



Continuous Isothermal Titration Calorimetry - cITC - a new way to speed up and improve binding experiments

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INTRODUCTION

Microcalorimetric instruments and methods are now essential tools for the general understanding of binding thermodynamics of biological macromolecules and specific biological systems. Provided that the enthalpy of binding is of appropriate magnitude, it is possible to determine affinities, K_D , in the order of 10 nM ($K=10^8 \text{ M}^{-1}$) for 1:1 complexes. One of the most important issues with ITC is the time needed to obtain a full binding isotherm. ITC inherently suffers from the relatively small number of binding experiments that are possible to perform per day. The focus has so far been on improving calorimetric response time of the instruments by various means, like assigning instrumental time constants and applying Tian's equation to dynamically correct the raw calorimetric signal from a fast step-wise titration¹. Notably, nothing has so far been done to improve the experimental procedure. At the moment 2-3 h is needed to complete an ITC binding experiment. The number of data points that can be obtained, 20-30 points, limits the range of equilibrium constants that can be resolved from the data. For ITC this range is $1 \leq KC_M \leq 1000$,^{2,3} where K is the equilibrium constant and C_M is the concentration of the reactant in the vessel. In this *Application Note* we show the possibility to shorten the time of an ITC experiment by slow continuous titration into the calorimetric vessel, cITC.

We have applied the methodology to systems that have been suggested as calibration and test reactions for titration calorimetry⁴, the binding of Ba^{2+} to 18-crown-6 and the binding of 2'CMP to RNase A.

EXPERIMENTAL

Aqueous solutions of BaCl_2 and 18-crown-6 were prepared at 49.7 mM and 3.1 mM respectively. RNase A from bovine pancreas (EC 3.1.2.7.5), was dialyzed overnight at 4°C against 1000-fold excess of the buffer (20 mM KAc, 20 mM KCl, pH 5.5), The concentration of RNase A was 23 μM and 2'CMP was 291 μM . We used a Thermometric 2277 TAM equipped with a titration unit with a 1 ml stainless steel vessel and a golden propeller stirrer. The stirring speed was adjusted to 60 rpm. A 250- μl Hamilton syringe mounted onto a pump (Thermometric 6120 Lund Pump) was used for the injections. The tip of the injection needle was positioned 1 mm above the upper part of the propeller stirrer. In the experiments 900 μl of reactant solution (18-crown-6 or RNase A) was loaded in the vessel and stirred at 60 rpm. A non-stirred titration unit charged with 900 μl of water was used on the reference side of the microcalorimeter. With the help of the highly flexible injection system from Thermometric the injection rate was optimized to 0.135 $\mu\text{l/s}$. In the analysis of a cITC experiment we use the thermal power, P , which is related to the enthalpy of binding, ΔH , for a 1:1 binding through:

$$P = \Delta H \cdot V_i \quad (1)$$

where P is the thermal power, $[ML]$ denotes the molar concentration of the complex and V_i is the current volume of the solution in the calorimetric vessel.

RESULTS

In a cITC experiment the shape of the thermal power vs. time curve is crucial for an accurate determination of the binding parameters. Therefore the quality of the dynamic correction for the thermal lag of the instrument must be high. Time constants are normally evaluated from electrical calibration of the calorimetric vessel. Here we have also performed a chemical steady-state calibration to obtain the time constants, by continuous injection of $BaCl_2$ into the calorimetric vessel containing a large excess of 18-crown-6. The conditions ensured constant nearly 100% binding of $BaCl_2$ throughout the titration so that a nearly steady-state signal was obtained. The cooling part of the calorimetric curve was used to calculate the time constants of the calorimeter. One time constant was sufficient to describe the calorimetric signal with good accuracy. In our case the quality of the dynamic correction was significantly improved when the time constant obtained from the chemical calibration was used.

Figure 1 shows calorimetric data and the best-fit curve from the non-linear regression of the experimental data from continuous addition of $BaCl_2$ to 18-crown-6. Figure 2 presents the results of the step-wise and continuous titration of RNase A with 2'CMP.

The calculated values of K and ΔH for the binding reactions are summarized in Table 1. The input parameters for the simulation and the results from non-linear regression for ITC and cITC are shown in Table 2. The values obtained by cITC data agree very well with the values obtain by ITC. Notably, the statistical uncertainties are improved for the values obtained by cITC.

Due to the dramatic in-

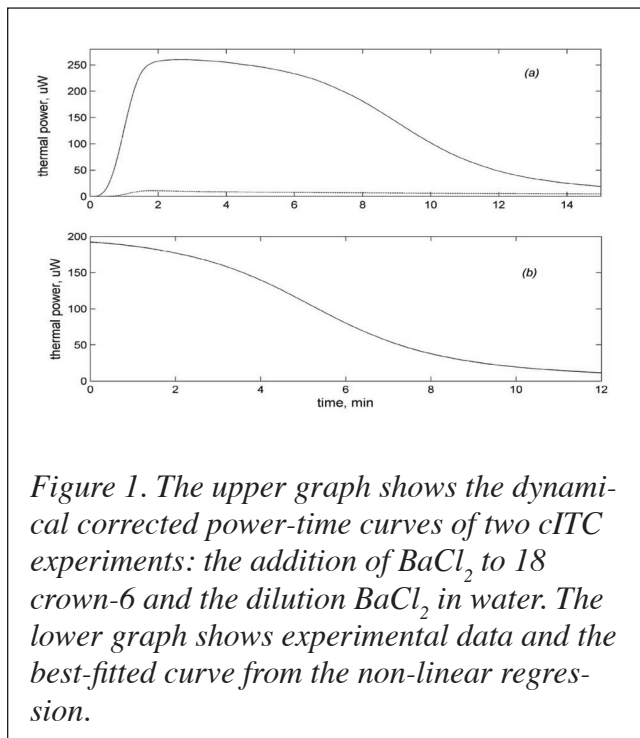


Figure 1. The upper graph shows the dynamical corrected power-time curves of two cITC experiments: the addition of $BaCl_2$ to 18 crown-6 and the dilution $BaCl_2$ in water. The lower graph shows experimental data and the best-fitted curve from the non-linear regression.

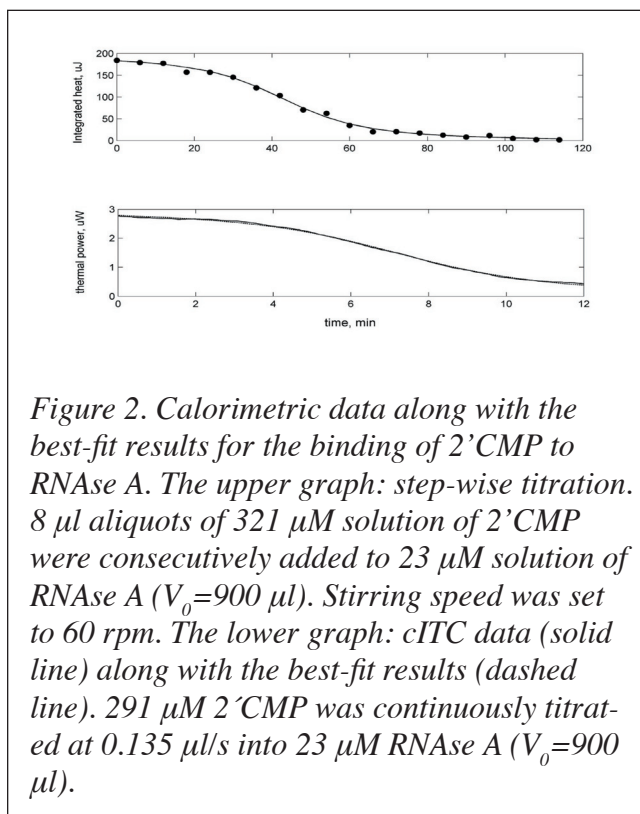
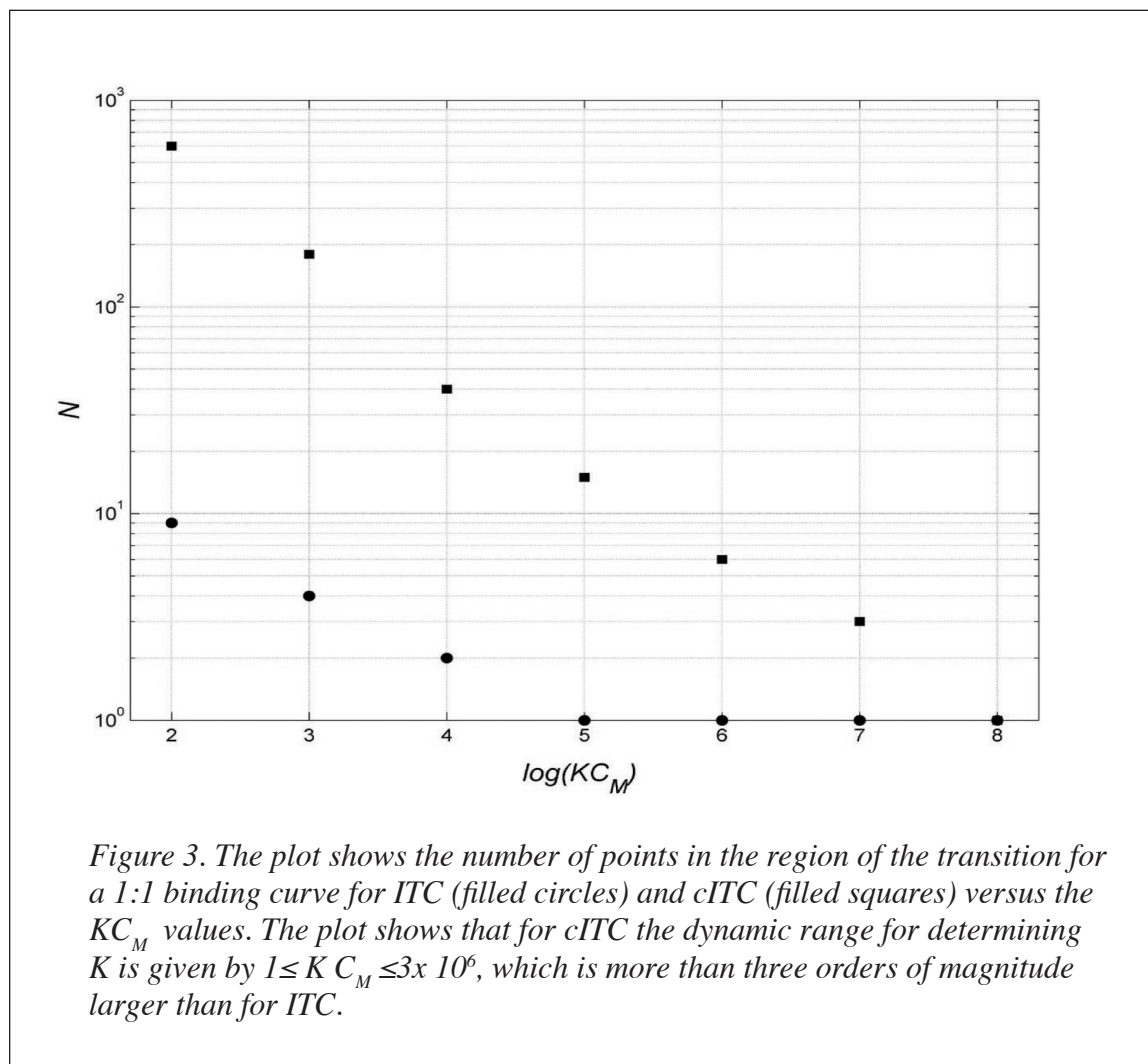


Figure 2. Calorimetric data along with the best-fit results for the binding of 2'CMP to RNase A. The upper graph: step-wise titration. 8 μl aliquots of 321 μM solution of 2'CMP were consecutively added to 23 μM solution of RNase A ($V_0=900 \mu l$). Stirring speed was set to 60 rpm. The lower graph: cITC data (solid line) along with the best-fit results (dashed line). 291 μM 2'CMP was continuously titrated at 0.135 $\mu l/s$ into 23 μM RNase A ($V_0=900 \mu l$).

crease in data density, in particular in the transition area of the binding isotherm, we extend the dynamic range of equilibrium constant that is possible to determine by calorimetry, Figure 3. With cITC the dynamic range is improved by more than three orders of magnitude, $1 \leq K C_M \leq 3 \times 10^6$. With cITC one should be able to determine K_D values down to pM range. Another important effect of the increase in data density is the improved possibility to discriminate between different thermodynamic models of binding. To visualize this we have simulated ITC and cITC data for an imaginary protein system where an endogenous ligand is bound to the protein at the site at which the added ligand binds. We have examined a case when the ligand that is added competes with the endogenous ligand bound to a portion of the total binding sites. The simulated data were fitted to three different models; 1:1 binding model, 2:1 binding model, and 1:1 competition binding model with an endogenous ligand present. The standard error of point from the fit of the cITC simulated data to the correct binding model, 1:1 competition binding model with an endogenous ligand present, was close to the applied noise, while standard error of point for the incorrect models, 1:1 binding model and 2:1 binding model, were three times than the applied noise. In addition, the residuals from the fit of the correct model showed random scattering, while the residuals from the fits to the incorrect models showed pronounced non-random scattering. For the ITC simulated data the standard error of point from the fits to the correct and incorrect models did not differ enough to conclusively distinguish between the binding models. The residuals from the fits of the ITC simulated data did not differ to the same extent as for



Reaction	Ba ²⁺ + 18-Crown-6		2'CMP + RNase A	
	cITC	ITC	cITC	ITC
K, M ⁻¹	5775±14	5900±200	(0.865±0.010).10 ⁶	(0.852±0.08).10 ⁶
-ΔH, kJ mol ⁻¹	31.19±0.04	31.42±0.20	75.62±0.20	75.45±0.95

Table 1. Comparison of *K* and ΔH for two reactions obtained by cITC and ITC at 25°C, Ref 5.

	Input data	ITC	cITC
K _{Ligand} M ⁻¹	1.10 ⁷	(1.09±0.79).10 ⁷	(1.00±0.35).10 ⁷
ΔHLigand kJ mol ⁻¹	50	50±26	49.6±0.7
K _{Endogenous} M ⁻¹	1.10 ⁵	(0.9±1.1).10 ⁵	(1.1±0.4).10 ⁵
ΔH _{Endogenous} kJ mol ⁻¹	70	67±82	63±48
[Endogeneous] μM	5	5±7	5±3
ms/applied noise		5.5/4 μJ	42/40 nW

Table 2. Input parameters for the simulation and results from non-linear regression for ITC and cITC. In the simulation the protein concentration in the vessel was 30 μM, the initial volume in the vessel was 900 μl and the ligand concentration in the syringe was 300 μM. In the ITC simulated experiment 10 μl of the ligand was added at each step and 25 data points were calculated. In the cITC simulated experiment the injection rate was 0.18 μl/s and 1200 data points were calculated.

CONCLUSION

cITC is a powerful microcalorimetric titration method that shortens the experimental time by a factor of ten while the number of experimental data points is increased more than 30 times. The dramatic increase in data density makes it possible to extend the dynamic range of attainable affinities by more than three orders of magnitudes compared to traditional ITC. Another effect of the increased data density is the improved possibility to distinguish

between different binding models. For a more comprehensive description of cITC please refer to reference 6.

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