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Thermochimica Acta 253 (1995) 275–283

thermochimica acta

Application of a reduced-extent method to thermokinetic studies of enzyme-catalyzed reactions ☆

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Received 22 August 1994; accepted 23 August 1994

Abstract

The relationship between the rate of enthalpy change of a chemical reaction and the formation rates of its constituents is derived, and then transformation equations for the thermokinetics of single-substrate enzyme-catalyzed reactions are deduced, and the validity and prerequisite of application of the chemical amplification of enthalpy changes to thermokinetic investigations are proved.

A reduced-extent method is applied to thermokinetic studies of enzyme-catalyzed reactions, and then a reduced-extent equation for the kinetics and a mathematical model for the thermokinetics of the single-substrate enzyme-catalyzed reaction are suggested. According to this model, the kinetic parameters and molar enthalpy can be calculated simultaneously with the data from a single thermogram. Three enzymatic reaction systems have been investigated with a heat conduction calorimeter, and the thermokinetic research method in this paper is discussed in detail.

Keywords: Calorimetry; Conduction calorimetry; Kinetics; Reduced-extent method; Thermokinetics

1. Introduction

It is significant in both theory and practice to investigate enzyme-catalyzed reactions. For any enzymatic reaction, heat is generated more or less as the reaction

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☆ Presented at the International and III Sino-Japanese Symposium on Thermal Measurements, Xi'an, 4–6 June 1994.

SSDI 0040-6031(94)02080-9

proceeds, so calorimetry is a realistic approach in the study of enzymatic reactions. There is no constraint or interference on the reactants, products or media of the reacting systems using this approach, and a continuously recorded curve can give lots of information on both the kinetics and thermochemistry of an investigated reaction.

In this paper the relationship between the rate of enthalpy change of a chemical reaction and the formation rates of its constituents is derived, and then transformation equations for the thermokinetics of single-substrate enzyme-catalyzed reactions are deduced, and the validity and prerequisite of application of chemical amplification of enthalpy changes to thermokinetic investigations are proved. The reduced-extent method, which was proposed in a previous paper [1], is applied to analyze thermograms of enzyme-catalyzed reactions. According to the Michaelis–Menten equation, the reduced-extent equation for kinetics and the mathematical model for thermokinetics of single-substrate enzyme-catalyzed reactions are suggested. Three enzymatic reactions have been investigated with a conduction calorimeter. The experimental results and the characteristics of the thermokinetic research method described here are discussed in detail.

2. Theory and method

2.1. Relationship between the rate of enthalpy change of a chemical reaction and the formation rates of its constituents

For a homogeneous closed system within a volume V in which a chemical reaction is taking place, the reaction is described by a set of M stoichiometric equations involving N constituents ($A_1, A_2, \ldots, A_N$) assumed to be present in the reacting mixture. These equations will be written in the form [2]

$$0 = \sum_{n=1}^{N} (v_{mn} A_n) \qquad m = 1, 2, \ldots, M \tag{1}$$

where A_n is nth component and v_{mn} is the stoichiometric coefficient of A_n in the mth stoichiometric equation, which is conventionally positive for a product, zero for an inert substance and negative for a reactant.

At constant temperature and pressure, the rate of enthalpy change of the reaction Ω can be written as

$$\Omega = V \sum_{m=1}^{M} (r_m \Delta H_m) \tag{2}$$

where r_m is the rate of the mth elementary reaction and ΔH_m is its molar enthalpy expressed as a function of the molar formation enthalpies h_n of the constituents A_n, using the relationship

$$\Delta H_m = \sum_{n=1}^{N} (v_{mn} h_n) \tag{3}$$

and so we have

$$\Omega = V \sum_{m=1}^{M} \left[r_m \sum_{n=1}^{N} (v_{mn} h_n) \right] \tag{4}$$

Eq. (4) is equivalent to

$$\Omega = V \sum_{n=1}^{N} \left[\left(\sum_{m=1}^{M} v_{mn} r_m \right) h_n \right] \tag{5}$$

We define

$$R_n = \sum_{m=1}^{M} (v_{mn} r_m) \tag{6}$$

where R_n is called the algebraic rate or total rate of production of the constituent A_n.

Inserting Eq. (6) into Eq. (5), we obtain

$$\Omega = V \sum_{n=1}^{N} (R_n h_n) \tag{7}$$

From Eq. (7), the rate of enthalpy change of the reaction Ω is, in essence, equal to the sum of the product of multiplication of the total production rates of the constituents respectively by their molar formation enthalpies.

According to the quasi-stationary state approximation, the total production rates R_n are equal to zero for these very reactive and short-lived intermediates, and so do not appear in the calculation of Ω.

2.2. Transformation equations for thermokinetics of single-substrate enzyme-catalyzed reactions

For a single-substrate enzyme-catalyzed reaction, according to the Michaelis–Menten mechanism [3], the reaction of a substrate S with an enzyme E forms an enzyme–substrate complex E · S, and then decomposes into a product P and the original enzyme E. The molar enthalpy of the reaction is expressed as ΔH

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_2} P + E \tag{8}$$

In accordance with the steady-state approximation proposed by the Michaelis–Menten mechanism, the total production rate of the intermediate complex E · S is equal to zero; thus it is not involved in the calculation of the rate of enthalpy change of the reaction Ω with Eq. (7).

Therefore we have

$$\Omega = VR_S h_S + VR_P h_P = Vr_P(h_P - h_S) = V\Delta H r_P \tag{9}$$

where r_P is the generation rate of product P.

If the rate of the reaction is expressed as the derivative of the reaction extent χ with respect to time t, i.e. $r_P = d\chi/dt$, thus

$$\Omega = V\Delta H(d\chi/dt) \tag{10}$$

Eq. (10) shows that the rate of enthalpy change of a single-substrate enzyme-catalyzed reaction is proportional to the generation rate of product P.

On integrating Eq. (10) with respect to time t, we obtain

$$Q = V\Delta H\chi \tag{11}$$

$$Q_\infty = V\Delta H\chi_\infty \tag{12}$$

where Q and Q_∞ are the heat effects of the reaction up to time t and the total heat effect respectively, with χ_∞ being the final extent of reaction.

From Eqs. (11) and (12) it is easy to deduce that

$$Q/Q_\infty = \chi/\chi_\infty \tag{13}$$

$$\Omega/Q_\infty = (\mathrm{d}\chi/\mathrm{d}t)/\chi_\infty \tag{14}$$

Eqs. (13) and (14) are called transformation equations for the thermokinetics of single-substrate enzyme-catalyzed reactions, which are the basic theoretical equations in the study of the thermokinetics of enzymatic reactions.

If an enzymatic reaction does not conform to the steady-state assumption, these equations could not be derived. In fact, modern physical techniques have indicated that the production rates of the intermediate complexes of a great many enzymatic reactions are equal to zero.

2.3. Theoretical basis for the chemical amplification of enthalpy changes

In thermochemistry, some reacting systems, especially biochemical systems, involve small heat effects. For one of these reactions, called the primary reaction, one or more secondary reactions (concurrent reactions) can be used to increase the signal and, thus, improve the sensitivity of the calorimetric determination. The procedure may be called chemical amplification of enthalpy changes [4]. In some earlier work, it was used in studies of thermostatics, and later, of thermokinetics.

It is required that only one of the products of the primary reaction is involved in the secondary reaction, that it is consumed stoichiometrically and instantaneously, and that a much greater heat is liberated. Let us consider the following example

primary reaction

$$\mathrm{A} + \mathrm{B} \xrightarrow[\Delta H_1]{r_1} \mathrm{C} + \mathrm{D} \tag{15}$$

secondary reaction

$$\mathrm{F} + \mathrm{C} \xrightarrow[\Delta H_2]{r_2} \mathrm{G} \tag{16}$$

Because the product C of the primary reaction can be consumed stoichiometrically and instantaneously by reacting with F, the secondary reaction will be linked stoichiometrically and kinetically to the primary reaction, thus it will be comparable with the extent and rate of the primary reaction. Therefore, $r_2 = r_1$ and the total

formation rate of substituent C in the reaction system, R_C, is equal to zero. According to Eq. (7)

$$\Omega = (R_A h_A + R_B h_B + R_D h_D + R_F h_F + R_G h_G)V$$
$$= (-r_1 h_A - r_1 h_B + r_1 h_D - r_2 h_F + r_2 h_G)V$$
$$= r_1 V(-h_A - h_B + h_C + h_D) - r_1 V(-h_C - h_F + h_G)$$

and consequently

$$\Omega = (\Delta H_1 + \Delta H_2) V r_1 \tag{17}$$

From Eq. (17), secondary reactions have no influence on the calorimetric determination of the rate of the primary reaction; only the proportional coefficient of the rate of heat generation to the reaction rate is increased. The calorimetric signals of an enthalpy change process are amplified, and then the sensitivity of the procedure is improved. Eq. (17) is the theoretical basis of the chemical amplification of enthalpy changes.

2.4. Reduced-extent equation for kinetics of single-substrate enzyme-catalyzed reactions

If the initial concentration of substrate S, enzyme E and product P are S_0, E_0 and zero respectively, and the extent of reaction at time t and the final extents of reaction are χ and χ_∞ respectively, according to the Michaelis–Menten mechanism and steady-state approximation, we can prove that

$$d\chi/dt = (V_m S)/(K_m + S) \tag{18}$$

where $K_m = (k_{-1} + k_2)/k_1$ and $V_m = k_2 E_0$, and K_m and V_m are the Michaelis–Menten constant and maximum reaction rate, respectively.

On integrating Eq. (18), and defining

$$\Phi = \chi/\chi_\infty \tag{19}$$

we can prove that

$$\Phi - (K_m/S_0)\ln(1 - \Phi) = (V_m/S_0)t \tag{20}$$

Eq. (20) is called the reduced-extent equation for kinetics of single-substrate enzyme-catalyzed reactions.

2.5. Mathematical model for thermokinetics of single-substrate enzyme-catalyzed reactions

According to the transformation equation for the thermokinetics of enzyme-catalyzed reactions and the thermogram reconstruction method as given in a previous paper [5], the reduced-extent Φ_i at any time t_i can be calculated from thermogram data, and then we can obtain three sets of data (Φ_1, Φ_2, Φ_3) at fixed time intervals, $\Delta t = t_3 - t_2 = t_2 - t_1$. On the basis of Eq. (20), we can prove that

$$K_m = \frac{(2\Phi_2 - \Phi_1 - \Phi_3)}{2\ln(1-\Phi_2) - \ln(1-\Phi_1) - \ln(1-\Phi_3)} S_0 \quad (21)$$

$$V_m = \frac{K_m}{\Delta t}\left[\frac{(\Phi_3 - \Phi_1)\ln(1-\Phi_2) - (\Phi_3 - \Phi_2)\ln(1-\Phi_1) - (\Phi_2 - \Phi_1)\ln(1-\Phi_3)}{(2\Phi_2 - \Phi_1 - \Phi_3)}\right] \quad (22)$$

If the reaction is undertaken in a heat conduction calorimeter, its molar enthalpy ΔH can be determined from the total area under the measured curve A according to the formula

$$\Delta H = (KA)/(VS_0) \quad (23)$$

where K is the heat-loss constant of the calorimeter.

Eqs. (21), (22) and (23) are called the mathematical model for the thermokinetics of single-substrate enzyme-catalyzed reactions.

3. Experimental

3.1. Reaction systems

In order to test the validity of application of the reduced-extent method to thermokinetic studies of enzyme-catalyzed reactions and the correctness of the mathematical model for thermokinetics in this paper, the thermokinetics of the three enzymatic reactions, as shown below, have been studied:

(1) Hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (ATEE) catalyzed by α-chymotrypsin in pH 7.8 Tris buffer at 25.0°C.

(2) Hydrolysis of isopropyl hippurate (IPH) catalyzed by α-chymotrypsin in pH 7.0 Tris buffer at 25.0°C.

(3) Hydrolysis of L-arginine catalyzed by L-arginase in pH 9.5 glycine–NaOH buffer at 25.0°C.

3.2. Apparatus

The apparatus for measuring the thermograms of enzymatic reactions is a twin heat conduction calorimeter, which was described in detail in a previous paper [6]. Except for the enzymes, the contents of the reference cell are the same as in the reacting cell, so that the heat effects of dilution, mixing, stirring, and evaporation in the reacting cell can be compensated for by similar heat effects produced in the reference cell.

4. Results and discussion

After a calorimetric curve of a reaction is measured, according to the method reported in a previous paper [5], it can be reconstructed into an ideal adiabatic

caloric curve, and then the reduced-extent Φ_i at any time t_i can be obtained. During the initial period of the reaction, $d\Omega/dt \approx 0$, i.e. the initial part of the reconstructed adiabatic curve presents an oblique line while the reaction is in a zero-order mode and, moreover, the calculating error should be considered. Hence, the data Φ_i for the curve analysis should be extracted in the range from 0.40 to 0.90. The experimental conditions and results are summarized in Tables 1, 2 and 3.

For the hydrolysis of ATEE and IPH, Tris buffer not only provides constant pH, but also amplifies the observed enthalpy change process through its enthalpy of protonation. The enthalpy changes associated with the enzymatic hydrolysis of ATEE or IPH alone are very small. However, the protons produced by these primary reactions can be trapped by Tris buffer with the concurrent evolution of much more heat. According to the literature [4], the enthalpy change accompanying

Table 1
Results of the hydrolysis of ATEE catalyzed by α-chymotrypsin at 25.0°C ($E_0 = 0.1$ mg ml^{-1}, medium, pH 7.8 Tris buffer containing 3% EtOH)

$V(E_0)$/ml	No.	$10^2 S_0$/ (mol l^{-1})	$10^3 K_m$/ (mol l^{-1})	$10^6 V_m$/ (mol l^{-1} s^{-1})	$-\Delta H$/ (kJ mol^{-1})
0.5	1	1.996	1.38	2.42	47.8
	2	1.857	1.51	2.56	48.4
	3	1.857	1.44	2.50	48.5
	4	1.420	1.20	2.75	48.1
	5	1.343	1.21	2.47	48.6
		Average	1.35	2.54	48.3
1	I	1.696	1.04	5.79	48.7
	II	1.557	1.22	5.47	48.5
	III	1.517	1.73	5.28	49.1
	IV	1.311	1.52	5.02	48.1
	V	1.134	1.35	5.02	48.3
		Average	1.37	5.32	48.5
		Literature [7]	1.43		

Table 2
Results of the hydrolysis of IPH catalyzed by α-chymotrypsin at 25.0°C ($E_0 = 0.2$ mg ml^{-1}, $V(E_0) = 2$ ml, medium, pH 7.0 Tris buffer containing 3% EtOH)

No.	$10^2 S_0$/ (mol l^{-1})	$10^3 K_m$/ (mol l^{-1})	$10^5 V_m$/ (mol l^{-1} s^{-1})	$-\Delta H$/ (kJ mol^{-1})
1	1.603	2.15	1.06	49.4
2	1.603	2.62	1.53	49.1
3	1.112	2.07	1.07	48.5
4	0.9845	2.26	1.51	48.9
5	0.9845	2.41	1.53	48.8
	Average	2.30	1.34	48.9
	Literature [8]	2.3		

Table 3
Results of the hydrolysis of L-arginine catalyzed by arginase at 25.0°C ($E_0 = 1.17$ mg ml^{-1}, $V(E_0) = 2$ ml, medium, pH 9.5 glycine–NaOH buffer)

No.	$10^2 S_0$/ (mol l^{-1})	$10^3 K_m$/ (mol l^{-1})	$10^5 V_m$/ (mol l^{-1} s^{-1})	$-\Delta H$/ (kJ mol^{-1})
1	2.057	4.92	1.94	18.2
2	2.656	5.30	1.79	18.0
3	3.613	5.61	1.26	18.3
4	4.086	5.12	1.83	18.1
5	4.086	5.26	1.77	17.9
	Average	5.24	1.72	18.1
	Literature [9]	5.6		

the protonation of Tris buffer is -47.3 kJ mol^{-1}; therefore, the enthalpy changes of hydrolysis of ATEE and IPH alone are -1.1 and -1.6 kJ mol^{-1} respectively.

In enzymatic reactions, the concentrations of the substrates are generally small, and thus the heat effects generated by these reactions are smaller. However, in general, when an enzymatic reaction is undertaken in a constant pH buffer, the protonation or deprotonation of the buffer can proceed stoichiometrically and swiftly with the evolution of great heat; thus it can amplify successfully the enthalpy change process without introducing additional reagents. The so-called chemical amplification of enthalpy change [4] is an effective means of extending the scope of thermokinetic research.

From the reduced-extent equation, Eq. (20), a single-substrate enzyme-catalyzed reaction can be regarded as a mixed-order reaction composed of zero-order and first-order reactions. Under the condition that the initial concentration of substrate S_0 is very much greater or smaller, the reaction can be treated by the reduced-extent method of zero-order or first-order reactions respectively.

On the basis of the mathematical model suggested in this paper, the kinetic parameters (K_m, V_m) and the molar enthalpy (ΔH) of a single-substrate enzyme-catalyzed reaction can be calculated simultaneously using data from a single experiment.

In contrast to the traditional initial rate method, the reduced-extent method is advantageous because it analyzes the total progress curve (not only the initial part) of an enzyme-catalyzed reaction, thus yielding much more information. This method results in a reduction in the number of experiments needed for determination of kinetic parameters, and in addition, while an enzymatic reaction is being investigated by microcalorimetry, a continuous progress curve of the reaction can be obtained; thus this method is especially useful. The correctness of the mathematical model for thermokinetics and the validity of application of the reduced-extent method for thermokinetic studies of enzyme-catalyzed reactions have been verified by the experimental results in this paper.

Certainly, from the prerequisite of the Michaelis–Menten equation, we know that this model is only adaptable for a single-substrate reaction proceeding to

completion. This method can be applied in a similar way to more complex enzyme-catalyzed reactions. Therefore, analyzing the progress curve of enzyme-catalyzed reactions with the reduced-extent method is another very useful way to determine simultaneously its kinetic parameters and thermochemical data.

Acknowledgment

This work was supported by the National Natural Science Foundation of China.

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