



Use of Isothermal Titration Calorimetry to Measure Enzyme Kinetics Parameters

ITC Application Note

Introduction

A key focus of biochemistry is determining the activity and function of enzymes, since every biochemical pathway depends on enzymes for catalysis. Enzymes are also important in drug discovery and development. Approximately half of current drug targets are enzymes, and researchers are discovering new enzymes and developing drugs that interact with enzymes.

Enzyme analysis requires measurement of catalytic activity (conversion of substrate to product), as well as determination of the substrate's affinity for enzyme. We need to understand how enzymes work with natural substrates before we can develop new therapeutic agents. Traditional assays use spectrophotometric or chemical detection methods. However, there are many enzymes that cannot be assayed using standard methods.

This application note describes the use of Isothermal Titration Calorimetry (ITC) as a universal assay to measure enzyme kinetic parameters.

Overview of Enzyme Kinetics

When an enzyme (E) interacts with its substrate (S), an enzyme-substrate complex (ES) is formed. This complex is converted to its transition state (ES*), then enzyme-product complex (EP). Finally, the enzyme-product complex dissociates to give free enzyme and product. In biological systems, where substrate concentration is much higher than enzyme concentration, the reaction is simplified to:



where k_1 is rate constant for formation of ES and k_{-1} is rate constant for dissociation of ES. $[E]$ is the total enzyme concentration in the reaction. The catalytic rate constant is k_{cat} .¹ Reaction rate R_i is determined by the Michaelis-Menten equation:

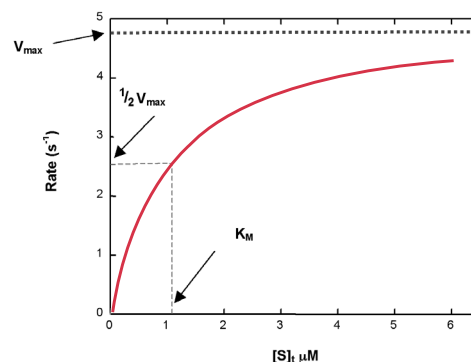
$$R_i = \frac{V_{max} \cdot [S]_t}{K_M + [S]_t} \quad [2]$$

where V_{max} is maximum observable velocity, $[S]_t$ is instantaneous substrate concentration, and K_M is Michaelis constant. The lower the K_M , the greater the affinity between enzyme and substrate. Since V_{max} is equal to $k_{cat} \cdot [E]$, Equation 2 is rewritten as:

$$R_i = \frac{k_{cat} \cdot [E] \cdot [S]_t}{K_M + [S]_t} \quad [3]$$

A hyperbolic rate plot is generated in a graph of R_i vs. $[S]_t$ (Figure 1). V_{max} is the rate when the curve plateaus, and K_M is equal to $[S]_t$ at $1/2 V_{max}$, where half of the enzyme molecules are in ES form.

FIGURE 1



Simulated Michaelis-Menten curve generated with Equation 3. $K_M = 1 \mu\text{M}$, $k_{cat} = 5 \text{ sec}^{-1}$, $[E] = 1 \text{ nM}$.

In the presence of a competitive substrate inhibitor, the rate is:

$$R_i = \frac{k_{cat} \cdot [E] \cdot [S]_t}{[S]_t + K_M \left(1 + \frac{[I]}{K_i}\right)} \quad [4]$$

where $[I]$ is inhibitor concentration and K_i is inhibitor constant.

Traditional enzyme assay methods include:

- **Continuous assays:** Rate determined by continuous measurement of product generation (or substrate depletion). Requires product or substrate to be detected with a spectrophotometer.
- **Discontinuous assays:** Reaction is stopped at fixed time point(s) and amount of product quantified using mass spectrometry, chromatography, gel electrophoresis, or other analytical methods.
- **Coupled assays:** Product of enzymatic catalysis is substrate for a coupled assay. The product can be monitored by continuous or discontinuous assay.

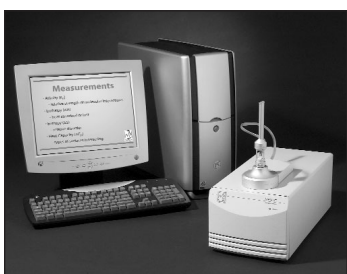
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However, assays do not exist for many enzymes. It is not always possible or straightforward to measure changes in substrate or product concentration, because:

- Opaque or turbid solutions interfere with spectrophotometric detection.
- Native, recombinant and/or mutant enzyme activity is below detection limit of assay.
- Substrate or product does not have a chromophore or fluorophore, and too costly or time-consuming to label.
- Discontinuous assays require multiple steps.
- No straightforward coupled reaction exists.
- Coupled assays introduce inaccuracies.
- Substrate/enzymatic activity is unknown.
- Protein function is unknown.

What is needed is a universal enzyme assay that is rapid, precise, can be used for activity screening, and is directly applicable to any enzyme-substrate system without the need for chemical tagging. ITC meets these criteria for a universal enzyme assay.

ITC and Enzyme Kinetics



It is well-established that enzyme reactions can be monitored by ITC.^{2,9} ITC is used to measure enzyme kinetics parameters using thermal power generated by the enzymatic conversion of substrate to product. The rate of reaction is

directly proportional to thermal power:

$$\text{Power} = \frac{dQ}{dt} \quad [5]$$

where Q is heat, and t is time. This thermal power change is detectable and measurable, and the VP-ITC is sensitive enough that enzyme requirements are similar to those of spectrophotometric assays.^{7,8}

Todd and Gomez⁷ have demonstrated that the amount of heat involved in converting *n* moles of substrate to product is expressed by:

$$Q = n \cdot \Delta H_{app} = [P]_{total} \cdot V_0 \cdot \Delta H_{app} \quad [6]$$

where ΔH_{app} is total molar enthalpy for the reaction (in calories/mole of substrate, determined experimentally by ITC), $[P]_{total}$ is concentration of product generated and V_0 is volume of the ITC cell. It follows that thermal power generated by the enzyme during conversion of substrate to product is:

$$\text{Power} = \frac{dQ}{dt} = \frac{d[P]_{total}}{dt} \cdot V_0 \cdot \Delta H_{app} \quad [7]$$

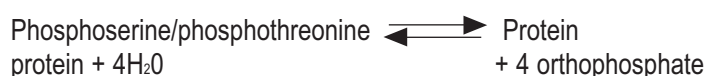
where $d[P]_{total}/dt$ is equal to rate of product formation (rate of reaction), and rearrangement of equation 7 gives:

$$R_t = \frac{1}{V_0 \cdot \Delta H_{app}} \cdot \frac{dQ}{dt} \quad [8]$$

A complete Michaelis-Menten curve is generated from one set of ITC titrations, because one experiment has different $[S]_t$ values. $[S]_t$ and $[E]$ are calculated after each ITC injection, using initial substrate and enzyme concentrations and ITC injection volumes. dQ/dt for each $[S]_t$ are determined as described below. R_t (from equation 8), $[S]_t$ and $[E]$ are fit to equation 3 to solve for K_M and k_{cat} .⁷ **Origin® 7.0 software for ITC automatically performs data analysis of enzyme-substrate ITC experiments, and calculates K_M and k_{cat} using non-linear least-squares regression analysis.** Analyses assume no significant product inhibition.⁷

This application note discusses two enzymes as examples:

Serine/threonine phosphatase catalyzes the hydrolytic removal of phosphate group of protein:



Protein phosphatases are ubiquitous, and protein phosphorylation states are balanced with competing actions of kinases and phosphatases. Phosphatases have important roles in glycogen metabolism, muscle contraction, protein synthesis, cell cycle regulation, and other metabolic pathways. A complete understanding of protein phosphatase mechanism is being explored to examine the enzyme's ability to serve as drug target. Haq⁸ used ITC to study the thermodynamics and kinetics of PP1- γ , using p-nitrophenyl phosphate (PNPP) as a model substrate. PNPP is used as a substrate for a spectrophotometric assay, thus Haq was able to validate ITC data with spectrophotometric data.

Hexokinase from Baker's Yeast (*Saccharomyces cerevisiae*) catalyses the phosphorylation of glucose:



Hexokinases are involved in the control in yeast metabolism. Hexokinase is an example of a reaction where neither the substrates (glucose and ATP) nor products (glucose 6-phosphate and ADP) are detectable with a spectrophotometer. The traditional hexokinase assay measures the formation of NADH at 340 nm in the coupled reaction with glucose-6 phosphate dehydrogenase and NAD⁺.

Experimental design for enzyme kinetics analysis with ITC

- Enzyme and substrate are prepared in identical buffer solutions, including matched ionic strength and pH. Buffer concentration must be high enough to ensure good buffering and prevent pH changes during experiment.
- If the enzyme reaction requires any cofactors, the concentration of cofactor is matched in substrate and enzyme solutions.

- Temperature: use typical temperatures for kinetics assays (20-37 °C). May do experiment at different temperatures.
- Recommendations for initial enzyme and substrate concentrations are discussed below.
- Total enzyme concentration in the ITC cell should be much less than total substrate concentration in the syringe, and initial substrate concentration greater than K_M .
- k_{cat} should be greater than 1 min^{-1} .
- Reaction must generate at least $0.007 \mu\text{M}/\text{sec}$ of product at 10% V_{max} to generate enough heat in ITC cell (at least $0.5 \mu\text{cal}/\text{sec}$ at V_{max}). ITC signal to noise ratio should be at least 100:1.⁷

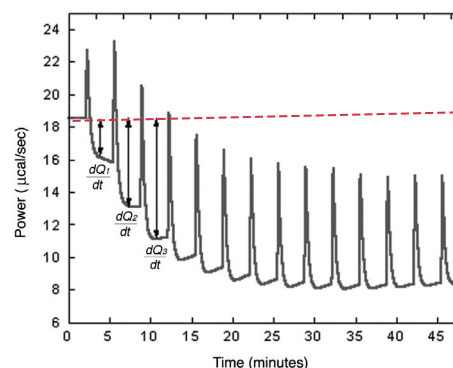
There are two methods that can be used to measure enzyme kinetic parameters with ITC: pseudo-first order conditions, and continuous assay conditions. Descriptions for each method are given in this application note. For more details on experimental design, refer to References 7, 8 and 9.

Calorimetric measurement of pseudo-first-order enzyme kinetics (multiple injections method)

An ITC experiment is performed to measure dQ/dt after each substrate addition, using conditions to maintain steady state. For this method, it is recommended that K_M be greater than $10 \mu\text{M}$ – if lower than that, the continuous assay is suggested.⁷

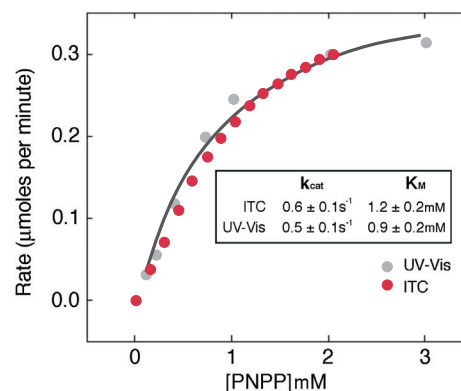
- **Recommended enzyme concentration in ITC cell:** 25 pM to 1 mM. In general, the higher the enzyme-substrate affinity, the lower the enzyme concentration.
- **Recommended substrate concentration in ITC syringe:** 10 μM to 100 mM, above K_M and in excess of enzyme concentration in cell. Do not start off with too high a substrate concentration; after several substrate injections, concentration of substrate in cell should not be above K_M .
- **Injection parameters:** 2-20 μl per injection, 15-30 injections (total available volume of VP-ITC syringe is ca. 300 μl).
- Suggested interval between injections is 3 minutes, to establish new thermal power baseline after each injection. (Figure 2). This change in thermal power baseline is due to heat produced (or absorbed) from the enzymatic reaction, and the ITC cell requires different power level to maintain thermal equilibrium.⁷
- As a general guideline, no more than 5% of substrate should be converted before the next injection.
- After completion of experiment, Origin 7.0 for ITC automatically performs data analysis.
- dQ/dt is determined by measuring the difference between original baseline and new baseline after each injection (Figure 2).⁷ $[S]_i$ and $[E]$ are calculated after each injection. ΔH_{app} is determined in a separate ITC experiment (see below). Using Equation 3, R_i is determined for each $[S]_i$ and data fit to Equation 8 to solve for k_{cat} and K_M .⁷ See Figure 3 for experimental Michaelis-Menten plot.

FIGURE 2



Raw ITC data for measurement of reaction rate for hydrolysis of PNPP by PP1- γ phosphatase.⁸ In ITC cell: $0.68 \mu\text{M}$ PP1- γ phosphatase, in 100 mM 2-amino-2-methyl-1, 3-propanediol, 150 mM NaCl, 20 mM MgCl_2 , 0.25 mM MnCl_2 , 0.5 mM OTT, 5% w/v glycerol, pH 8.5. In ITC syringe: 100 mM PNPP in same buffer. The drop in baseline power (dQ/dt) is indicated for the first three injections. Red dotted line is a linear least squares best fit to the pre-injection baseline.

FIGURE 3



Michaelis-Menten curve for hydrolysis of PNPP by PP1- γ hydrolase. Rate data from ITC from Figure 2 (red) and spectrophotometric data (gray) were normalized for enzyme concentration. Black line is non-linear least squares best fit to the spectrophotometric data.⁸

Figure 2 shows ITC data for the titration of PP1- γ phosphatase ($0.68 \mu\text{M}$) with PNPP (100 mM). The PNPP injections were $2 \mu\text{l}$, spaced at 3 minute intervals. There was an initial endothermic peak after each injection, which corresponds to heat of dilution. The thermal baseline then stabilized to a lower power level, due to exothermic catalysis of PNPP by PP1- γ . The power generated (dQ/dt) at each substrate concentration was proportional to the rate of reaction.

Data from Figure 2 were used to determine the reaction rate at different substrate concentrations, and these are plotted in Figure 3. This figure also shows data generated from spectrophotometric assays.⁸ Since different enzyme concentrations were used for the two sets of assays, the results in Figure 3 were normalized for enzyme concentration used in the spectrophotometric assays. k_{cat} and K_M values are shown in the inset. Kinetic parameters obtained by the two assay methods were in excellent agreement. Since many substrates of protein phosphatases cannot be monitored with a spectrophotometer, ITC can be useful to study enzyme kinetics and mechanisms as well as in the design of therapeutic agents.

The steady-state ITC method has been used to determine the enzyme kinetics parameters of several enzymes, including *H. pylori* urease,⁷ *E. coli* GroEL chaperonin,⁷ heparinase I,⁷ yeast hexokinase,⁷ HIV protease,⁷ pyruvate carboxylase,⁷ and dinucleoside polyphosphate hydrolase.⁸ Values determined by ITC agreed with enzyme parameters determined by other methods (Table 1).

Calorimetric measurement for continuous assay enzyme kinetics (single injection method)

An alternative strategy for obtaining enzyme kinetics parameters involves continuous rate measurements after a single injection of substrate. When substrate is completely depleted, thermal power returns to pre-injection baseline (Figure 4). ΔH_{app} is determined by integration of area under peak. At any given time the concentration of substrate is calculated and rate vs. substrate concentration is plotted to give a continuous kinetic curve.

- **Recommended enzyme concentration in ITC cell:** 1-25 nM.
- **Recommended substrate concentration in ITC syringe:** 10 μ M to 100 mM, above K_M and in excess of enzyme.
- **Injection parameters:** single injection of 25 μ l, followed by data collection for 1200-2000 sec, until thermal power baseline returns to pre-injection level (Figure 4).
- After completion of experiment, Origin 7.0 for ITC automatically performs data analysis.
- Integrate area under peak, and divide amount of substrate depletion, to determine ΔH_{app} .⁷
- Enzyme rate at time t calculated from Equation 8, and instantaneous substrate concentrations are determined by:

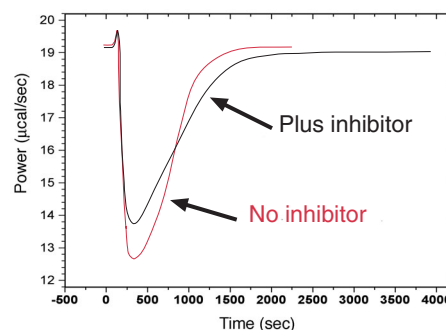
$$[S]_t = [S]_{t=0} - \frac{\int_0^t (Power) dt}{V_0 \cdot \Delta H_{app}} \quad [9]$$

Data are fit using Equation 3 to calculate k_{cat} and K_M .⁷

The continuous enzyme assay method was used to calculate kinetic parameters for yeast cytochrome c oxidase,⁴ chitinase,⁶ bovine dihydrofolate reductase,⁷ and trypsin.⁷ ITC results agreed with data from other assays (Table 1).

Bianconi⁹ used the continuous method to measure enzyme kinetics of yeast hexokinase isomers I and II, and parameters from ITC agreed with those previously published. Bianconi noted that the ITC assay was direct, and there was no need to couple the enzyme to glucose-6-phosphate dehydrogenase. For studies with inhibitors or activators, a direct assay is preferred, since additives may interfere with the coupled assay.

FIGURE 4



Raw ITC data for continuous assay. Red: absence of inhibitor; black: presence of inhibitor. The area under each curve was identical.

TABLE 1. Kinetic parameters of enzymes determined by ITC

Enzyme	K_M by ITC	K_M by non-ITC ^a	k_{cat} by ITC	k_{cat} by non-ITC ^a
Cytochrome c oxidase ^a	nd	nd	$7.48 \times 10^2 \text{ min}^{-1} \text{ mg}^{-1}$	$7.28 \times 10^2 \text{ min}^{-1} \text{ mg}^{-1}$
Chitinase A (<i>Arthobacter</i>) ⁶	nd	nd	103 min^{-1}	nd
Chitinase B (<i>Arthobacter</i>) ⁶	nd	nd	$81 \text{ min}^{-1} (15 \text{ }^\circ\text{C})$ $227 \text{ min}^{-1} (25 \text{ }^\circ\text{C})$	$207 \text{ min}^{-1} (25 \text{ }^\circ\text{C})$
Chitinase A (<i>S. marcescens</i>) ⁶	nd	nd	235 min^{-1}	nd
Urease (<i>H. pylori</i>) ⁷	0.79 mM	0.17 mM	1400 s^{-1}	2700 s^{-1}
GroEL (<i>E. coli</i>) ⁷	3 μ M	5 μ M	0.052 s^{-1}	0.08 s^{-1}
Heparinase (<i>F. heparinum</i>) ⁷	1.8 μ M	10.2 μ M	0.059 s^{-1}	92 s^{-1}
HIV protease ⁷	5-300 μ M (NaCl dependent)	15 μ M	10 s^{-1}	45 s^{-1}
Pyruvate decarboxylase Substrate = ATP ⁷	85 μ M	58 μ M	nd	nd
Pyruvate decarboxylase Substrate = pyruvate ⁷	105 μ M	440 μ M	nd	nd
Dihydrofolate ⁷	1.2 μ M	6 μ M	6 s^{-1}	3 s^{-1}
Hexokinase (yeast) ⁷	72 μ M	100 μ M	270 s^{-1}	450 s^{-1}
Trypsin ⁷	4 μ M	5 μ M	15 s^{-1}	22 s^{-1}
AP ₅ A hydrolase (<i>C. elegans</i>) Substrate=AP ₅ A ⁸	2.68 μ M	nd	2.76 s^{-1}	nd
AP ₄ A hydrolase (<i>C. elegans</i>) Substrate=AP ₄ A ⁸	35.5 μ M	nd	0.05 s^{-1}	nd
Hexokinase I (yeast) ⁹	155 μ M	nd	nd	nd
Hexokinase II (yeast) ⁹	210 μ M	nd	nd	nd

See cited references for experimental details and discussion of results.

- Parameters determined by non-ITC methods were either measured in the reference cited in this table, or from another reference. See cited references for details.

nd: Not determined or not documented.

Competitive Inhibitors

Enzyme kinetics experiments can be performed in the presence of a competitive inhibitor (1-100 mM) in the ITC cell, using the multiple injection or continuous method.

Preliminary experiments in the absence of inhibitor are required, and ΔH_{app} and k_{cat} do not change in the presence of a competitive inhibitor. See Figure 4 for continuous assays in presence and absence of inhibitor. Origin 7.0 software for ITC calculates K_i and K_M , using Equation 4.

The ITC method has been used to look at 2-mercaptoethanol inhibition of *H. pylori* urease (K_i of 4.1 mM),⁷ and benzamide inhibition of trypsin (K_i of 16 μ M).⁷

Calorimetric determination of ΔH_{app} for enzyme-substrate binding reaction

Equation 8 uses ΔH_{app} for the enzyme and substrate reaction. To measure ΔH_{app} by ITC, there has to be sufficient enzyme in the cell to convert all injected substrate into product in a given time period, so baseline response returns to the same value after substrate injection as it was before injection. This experiment is similar as described above for the continuous assay method.

- **Recommended enzyme concentration in ITC cell:**
1 nM – 10 μ M.
- **Recommended substrate concentration in ITC syringe:**
10 μ M to 100 mM, above K_M and in excess of enzyme.
- **Injection parameters:** single injection of 10 to 50 μ l, followed by data collection for 1200-2000 sec, or until thermal power baseline returns to pre-injection level.
- Integration of area under peak with respect to time yields the total heat produced by the reaction. Dividing total heat by the amount of product generated gives ΔH_{app} .⁷

An alternative method is to have the enzyme in the syringe, and a limited amount of substrate in the ITC cell. Bianconi⁹ used this technique to determine ΔH_{app} for yeast hexokinase I and II. ATP concentration in the ITC cell was limiting, while glucose was not limiting. After injection of hexokinase into the substrate solution, there was an exothermic reaction. As ATP level was depleted, the thermal power baseline returned to pre-injection level. Bianconi measured glucose-6-phosphate production in a separate experiment, and calculated ΔH_{app} .

ΔH_{app} and buffer effects

ΔH_{app} is the sum of ALL heats, and is dependent on reaction conditions.^{7,9,10} If different reaction buffers and/or temperatures are used for the enzyme-substrate reaction, ΔH_{app} needs to be experimentally determined for each set of conditions.

If the enzymatic reaction involves a release or uptake of protons, ΔH_{app} is a combination of intrinsic enthalpy of the reaction (ΔH_{int}), and enthalpy for ionization of buffer (ΔH_{ion}^{buff}), ionization of protein (ΔH_{ion}^{prot}) and the number of protons involved.¹⁰ When a reaction is performed using different buffers at the same pH, there is a correlation between ΔH_{app} and ΔH_{ion} . Using published values of buffer heats of ionization,¹¹ the number of protons and ΔH_R without buffer effects can be determined from ΔH_{app} values.¹⁰

Todd and Gomez⁷ studied urease activity in different buffers, and showed that ~1 proton was taken up during urea turnover. Bianconi⁹ demonstrated the buffer effects on the ΔH_{app} of yeast hexokinase isomers I and II, and confirmed that one proton was released during the enzymatic reaction. Bianconi observed that there was a difference of 1.8 kcal/mole in ΔH_R of hexokinase I and II, indicating that there was another reaction, besides glucose phosphorylation, causing a change in enthalpy.

Summary

Results shown here and in cited publications demonstrate that ITC:

- Involves minimal method development
- Is universal for any enzymatic reaction where heat is produced
- Is a sensitive technique – noise level of <0.01 $\mu\text{cal}/\text{sec}$ results in high signal to noise ratios with modern ITC instruments^{7,8}
- Does not require chromogenic or fluorogenic substrates
- Generates data from a single experiment
- Is appropriate for enzymes from every EC classification⁷
- Can be used with opaque solutions⁷
- Generates data comparable to other assay methods^{7,8,9}
- Generates thermodynamic information as well as kinetics data^{7,8,9}

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