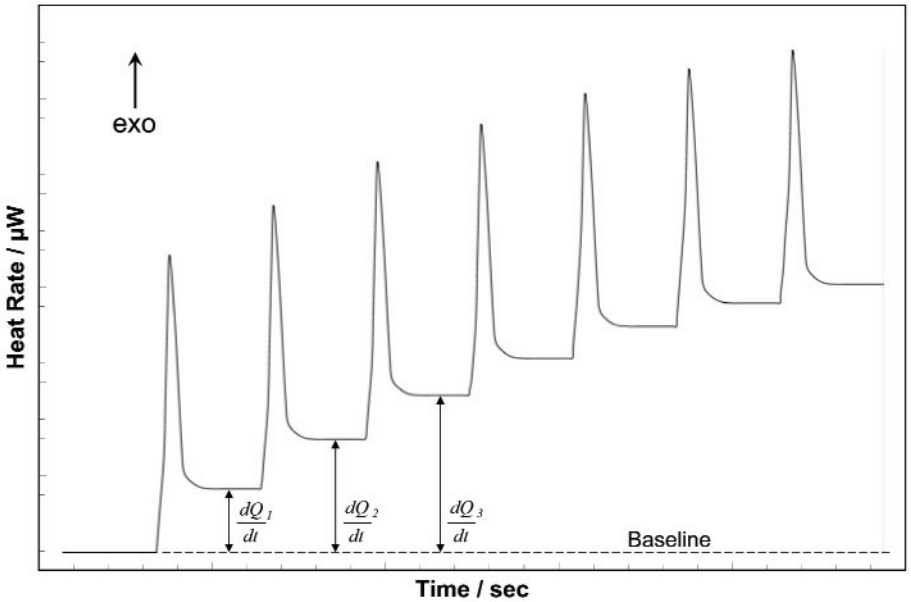
Characterizing Enzyme Kinetics by ITC

Christin T. Choma
TA Instruments, 109 Lukens Drive, New Castle, DE 19720, USA



ITC can characterize the kinetics of essentially any enzymatic reaction.

All enzymatic reactions generate heat, so all enzymatic reactions can in principle be directly studied by calorimetry. Calorimetric experiments do not require extensive protocol development, and measurements are direct, rapid, non-destructive and sensitive. In addition to kinetics data, ITC also generates thermodynamic information which can help correlate enzyme stability to reaction rates and substrate specificity.

It has long been believed that enzymes catalytically control all biochemical processes, and that each enzyme is specific for a particular substrate and catalyzes only one reaction. While the first belief remains true, recent proteomics and functional genomics studies have shown that many enzymes have capabilities seemingly unrelated to their primary catalytic function, and that some of these secondary capabilities may be connected to various diseases. For example, some enzymes can accept a range of substrates and direct seemingly unrelated catalytic chemistries ('promiscuity'; Bornscheuer and Kazlauskas, 2004). In addition, enzymes can exhibit structural or regulatory functions that are unrelated to their primary catalytic role ('moonlighting'; Copley, 2003). These findings, coupled with an evolving understanding of the chemical basis for enzyme catalysis (Benkovic and Hammes-Schiffer, 2003) highlight the necessity of understanding not only *how* enzymes catalyze reactions, but also *why*. How does thermodynamics drive binding and specificity? What thermodynamic processes control the conformational rearrangements necessary to produce the transition state? What factors control an enzyme's 'promiscuity', and determine whether or not it engages in 'moonlighting'? If the thermodynamic basis for these various functions could be established, it might be possible to control functionality (particularly of enzymes involved in diseases) using, for example, rationally-designed function-specific inhibitors.

The internal motions of an enzyme are reduced upon substrate binding and transition state formation: hydrogen bonds form and are optimized between the binding site and the substrate, thus reducing the internal motions of the enzyme-substrate complex. Importantly, transition state formation improves non-covalent bonds throughout the entire enzyme, stabilizing the structure. It appears that this positive change in enthalpy is sufficient to offset the entropic penalty of reducing the dynamic behavior of the enzyme in the transition state. Enzymes for which data are available show that enzyme-catalyzed reactions are greatly favored in enthalpy, and that enthalpic factors are largely responsible for the observed catalytic rate enhancement (Williams *et al*., 2004).

Calorimetry is a direct approach for studying the thermodynamics of enzyme function. There are two general types of calorimetry: differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC). DSC, described in an accompanying set of application notes, is a powerful approach for determining the stability of macromolecules such as enzymes and enzyme-inhibitor complexes. In contrast, ITC is particularly suited to measuring dynamic events such as binding and kinetics. This Application Note examines the utility of ITC for the analysis of enzymatic reactions. For a general description of the principles behind ITC, its versatility and the types of biological problems that can be addressed by this technique, please see CSC's Overview Note entitled 'Life science applications of ITC'.

ITC is widely applicable to the study of enzyme kinetics:

The power of ITC derives from the universality of the technique: every reaction generates or absorbs heat, so every reaction can in principle be studied by calorimetry. In practice it has been shown that representative enzymes from every EC classification can be analyzed kinetically using ITC (Todd and Gomez, 2001). In addition, ITC analyses are rapid, precise, nondestructive, compatible with both physiological and synthetic substrates, and are as sensitive as spectroscopic techniques but do not require a spectroscopic label or chemical tag.

Importantly, ITC analyses of enzyme kinetics are also straightforward. The sum of the enthalpy (ΔH) and entropy (ΔS) of a spontaneous reaction such as enzyme catalysis must result in a decrease in the free energy (ΔG) of the system:

$$\Delta G = \Delta H - T\Delta S$$

where the enthalpic component is observed as heat. ITC experiments are designed to measure the rate of heat generation (*i.e.,* dQ/dt, or the thermal power) thus allowing the rate of the reaction to be calculated:

$$Rate = \frac{1}{V_o \bullet \Delta H_{app}} \bullet \frac{dQ}{dt} \qquad \text{(Todd and Gomez, 2001)}$$

where V_o is the volume of the sample in the reaction cell and ΔH_{app} is the experimentally-determined change in enthalpy. In a typical ITC experiment, buffered substrate is injected into enzyme (dissolved in the same buffer) in the sample cell. Catalytic conversion of the substrate into product generates heat, which is detected and

compensated for by the calorimeter. ΔH_{app} is determined by integrating the total heat produced during catalysis of a known amount of substrate to product. Since the rate of heat production for a given amount of substrate is known, the rate of the reaction can be calculated. The reaction kinetic parameters can then be calculated from the Michaelis-Menton equation:

$$Rate = \frac{k_{cat} \bullet [E_{Total}] \bullet [S]}{K_M + [S]}$$

where k_{cat} is the turnover number of the enzyme, K_M is the substrate concentration at which the reaction velocity is half-maximal, $[E_{Total}]$ is the total amount of enzyme present and [S] is the concentration of substrate.

There are two approaches for studying enzyme kinetics by ITC: the single injection (continuous assay enzyme kinetics) method, and the multiple injection (pseudo-first-order enzyme kinetics) method.

a) The single injection method:

The single injection method is a versatile and straightforward approach that can be used to study both fast and slow kinetic reactions. A single injection of substrate (10-30 µL at 10-100 µM, depending on the reaction) at concentrations higher than K_M and in excess of the enzyme, is delivered to the sample cell; in some cases, for example with self-hydrolytic or temperature-unstable enzymes, or where catalysis is being conducted at an elevated temperature, it may be advantageous to titrate the enzyme into the substrate. Fig. 1A shows the hydrolysis of penicillin G by penicillinase. The thermal power is monitored continuously as the substrate is converted to product, and continues until the substrate is depleted and the thermal power returns to the initial baseline. A continuous curve is obtained when the rate is plotted as a function of substrate concentration normalized for the concentration of enzyme (Fig. 1B). Plotting 1/velocity *vs.* 1/[substrate] yields the Lineweaver-Burk plot, allowing the maximum velocity to be determined from the *y* intercept and K_M from the *x* intercept. Under saturating conditions, the maximum turnover number of the enzyme, k_{cat}, can be determined from $V_{max}/[E_{Total}]$.

Product inhibition can be easily monitored by making a second injection of substrate: if the same response curve is obtained, the reaction does not experience product inhibition at that concentration of substrate and product. The effect of a competitive inhibitor on catalysis can be determined by conducting catalysis in the presence of a known concentration of the inhibitor. Figure 2 shows the hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester (BAEE) by trypsin in the presence and absence of benzamidine, a competitive inhibitor.

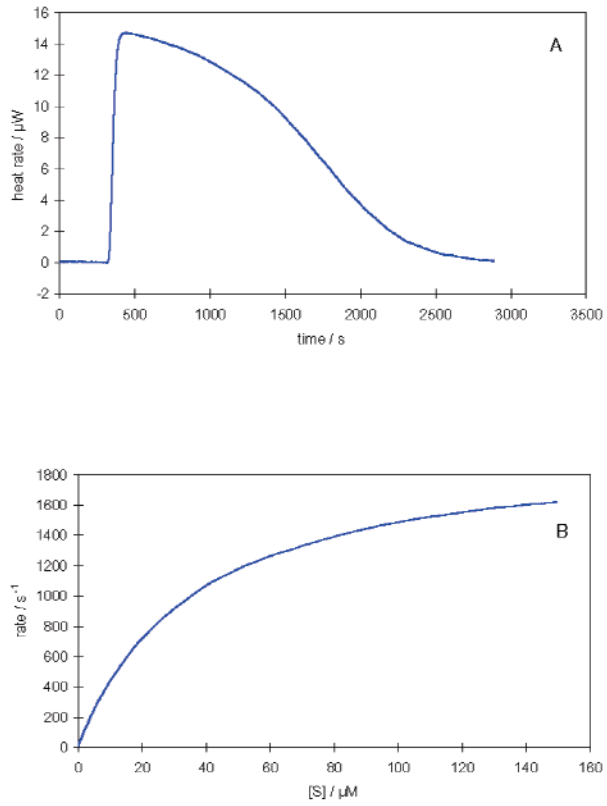


Fig. 1. Catalysis of penicillin G by penicillinase using the single injection method. Penicillinase (950 µL 7.7 x 10^{-11} M in 50 mM phosphate, pH 7.0, 150 mM KCl) was equilibrated in the sample cell at 30 °C. Penicillin (15 µL 10.2 mM) was injected and data were collected until the instrument signal returned to baseline. A): Raw data showing instrument response. B): Data converted to rate of hydrolysis (normalized for enzyme concentration) vs. substrate concentration. Replotting B in the format of the Lineweaver-Burk double reciprocal plot yielded K_M = 30.7 µM, V_{max} = 1.4 x 10^{-10} mol s^{-1}, and k_{cat} = 1950 s^{-1}, in good agreement with the literature values reported in Table 1 in Todd and Gomez (2001).

The difference in K_M in the presence (observed K_M) and absence (true K_M) of the inhibitor permits the inhibition constant, K_i, to be calculated from:

$$K_i = \frac{[inhibitor]}{\frac{K_{M_{obs}}}{K_M} - 1.0}$$

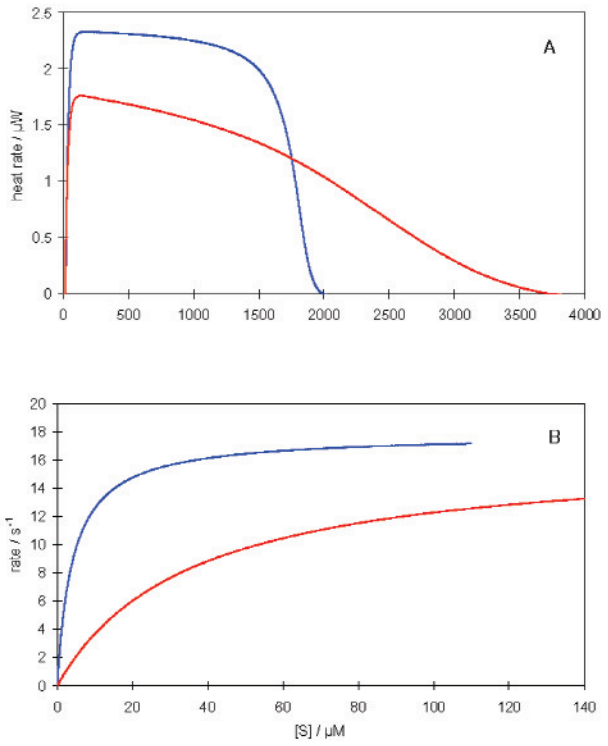


Fig. 2. Trypsin hydrolysis of N- -benzoyl-L-arginine ethyl ester (BAEE) in the absence (blue) and presence (red) of benzamidine, a competitive inhibitor. All solutions were prepared in 200 mM Tris-HCl, pH 8.0, 50 mM $CaCl_2$, 0.2% PEG-2000. After 300 seconds thermal equilibration at 25 °C, trypsin (10 µL 5.1 X 10^{-7} M) was injected into 950 µL 1.44 x 10^{-4} M BAEE ± 1.36 x 10^{-4} M benzamidine. A): A representation of the change in instrument thermal power as the substrate is completely hydrolyzed and the original baseline is reestablished. The area under both curves (representing the total heat output for complete conversion of substrate to product) is the same either in the presence or absence of inhibitor (H = -6.33 kcal/mole BAEE). B): The data were converted to rate of substrate hydrolysis vs. substrate concentration. Rearranging into the Lineweaver-Burk plot yielded (in the absence of inhibitor) K_M = 4.17 µM, V_{max} = 0.091 µMol/s, k_{cat} = 17.8 s^{-1}. In the presence of inhibitor, K_M = 35.1 µM, V_{max} = 5.9 x 10^{-4} µMol/s, k_{cat} = 0.11 s^{-1}, K_i = 18.4 µM.

Detailed examples of the single injection method are described in Morin and Freire (1991), Todd and Gomez (2001) and Bianconi (2003), and a clear discussion of practical issues that should be addressed when designing an ITC kinetics experiment is presented in O'Brien *et al.* (2001).

b): The multiple injection method

The multiple injection method is an alternate approach for studying kinetic reactions (Todd and Gomez, 2001). Enzyme (in the range 50 pM to 20 nM, depending on the reaction) in the 1 mL sample cell is titrated with 2 – 10 µL volumes of substrate (20 µM to 400 mM, again depending on the reaction, but above K_M and far in excess of the enzyme). Enzyme reaction rates are determined by measuring the change in instrument signal following the addition of substrate. Each addition gives rise to a spike due to the heat of dilution of the substrate (Fig. 3), then the instrument signal rapidly decreases and approaches a new steady state. Negligible substrate is converted to product in the next 2-3 minutes (the time typically taken for a new stable heat rate to be established). The reaction proceeds at a steady state until a second aliquot of substrate is added, increasing

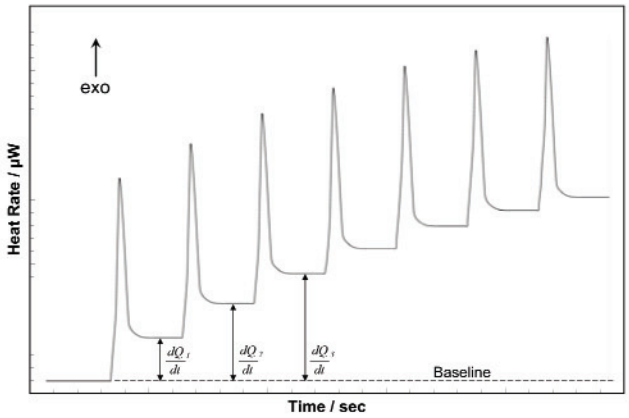


Fig. 3. Simulated data obtained using the multiple injection method. Successive injections of substrate are made into the enzyme solution in the sample cell, producing dilution heat (spike) and reaction heat (increasing heat rate with each subsequent injection). The change in thermal power due to catalysis is obtained from the shift in the heat rate, converted to reaction rate and corrected for enzyme concentration dilution. 'Exo' depicts the sign convention for an exothermic reaction.

both the concentration of substrate and the thermal power generated by the enzymatic reaction (dQ_2/dt). Typically 10 – 30 injections are made at 2 – 5 minute intervals, and the enzyme reaction rate is calculated as above. The catalysis of penicillin G by penicillinase, followed using the multiple injection method (Fig. 4), provided a K_M value of 101μM. This is very similar to the literature value (120 μM) obtained using this technique; see Todd and Gomez (2001) for a complete description of experiment design and data analysis using the multiple injection method.

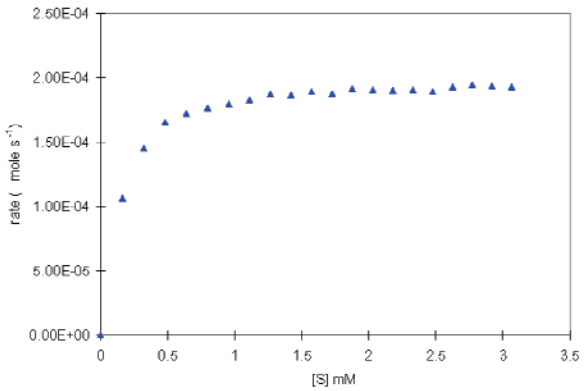


Fig. 4. Catalysis of penicillin G by penicillinase using the multiple injection method. Penicillinase (950 µL 7.7 x 10^{-11} M in 50 mM phosphate, pH 7.0, 150 mM KCl) was equilibrated in the sample cell at 30 °C. Penicillin (30.6 mM) was injected in 20, 5 µL aliquots at 5 min intervals. Making the conversions described in Fig. 3 transformed the data into the format shown in Fig. 4, yielding a K_M of 101 µM, similar to the value (120 µM) obtained by the same technique by Todd and Gomez (2001).

Summary:

Essentially any catalytic system can be studied by ITC. Measurements are direct, fast, reliable and straightforward. The amount of biological material required is similar to that for spectroscopic methods, but there is no need for optical transparency or sample homogeneity, and impurities are tolerated. Importantly, thermodynamic information is generated in addition to kinetics data. As ITC becomes increasingly prominent in the study of enzyme kinetics, the correlation of stability and reaction rates will help elucidate the subtle structural changes that thermodynamically control the (often multiple) functionalities of an enzyme.

References:

(Preference has been given to current references. Citation does not imply that a paper is necessarily the original reference to a study.)

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