Experimental Design for the Determination of Kinetic Parameters Using the Multiple Injection Method (MIM) with Isothermal Titration Calorimetry (ITC)

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ABSTRACT

Reactions, including enzymatic reactions, produce or absorb heat. This heat can be directly related to the product formation in an enzymatic reaction. The advantage to using heat as a metric is that the materials don't require modification, such as the addition of a chromophore, and the assay is continuous without the need to quench the reaction at various points for product quantification. One of the only requirements is that the substrate and enzyme buffers are matched, which is ideally accomplished through dialysis of the enzyme and substrate in the same buffer.

THEORY

Studying Michaelis-Menten kinetics via ITC has been established as a straight-forward way to evaluate simple systems that follow a pseudo first order rate (1, 2). The rate limiting step for this type of interaction is k_2, the dissociation of the enzyme-substrate complex [ES] (equation 1).

$$Rate = k_2[ES] \quad eq.\ 1$$

It is assumed in analysis that the reverse of this reaction, the complex formation, (k-2) is negligible. The rate or velocity (v) of the reaction is given by the Michaelis-Menten relationship (equation 2).

$$v = d[P]/dt = (v_{max}[S])/(K_M + [S]) \quad eq.\ 2$$

In equation 2, [P] is the concentration of the product released, v_{max} is the maximum velocity at saturating substrate concentrations, [S], and K_M is the Michaelis-Menten constant, which is the value of [S] at which $v = ½v_{max}$. Another parameter that is often acknowledged is the enzyme turnover number, k_{cat}, which is the product of v_{max} and $[E]_{total}$.

The amount of heat (Q) involved in converting n moles of substrate to product is simply the number of moles produced times the enthalpy for this reaction (dH_{app}).

$$Q = n*dH_{app} = [P]*V_0*dH_{app} \quad eq.\ 3$$

Where V_0 is the volume of the reaction cell.
Rearrange and apply the first derivative:

$$Rate = d[P]/dt = (1/(V_0*dH_{app}))*dQ/dt \quad eq.\ 4$$

The dQ/dt should be familiar. It is the Y-axis of an ITC thermogram, the differential power with respect to time.

Kinetic studies via the MIM in an ITC is a straightforward assay that requires the operator to perform two experiments, one to determine the differential power effects arising from the continuous turnover of substrate to product and the other to determine the enthalpy from the total conversion of all the substrate converted into product.

SETTING-UP THE MIM EXPERIMENT

A. DETERMINATION OF dQ/dt

The first experiment of a MIM kinetic study is determination of the differential power change (dQ/dt), which is proportional to the turn-over of substrate into product. An acceptable MIM assay is shown in Figures 1 and 2.

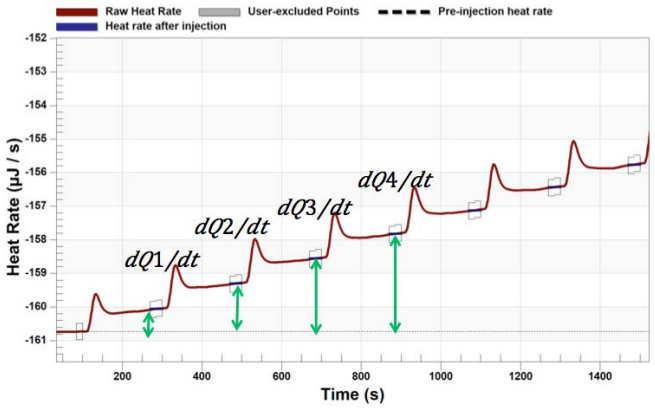

Figure 1: Heat rate (dQ/dt) shift for an enzymatic assay where [S]total>>[E]total.

Steady state conditions are required after each injection and ideally < 5% of the substrate is depleted prior to the next injection. Experimentally this means working at a low $[E]_{total}$ and having a minimal time between injections, 2-4 minutes.

Substrate is loaded into the injection syringe at a concentration 5-10 times greater than the K_M. This will ensure that the final concentration of the substrate in the cell is greater than the K_M. If the K_M is not known, then a high micromolar concentration is initially suggested. If the entire volume of either the 250 µL (3-6 µL aliquots) or the 50 µL (1-2.5 µL aliquots) syringe is delivered into the contents of the cell, then the final concentration of the titrant in the cell will be ⅕ to ¼ of $[S]_{initial}$ syringe. The exact value depends on the active cell volume.

Enzyme is loaded into the cell at a low nanomolar to high picomolar concentration. If the heat after each injection decays rapidly, then steady state is not achieved and the concentration of the enzyme should be decreased or the injection volume of substrate increased.

Ideally, $dQ_n/dt = dQ_{n-1}/dt$ is reached where additional aliquots of substrate will not yield an increase in the turn-over (v_{max}). Under these conditions the concentration of substrate in the cell will be in excess of the K_M. In Figure 2, a plateau has been reached. If a plateau is not reached, increase the substrate concentration.

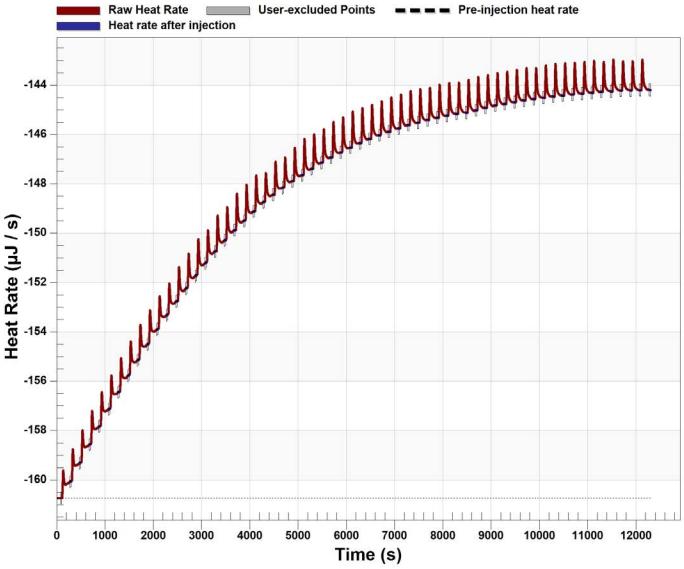

Figure 2: Full thermogram for enzymatic assay of substrate titrated into enzyme

B. ENTHALPY

Determination of the enthalpy is the second experiment required for the MIM study. For this assay, the enzyme is not limited and all of the substrate is converted into product.

A low substrate concentration, < K_M, is loaded into the syringe. Initially try a concentration 10-100 times lower than the conditions in first titration described above. A high concentration of enzyme is loaded into the cell. A starting point is 100-1000 times the concentration used in the dQ/dt experiment.

A minimum of three injections should be performed. The first injection is discarded and the latter two should agree with each other. If the normalized heat isn't consistent, either increase the concentration of enzyme or decrease the concentration of substrate. An alternative optimization is to try smaller injection volumes. The suggested injection volume used is 1-3 µL for the low volume (190 µL) instrument and 3-8 µL for the standard volume (1 mL).

A background titration of substrate into buffer should also be collected and this heat subtracted from the enthalpy of the substrate-enzyme titration.

MICHAELIS-MENTEN ANALYSIS

The information collected from these two experiments are plotted on each axis. The product of the reaction volume (V_0) and enthalpy (dH_{app}), both constants, along with each dQ/dt for each injection is the rate, d[P]/dt, plotted on the y-axis. And the substrate concentration in the cell, [S], is plotted on the x-axis. In this form the resulting graph is a Michaelis-Menten plot and the alternative Lineweaver-Burk, a double reciprocal plot.

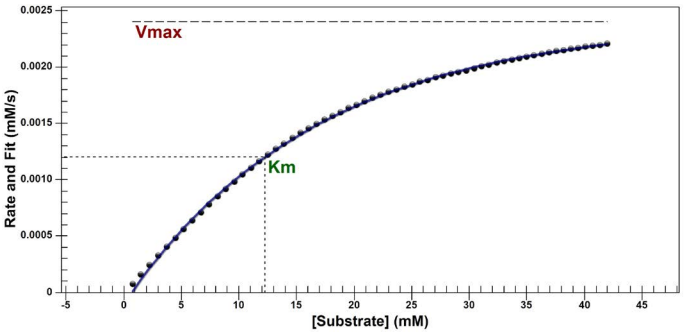

Figure 3: Michaelis-Menten Plot.

PRECAUTIONS

ITC is a global technique and if secondary chemistries such as proton-coupled events are occurring along with the turnover, these will be detected in the total heat. Data can be deconvoluted so that only the binding event is used in the kinetics analysis or reported as condition specific.

The turnover is also an important parameter and k_{cat} > 1/min (1). This is because the heat generated would be spread over a larger time interval and may become undetectable.

When the shift in dQ/dt is small, then the MIM is not recommended. Small is a relative term and is dependent on the stability of the baseline slope of the calorimeter that is used. As a general rule, a K_M lower limit that has been previously recommended is ~10 µM (1). For these systems it is suggested to operate under the single injection method (SIM).

CONCLUSION

The multiple injection method (MIM) provides a way to obtain pseudo first order kinetic information using ITC. After collecting data under two separate conditions the turnover and enthalpy are determined and then plotted to obtain Michaelis-Menten kinetic parameters. These experiments directly and continuously monitor the conversion of substrate into product.

REFERENCES

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All figures were created in NanoAnalyze.