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Enzyme kinetics determined by single-injection isothermal titration calorimetry

Mark K. Transtrum [a], Lee D. Hansen [b,*], Colette Quinn [c]

[a] Department of Physics and Astronomy, Brigham Young University, Provo, UT 84602, USA
[b] Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602, USA
[c] Applications Lab, TA Instruments, Lindon, UT 84042, USA

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ABSTRACT

The purposes of this paper are (a) to examine the effect of calorimeter time constant (τ) on heat rate data from a single enzyme injection into substrate in an isothermal titration calorimeter (ITC), (b) to provide information that can be used to predict the optimum experimental conditions for determining the rate constant (k_2), Michaelis constant (K_M), and enthalpy change of the reaction ($\Delta_R H$), and (c) to describe methods for evaluating these parameters. We find that K_M, k_2 and $\Delta_R H$ can be accurately estimated without correcting for the calorimeter time constant, τ, if (k_2E/K_M), where E is the total active enzyme concentration, is between $0.1/\tau$ and $1/\tau$ and the reaction goes to at least 99% completion. If experimental conditions are outside this domain and no correction is made for τ, errors in the inferred parameters quickly become unreasonable. A method for fitting single-injection data to the Michaelis–Menten or Briggs–Haldane model to simultaneously evaluate K_M, k_2, $\Delta_R H$, and τ is described and validated with experimental data. All four of these parameters can be accurately inferred provided the reaction time constant (k_2E/K_M) is larger than $1/\tau$ and the data include enzyme saturated conditions.

1. Introduction

Mass-action kinetic models, of which Michaelis–Menten (or Briggs–Haldane) is one example, Eq. (1),

$$dS/dt = -k_2 ES/(K_M + S) \quad (1)$$

describe the kinetics of reactions catalyzed by a single enzyme and provide an approximation to the kinetics of processes involving a network of enzymes [1]. These models express the reaction rate as a function of the total concentration of active enzyme (E) and the concentration of substrate (S) with rate constants (k_2 or k_{cat} in some literature) and mass-action constants (K_M) as parameters to be evaluated from the data. These functions do not have a simple closed form for the expression for $dS/dt = f(t)$ where $f(t)$ is a function of time. Therefore, equations with rate expressed as a function of time, which is the data form produced by heat-conduction and power-compensation isothermal titration calorimetry (ITC), cannot be obtained. This makes ITC data analysis with mass-action kinetic models particularly challenging.

Two ITC methods for determining enzyme kinetics have been described in the literature [2–4]; a multiple-injection method and a single-injection method. In the multiple injection method, the steady-state heat rate is measured after each injection of substrate into an enzyme solution. The data produced is a plot of heat rate versus concentration of substrate, typically with (20–40) data points. Since each data point takes 3–5 min, a single experiment takes 1.5–3.5 h. For this method to work, concentrations of enzyme and substrate must be adjusted so that heat rates change significantly with each injection of substrate but are also constant after each injection. The inverse titration is not practical because of leakage of enzyme from the burette after the first injection. In addition to the step-wise heat rate measurements, an additional single injection experiment must be done to determine the enthalpy change for the catalyzed reaction ($\Delta_R H$). Because τ does not enter into the calculation, after correction for the baseline and determination of $\Delta_R H$, evaluation of K_M and k_2 from multiple-injection data can be done in a spreadsheet by the traditional methods used with the Michaelis–Menten model.

In the single-injection method, a single injection of enzyme is made into a solution of substrate with the substrate concentration adjusted so that the substrate is mostly consumed in 30 min to an hour. The data produced thus consists of several hundred measurements of heat rate versus time. Data from a single injection exper-

* Corresponding author. Fax: +1 801 422 0153.
E-mail addresses: mktranstrum@byu.edu (M.K. Transtrum), lee_hansen@BYU.edu (L.D. Hansen), cquinn@tainstruments.com (C. Quinn).

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iment are shown by the solid line in Fig. 1. Since only one injection is necessary, leakage from the burette prior to injection can be prevented by filling the tip with a small amount of buffer. Although the single-injection method requires only one experiment, is significantly faster, and requires less enzyme, it has been little used because data analysis is significantly more challenging than analysis of multiple-injection data. Accurate analysis of single-injection data requires recursive, simultaneous fitting of the entire curve with K_M, k_2, $\Delta_R H$, τ and possibly the baseline heat rate, ϕ_B, as fitting parameters.

The traditional method for correcting for instrument time constant by use of the Tian equation, e.g. [5,6],

$$\phi_{\text{corrected}} = \phi_{\text{measured}} + \tau(\mathrm{d}\phi_{\text{measured}}/\mathrm{d}t) \tag{2}$$

where τ is defined by the function $1 - e^{-t/\tau}$ for an increasing response and $e^{-t/\tau}$ for a decreasing response and ϕ is heat rate, presupposes an accurate value for τ that is not easily measured in heat-conduction and power-compensation calorimeters [7]. There is no universal value of τ for a particular calorimeter design, and since τ depends on the mixing time, thermal conductivity of the solution, and thermal time constants of all the parts of, and connections to, the reaction vessel, the value of τ is not the same for all calorimeters of the same design, and can vary from experiment to experiment even in the same calorimeter. Determination of τ by injection of methanol in a separate experiment or with a heater pulse prior to or post experiment produce values that differ significantly from the applicable value of τ. For example, a heater pulse in the ITC model 2G used to collect the data in Fig. 1 gives $\tau = 12$ s, but analysis of the data by fitting to the model gives $\tau = 37$ s. In another example, the "high feedback response time" in the specifications for the MicroCal ITC 200 is 10 s (i.e., $\tau = 2$ s), but Burnouf et al. [8] report finding $\tau = 3.5$ s which gives a 99% response time of 18 s. Demarse et al. [9] found $\tau = 14.5$ s by fitting single injection data for sucrose–invertase from a NanoITC Low Volume instrument, but electrical heater pulse gave $\tau = 2.2$ s. Note that in every case, the value of τ obtained from a heater pulse is significantly shorter than the value of τ obtained from fitting kinetic data.

The mathematics necessary for multi-parametric fitting of single-injection data to a Michaelis–Menten model, Eqs. (3)–(6),

$$\phi_r(t) = -\Delta_R H V k_2 E S(t)/[K_M + S(t)] \tag{3}$$

$$-K_M \ln S - S = k_2 E t - K_M \ln S_0 - S_0 \tag{4}$$

$$t(S) = -(k_2 E)^{-1}[(S - S_0) + K_M \ln(S/S_0)] \tag{5}$$

$$\phi_{\text{cal}}(t) = \tau^{-1} e^{-t/\tau} \int e^{S/\tau} \phi_r(S)\mathrm{d}S \tag{6}$$

where ϕ_r is the heat rate from the reaction and ϕ_{cal} is the heat rate measured by the calorimeter, has been published [7,9] along with the process for use of these equations. The model in Eqs. (3)–(6) is fit to data by nonlinear least squares. First, the parameters k_2, K_M, $|\Delta_R H|$, and τ are log transformed. This guarantees that the parameter values remain positive and improves the efficiency of the fitting procedure. The resulting model is fit using the geodesic Levenberg–Marquardt algorithm [10,11]. The data to be fit do not contain error bars since each point consists of a single measurement. Assuming the error in each data point is from a Gaussian distribution with variance σ^2, we estimate σ using a maximum likelihood method. If SS represents the sum of squares error from fitting the model, $\sigma^2 = \text{SS/M}$, where M is the number of independent data points in the sample. We find that $\sigma \approx 0.05$ for the data in Fig. 1. An alternative to this method uses the Lambert $W(x)$ (or Omega) function [12].

Use of ITC for determination of the kinetics of enzyme catalyzed reactions is increasing [13], but programming this process is challenging. The purposes of this paper are (a) to examine the effect of calorimeter time constant on single-injection ITC kinetic data, (b) to provide the user with information that can easily be used to predict the experimental conditions for optimum results, and (c) to describe methods and software for evaluating model parameters in mass-action kinetic models.

2. Effect of calorimeter time constant

The rate at which heat is generated by the reaction (ϕ_r) is directly proportional to the reaction rate with $\Delta_R H$ as the proportionality constant. Observation of this heat rate by the calorimeter is delayed due to the effects of the time constant of the instrument as illustrated in Fig. 2. This delay manifests itself as the rising curve at small times and an elongation of the curve's tail at long times. For instruments with small time constants, this initial rise is sharp and brief and the exponential tail is mostly unaffected. However, for large time constants the initial rise can take a much longer time, resulting in a large elongation of the curve's exponential tail.

Often, only the decaying portion of the data in curves such as those in Fig. 2 have been analyzed to obtain kinetic constants with $\Delta_R H$ being determined from the area under the curve [2,4–6,8,14]. Only the exponential tail of the curve is fit because the instrument time constant is necessary to replicate the initial rising portion of the curve. This practice introduces new complications: How does one determine which data to ignore? And how much does the time constant affect these data where the signal is changing relatively rapidly? We illustrate this dilemma in Table 1 in which different portions of the real data in Fig. 1 are fit to Eqs. (3)–(6) with τ included or excluded as a fitting parameter. The second column

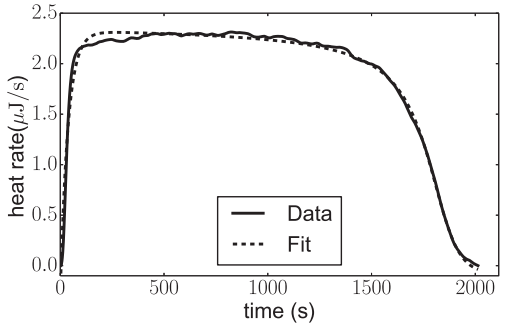

Fig. 1. Solid line – heat rate data from a single injection of 10 μL of 511 nM trypsin into 950 μL of 144 μM N-α-benzoyl-L-Arginine Ethyl Ester (BAEE) in 200 mM Tris–HCl buffer pH 8.0, 50 mM $CaCl_2$, and 0.2% PEG-2000 in an ITC model 2G (TA Instruments, Lindon, UT) [4]. Dashed line – fit to the data with Eqs. (3)–(6).

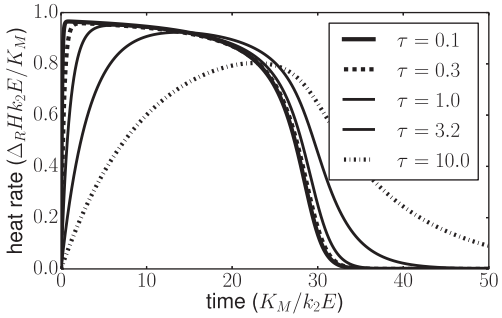

Fig. 2. Simulated Michaelis–Menten data for a single injection experiment in calorimeters with different time constants. Eqs. (3)–(6) were used to generate the curves.