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Binding curves by continuous gradient flow-mix calorimetry

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Abstract

Steady-state flow-mix calorimetric methods require large amounts of sample and time to obtain data at variable reactant ratios. The use of continuous concentration gradients allows rapid generation of data with chemicals consumption comparable to other calorimetric techniques. An experimental set-up able to generate a linear concentration gradient of one reactant, while keeping the other concentration constant during the experiment, permits a complete titration in a single experiment. Since the calorimeter is not operating under steady-state conditions, the output is affected by the dynamics of the instrument. Therefore, it is necessary to correct the experimental curve by deconvolution to obtain the true signal. Titration of the carboxyl group of glycine with hydrochloric acid, as well as the binding of ribonuclease A to two different nucleotides prove the efficacy of this new methodology.

Keywords: Flow calorimetry; Binding; Thermodynamics; Ribonuclease A; Protein–ligand interactions

1. Introduction

Titration calorimetry can provide a complete description of the thermodynamics of macromolecule–ligand binding through a single experiment. From this experiment, the heat of binding versus ligand concentration is obtained [1]. However, in steady-state flow calorimetry only a single data point on the binding curve is obtained in each experiment corresponding to one ligand concentration and, since several different concentrations are needed to obtain the complete binding curve, the amount of sample and time required are much higher than with titration calorimeters (ITC) [2].

This work describes a new methodology employing a flow-mix calorimeter in a continuous mode that obtains a complete binding curve with similar sample and time requirements as for actual titration calorimeters.

2. Materials and methods

2.1. Chemicals and sample preparation

Bovine pancreatic ribonuclease A (RnaseA) was purchased from Sigma. The lyophilised powder was dissolved in the experimental buffer and extensively dialysed against the buffer for at least 24 h, using Spectra/por dialysis tubing of 3.5 kDa cut-off. The nucleotides cytidine-2′-monophosphate (2′CMP) and cytidine-3′-monophosphate (3′CMP) were also purchased from Sigma. Their solutions were prepared by dissolving the powder in the corresponding dialysis buffer. All the solutions were filtered immediately before the experiment. The sample concentration of the samples was measured spectrophotometrically after filtration through a 0.22-μm filter (Millipore), using molar extinction coefficients of 9800 cm^{-1} M^{-1} at 278 nm for RnaseA [3], 7400 cm^{-1} M^{-1} at 260 nm for 2′CMP [4] and 7600 cm^{-1} M^{-1} for 3′CMP [5].

Hydrochloric acid (HCl), sodium hydroxide (NaOH) and glycine were purchased from Merck. Glycine and NaOH

Abbreviations: 2′CMP, cytidine-2′-monophosphate; 3′CMP, cytidine-3′-monophosphate; HCl, hydrochloric acid; ITC, isothermal titration calorimetry; NaOH, sodium hydroxide; RnaseA, bovine pancreatic ribonuclease A

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solutions were prepared by weighing the solid compound and dissolving in double-distilled water. HCl solutions, prepared by dilution of the concentrated acid, were standardized with calcium carbonate [6].

2.2. Experimental arrangement of the flow calorimeter

Calorimetry experiments were carried out with an LKB 2277 Bio Activity Monitor equipped with a flow-mix unit. Both input flow lines, driven by a pump with two channels, were connected to two electro-valves that allow switching between buffer or ligand and buffer or macromolecule. The electronic signal of the calorimeter was recorded with a sampling period of 4 s.

To generate the gradient concentration of ligand an appropriate sequence of switching pulses was programmed. In all the experiments, the solutions were introduced into the calorimeter cell at a constant and equal flow from both lines. The reaction was initiated by switching both valves from buffer to macromolecule or ligand solution, which can be done at the same time or sequentially in order to record the dilution heat in a single experiment and to minimize the consumption of reactants. A linear gradient of ligand concentration is achieved by switching the electro-valve between the ligand solution and the buffer with duration pulses being increased lineally.

2.3. Instrument characterization

Stability from a baseline recorded during 10 h under continuous flow of water gave a drift smaller than $1\,\mu W\,h^{-1}$. Three times the standard deviation of the noise gave a minimum detectable power of 0.3 μW. Applying several power pulses of 30 μW to the sample cell, and chemically by titration of NaOH solutions with HCl at appropriate concentrations to produce the corresponding power steps, both experiments reproduced 10 times in identical conditions gave a standard deviation of 1%. Calibration of the instrument with electrical and chemical pulses of different magnitude under continuous flow gave:

$$W_{\text{exp}}\,(\mu W) = (-0.42 \pm 0.78) + (1.02 \pm 0.01) W_{\text{theor}} \qquad (1)$$

From the slope is obtained the calibration constant of the instrument, *a*.

All the data analysis and figures of this manuscript were done with Microcal Origin 5.0 (Microcal Inc.) software.

2.4. Experimental design of the gradient flow calorimeter

The experimental set-up of the flow calorimeter to work in gradient mode was obtained by substitution of a manual valve by an electro-valve connected to a digital output of the data acquisition card DT-2801 (Data Translation Co.). This valve is used as a concentration-gradient generator and the computer controls its switch position. One experiment can be made using as inputs only three parameters: initial equilibration time (baseline), gradient time and final equilibration time (baseline). During the gradient time the electro-valve opening–closing period for each reactant (ligand or buffer) is controlled to obtain a linear increasing of the ligand concentration in the cell from zero (ligand line fully closed) to the maximum (ligand line fully opened).

3. Results and discussion

3.1. Dynamic characterization of the system

The use of continuous concentration gradients using isothermal flow-mix calorimetry requires dynamic characterization of the calorimeter since the instrument does not operate under steady-state conditions [2,7–10]. For this reason the reaction heat cannot be directly calculated from the calibration constant determined in Section 2.3. This correction implies a deconvolution of the experimental data by a mathematical representation of the system, i.e. a "transfer function" [11]. For our purpose, a transfer function with two time constants gave a good description of the system. The corrected power, W_{real}, is obtained by a second-order deconvolution of experimental power, W_{exp}, with the equation:

$$W_{\text{real}} = \frac{1}{a}\left[W_{\text{exp}} + (\tau_1 + \tau_2)\frac{\mathrm{d}W_{\text{exp}}}{\mathrm{d}t} + \tau_1\tau_2\frac{\mathrm{d}^2 W_{\text{exp}}}{\mathrm{d}t^2}\right] \qquad (2)$$

where "*a*" is the calibration constant (see Section 2.3.), and τ_1 and τ_2 are the time constants. These constants are obtained by fitting the decay portions of the instrument response to a rectangular electrical power step or by fitting the response peak to a finite width power pulse to the corresponding second-order exponential functions:

$$\begin{aligned} &\text{For power steps :} \\ &W(t) = A + B\,\exp\left(-\frac{t}{\tau_1}\right) + C\,\exp\left(-\frac{t}{\tau_2}\right) \\ &\text{For power pulses :} \\ &W(t) = A\,\exp\left(-\frac{t}{\tau_1}\right) + B\,\exp\left(-\frac{t}{\tau_2}\right) \end{aligned} \qquad (3)$$

The average values obtained from a total of six curves from both types of power inputs are $\langle\tau_1\rangle = 95.3 \pm 0.4$ s and $\langle\tau_2\rangle = 11.0 \pm 0.6$ s. The data in Fig. 1A proves the ability of the second-order dynamic deconvolution to regenerate the real power of an electrical-step pulse from the experimental signal.

When heat is generated in the flow cells from a chemical reaction in solution, a new time response constant, τ_3, must be considered to account for the heat transfer through the solution. Eq. (2) must be rewritten to consider a dynamic

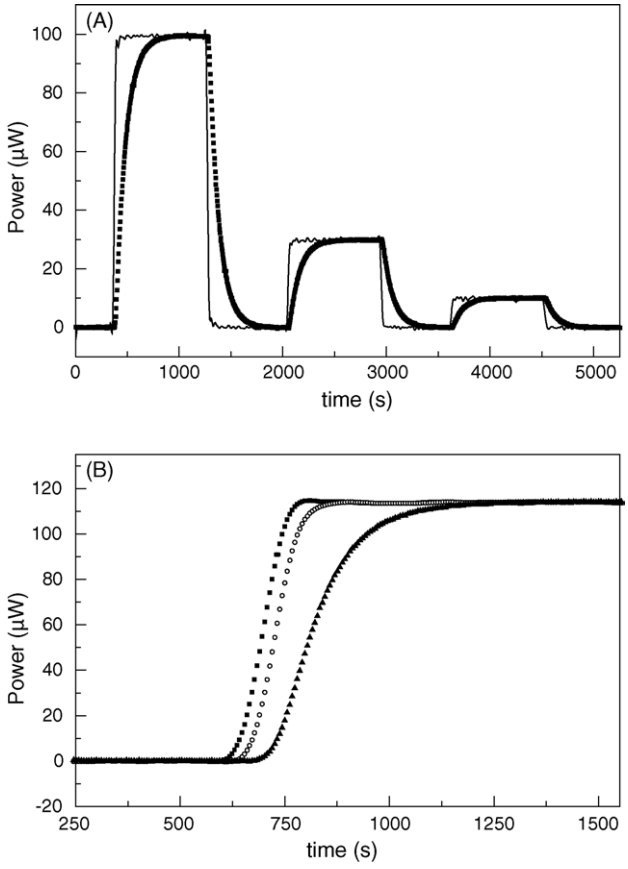

Fig. 1. (A) Application of the second-order dynamic deconvolution (continuous line) of a curve corresponding to three electrical steady-state calibration step pulses of 100, 30 and 10 μW (dots). The deconvolution was performed using Eq. (2), where the values of τ_1 and τ_2 were obtained by fitting the transient part of these curves to Eq. (3). (B) Application of the dynamic deconvolution to a chemical steady-state step generated from the protonation reaction of glycine 100 mM with HCl 12 mM (filled triangles). The second-order dynamic deconvolution is represented by open circles and the third-order dynamic deconvolution by filled squares.

deconvolution of third-order:

$$W_{\text{real}} = \frac{1}{K}\left[W_{\text{exp}} + (\tau_1+\tau_2+\tau_3)\frac{\mathrm{d}W_{\text{exp}}}{\mathrm{d}t} + (\tau_1+\tau_2)(\tau_1+\tau_3)(\tau_2+\tau_3)\frac{\mathrm{d}^2W_{\text{exp}}}{\mathrm{d}t^2} + (\tau_1\tau_2\tau_3)\frac{\mathrm{d}^3W_{\text{exp}}}{\mathrm{d}t^3}\right] \quad (4)$$

The time constant τ_3 was calculated by fitting the transient portion of a chemical step generated mixing aqueous solutions of NaOH and HCl, to a third-order exponential function using the fixed values of τ_1 and τ_2 calculated previously. The average value of τ_3 obtained from eight experiments was $\langle\tau_3\rangle = 33.5 \pm 1.0$. The need for a third time constant is clearly observed in Fig. 1B where second- and third-order deconvolutions over a chemical reaction heat step are compared.

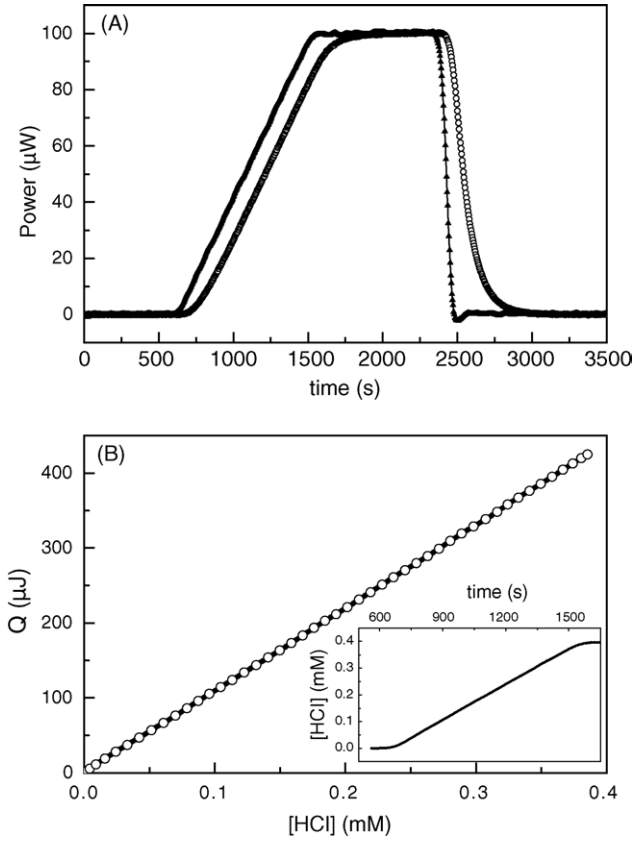

Fig. 2. (A) Curve generated by a linear gradient of HCl (0.6 mM) reacting with a constant flow of NaOH (3.0 mM). The experimental design was: 10 min of NaOH–water baseline → 15 min of HCl gradient → 15 min of NaOH–HCl final concentration → 20 min of NaOH–water baseline. Open symbols show the experimental data and filled symbols represent the dynamically deconvoluted data using the three time constants τ_1, τ_2 and τ_3, according to Eq. (4). (B) The heat versus HCl concentration obtained from the linear gradient titration of HCl/NaOH (open circles). The continuous line represents the linear regression of the data. The inset shows the conversion time–concentration done as described in the text.

3.2. Gradient characterization

The linearity of the gradient was tested with the neutralization of NaOH with HCl. NaOH is kept in excess and at a constant concentration while the continuous gradient concentration is generated with the HCl solution. Fig. 2A shows a typical gradient for this reaction and its dynamic deconvolution applying Eq. (4).

The method of continuous gradients concentration is based on the establishment of two correlations, power–heat and time–concentration (inset of Fig. 2B), to obtain heat versus HCl concentration from the experimental data power versus time (Fig. 2). The conversion of power to heat amount is easily done by taking into account the sampling period (SP):

$$Q\,(\mu\text{J}) = W_{\text{real}}\,(\mu\text{J/s})\,\text{SP}\,(\text{s}) \quad (5)$$

whereas the conversion of time to HCl concentration is done by normalizing the dynamically deconvoluted data with the

averaged W_{max} value obtained from the plateau in Fig. 2A. The real HCl concentration in the cell is obtained by:

$$[HCl]_{CELL} = \frac{\Phi}{\Phi_1 + \Phi_2}[HCl]_0 \quad (6)$$

where $[HCl]_0$ is the concentration of the HCl in the reservoir solution, and Φ_1 and Φ_2 are the flow rates of the peristaltic pumps for HCl and NaOH, respectively. Linear regressions of the gradients showed typically standard deviations below 1% (Fig. 2B).

3.3. Titration of the carboxyl group of glycine with HCl

The ability of the method to characterize reactions with small binding constants was tested with the titration of the carboxyl group of glycine with HCl. The small value of the binding constant requires an increased concentration of HCl to saturate the carboxyl group. This high HCl concentration generates a dilution heat that must be evaluated in a dilution experiment generated with the same gradient parameters as used in the titration experiment. The heat contribution due to HCl dilution is then subtracted from the titration heat after dynamical deconvolution (Fig. 3A).

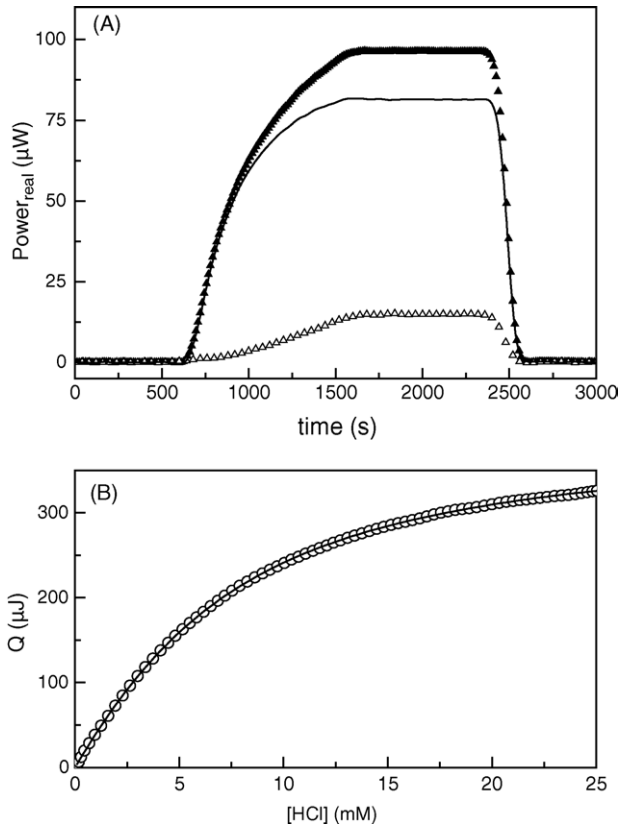

Fig. 3. (A) Protonation of glycine (10.0 mM) with HCl (50.0 mM) by a linear gradient. Filled triangles show the third-order deconvoluted data, and open triangles the profile of HCl dilution heat under the same gradient parameters. The continuous line corresponds to the difference, i.e. the protonation reaction heats. (B) The titration curve, heat versus HCl concentration, obtained from the linear gradient titration of glycine/HCl (open circles). The continuous line represents the nonlinear fit to a model of n identical and independent binding sites.

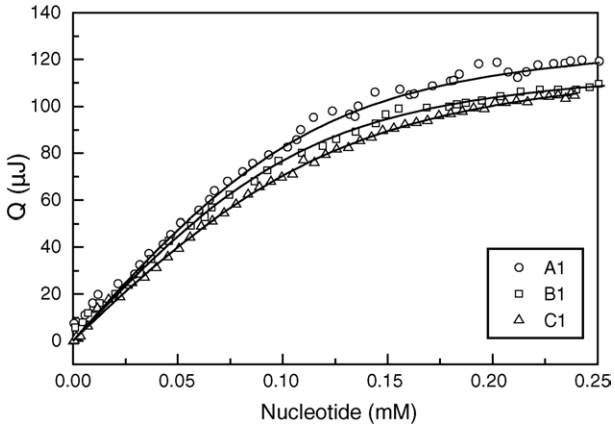

Fig. 4. The titration curve, obtained from the linear gradient titration of Rnase A/nucleotide (open symbols). Open squares (0.214 mM/0.853 mM) and circles (0.208 mM/0.862 mM) show the binding curve of RnaseA/3′CMP corresponding to experiments A1 and B1, respectively, of Table 1, and open triangles (0.223 mM/0.49 mM) the binding curve of RnaseA/2′CMP from experiment C1. Continuous lines represent the nonlinear fit to a model of n identical and independent binding sites.

Fig. 3B shows the plot of the corrected data. These data were fitted to a binding model of n identical and independent sites according to [1].

For 10 experiments, ΔH_B = 4.04–4.09 kJ/mol; K = 196.5–222.3 M^{-1}; n = 0.96–1.1. These values are in good agreement with the reported values of 3.99 kJ/mol, 199.52 M^{-1} and 1 [12].

3.4. Binding of ribonuclease A to 2′CMP and to 3′CMP

To evaluate the efficiency of this method, several experiments were done with the same conditions previously described for ITC experiments [4,5], i.e. 200 mM potassium acetate, 200 mM KCl at pH 5.5 for RnaseA–2′CMP and 50 mM sodium acetate at pH 5.5 for RnaseA–3′CMP at 293.5 K.

Table 1
Experimental results obtained by flow-mix calorimetry on the binding of bovine pancreatic ribonuclease A with 2′CMP and 3′CMP at 293.5 K

	ΔH (kJ/mol)	$K \times 10^{-3}$ (M^{-1})	N
RnaseA–3′CMP[a]			
A1	71.4 ± 1.5	60.4 ± 4.4	1.02 ± 0.02
A2	71.4 ± 2.0	46.6 ± 2.8	1.02 ± 0.02
A3	76.3 ± 4.5	64.3 ± 7.6	1.02 ± 0.04
B1	69.2 ± 2.0	49.4 ± 3.6	0.99 ± 0.02
B2	70.8 ± 4.0	59.7 ± 4.6	0.96 ± 0.05
RnaseA–2′CMP			
C1	57.5 ± 1.3	31.3 ± 2.4	1.00 ± 0.02
C2	57.5 ± 1.5	30.0 ± 2.5	1.00 ± 0.02

[a] The experiments labelled “A” were performed by titrating a constant RnaseA concentration with linearly increasing concentrations of 3′CMP, whereas the experiments labelled “B” are the results of titrating the protein with decreasing concentrations of the ligand.

The binding curves are presented in Fig. 4. In the case of RnaseA–3′CMP binding experiments, different concentrations of protein and nucleotide were used to check the sensitivity of the method. Furthermore, experiments were done with both increasing and decreasing ligand concentration. An example of each type of experiment is shown in Fig. 4 and the results of the fit to an *n* identical and independent binding sites model are summarized in Table 1. These results are in good agreement with previously published data [4,5].

4. Conclusions

Comparison of this method with ITC shows two clear advantages. First, the larger number of data points obtained, i.e. by using a sampling period of 4 s we can obtain 225 data points in a single experiment in 15 min. In a typical titration no more than 30 data points are collected. Second, the time is much shorter. The dynamic analysis avoids the time required for baseline stabilisation after each ligand addition, as happens in steady-state flow experiments and ITC. Binding curves can be obtained with micromoles of RnaseA, an amount comparable to that needed for ITC.

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