TA Instruments – Application Note

Advanced ITC Techniques: The Reverse Titration

Colette F. Quinn, Ph.D.

TA Instruments, 890 West 410 North, Lindon, UT 84042, USA

Introduction

When using isothermal titration calorimetry (ITC), the technique of the reverse titration is based up the idea of microscopic reversibility. The potential need for a reverse titration can emerge when the fit of a thermogram produces unexpected stoichiometry (n). Typically, the forward titration is defined as the one in which the ligand (L) is in the syringe, as the titrant and macromolecule (M) is in the cell, as the titrand. Some choose to further define the ligand as the higher complexing species, for example, in the case of ML_2, L is the ligand and in case of M_2L, M is the ligand. For this study, L into M is defined as the forward titration, regardless of the relative ratio. Although the designation of L or ligand is used here, the technique is not limited to small organic compounds and can be used for macromolecule-macromolecule interactions as well.

For an ideal reverse titration, the concentrations are readjusted so that L as the titrand will become fully saturated (1,2). However, some limitations may exist where this is not possible, for example, when M cannot be concentrated further. For this case, as long as the heat generated is statistically larger than the background heat, the dilute M can be titrated in the concentrated L. This means that the exact solutions used for the forward titration can be used for the reverse titration. Another advantage with this experimental set-up is that pipetting errors are minimized.

In order for this application to work, the concentration of the one of the species, M or L must be known. If the concentration of M is known, then the $\Delta H_{reverse}$ can be used to normalize the $\Delta H_{forward}$ by changing the concentration of the titrant, L in the forward titration. If the concentration of L is known, then the $\Delta H_{reverse}$ is normalized to the $\Delta H_{forward}$ by changing the concentration of the titrant, M. Later this new concentration of M can be inserted into the forward titration and the data can be refitted. Here, the concentration of M is known and there is uncertainty in the concentration of L. Error in the concentration could arise from several situations, including an unknown hydration state of a metal ligand, partial insolubility of a hydrophobic small molecule, or partial inactivity of a biological molecule.

Experimental

A low volume isothermal titration calorimeter (LV ITC, TA Instruments) was equilibrated to 25 ˚C and loaded with 300 µL of titrand and the titration syringe was loaded with a working volume of 52 µL of titrant. Prior to the beginning of each titration the calorimeter was allowed to automatically equilibrate while stirring at 350 rpm to a baseline slope of less than 0.3 µW/hr and a standard deviation of less than

0.03 µW. All experiments were carried out at minimum in duplicate. The solutions used in the forward titration were set-up in saturating conditions, where L would oversaturate M in cell. The identical solutions M and L were used in the reverse titration without modification. Therefore, the reverse titration was not set-up in saturating conditions; M did not saturate L by the end of the titration. Background titrations of titrant, M into buffer and L into buffer were also collected and data was baseline resolved using the appropriate background titration.

Results

The forward titration yielded questionable stoichiometry, as it was expected to be 1:1 To be certain of the possible new stoichiometry, the reverse titration was performed using the identical solutions (Figure 1). Even with a low concentration of the titrant, 30 µM, the signal (black) was statistically different from the background titration (red). The first step was to background resolve the unsaturated reverse titration data (black) with the M into buffer (red) background data set (Figure 1, Top thermogram). The two enthalpies, $\Delta H_{forward}$and $\Delta H_{reverse}$, were compared and the $\Delta H_{forward}$ was approximately 2x greater than $\Delta H_{reverse}$.

Because the concentration of M was known with certainty, $\Delta H_{reverse}$ was used to correct the concentration of the ligand, L, for the forward titration, by changing this value until $\Delta H_{reverse} = \Delta H_{forward}$. After normalizing $\Delta H_{forward\ to}\ \Delta H_{reverse}$ for two sets of data, the overlay thermogram to the right is produced. Rather than plotting heat, Q(µJ) in NanoAnalyze © has the option to plot the normalized heat flow (kJ/mol)(Figure 1, Bottom). From the raw heat measurement, the duplicate run values differ by only 0.08 µJ, corresponding later to a standard deviation of less than 1 kJ/mol, with an average value of $\Delta H_{reverse}$ = 24.6 ±- 0.9.

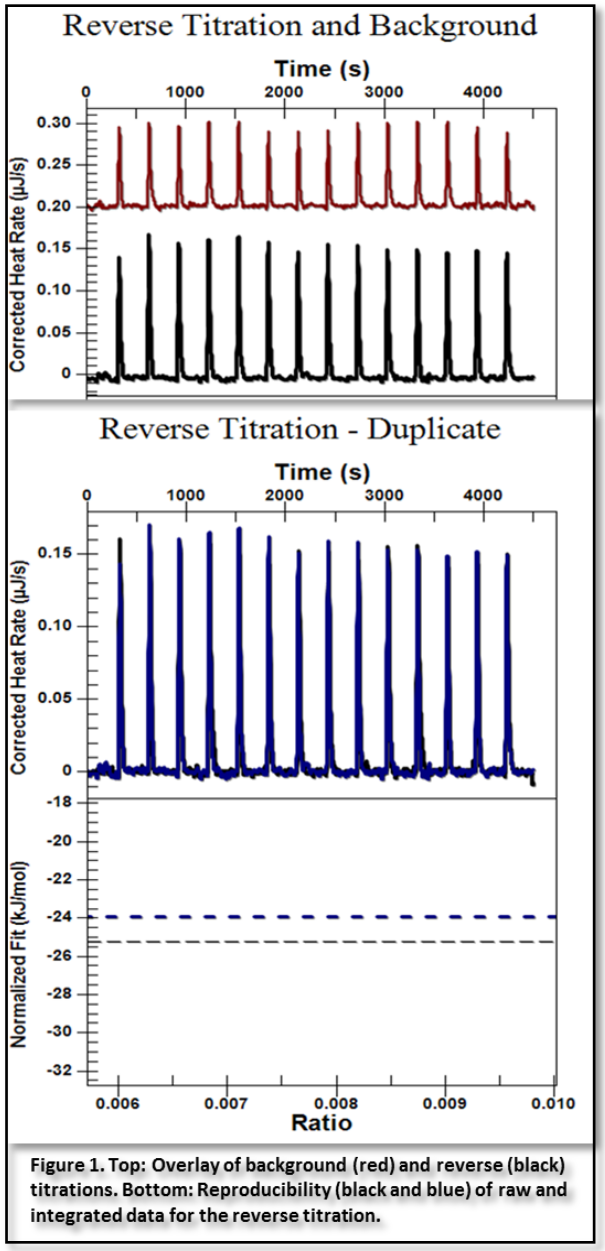

Figure 1. Top: Overlay of background (red) and reverse (black) titrations. Bottom: Reproducibility (black and blue) of raw and integrated data for the reverse titration.

The final concentration of the ligand was adjusted to 0.41 mM (Figure 2, right) from 0.25 mM (Figure 2, left). This change is also consistent with the chemistry that was used, Cu(II) into bovine serum albumin. The ligand, CuSO4, was stored in a desiccator, and it was unknown whether or not the pentahydrate complex was present. For the initial calculations, the molecular weight of the pentahydrate was used. The percent molecular weight difference between $CuSO_4(H_2O)_5$ and $CuSO_4$ is 64% and the concentration of the ligand was increased by 61%, indicating dry $CuSO_4$.

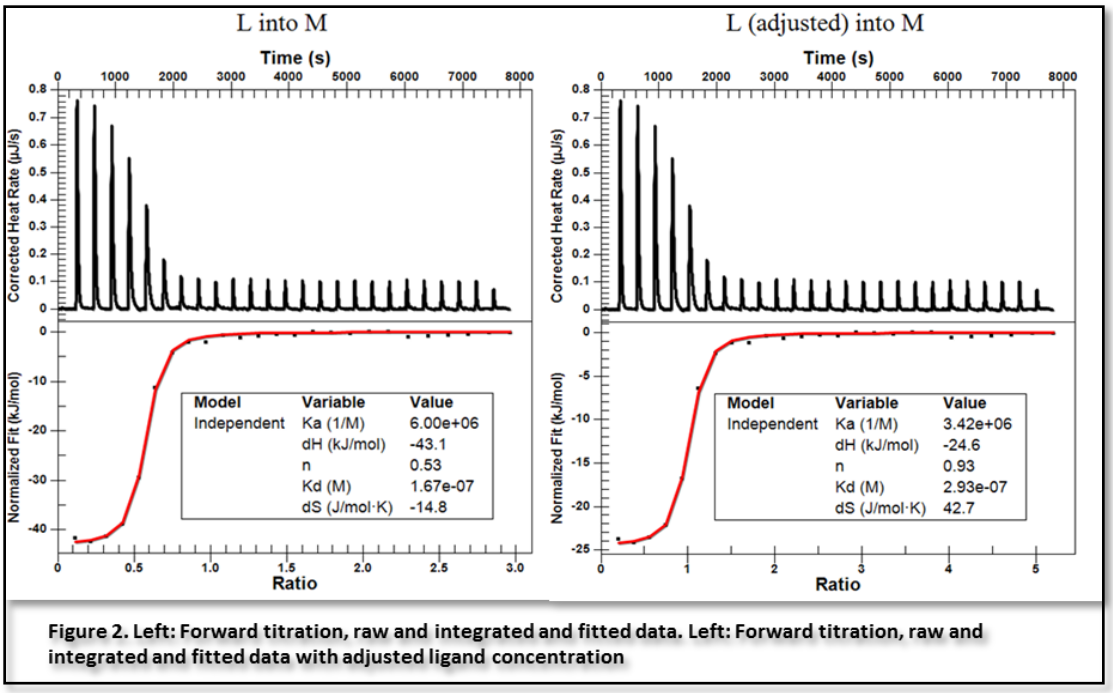

Figure 2. Left: Forward titration, raw and integrated and fitted data. Left: Forward titration, raw and integrated and fitted data with adjusted ligand concentration

Conclusion

The reverse titration should be considered for use when a titration yields unexpected stoichiometry or when there is concern for one of the concentrations of the components. An ideal reverse titration is one in which the titrant saturates the titrand. If this is not a possibility due to material consumption or concentration limitations, then a reverse titration where the experiment is conducted under non-saturating conditions can still yield valuable data. With this data in hand, the enthalpy with the known concentration of the titrant, L or M, can be used to normalize the enthalpy of the titrant, L or M, with the unknown concentration. It is important that one concentration is known. If both concentrations are unknown, the data from a reverse titration will not help in determining the concentrations because a low concentration of macromolecule or a high concentration of ligand will have the same effect on the trend observed with the reverse titration.

References

1. Spuches AM, Kruszyna HG, Rich, AM, Wilcox DE (2005) Inorg Chem 44:2964-2972
2. Velazquex-Campoy A, Friere E (2006) Nat Protoc 1:186-191