Determination of a protein ligand interaction via continuous isothermal titration calorimetry

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Introduction

Isothermal titration calorimetry (ITC) is a straightforward method to determine basic chemical details of a binding interaction (affinity, thermodynamics and stoichiometry) in a single experiment and under native conditions [1, 2]. Traditionally, ITC experiments are performed using the method of incremental titration, whereby a precise volume of titrant is added to a solution of titrand at discrete time-intervals (Figure. 1). The area under each peak is then integrated, normalized and fit to a model to calculate affinity, enthalpy and stoichiometry for the interaction.

The method for incremental titration, although popular, is time consuming, and yields relatively little data that could possibly complicate model fitting and analysis. In addition, when testing unstable samples, reducing the time of an experiment is essential. By implementing the method of continuous-ITC, a researcher can reduce experiment time and improve model accuracy. To illustrate the effectiveness of this technique, binding of the ligand $(\text{N-acetyl-D-glucosamine})_3$ or chitotriose to the protein lysozyme was analyzed and compared to data collected via incremental-ITC.

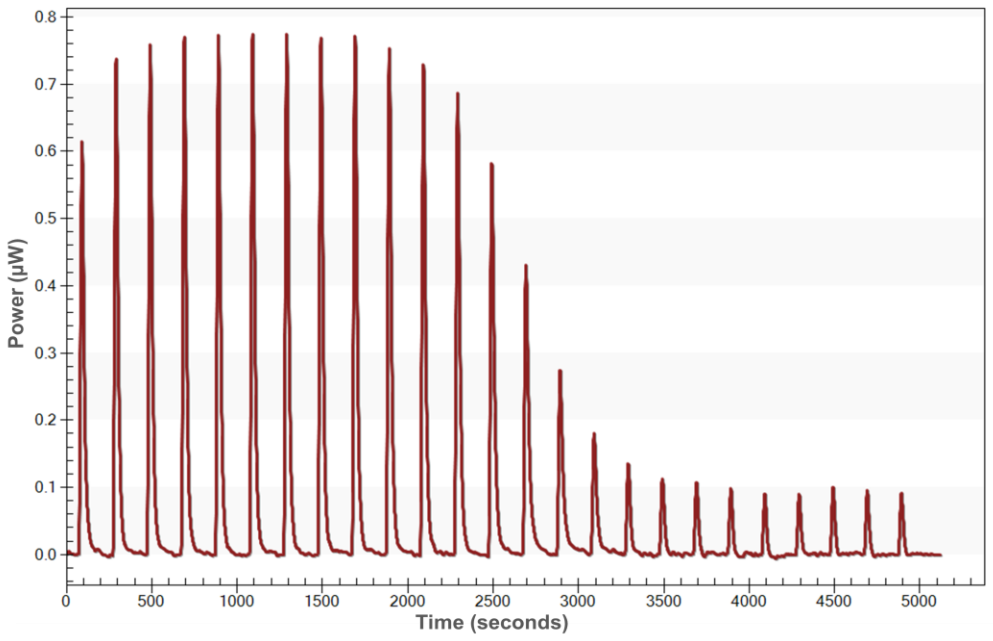

Figure 1. Incremental titration of 1mM HCl into 0.18 mM $KHCO_3$: Each peak corresponds to a single titration of HCl into a solution of $KHCO_3$ at specific time intervals. The titration data is measured in µW.

Experiment Method and Setup

Chitotriose, $(NAG)_3$, was purchased from Sigma and hen egg white (HEW) lysozyme purchased from Hampton Research were prepared in an aqueous solution containing 100mM Sodium Phosphate, pH 3.0. The ITC binding experiments were performed in the Nano ITC Standard Volume with a fixed gold cell by titrating 3 mM $(NAG)_3$ into 0.3 mM lysozyme solution. The data were acquired using ITCRun data acquisition software. The incremental-ITC experiments consisted of thirty-one, 8µL injections at 350-second intervals with stirring speed of 300 revolutions per minute (rpm). The continuous-ITC experiments consisted of a single 250 µL injection delivered over an interval of 1800 seconds (or 30min) stirring at 300 rpm. The duration for this injection was determined by looking taking into consideration when each injection from the incremental titration was baseline resolved and then this time was then cut in half. For this example, after 120 seconds each 8 µL injection was baseline resolved therefore the total time was 3720 seconds and half of this is 1860 seconds. To perform the continuous-ITC experiment, the "Continuous Titration" Experiment Type was selected from the ITCRUN "Setup tab". The experiment parameters were then set (Figure 2). The user adjustable Auto Equilibrate function allowed for equilibration of the baseline to a peak-to-peak standard deviation of less than 10nW. The Auto Equilibrate function ensures a stable baseline prior to injection of titrant (Figure 2). ITC data were analyzed with Nano Analyze Software.

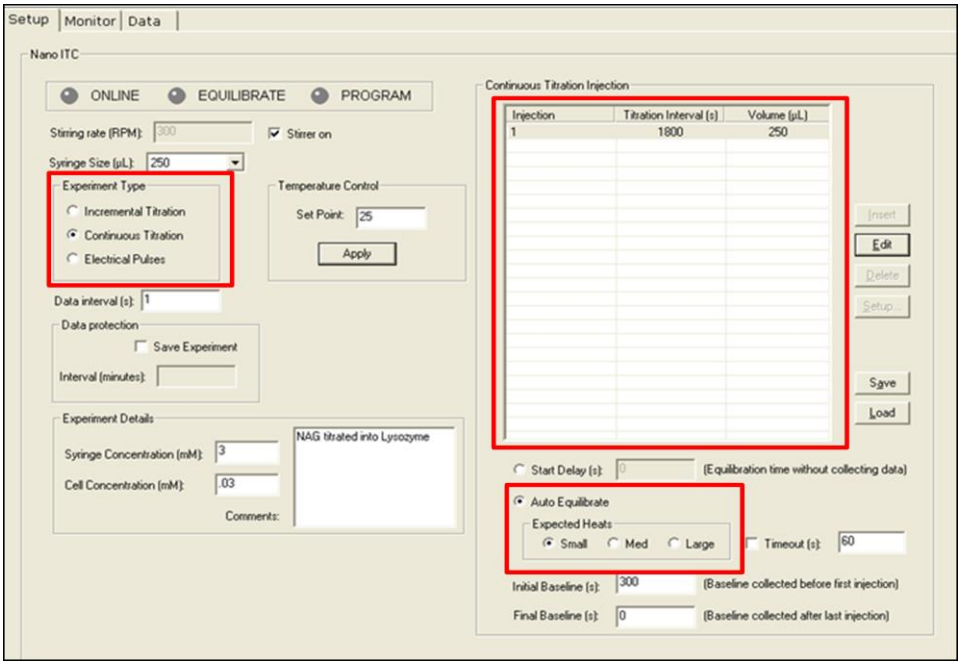

Figure 2. Continuous-ITC Experiment Setup: "Continuous Titration" Experiment Type was selected (red box, left) and then the Continuous Titration Injection parameters set (red box, right). Auto Equilibrate function (red box, bottom) has three settings pertaining to the Expected Heat (Small, Medium and Large) for each injection.

Results and Discussion

In the traditional incremental-ITC experiment, titration of $(NAG)_3$ into lysozyme elicits an exothermic reaction. The relatively low heat produced from the first titration was due to diffusion of titrant into the titrand during equilibration. Thus, this point was masked during data analysis. The following two titration peaks appear similar in magnitude and then gradually decrease until saturation is reached (Figure 3), at which point only heat of dilution is measured. The area under each peak integrated and plotted against the molar ratio of titrant to titrand were fit to a one-site Independent binding model and Blank (constant) (Figure 4). The Blank (constant) model takes into consideration the heat of dilution. The data analysis revealed an association constant (K_a) of 3.23 $\times 10^4$ (dissociation constant (K_d) of approximately 32 µM) that corresponds to the published value [3, 4]. The enthalpy (ΔH) of binding is -37.9 kJ/mol and stoichiometry of binding is 1:1 (n = 1.0). The value for the Blank (constant) is -4.08 µJ.

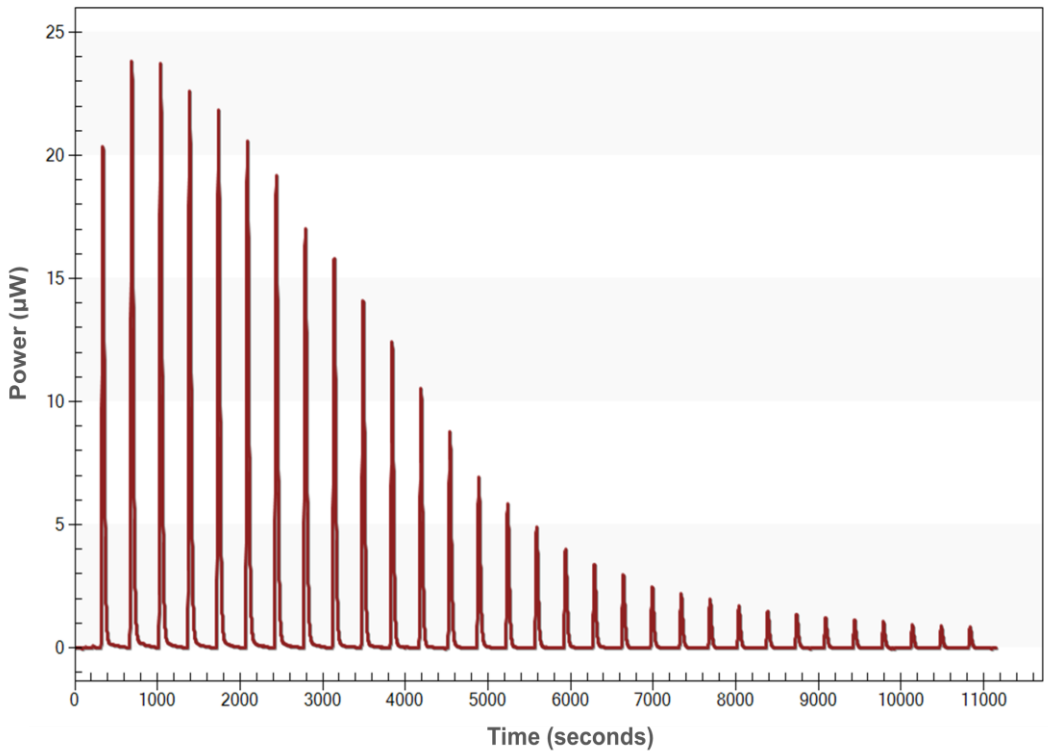

Figure 3. Binding analysis of $(NAG)_3$ to Lysozyme using Incremental Injection ITC: Raw incremental-titration data plotted as power (µW) versus time (sec). Each peak corresponds to injection of NAG_3 into the lysozyme solution

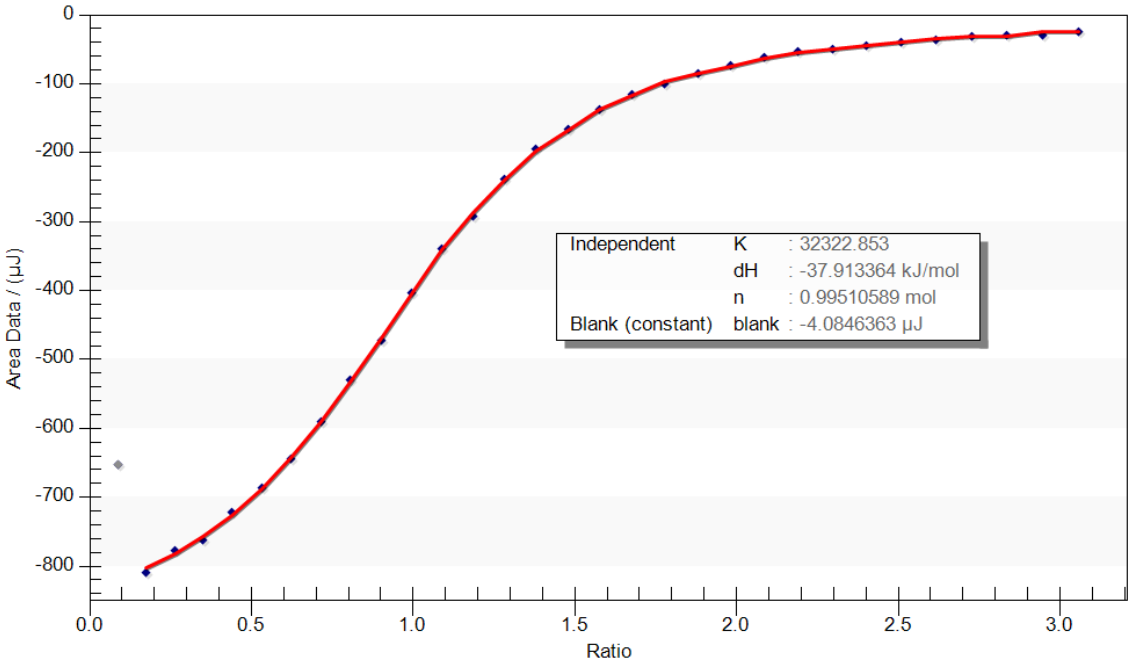

Figure 4. Binding analysis of $(NAG)_3$ to Lysozyme using Incremental Injection ITC: The area under each peak integrated and fit to a one-site Independent binding model and a Blank (constant) (red) to determine the binding affinity (K) 3.23 x 10^4, enthalpy (dH) -37.91 kJ/mol and stoichiometry (n) 0.995 mol. The heat of dilution was subtracted from the integrated peak area and is represented by the Blank (constant) (blank) -4.08 µJ.

In the continuous-ITC experiment, titration of $(NAG)_3$ into lysozyme also exhibited an exothermic titration profile. The increasing heat from the baseline to the maximum heat (~14 µW), at the start of the titration, represents the initial binding events of $(NAG)_3$ to lysozyme. These points were masked during data analysis. As the titration continues, heat released steadily decreases until saturation is reached at approximately 1800 seconds (Figure 5A). The raw data, converted to µJ, normalized and plotted versus time were fit similarly to the incremental titration experiment. Data analysis revealed an association constant (K_a) of 3.51 ×10^4 (dissociation constant (K_d) of approximately 35 µM), enthalpy (ΔH) of binding is -36.4 kJ/mol and stoichiometry of binding (n = 1.0) (Figure 5B). These values are consistent with the values measured in the incremental-ITC (Figure 4) experiment as well as the published values [3].

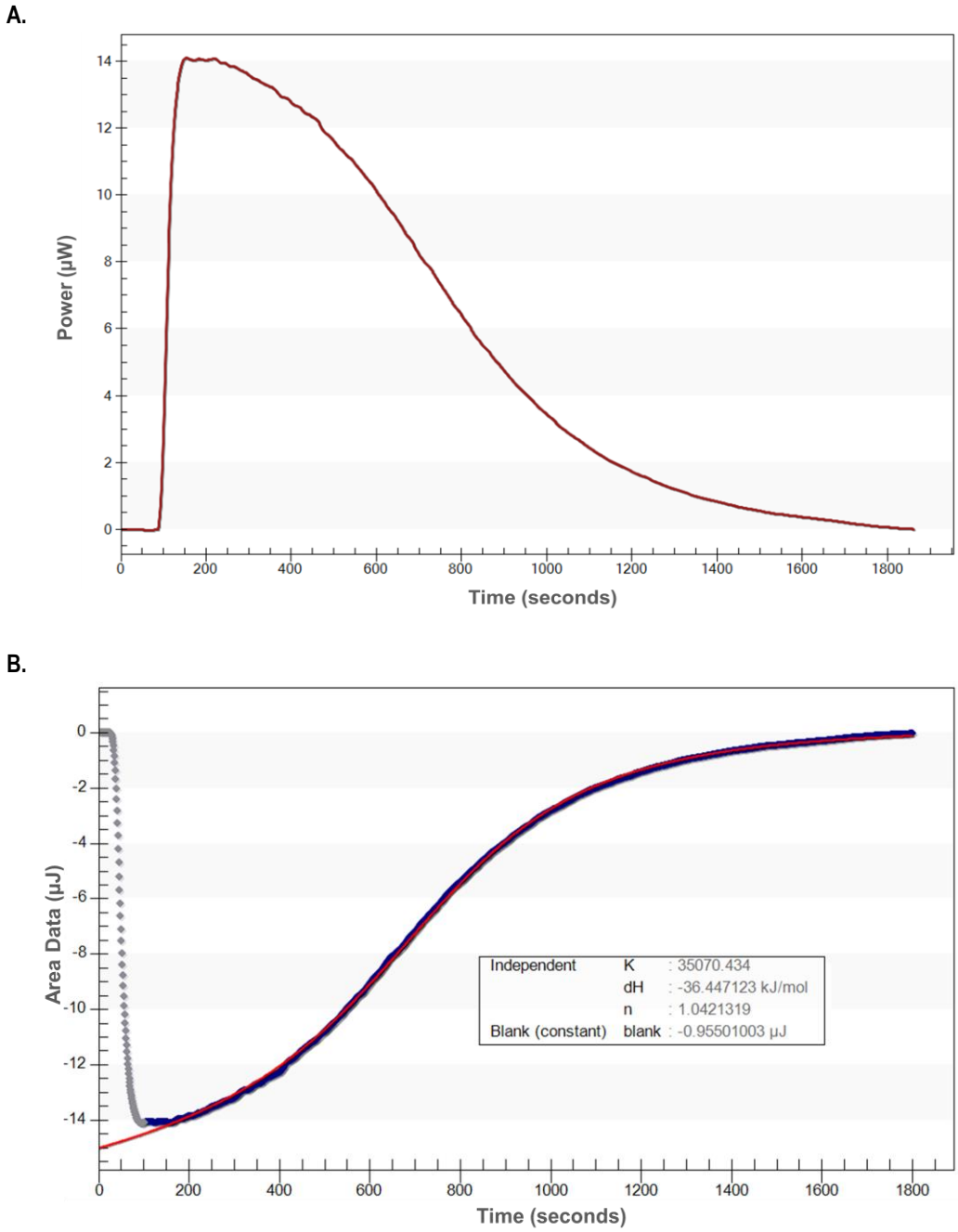

Figure 4. Binding analysis of $(NAG)_3$ to Lysozyme using Continuous Injection ITC: A. Raw continuous-titration data in µW. **B.** The points were then fit to a one-site Independent binding model and a Blank (constant) (Red) was used to determine the binding affinity (K) 3.51 x 10^4, enthalpy (dH) -36.45 kJ/mol and stoichiometry (n) 1.04 mol. The Blank (constant) value subtracted from each data point was -0.96 µJ.

Conclusion

Continuous-ITC is an extremely versatile and sensitive technique that allows for the simultaneous determination of binding affinity, enthalpy and stoichiometry of molecular interactions. Ultrasensitive ITC instruments such as the Nano ITC are capable of easily executing the required continuous injection accurately. The continuous-ITC data reported in this study was generated by titrating chitotriose into lysozyme using a Nano ITC from TA Instruments. The ITCRun data acquisition software interface allowed easy continuous-ITC experiment parameter setup. Furthermore, the user adjustable pre-injection baseline autoequilibrate function in ITCRun ensured that at least one important potential user induced variable, stability of baseline when the experiment starts, would not be a significant variable in either data set. The results of this study indicate that for $(NAG)_3$ titrated into Lysozyme in a continuous-ITC experiment (K_a = 3.23×10^4, ΔH = -37.9 kJ/mol and n = 1) is comparable with previously published results and the results obtained with the same reagents on the same Nano ITC instrument using incremental-ITC. The actual data acquisition for the continuous-ITC was completed in approximately 20% of the time required for the incremental-ITC experiment. For many applications such as ligand screening or rank order determinations of binding reactions where experiment time is an important consideration, continuous-ITC experiments performed on a Nano ITC will typically allow accurate data acquisition of a binding isotherm to be completed in much less time than that required for incremental titration ITC experiments without sacrificing quality.

References

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