Investigation of Ribonuclease-Catalysed Kinetics by a Micro-Calorimetric Method

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A rapid micro-calorimetric method for the simultaneous determination of the Michaelis-Menten parameters and the enthalpy of enzymic reactions is developed. The hydrolysis of 2':3'-cyclic CMP by ribonuclease A is studied to test the proposed method; values obtained are in good agreement with already published data. Enzymic hydrolysis of yeast RNA, unlike that of cyclic phosphates, is shown to be endothermic. This result is explained by the two-step mechanism of this reaction.

Many methods are used to estimate kinetic parameters of enzymic reactions. Thermodynamic parameters, such as the enthalpy of reaction, are less frequently considered. Their evaluation is generally performed by calorimetric measurements (Greengard *et al.*, 1969; Konickova & Wadsö, 1971; Rudolph *et al.*, 1971). Accurate determination of both kinetic and thermodynamic parameters should be possible by using flow micro-calorimetry. This method is very attractive because one type of experiment provides information on the interactions of any particular solute with the enzyme or the substrate and their resulting effect on the enzymic reaction.

Bovine pancreas RNAase A* was used in the micro-calorimetric experiments. This enzyme hydrolyses polynucleotides in a two-step mechanism: first, a transesterification to yield a polynucleotide fragment with a terminal pyrimidine nucleoside 2':3'-cyclic phosphate, and secondly by a hydrolysis of this cyclic ester to a terminal pyrimidine nucleoside 3'-phosphate (Witzel, 1963). This second step has been extensively analysed, with pyrimidine 2':3'-cyclic phosphates as substrates (Westheimer, 1962; Witzel, 1963; Witzel & Barnard, 1962*a,b*). In the present work, the reaction of RNAase with 2':3'-cyclic CMP was studied to substantiate the validity of the micro-calorimetric method proposed. Preliminary results obtained for the hydrolysis of yeast RNA are also reported.

Experimental

Materials

RNAase A (type 1A; 5-times crystallized) and 2':3'-cyclic CMP were obtained as sodium salts from Sigma Chemical Co. (St. Louis, Mo., U.S.A.); yeast

RNA was obtained from Fluka A.G. (Buchs, Switzerland). All the solutions were prepared in acetate buffer at pH 5 and 1.0 mol/l, just before use. Experiments were performed at 25°C.

Micro-calorimetric method

The flow micro-calorimeter, LKB 10007-1, described by Monk & Wadsö (1968), was connected to a Keithley 150B microvolt ammeter (for the experiments reported here, the 10 μ V and 30 μ V scales were used). Electrical calibration was performed after each experiment. The calorimeter signal was recorded by means of a Servogor RE 511 recorder (100 mV range). The solutions were pumped through the reaction cell by two peristaltic pumps (Varioperpex 12000; LKB) at constant flow (V_E kg·s⁻¹ and V_S kg·s⁻¹ for the enzyme and the substrate solutions respectively). Mathematical analysis was performed with a Hewlett-Packard calculator model HP 9820 linked to a plotter.

Each experimental run was started by establishing the instrumental baseline with the buffer solution flowing through the reaction cell. The enzyme and the substrate (C_{1s}^0) solutions were then pumped through the cell for 15 min; then, buffer or another substrate solution of different concentration (C_{2s}^0) was connected to the first substrate flask. In this way the concentration of the substrate solution injected into the reaction cell changed continuously. With this modification of the technique of Monk & Wadsö (1969) more results could be obtained in a rather short time. The substrate concentration (C_s^0) at any time (t in s) after the dilution was started is given by the equation:

$$C_s^0 = (C_{1s}^0 - C_{2s}^0) \exp(-V_s t/m) + C_{2s}^0 \quad (1)$$

where m is the weight (in g), at zero time, of the initial substrate solution; the concentrations are

* Abbreviation: RNAase A, ribonuclease A.

expressed in mol/kg of solution or, when the molecular weight is unknown, in g/kg of solution.

To avoid significant effects of the apparatus inertia, the heat must vary slowly during the experimental run, which is obtained by a proper selection of m and $(C_{1s}^0 - C_{2s}^0)$; to check the validity of the heats recorded, any fixed concentration range was always covered by two runs, one with increasing concentrations ($C_{1s}^0 < C_{2s}^0$), the other with decreasing concentrations ($C_{1s}^0 > C_{2s}^0$).

Heats of dilution of the enzyme and the substrate were measured under the same conditions, and subtracted from the total heat to provide the reaction heat.

Results and Discussion

Quantitative analysis

The disappearance of substrate molecules due to the enzymic reaction results in a decreased flow of these molecules through the calorimeter cell during the transition. The heat of reaction (Q_R in $\mu\text{J}\cdot\text{s}^{-1}$) is proportional to that flow modification; Q_R can thus be related to the reaction velocity (v in $\text{mol}\cdot\text{kg}^{-1}\cdot\text{s}^{-1}$) by the equation:

$$Q_R = AV_s t_s v \quad (2)$$

where t_s (in s) is the time needed by the solution to pass through the cell, at a total flow of $(V_E + V_S) \cdot A$, the proportionality factor between Q_R and the substrate flow change ($V_S t_s v$), represents the heat evolved when 1 mol of substrate has disappeared by reaction with the enzyme. A is thus the enthalpy of reaction (ΔH in $\text{J}\cdot\text{mol}^{-1}$).

The part of the injected substrate reacting in the calorimeter cell depends on the flow time t_s and on the concentration ratio C_E/C_S (C_E and C_S are the concentrations of the enzyme and the substrate after mixing of the two reactants in the calorimeter cell). At the limit, when one of these factors gets very large, all the substrate injected will disappear by reaction:

$$\Delta H = \lim_{(C_E t_s / C_S) \rightarrow \infty} \left[\frac{Q_R}{C_S (V_E + V_S)} \right] \quad (3)$$

By using the reaction scheme:

and the Michaelis–Menten equation:

$$v = \frac{k_p C_E C_S}{K_m + C_S} \quad (4)$$

it is possible to calculate k_p and K_m . Expressing v

according to eqn. (2), the Michaelis–Menten equation may be formulated as follows:

$$\frac{\Delta H V_s t_s C_E C_S}{Q_R} = \frac{K_m}{k_p} + \left(\frac{1}{k_p} \right) C_S \quad (5)$$

Values of k_p and K_m are obtained by plotting the left-hand side of eqn. (5) as a function of C_S . However, as C_S is the substrate concentration before the reaction occurs, the use of eqns. (2) and (5) presupposes that the reaction heat is related to the initial velocity, i.e. that the substrate concentration decreases linearly in the reaction cell. This condition will be fulfilled at the lowest flow time, t_s . This assumption represents the most serious limitation to the application of the proposed method; the highest total flow allowed with the micro-calorimetric unit used is about $1.4 \times 10^{-5} \text{ kg}\cdot\text{s}^{-1}$ at 25°C , which corresponds to a minimum flow time of 30 s.

The linear decay of the substrate concentration in the reaction cell can be checked by repeating the experiments at different t_s values. For the reactions analysed in this present work, published data (Crook *et al.*, 1960; Zöllner & Hobon, 1963) indicate that, in the concentration range considered, this assumption is valid during the first 4 min (which is at least 4 times the t_s values used).

Hydrolysis of 2':3'-cyclic CMP

In these experiments, t_s and C_E are respectively 37 s and $1.16 \times 10^{-5} \text{ mol}\cdot\text{kg}^{-1}$; the substrate concentration ranges from 4.5×10^{-3} to $5 \times 10^{-5} \text{ mol}\cdot\text{kg}^{-1}$. A value of $-33.9 \text{ kJ}\cdot\text{mol}^{-1}$ ($-8.1 \pm 0.1 \text{ kcal}\cdot\text{mol}^{-1}$) is obtained for ΔH (eqn. 3), which is in very good agreement with the value proposed by Rudolph *et al.* (1971). Values of k_p and K_m , calculated from eqn. (5), are respectively 0.96 s^{-1} and $4.6 \times 10^{-4} \text{ mol}\cdot\text{kg}^{-1}$, with a precision of 5–6%. The same reaction followed spectrophotometrically (at 27°C , pH 5.8 and 0.2 ionic strength) leads to values of 2 s^{-1} and $4 \times 10^{-4} \text{ M}$ respectively for the same parameters (Witzel & Barnard, 1962a). The small variation of these values can be accounted for by the differences in the experimental conditions (Kalnitsky *et al.*, 1959; Westheimer, 1962; Witzel, 1963).

The results presented show thus the perfect adequacy of the micro-calorimetric method for following an enzymic reaction.

Hydrolysis of RNA

To study this reaction, experiments were performed at two t_s values, 61 s and 43 s, with C_E respectively 3.19×10^{-7} and $3.29 \times 10^{-7} \text{ mol}\cdot\text{kg}^{-1}$, and C_S ranging from 4.1 to $0.2 \text{ g}\cdot\text{kg}^{-1}$. In an additional run, the substrate concentration was modified discontinuously from 8.74 to $0.04 \text{ g}\cdot\text{kg}^{-1}$ (t_s and C_E were respectively

43 s and $3.03 \times 10^{-7} \text{ mol} \cdot \text{kg}^{-1}$); the molecular weight of the substrate being unknown, C_s is expressed in $\text{g} \cdot \text{kg}^{-1}$.

The calculated enthalpy of reaction is $35.1 \text{ J} \cdot \text{g}^{-1}$ ($8.4 \pm 0.1 \text{ cal} \cdot \text{g}^{-1}$); the parameters k_p and K_m calculated from eqn. (5) are $2.2 \times 10^4 \text{ g} \cdot \text{s}^{-1} \cdot \text{mol}^{-1}$ and $0.28 \text{ g} \cdot \text{kg}^{-1}$ respectively.

The hydrolysis of RNA occurs in a two-step mechanism (Witzel, 1963); when this reaction is followed spectrophotometrically, one observes at first a decrease in the absorption in the course of the transesterification step, and later, with the hydrolysis of the cyclic ester, an increase to the original value. The same trend was observed with dinucleoside (3':5')-phosphates (Witzel & Barnard, 1962b). The time t_s being very short in comparison with the time elapsed before the minimum in spectral absorbance is observed (about 2 h in the same concentration range), one may consider that the heats recorded correspond chiefly to the first step of the hydrolysis mechanism.

Published data on the reaction of dinucleoside (2':3')-phosphates (Witzel, 1963; Witzel & Barnard, 1962b) show that K_m does not depend on the nature of the second nucleoside, and its value is about the same as that for cyclic esters; on the other hand, k_p seems to be strongly affected by the second nucleoside. Assuming a K_m value of $4.6 \times 10^{-4} \text{ mol} \cdot \text{kg}^{-1}$ (obtained for 2':3'-cyclic CMP) for the first step of RNA hydrolysis, it is found that 1 g of this substrate contains 1.65×10^{-3} dinucleoside (3':5')-phosphate bonds hydrolysed by RNAase; this leads to an average k_p value of 36.5 s^{-1} and a ΔH of 21.3 kJ (5.1 kcal) per mol of the bond. This positive enthalpy of reaction stands in contrast with the negative value found for the cyclic ester. On hydrolysis of RNA to polynucleotide fragments, more nucleosides are exposed to the solvent, and the base-stacking possibilities decrease. This results in a positive contribution to the reaction enthalpy measured (De Voe, 1969). Another positive contribution arises from a ring strain in cyclic phosphates (Cox *et al.*, 1959),

resulting in an energy difference of $29\text{--}38 \text{ kJ} \cdot \text{mol}^{-1}$ ($7\text{--}9 \text{ kcal} \cdot \text{mol}^{-1}$) between a cyclic ester and its open-chain analogues. Experiments performed at different temperatures should supply important information about the entropic contribution to the free energy of each step in the RNA hydrolysis mechanism.

It is thus shown in this present work how thermodynamic and kinetic parameters can be obtained by flow micro-calorimetric measurements. An important argument in favour of this technique is that the recorded heats are not related to a particular property of one of the reagents; they are a direct consequence of the chemical process occurring in the reaction cell. Owing to its high sensitivity and reproducibility, this method can promote the elucidation of many important problems concerning enzymic kinetics.

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