

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

## 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=108 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 point, limits the range of equilibrium constants that can be resolved from the data. For ITC this range is  $1 \le K C_M \le 1000$ , <sup>2,3</sup> 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 calorimetry<sup>4</sup>, the binding of Ba<sup>2+</sup> to 18-crown-6 and the binding of 2'CMP to RNAse A.

# Experimental

Aqueous solutions of BaCl<sup>2</sup> and 18crown-6 were prepared at 49.7 mM at 3.1 mM respectively. RNAse A from bovine pancreas (EC 3.1.27.5), 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 23µM 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 ml 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 µl/s. In the analysis of a cITC experiment we use the thermal power, P, which

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Fields of application Life Sciences Pharmaceuticals Chemistry

# Instrument configuration

2277 Thermal Activity Monitor Equipment for Isothermal Titration Calorimetry

Matlab 6.5 (The Math Works, USA) was used to fit and to generate simulated ITC and cITC data.

## References

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(3) Hallén, D. Pure Appl. Chem. 1993, 65, 1527-1532.

(4) Wadsö, I.; Goldberg, R. N. Pure Appl. Chem. 2001, 73, 1625-1639.

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(6) N. Markova and D. Hallén, "The development of a continuous isothermal titration calorimetric method for equilibrium studies", Analytical Biochemistry 331 (2004) 77–88 is related to the enthalpy of binding,  $\Delta H$ , for a 1:1 binding through:

$$P = \Delta H \cdot \frac{d([\text{ML}])}{dt} \cdot V_i \qquad (1)$$

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, into the calorimetric vessel containing a large excess of 18-crown-6. The conditions ensured constant nearly 100% binding of BaCl, 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  $\Delta H$  for the binding reactions are summarized in Table 1. The input pa-



**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 µl). 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 µl/s into 23 µM RNAse A ( $V_0$ =900 µl).

rameters 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 increase in data density, in particular in the transi-

tion 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,  $1 \le K C_M$  $\le 3 \ 10^6$ . 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 visualise 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



**Figure 3.** The plot shows the number of points in the region of the transition for a 1:1 binding curve for ITC (filled circles) and cITC (filled squares) versus the  $KC_M$  values. The plot shows that for cITC the dynamic range for determining K is given by  $1 \le K C_M \le 3 \ 10^6$ , which is more than three order of magnitudes larger than for ITC.

Reaction	Ba <sup>2+</sup> + 18-Crown-6		2'CMP + RNAse A	
Method	cITC	ITC	cITC	ITC
K_M <sup>-1</sup>	5775±14	5900±200	$(0.865 \pm 0.010)$ 10 <sup>6</sup>	$(0.852\pm0.08)$ 10 <sup>6</sup>
- $\Delta H$ , kJ mol <sup>-1</sup>	31.19±0.04	31.42±0.20	75.62±0.20	75.45±0.95

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

	Input data	ITC	cITC
K <sub>Ligand</sub> M <sup>-1</sup>	1.107	$(1.09\pm0.79)10^7$	$(1.00\pm0.35)10^7$
$\Delta H_{Ligand}$ kJ mol <sup>-1</sup>	50	50±26	49.6±0.7
K <sub>Endogenous</sub> M <sup>-1</sup>	1.105	$(0.9\pm1.1)$ 10 <sup>5</sup>	$(1.1\pm0.4)10^5$
$\Delta H_{Endogenous}$ kJ mol <sup>-1</sup>	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  $\mu$ M, the initial volume in the vessel was 900  $\mu$ l and the ligand concentration in the syringe was 300  $\mu$ M. In the ITC simulated experiment 10  $\mu$ l 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  $\mu$ l/s and 1200 data points were calculated.

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 cITC. Thus determination of the most probable binding model is complicated.

#### 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. is related to the enthalpy of binding,  $\Delta H$ , for a 1:1 binding through:

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