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Calorimetry of enzyme-catalyzed reactions

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Abstract

This mini-review shows the valuable contributions of Professor Julian Sturtevant to the current applications of calorimetry to the study of enzyme-catalyzed reactions. The more recent applications of calorimetric techniques such as isothermal titration calorimetry and flow calorimetry to the study of enzyme kinetics, as well as the advantages on using calorimetric techniques in the determination of kinetic parameters of enzymes, is also discussed here.

Keywords: Calorimetric enzyme assays; Thermodynamic of enzyme reactions

1. Introduction

The proper characterization of an enzyme that follows the Michaelis–Menten mechanism requires the determination of the Michaelis constant, K_m, and the enzymatic conversion rate constant or turnover number, k_{cat}. The k_{cat}/K_m ratio is referred as the catalytic efficiency of an enzyme. These kinetic parameters are usually determined from a set of experiments where the kinetics of the enzyme-catalyzed reaction is followed by optical or electrochemical measurements. Since most substrates and/or the products do not possess suitable properties, enzyme-catalyzed reactions are often studied by using modified substrates or a coupled enzyme assay. These strategies can introduce a number of experimental errors in the determination of K_m and k_{cat}. Virtually all chemical reactions, and therefore enzyme-catalyzed reactions, occur with some heat effect (heat release or heat absorption) making calorimetric techniques very suitable for following such reactions. In this review, the applications of isothermal titration calorimetry (ITC) and flow calorimetry to the study of enzyme-catalyzed reactions will be discussed as alternative (and advantageous) ways to the direct measurement of enzyme kinetics.

2. The early days

The application of calorimetric techniques to the study of enzyme-catalyzed reactions has increased in the past few years with the improvement of available instruments. Julian Sturtevant was the pioneer in the field [1–13] and his first contributions to the study of enzyme kinetics dates from the early 1950's [1–3]. Long before that, Julian Sturtevant published the series "Calorimetric Investigations of Organic Reactions" [14–17] showing that calorimetry was a powerful technique to follow reaction kinetics. In the first article of this series, published in 1937, Julian Sturtevant called the attention to the fact that many reactions can not be followed by standards physical methods based on changes in density, optical rotatory power, refractive index and electrical conductivity as most of them "are rather restricted in applicability". For the first time, Sturtevant discussed that "following the evolution or absorption of heat during reaction should be much more widely applicable, since nearly all reactions proceed with an appreciable change in heat content" [14].

In the case of enzyme-catalyzed reactions, a calorimetric procedure for the determination of the heats of hydrolysis of amides and peptides was first described by Dobry and Sturtevant [1]. In the following article [2], the thermodynamics of hydrolysis of peptide bonds was further assessed by Sturtevant. Although the main interest was related to the

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determination of the enthalpy of reaction, the authors also calculated the rate of the heat evolution which followed the first-order kinetics [1,2] allowing the calculation of the specific rate for the conversion of the enzyme–substrate complex into products (k_3). For the first time, Julian Sturtevant shows the importance of calorimetry for the study of enzyme kinetics. As commercial calorimeters were not available at that time, these studies were carried out in the first isothermal calorimeter developed by Anne Buzzell and Julian Sturtevant [18]. This calorimetric apparatus constituted of twin calorimeters working in thermal balance "to compare the chemical energy evolved or absorbed with automatically adjusted electrical energy" [18]. During an exothermic reaction, the chemical energy released in one calorimeter was electrically duplicated in the other, while in endothermic processes the electrical energy was introduced in the cell where the reaction was taking place. In this way, an electrical feedback maintained the same temperature in both calorimetric cells, one way or the other [18].

The twin calorimeter apparatus was used in a series of studies of enzyme-catalyzed reactions by Sturtevant and co-workers [1–9], with special attention to the hydrolysis of peptide bond. The first two articles describe the chymotrypsin-catalyzed hydrolysis of benzoyl-L-tyrosinamide [1] and benzoyl-L-tyrosylglycinamide [2]. Carboxypeptidase was used for the hydrolysis of different substrates, i.e., carbobenzoxyglycyl-L-phenylalanine [1], Carbobenzoxyglycyl-L-leucine [3], and benzoyl-L-tyrosylglycine [4]. Other enzymes were also employed for the study of peptide bonds hydrolysis, such as chatepsin C [3], leucineaminopeptidase [4], trypsin [5], and α-renin [6].

The twin calorimeter apparatus presented two major problems — it required a long equilibration time and large amount of samples: "In each run, 12.5 ml. of an appropriate enzyme solution were placed in the lower half of each calorimeter, and 12.5 ml. of substrate solution in the upper half. The apparatus was assembled and allowed a period of approximately 15 hours to reach thermal equilibrium" [2]. The long equilibration time was possibly very harmful to the samples, specially enzymes that could be denatured. The amount of sample used was also a negative aspect of the twin calorimeters method. Each cell was filled with 12–20 mL of solution, which certainly required a substantial amount of substrates and enzymes, even if the later was used very diluted. Although this home-made calorimeter had much lower sensitivity than the new calorimeters available today, the results obtained by Sturtevant and co-workers were found to be very accurate, such as in the case of the very low enthalpy determined in the hydrolysis of peptide bonds [1–6] corroborated by Williams and Toone in 1993 [19] by using a Microcal Omega calorimeter.

In 1955, Podolsky and Sturtevant [9] showed that the enthalpy of ATP hydrolysis, catalyzed by myosin from rabbit muscle was "approximately half the value 12 kcal/mol which has been hitherto accepted". In fact, in 1979, by using a microcalorimeter with improved time response, Kodama and Woledge [20] showed that the individual steps of the ATP hydrolysis by Myosin, i.e., ATP binding, ATP hydrolysis, Pi release, and ADP release, contributes to either a heat release or heat absorption. The changes in enthalpy observed for the overall reaction is actually very low, in the order of 2 kcal/mol [20], in good agreement with the low enthalpy change found by Podolsky and Sturtevant [9].

Later on, Sturtevant helped in the development of a flow calorimeter from Beckman, which was described in the determination of the enthalpies of mixing of pairs of liquids at 25 °C [10]. This calorimeter was used to determine the enthalpy of hydrolysis of cyclic AMP and cyclic GMP catalyzed by bovine heart phosphodiesterase [11], of the activation of chymotrypsinogen A by trypsin [12], and the phosphohydrolase-catalyzed hydrolysis of acyclic, monocyclic, and glycoside cyclic phosphate diesters [13].

3. Isothermal titration calorimetry (ITC)

The availability of more sensitive isothermal titration calorimeters had an important consequence on the application of ITC in enzyme assays [18–26], recently reviewed by Lonhienne and Winzor for enzyme kinetics in crowded systems [27]. The principles of implementation of ITC were described by Wiseman et al. [28], and the application to enzyme kinetics is described here according to Todd and Gomez [22] and Bianconi [26]. ITC has also being used to study the kinetics of more complex systems such as the determination of the heat production in sarcoplasmic reticulum vesicles [29] or in mitochondrial respiration upon activation of an ATP-insensitive K^+ channel [30].

Calorimetry can be used to study enzyme kinetics given that the heat (Q) evolved is proportional to the molar enthalpy of the reaction (ΔH) and, therefore, to the concentration of product ($[P]$) formed in a reaction volume V:

$$Q = n \cdot \Delta H = [P]^{\text{total}} \cdot V \cdot \Delta H \tag{1}$$

The isothermal power-compensation calorimeters operate in a wide temperature range (up to 80 °C) and consist of two cells (sample and reference) maintained at the same and constant temperature through a thermal power supply to the sample cell. This thermal power consists in the raw data obtained in this type of calorimeters and reflects the heat flow, i.e, the dissipation of heat (dQ) as a function of time (dt). The heat flow (dQ/dt) is directly proportional to the rate of product formation ($d[P]/dt$), and can be described as:

$$\frac{dQ}{dt} = \Delta H \cdot V \cdot \frac{d[P]}{dt} \tag{2}$$

Eq. (2) shows that in order to calculate the rate of an enzyme-catalyzed reaction by ITC, it is necessary to determine not only the heat flow but also the reaction enthalpy. For first-order reaction kinetics the heat flow is given by Eq. (3),

$$\frac{dQ}{dt} = \Delta H \cdot V \cdot k \cdot [C_0] \cdot \exp^{(-kt)} \tag{3}$$

where k is the rate constant and $[C_0]$ is the initial concentration of substrate [14].

The observed calorimetric enthalpy (ΔH^{cal}) for the reaction can be determined by dividing the total heat (Q_T) generated in the reaction by the amount of product formed when the substrate is totally consumed. The total heat is obtained by integrating the area under the peak of the calorimetric thermograms (dQ/dt as a function of time).

Fig. 1 shows the calorimetric thermograms for the hydrolysis of urea catalyzed by the *H. pylori* urease [22]. In Fig. 1A, a download displacement of the baseline occurs after urea is injected into the sample cell containing a solution of urease, indicating the exothermic nature of the reaction. The rate of heat generated by the enzyme corresponds to the decrease in instrumental thermal power (dQ_1/dt). A second injection of urea, indicated by an arrow at 1200 s (Fig. 1A) increases the concentration of substrate and therefore, the rate of the reaction as seen by an increase in dQ/dt. The conditions in Fig. 1A are such that the reaction kinetics can be followed at steady-state. The K_m of a reaction can be calculated in one experiment if several injections of substrate are done into the sample cell containing very low enzyme concentrations [22,26], or by doing separated measurements of the reaction kinetics [24], which can be helpful when the effects of product inhibition, for instance, should be avoided.

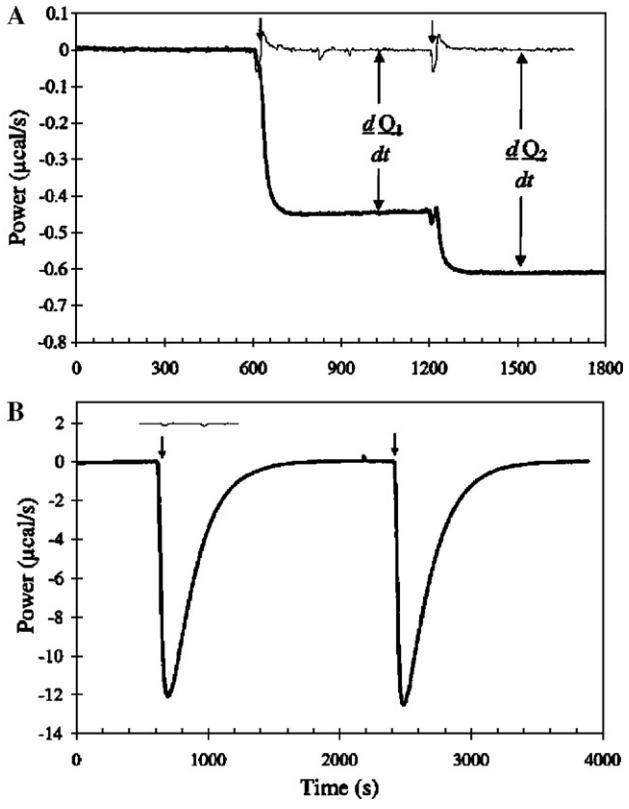

Fig. 1. ITC traces for the reaction catalyzed by urease at 37 °C. In (A), the power (or heat flow) as a function of time was measured in a condition of a steady-state kinetics, where the calorimetric cell was loaded with a solution containing enzyme (urease) and the substrate (urea) was injected as indicated by the arrows. In (B), after the substrate is completely consumed there is a return of the power to the baseline level, and the area under the peak corresponds to the total heat (Q_T) released in the hydrolysis of urea. Reprinted from Analytical Biochemistry 296, M.J. Todd and J. Gomez, Enzyme kinetics determined using calorimetry: a general assay for enzyme activity?, pages 179–187, copyright (2001), with permission from Elsevier.

In Fig. 1B, the thermogram shows a reaction where the substrate is completely consumed, allowing the calculation of Q_T and, consequently, ΔH^{cal}. There is, however, one care to be taken when studying enzyme-catalyzed reactions by calorimetry. It is well known that the calorimetric enthalpy is the sum of all the heat effects taking place during any reaction. If there is a release (or uptake) of protons, ΔH^{cal} will be a combination of the enthalpy of reaction (ΔH^R) and the enthalpy of protonation (or ionization) for each proton absorbed (or released) by the buffer. Fukada and Takahashi [31] determined the enthalpy of buffer protonation/ionization (ΔH^P) for a series of buffers in a wide temperature range. Nevertheless, ΔH^P can be easily determined in any buffer system, and ΔH^R can be calculated from the linear relationship between ΔH^{cal} and ΔH^P, expressed in Eq. (4):

$$\Delta H^{cal} = \Delta H^R + n\Delta H^P \tag{4}$$

where n represents the number of protons absorbed/released in the reaction.

4. Flow calorimetry

Flow calorimetry has also been used to the study of enzyme kinetics [32–38]. This technique was also successfully used in the measurement of dihydrofolate reductase in crude tissue homogenates [39], an enzyme that requires very sensitive methods for its determination due to the low levels found in human tissues, and in the study of immobilized glucoamylases [40].

The flow calorimeters can be used in two different modes: (i) in the flow through mode, where the heat effect is measured with the reacting solution is constantly pumped trough the calorimetric cell, and (ii) in mixing mode, where two separated reactant solutions are continously pumped and mixed into the cell [32].

As described by Eftink et al. [32], in the mixing mode, the heat effect for determined reacton (W_R) is given by:

$$W_R = \Delta H_R \cdot \Delta[R] \cdot f \tag{5}$$

where ΔH_R is the enthalpy change of the reaction $\Delta[R]$ is the change in concentration of the limiting reactant, and f is the total flow rate. $\Delta[R]$ can be substituted by the concentration of product formed during the residence time τ in the cell ($[P]\tau$) when the reaction does not go to completion, allowing the determination of the allowing the determination of the average velocity ($\overline{v}$), by:

$$\overline{v} = \frac{[P]\tau}{\tau} = \frac{\varepsilon V}{\Delta H_R \cdot f}\frac{1}{\tau} \tag{6}$$

where ε is a proportionality constant or calibration factor that can be experimentally determined [32].

Fig. 2 shows the result of the kinetics of hydrogen peroxide decomposition by catalase done in a flow-mix calorimeter by

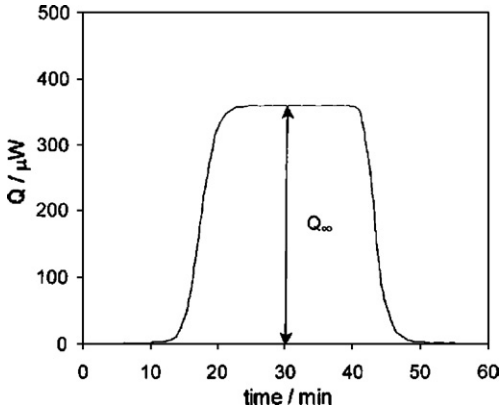

Fig. 2. Thermal power (Q) as a function of time in a flow-mix kinetic experiment at 15 °C. The reaction was started after baseline equilibration, by feeding the cell containing hydrogen peroxide with a catalase solution. The overall flow rate in the reaction unit was 0.23 $cm^3 min^{-1}$. Reprinted from Thermochimica Acta 402, M. Fidaleo and R. Lavecchia, Kinetic study in a flow-mix microcalorimetric system, pages 19–26, copyright (2003), with permission from Elsevier.

Fidaleo and Lavecchia [35]. After equilibration of the calorimeter, once a stable baseline was obtained, the substrate was introduced into the cell containing catalase. The thermal power (Q) progressively increases when the reaction started, reaching a steady-state (Fig. 2), and the reaction was stopped by removing the substrate which cause the return to the baseline level [35].

O'Neill et al. [36] showed the calorimetric outputs (Φ) for the reaction catalyzed by urease with increasing urea concentrations giving rise to kinetics of first-order (0.02–0.04 mM urea), mixed-order (up to 0.1 mM urea), and zero-order kinetics (0.2–0.4 mM urea) (Fig. 3). At low substrate concentration, the slope of lnΦ as a function of time equals to the first-order rate constant (k_1) for the reaction [36]:

$$\Phi = -\mathrm{FCH}(1 - e^{-k1\tau}) \cdot e^{-k1\tau} \quad (7)$$

where F is the flow rate of the flowing solution, C is the concentration of reagent in solution, H is the enthalpy of reaction, τ, the residence time of the solution in the calorimetric cell, and t is time. For zero-order kinetics, Eq. (7) can be rewritten as $\Phi = -k_0 H\tau \cdot F$ [36].

According to O'Neill et al. [36], the kinetic parameters found in the urea/urease system corroborates with early studies performed more than 30 years ago, and the reason for the equivalence in their numbers is that the rate constant is not governed by the thermodynamics of the system.

Hills et al. [38] pointed out the sources of errors as well as the corrections that can be used, in the calorimetric determination of kinetic parameters of enzyme-catalyzed reactions. If the upper calibration is incorrectly set, the thermal power measured is greater than that produced by the reaction. Baseline shifts are also sources of errors that can generate a non-uniform error if occurring together with an error in the upper calibration set. These errors are constant during the experiment and can be fixed [38]. Nevertheless, the same authors call the attention to time-dependent errors that cause distortions in the curve of thermal power as a function of time, due to time-dependent errors [38].

5. Advantages on using calorimetry in enzyme kinetic assays

The main advantage on using calorimetry in enzyme-catalyzed reaction is the possibility of doing a direct assay without the requirement of using modified substrates or coupled reactions. There is also no need to have a clear sample since calorimetric measurements do not involve absorption or emission of light. It has been shown a good correlation between calorimetric and spectrophotometric data for reaction kinetics [19,21,24].

The use of modified substrates by introducing an UV active or fluorescent group is very common, but it will certainly change the kinetic properties of the enzyme. This can be easily understood by comparing the enzyme affinity towards different (but similar) substrates. Hexokinases (HK), for instance, are found in several organisms and catalyze the phosphorylation of different hexoses, such as glucose and fructose. Despite their great structural similarity, the K_m for glucose and fructose can be quite different [41–43], and the affinity for glucose can be in the order of 100-fold higher than that for sucrose as observed for the plant HK [43]. If such significant differences are found for very similar substrates, one can only expect that the K_m for labeled substrates by incorporation of large probes will not reflect the affinity of the enzyme for the unmodified substrate but for the probe.

Calorimetry allows the accurate determination of kinetic parameters of enzyme-catalyzed reactions, as seen by the NAD^+-linked reaction of glucose-6-phosphate dehydrogenase (G6PD) from *Leuconostoc mesenteroides*, where the K_m values found in our group by ITC for glucose-6-phosphate (78±7 μM) and NAD^+ (89±10 μM) are very close to those found by Cosgrove et al. [44], i.e., 69±9 μM and 109±18 μM, respectively. The determination of K_m can be done in one

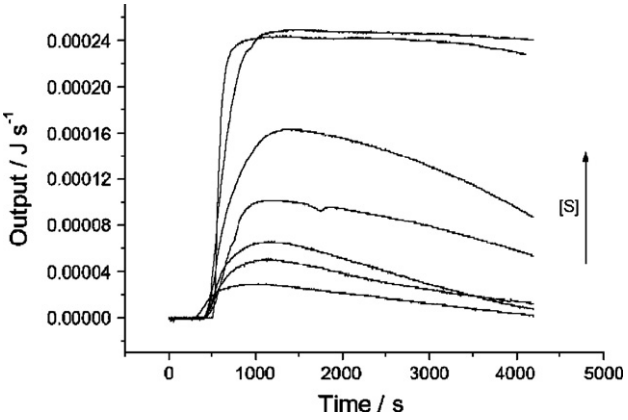

Fig. 3. Calorimetric outputs for first-order, mixed order and zero-order urea/urease enzymatic reactions. The substrate concentration varied from 0.02 to 0.4 mM as indicated by the arrow. The experiments were performed using an LKB 10700-1 flow operated at 25 °C and a flow rate of 7.17×10^{-6} $dm^3 s^{-1}$. Reprinted from Thermochimica Acta 417, M.A.A. O'Neill, A.E. Beezer, J.C. Mitchell, J. Orchard and J.A. Connor, K Determination of Michaelis–Menten parameters obtained from isothermal flow calorimetric data, pages 187–192, copyright (2004), with permission from Elsevier.

single experiment where several injections of substrate into the sample cell containing the enzyme [22,26] or in a set of separated experiments with determination of steady-state kinetics at variable substrate concentration [25].

In coupled assays, one needs to ensure that the studied reaction is not being affected by the substrates of the second enzyme or vice-versa. Again, hexokinase will be used as example as the HK reaction is studied by the coupled assay with G6PD. However, G6PD can be inhibited by glucose [45] and ATP [46], both substrates for HK reaction.

Another advantage of studying enzyme kinetics by calorimetry is the knowledge of the precise temperature of the reaction, allowing the accurate determination of thermodynamic parameters of activation [23,24].

In short, the study of enzyme kinetics by calorimetry can be helpful not only in the direct determination of the kinetic parameters but also in the determination of thermodynamic parameters of the reaction. This can be very useful to give insights in the reaction mechanisms of enzyme-catalyzed reactions.

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