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BINDING STUDIES ON 3'-CYTIDIN MONOPHOSPHATE AND RIBONUCLEASE A DERIVATIVES BY FLOW AND BATCH CALORIMETRY *

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SUMMARY

Isothermal batch calorimetry can give useful information on the biological activity of enzymes immobilized on solid supports. As a model system, the enthalpy change and the apparent equilibrium constant of the cytidine-3'-monophosphate binding to soluble ribonuclease A were evaluated with the batch and flow calorimeters and the results compared. The batch technique was then employed to study the same thermodynamic parameters with ribonuclease immobilized on Silica beads. Reproducible results were obtained after correction of the thermograms for the instrumental time response delay.

INTRODUCTION

Since immobilized enzymes became an important tool in biotechnology as biocatalysts (ref.1), information about the thermodynamic properties of the protein attached to the support are essential for their practical application at industrial level. Among the techniques used, isothermal calorimetry provides a direct, non-destructive method of gaining an insight about the thermodynamic quantities that are related to the enzyme biological activity, such as ligand binding or protein-protein interaction (ref.2). Therefore, since it does not need a transparent sample and it can be applied to suspensions, emulsions or even to heterophasic systems, it is a convenient technique for studying the change of the enzyme properties after immobilization on insoluble supports. In this case, isothermal batch calorimetry has to be employed because the samples to be analysed are often solid and difficulties may arise when the results are compared with those obtained with the flow isothermal technique, used for the free soluble enzymes.

In this report we studied the binding of the inhibitor cytidine-3'-monophosphate (3'CMP) to the soluble Ribonuclease A

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(RNase) with the flow and batch techniques. Since the results obtained with both methods agreed satisfactorily, we then extended the batch titration method to the immobilized enzyme. In order to mimic the chemical effect of the covalent attachment to the support, the soluble RNase was also chemically modified with valeraldehyde and its activity analysed in the same way. The enthalpy change of the binding reaction was calculated for the three RNase forms. In order to estimate the apparent association constant, the thermograms obtained from the batch titration experiments were mathematically corrected for the instrumental time response delay.

MATERIALS AND METHODS

Ribonuclease A (RNase) was purchased from Sigma Chemical Co. and used without further purification. After dialysis against 0.1 M acetate buffer, pH 5.0, the protein concentration of the solution was determined by uv absorption at 278 nm (ϵ = 9,800 $M^{-1}cm^{-1}$) on a Cary 219 spectrophotometer.

RNase modified (VA-RNase) with valeraldehyde (Fluka) was prepared by mixing the aldehyde solution (final concentration 10% v/v) with the enzyme solution (10 mg/ml), 0.1 M phosphate buffer, pH 7.0, at 20 °C. After 8 hours, the protein was dialysed (3 times x 3 hours) against the appropiate buffer in order to remove the excess aldehyde. The protein concentration was determined by Lowry-Folin method or by uv spectrophotometry, assuming the same extinction coefficient of the native RNase.

CPC-Silica (500 Å pore size, 30-45 mesh) was purchased from Fluka, hydrated extensively and washed with 0.1 M phosphate buffer, pH 7.0. The beads (5 g) were activated with glutaraldehyde (Fluka), 10% v/v for 30 min at 4 °C. The excess glutaralhyde was removed by washing the solid phase with the same buffer. The beads were gently agitated in the presence of RNase solution in 0.1 M phosphate buffer, pH 7.0 (8 mg/ml), for 8 hours at 20 °C. An estimation of the bound protein was obtained by determining the difference between the initial concentration of the added enzyme and the concentration of the unbound enzyme after 8 hours as measured by the absorbance changes of the supernatant and washing buffer. The concentration of the bound

protein per gram of CPC-silica (20 mg/g of dry beads) was confirmed by determining the amino acid composition, after hydrolysis at 110 °C (ref. 3).

3'Cytidine monophosphate (3'CMP) (Sigma Chemical Co.) (99% pure) was dissolved in 0.05 M acetate buffer, pH 5.0 and its concentration was estimated by its molar extinction coefficient (ϵ_{260} = 7,600 $M^{-1}cm^{-1}$, pH 7.0).

Flow calorimetry

Isothermal calorimetric titration of the reaction between 3'CMP and RNase or VA-RNase was carried out in a Bio Activity Monitor (BAM)(Thermometric AB, Jarfalla, S) using the flow system device at 25 °C, 0.05 M acetate buffer, pH 5.0. Solutions were pumped in the instrument by two twin LKB Microperpex peristaltic pumps at a flow rate of 3.5 μl/s and the corresponding power evolved was amplified and recorded. Analysis of the data were performed according the procedure reported by Bolen et al. (ref. 4). Power effects ranged from 0 to 40 μW.

Batch calorimetry

(i) RNase in solution. Batch calorimetric experiments were carried out in a BAM calorimeter using a twin titration cell. 3.9 x 10^{-4} M RNase samples (1 ml) in 0.05 M acetate buffer solution, pH 5.0, were thermally equilibrated in the instrument under a constant slow stirring (base line). After the addition of 1.5 mM 3'CMP solution by a LKB peristaltic pump (5 - 1.2 x 10^{-6} ml/s), through one of the inlet titration tubes, the evolved power was recorded. The pumping was prolonged for enough time in order to reach an inhibitor concentration largely in excess (5 times) with respect to the enzyme molarity assuming a 1:1 stoichiometry. Negligible power effects were observed during the dilution of RNase solution by pumping buffer in the titration cell or during dilution of inhibitor in buffer using the calorimetric amplification full scale of 100 μW. Powers were collected every 12 s.

(ii) RNase in eterophase. A fixed amount of CPC-silica / immobilized enzyme complex (0.23 g) supporting 5 mg of RNase and buffer (1 ml, 0.05 M acetate buffer, pH 5.0) were placed in the

titration cell at 25 °C. The agitation was started only after thermal equilibration of the instrument in order to avoid the grinding of silica beads. Furthermore the pales of the propeller were modified to stir the sediment in the cell from bottom to up. Such a modification was necessary in order to reach a closer fit between the pales and the round-edge contour of the reaction vessel bottom. Powers connected with stirring and dilution of reactants were neglibibles or subtracted from the total heat effect.

RESULTS

Fig. 1 shows the results obtained by flow calorimetry after mixing different concentrations of 3'CMP with a fixed amount of soluble RNase in the flow calorimeter at 25 °C. Curve a) refers to the native RNase and curve b) to VA-RNase.

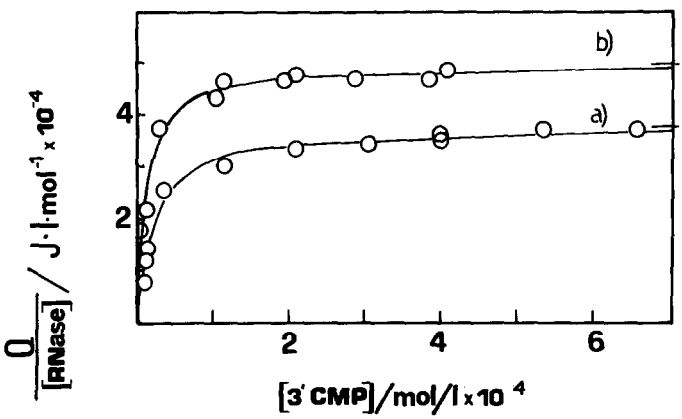

Fig. 1. Thermal titration of native (a) and chemically modified RNase (b) with 3'CMP by flow calorimetry in 0.05 M acetate buffer pH 5. at 25 °C. The solid lines represent the calculated binding curves obtained by least square treatment according to eqn.(1).

The enthalpy changes, ΔH, (Qmax/[RNase]) and of the apparent association equilibrium constant, Ka, can be calculated by an iterative least square treatment of the following equation (ref.4):

$$\frac{1}{Q_{exp}} = \frac{1}{\Delta H} + \frac{1}{\Delta H \; Ka} \cdot \frac{1}{[3'CMP]_f} \qquad (1)$$

where $[3'CMP]_f$ is the free inhibitor concentration and Q the molar heat of reaction. The double reciprocal plot of the two sets of data according to eqn. (1) is reported in fig. 2 .

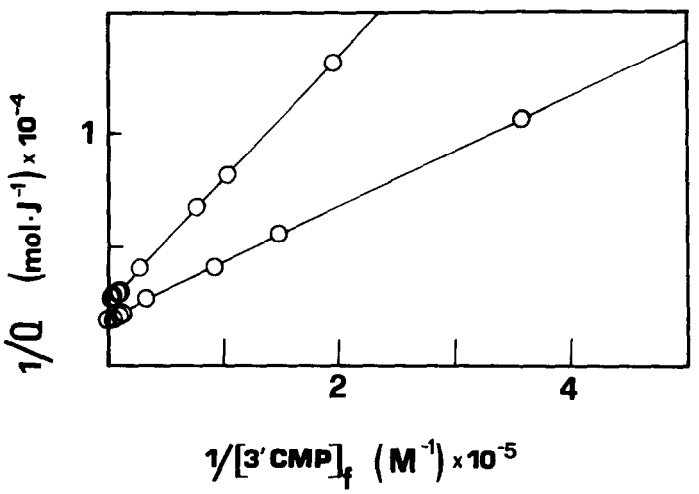

Fig. 2. Double reciprocal plot of data in Fig.1 according to eqn.(1).

The same binding reaction can be also studied with a batch calorimeter. To a preparation of immobilized RNase of known concentration, a solution of 3'CMP has been added. The total area of the power-time curve as a function of time, after subtraction of the baseline, represents the total heat of the reaction when the concentration of the ligand is stoichiometrically in large excess with respect to that of the protein binding sites. In order to calculate the apparent association equilibrium constant, Ka, of the binding reaction, it is necessary to make a correction of the thermogram because of the shape distortions (Tian effect) of the curve due to the time response delay of the instrument. The deconvolution analysis was carried out as suggested by Calvet et al. (ref. 5). The time constant of the calorimeter, τ = 192 ±5 sec^{-1} was determined by direct differentiation of the thermogram following the procedure reported by Randzio and Suurkuusk (ref.

6). The algorithm used for the calculation was developped by Kirchhoff (NBS, Gathersburg, MD, USA).
Only one time constant was necessary to obtain a precise correction of the data since the areas underneath the actual and corrected curve were equal.

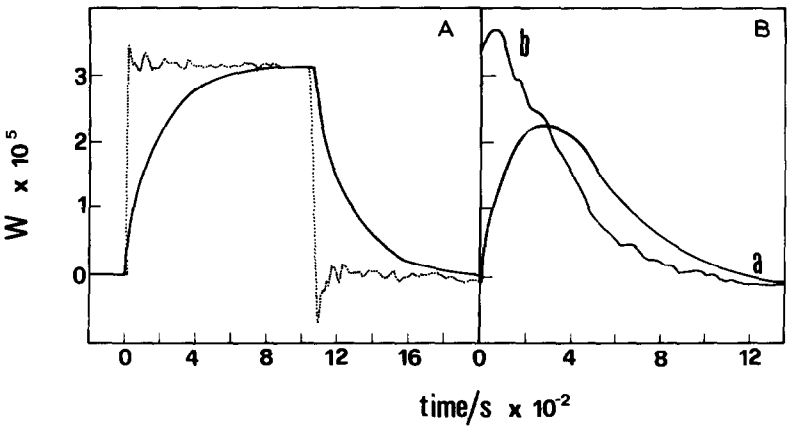

Fig. 3. A) Correction of a power-time curve obtained by electrical calibration of the batch calorimeter according to ref. 6. B) Power-time curve recorded after pumping a 1.5 mM 3'CMP solution at a flow rate of 1.2 μl/s in a titration cell of the calorimeter containing 1 ml of 38.6 μM native RNase. (curve a) After correction for the Tian effect, the curve a) is transformed in to curve b).

The measured and corrected calibration curves as well as the experimental and corrected binding thermograms are shown in fig. 3A and 3B respectively. As it can be seen, the corrections are significant, indicating that the rate of the heat evolution of the reaction is fast with respect to the time response of the instrument. By integration of the corrected curve of the power (joules/sec) evolved as a function of time, the heat of reaction (joules) as a function of the free 3'CMP concentration is calculated (fig. 4). The ligand concentration was calculated by taking in to account the 3'CMP concentration of the solution pumped into the calorimeter vessel, the flow rate constant of the pump and the continuous dilution of the ligand inside the vessel. The solid line in fig. 4 is a theoretical curve calculated assuming a molar enthalpy change of the reaction, ΔH, of 38,700 J/mol and an apparent equilibrium constant of 50,500 mol^{-1}.

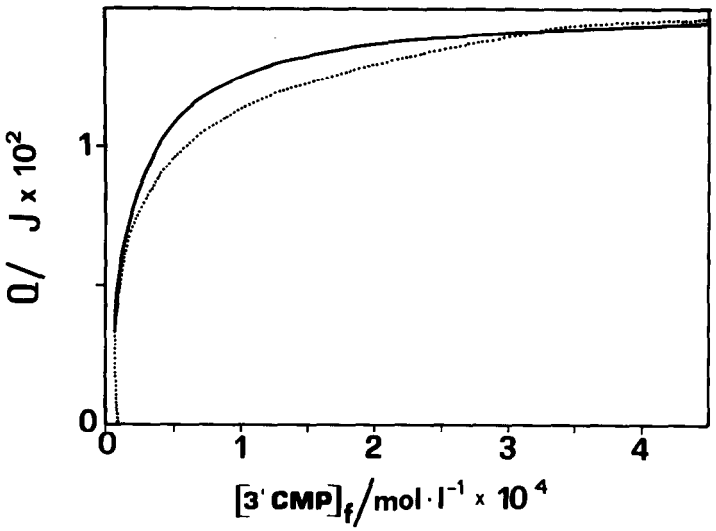

Fig. 4 Thermal titration of native RNase with 3'CMP by batch calorimetry. The curve (····) was obtained from experiment reported in fig. 3 B). The solid line represents the theoretical curve for the same experiment assuming a ΔH of binding of 38,700 J/mol and a Ka of 50,500 mol^{-1} which are the best approximations calculated from eqn. (1).

The same procedure was used to calculate the enthalpy change and Ka for VA-RNase with the batch technique. An experimental binding isotherm is shown in fig. 5. The binding isotherm of the reaction between 3'CMP and RNase immobilized on silica beads, and calculated as described above, is shown in fig 6. Although the curve appears somehow more 'noisy', a good reproducibility has been obtained among several enzymatic preparations. The fitting of the experimental curve is not accurate at the beginning of the titration curve. This may be due to the presence of either a non-specific interaction of the ligand with the support or some limited diffusion heat effects. However, the uncertainty on the total heat effect due to this initial perturbation is small.

The thermodynamic parameters associated with the binding reaction for all the RNase forms are listed in table 1.

TABLE 1

Enthalpy changes and apparent binding constants associated with the reaction 3'CMP and Ribonuclease A derivatives.

	ΔH kJ/mol	Ka 1/mol x 10^3
flow		
RNase	37.4 ±0.3	50.6 ±0.6
RNase-VA	49.5 ±0.3	85.0 ±0.4
batch		
RNase	38.7 ±0.5	50.5 ±1
RNase-VA	47.4 ±0.6	85.0 ±5
RNase-CPC-silica	35.6 ±1	76.0 ±5

Buffer: 0.05 acetate, pH 5.0, 25 °C.

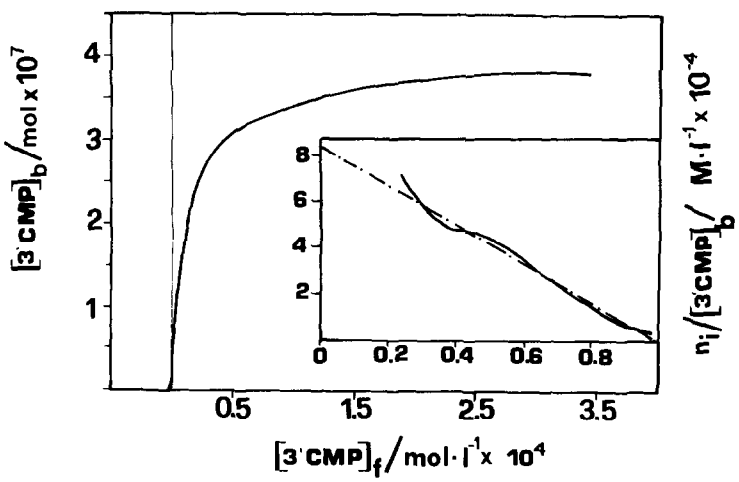

Fig. 5. Titration curve of RNase-VA with 3'CMP calculated with the same procedure reported for native RNase (titration cell), The inset shows the Schatchard plot of the experimental curve.

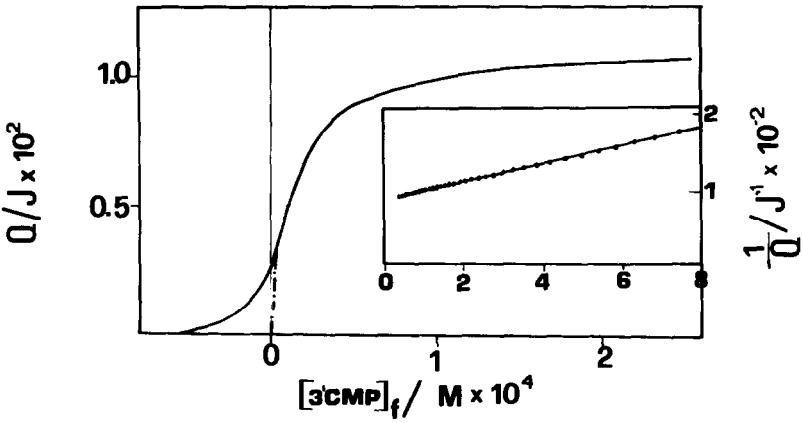

Fig. 6. Titration curve of immobilized RNase on CPC silica with 3'CMP by batch calorimetry. The inset shows the double reciprocal plot of data according to eqn. (1): 1/Q (1/J) x 10^{-2} vs 1/$[3'CMP]_f$ (1/mol) x 10^{-4}.

DISCUSSION

In order to gain correct thermodynamic information (ΔH, Ka) from the data obtained with batch calorimetry, a mathematical treatment of the thermogram appears to be necessary. Although the total heat effect can still be derivated from the experimental curve, this is not the case for Ka. The distortion of the curve due to the instrumental time response delay, related to a fast rate of heat evolution during the reaction, prevents its precise assessment. Such distortions can be taken into account by mathematical differentiation tecniques with a sufficient precision. In the case of the enzyme in soluble form, the agreement between the results obtained with the corrected data from the titration cell and those from the flow technique is satisfactory. This is the basis of the correct application of the thermal analysis to reactions taking place in the heterophasic system, such as enzymes immobilized onto insoluble supports.

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