



TA Instruments – Application Note

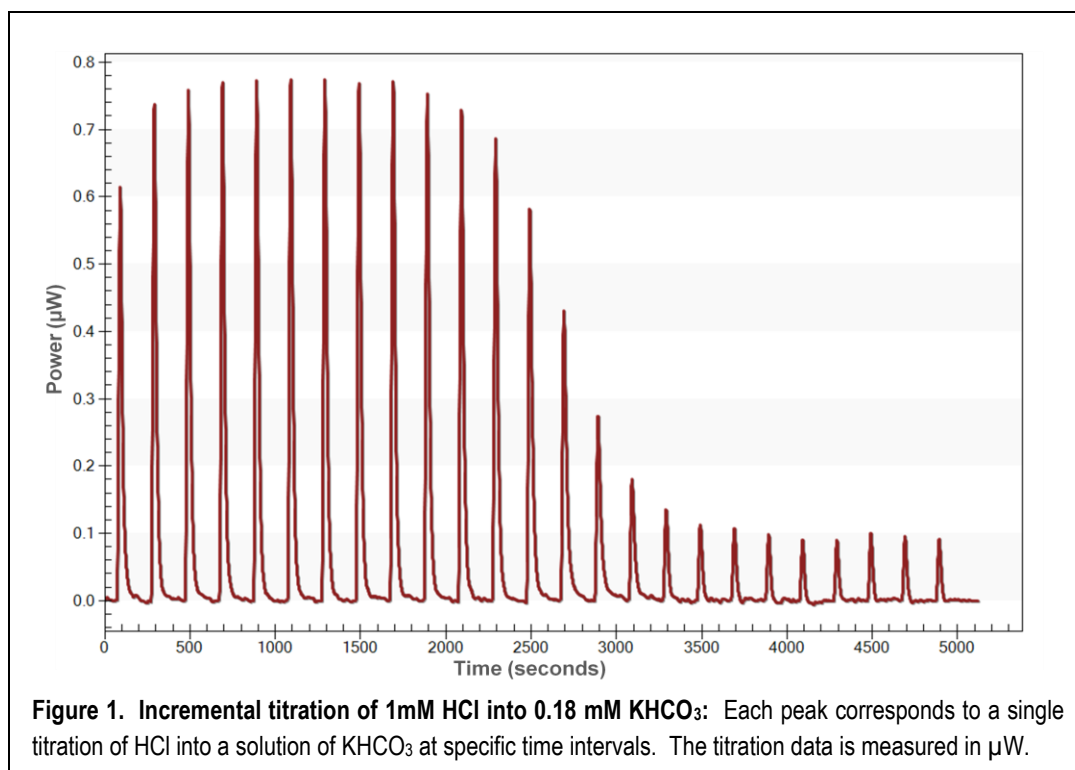
Determination of a protein ligand interaction via continuous isothermal titration calorimetry

Neil Demarse, Ph.D., Colette Quinn, Ph.D.
TA Instruments, 890 West 410 North, Lindon, UT 84042, USA

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 N-acetyl-D-glucosamine to the protein lysozyme was analyzed and compared to data collected via incremental-ITC.





TA Instruments – Application Note

Experiment Method and Setup

N-acetyl-D-glucosamine (NAG) 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 into 0.3 mM lysozyme solution. The data were acquired using ITCRun data acquisition software. The incremental-ITC experiments consisted of thirty-one, 8 μ L injections at 350-second intervals with stirring speed of 300 revolutions per minute (rpm). The continuous-ITC experiments consisted of a single 250 μ L 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 μ L 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.

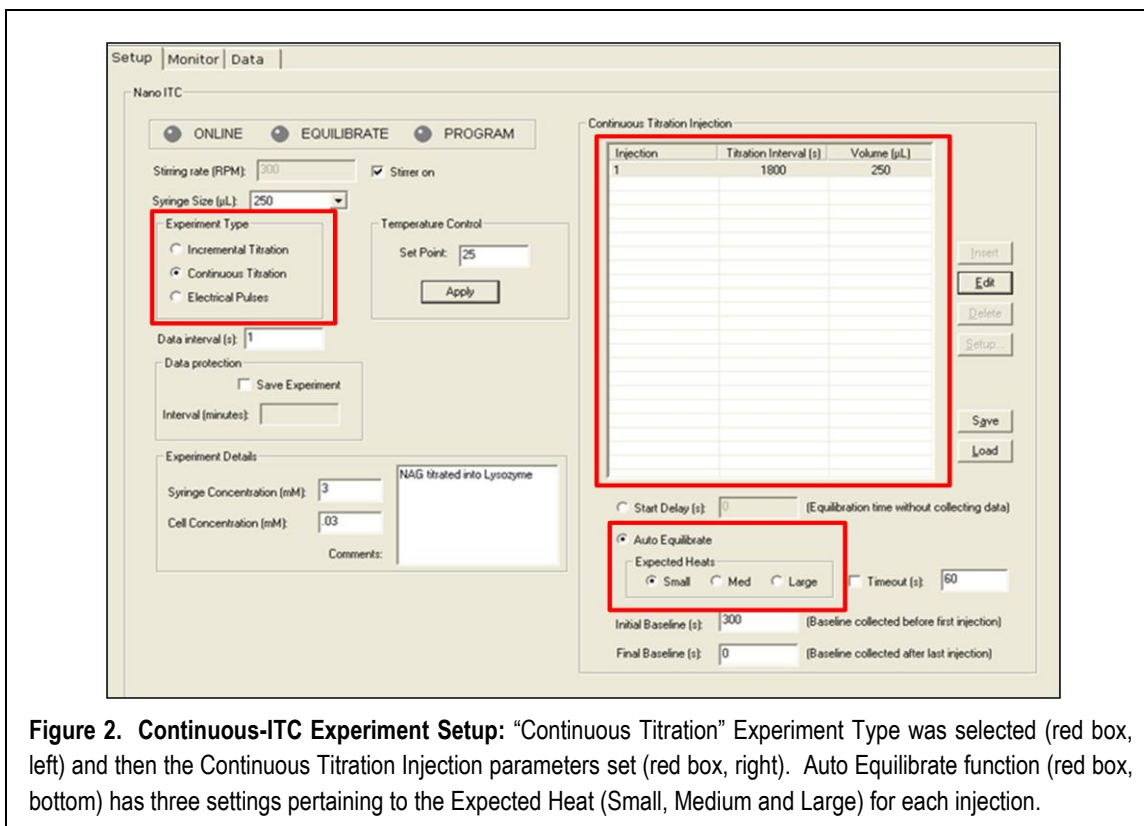


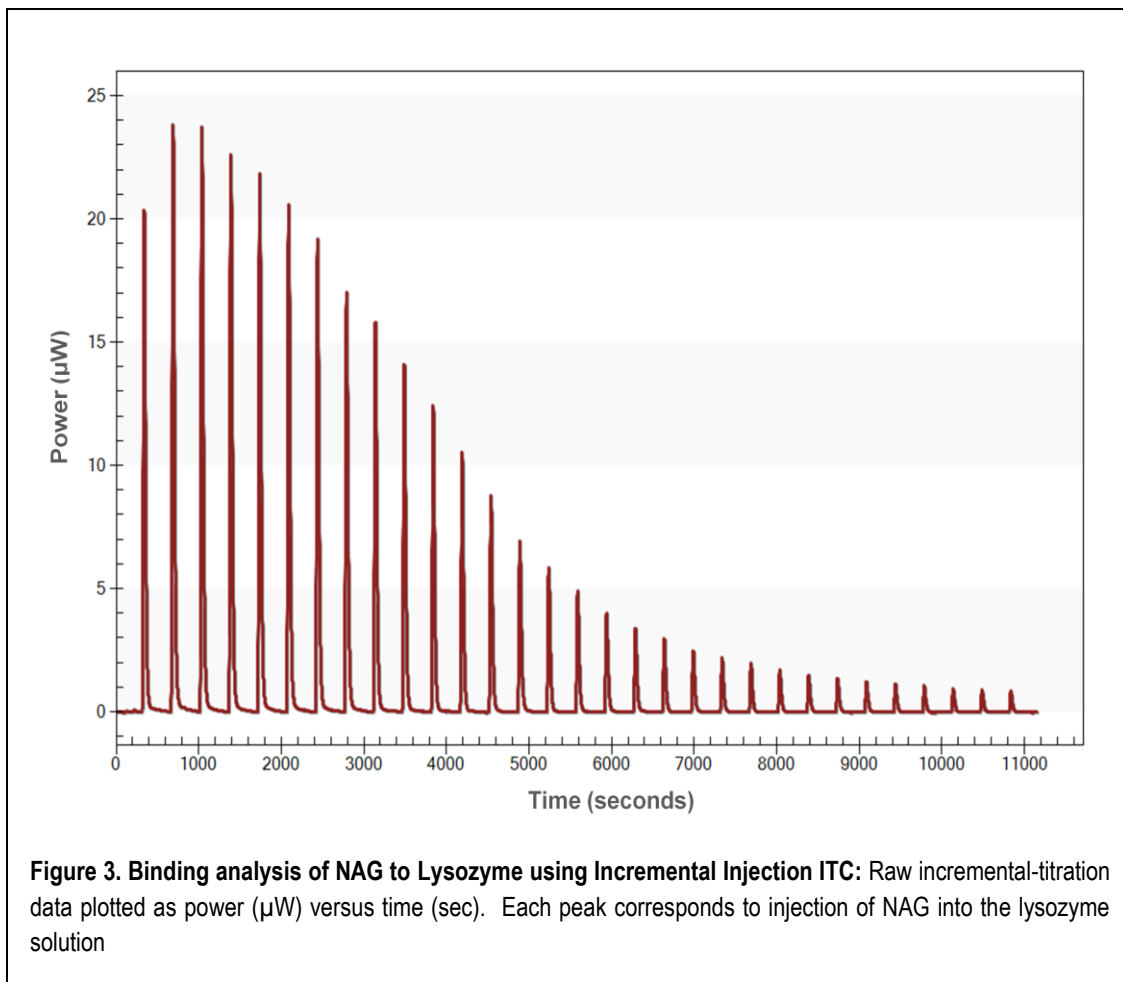
Figure 2. Continuous-ITC Experiment Setup: “Continuous Titration” Experiment Type was selected (red box, left) and then the Continuous Titration Injection parameters set (red box, right). Auto Equilibrate function (red box, bottom) has three settings pertaining to the Expected Heat (Small, Medium and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



TA Instruments – Application Note

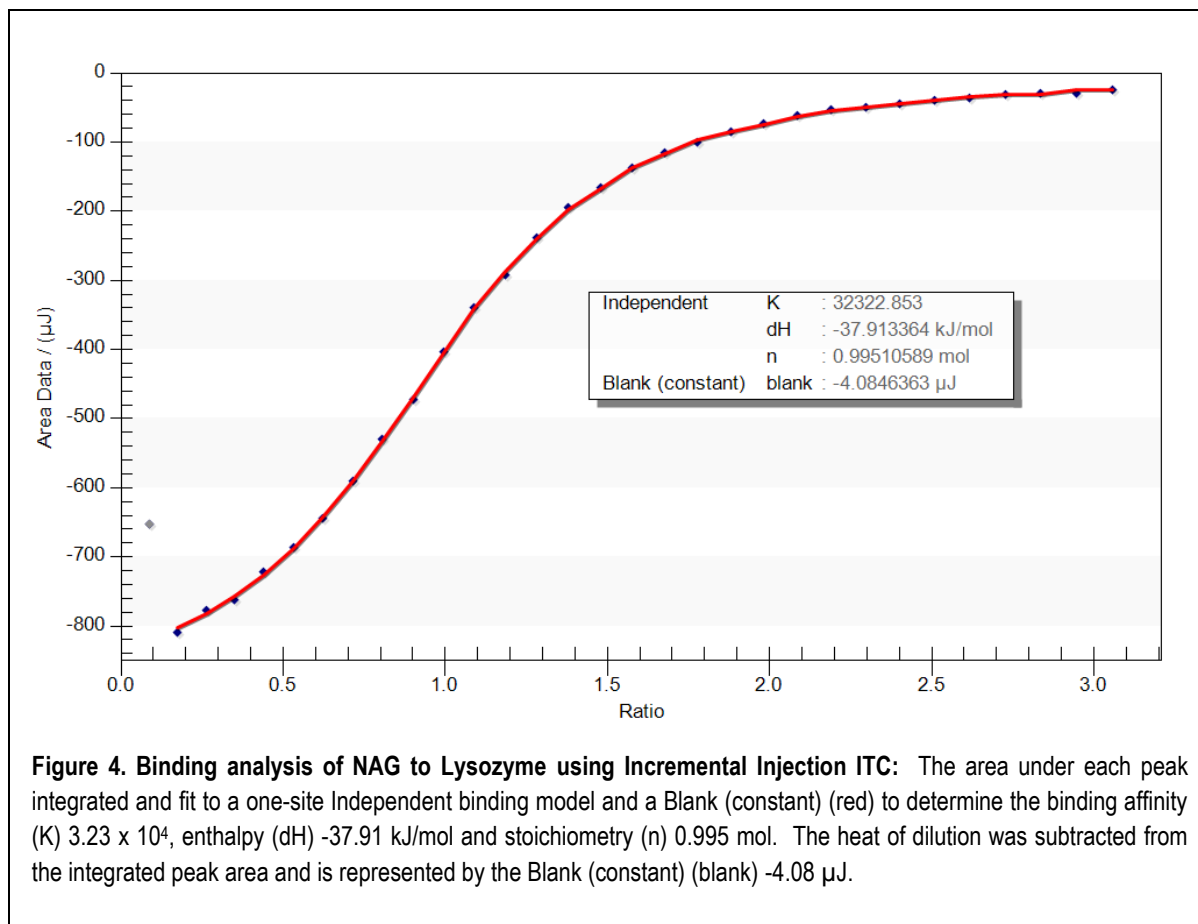
Results and Discussion

In the traditional incremental-ITC experiment, titration of NAG 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 3.23×10^4 (dissociation constant (K_d) of approximately 3.1 mM) 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 .





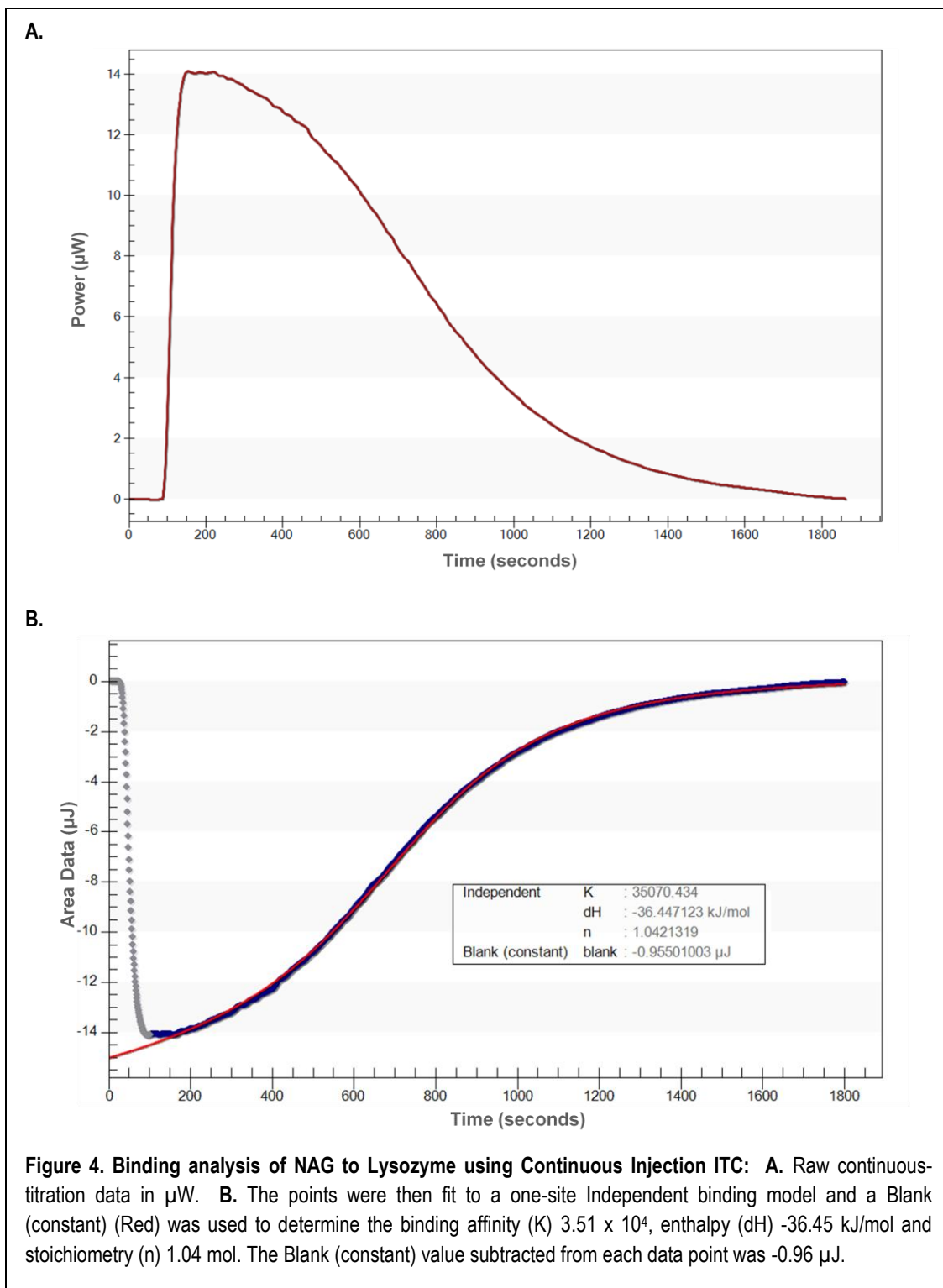
TA Instruments – Application Note



In the continuous-ITC experiment, titration of NAG 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 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 2.9 mM), 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].



TA Instruments – Application Note





TA Instruments – Application Note

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 N-acetyl-D-glucosamine 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 *N*-acetyl-D-glucosamine titrated into Lysozyme in a continuous-ITC experiment ($K_a = 3.23 \times 10^4$, $\Delta H = -37.9$ 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

1. Choma, C.T. Characterizing Binding using Continuous Isothermal Titration Calorimetry. [TA Instruments Microcalorimetry Compendium Vol I.](#)
2. Markova, N., Hallen, D. Continuous Isothermal Titration Calorimetry – cITC – a new way to speed up and improve binding experiments. [TA Instruments Microcalorimetry Compendium Vol I.](#)
3. Hall, M.L., Guth, C.A., Kohler, S.J., Wolfson, A.J. (2003) Advanced Instrumentation Projects for First Year Biochemistry Laboratory. *Biochemistry and Molecular Biology Education*. **31**, 115-118.
4. Banerjee, S.K., Rupley, J.A. (1973) Temperature and pH dependence of the binding of oligosaccharides to lysozyme, *J. Biol. Chem.* **248**, 2117–2124.