reactions; however, the details of substrate specificity or cofactor requirement usually require precise experimental analysis. The field of proteomics or functional genomics seeks to identify the function of new proteins rapidly, including the subtle activity variations of new protein isozymes or proteins that are tightly regulated. Calorimetric assays of proteins could easily fulfill this niche. In addition, the precise analysis of mutant proteins (as generated by site-directed mutagenesis or as revealed by mutations conferring resistance to current drug targets) requires careful correlation of subtle changes in structure to subtle changes in activity. The rapid, precise, complete kinetic characterization possible with calorimetry should also aid these critical analyses.

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