Chapter 9

Isothermal titration calorimetry

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9.1 Introduction

Isothermal titration calorimetry (ITC) is one of the physical techniques that directly measures the heat discharged or consumed all along a bimolecular reaction. It is an analytical method where the ligand comes in contact with a macromolecule under constant temperature [1]. It works on the basic principle of thermodynamics where contact between two molecules results in either heat generation or absorption, depending on the type of binding, that is, exothermic or endothermic [2]. The instrument consists of two cells; one is the main cell for the macromolecule of concern and the other cell is called a reference cell, which is meant for the solvent, as shown in Fig. 9.1.

Both cells are kept at steady temperature and pressure. The ligand is sucked through a syringe and titrated into the main cell. Macromolecular binding with the ligand results either in heat discharge or consumption, which causes the change of temperature within the main cell. However, the instrument will always maintain the constant temperature in the main cell equivalent to that of the reference cell. For maintaining the temperature, the instrument gives relevant power (higher or lower) depending on interactions (Fig. 9.2).

The heat change is then simply calculated by integrating the power over the time (seconds), which gives us the enthalpy of the reaction. The heat discharged or consumed all along the calorimetric reaction corresponds to the fraction of bound ligand and increased ligand concentration leads to saturation of substrate and finally less heat is discharged or consumed.

The amount of heat discharged upon inclusion of ligand is defined as follows [4]:

$$Q = V_o\ \Delta H_b\ [M]_t \left\{ \frac{K_a\ [L]}{1 + K_a\ [L]} \right\}$$

where Q is heat evolved/absorbed; V_o is sample cell volume; ΔH_b is enthalpy of binding per mole of ligand; $[M]_t$ corresponds to the total concentration of macromolecule in the sample cell; K_a is binding constant; $[L]$ is concentration of free ligand.

Accurate measurement of released/absorbed heat is then used to determine the binding constants (K_a), enthalpy (ΔH), entropy (ΔS), and the reaction stoichiometry (n), thereby an entire thermodynamic parameter of the molecular binding can be obtained in an individual analysis by applying this technique (Fig. 9.3).

A very important aspect that determines the success of an isothermal titration calorimetric attempt is deciding the actual concentration for protein and ligand respectively. The appropriate protein concentration relies on the parameter "c" value, which is defined as:

$$c = nP_t/K_d$$

where P_t is the concentration of protein kept in the measurement cell, "n" is the number of binding sites per protein molecule, and K_d is the affinity constant [5]. The c value determines the architecture of the binding isotherm. The higher c value ($\sim$1000) will lead to the generation of too-steep shaped curve, which will make it difficult to get an estimate of K_d, although other parameters such as ΔH and n can be determined. If the c value is <5, then

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architecture of the binding isotherm is too shallow, which does not allow precise determination of the thermodynamic parameters unless one of them is earlier known (such as the stoichiometry). Hence, to get a good sigmoidal shape of the binding isotherm so as to estimate the K_d, ΔH, ΔS, and n, it is necessary to keep the c value between 20 and 100.

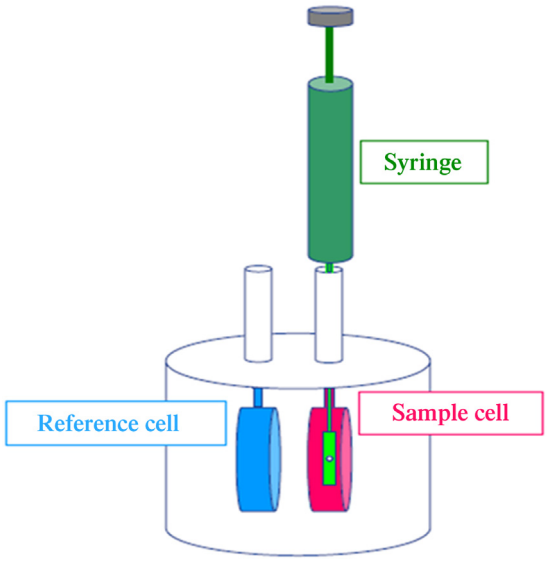


Figure 9.1 Instrumentation of the typical isothermal titration calorimetry [3].

9.2 Raw data

The raw signal in isothermal calorimetry is the power (μcal s^{-1} or μJ s^{-1}) used to control the heater to keep the temperature of cell the same as a function of time as represented in Fig. 9.4. Sample raw data is illustrated in Fig. 9.5.

To further determine the heat produced after every injection, the area under each peak is obtained by using the following equation [7]:

$$q_i = v \times \Delta H \times \Delta L_i$$

which demonstrates that the heat released or absorbed (q_i) during each injection of a reaction corresponds to the extent of ligand that interacts with protein in a precise injection ($v \times \Delta L_i$) and the distinctive binding enthalpy (ΔH) for the reaction. The quantity ΔL_i denotes the concentration difference between the bound ligand in the ith and $(i-1)$th injections. This is dependent upon the specific binding model, reflective of whether the interaction follows single, multiple, or cooperative binding model. The most common case is the protein with a single interaction site; the above equation evolves into:

$$q_i = v \times \Delta H \times [P] \times \left(\frac{K_a[L]_i}{1 + K_a[L]_i} - \frac{K_a[L]_{i-1}}{1 + K_a[L]_{i-1}} \right)$$

where K_a and $[L]$ is the binding constant and concentration of free ligand respectively [7].

Therefore, heat after every injection is determined by estimating the area under each peak. Since the amount of free protein (not bound to ligand) decreases

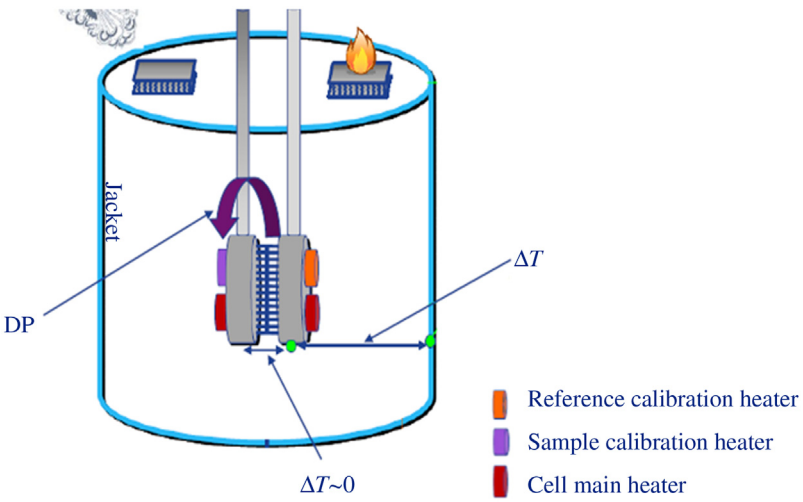


Figure 9.2 Representative model showing how ITC works [3].

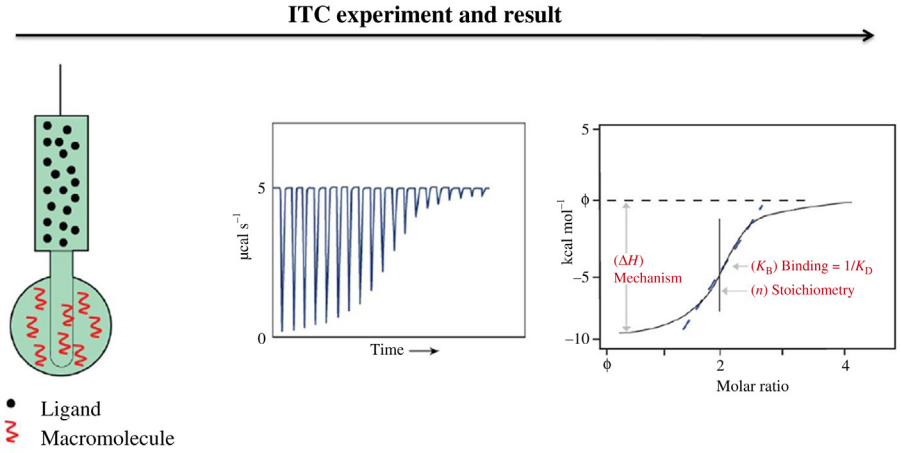


Figure 9.3 Pictorial representation showing the progress of ITC experiment with result [3].

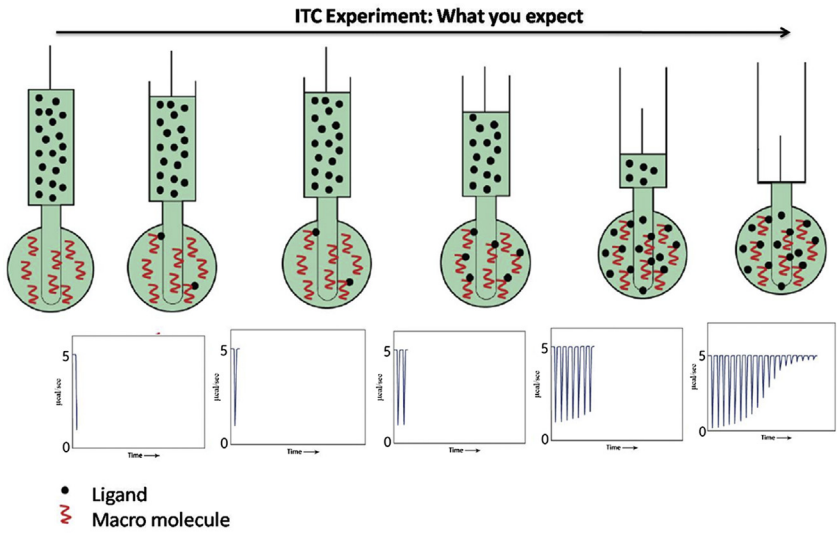


Figure 9.4 Pictorial representation showing the stepwise progress of ITC experiment result.

progressively after each successive injection as the reaction proceeds, accordingly the magnitude of the peak also reduces continuously until saturation is accomplished. Once the saturation is reached, consecutive injections produce the same peaks equivalent to the heat of dilution. To further obtain the heat of binding, the observed binding peaks are integrated and subtracted from the heat of dilution, which is identified by injecting the ligand solution into the buffer.

9.3 Data processing

To obtain the thermodynamic parameters, raw data is further processed for baseline correction, subtraction of heat of dilutions using the ITC software. In the subsequent discussion, we will explain all the steps involved in data processing using the Nanoanalyze software. Finally, the processed experimental data is fitted to a particular model

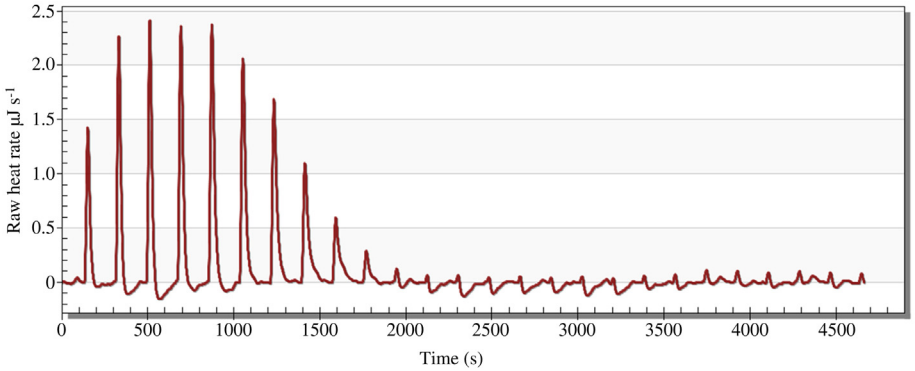


Figure 9.5 Calorimetric titration of the native vacuolar sorting protein 29 from *Entamoeba histolytica* with divalent metal Zn representing the raw ITC data acquired from 25 injections (1 μL each) of divalent metal that is Zn into 100 μM of protein (*Eh*Vps29) [6].

(independent model, multiple site model, cooperative model, etc.) given in the Nanoanalyze software. It is generally recommended to first fit the data in the independent model if you do not have prior information about the stoichiometry of the interaction. Then try to fit the data in other models as well and check the results. Finally, the correct model for the data is selected by looking at the mathematical analysis for thermodynamic parameters acquired from each of the binding models given in the Nanoanalyze software.

To identify the values of thermodynamic parameters, the thermograms are usually identified by a curve fitting process that utilizes the nonlinear regression analysis so as to obtain the best fit of the model. The analysis gives the values of stoichiometry (n), affinity constant (K), and enthalpy (ΔH) for the binding reaction. Then, free energy of binding is determined from these values using the equation:

$$\Delta G = -RT\ln K$$

The value of entropy is determined by the following equation:

$$\Delta G = \Delta H - T\Delta S$$

Statistical analysis for thermodynamic parameters can be executed using the program inbuilt in Nanoanalyze software.

9.3.1 Web-based sources

There are no freely available automatic software programs for processing the ITC data. These software programs are in-built programs provided by the manufacturer along with the instrument. Although, the data can be processed with any scientific program available for graphing and data analysis such as Origin, Sigma plot, etc. Here for further discussion, we will explain the ITC data processing using Nanoanalyze software. First of all, install the software and open it. The window will look as shown in Fig. 9.6.

Now, open both the raw data, interaction and control (heat dilution) by going to file and open option given in the software window. The raw data appears as shown in Fig. 9.7.

To further process the data it is necessary to correct the baseline of the data. This can be done by baseline correct option given in the software as shown in Fig. 9.7.

After baseline correction we need to subtract the heat of dilution data from the interaction data, which is very important to determine the actual heat of interaction. For this go to the Area option, which will show an option for area correction, as shown in Fig. 9.8.

The heat of dilution must be subtracted. Go to the Area option, drag and drop the heat of dilution data to blank (Figs. 9.9 and 9.10).

We are ready with the data for modeling it into particular model so as to determine the thermodynamic parameters of the interaction after subtracting the heat of dilution. For this purpose, go to the model section in the modeling area, as shown in Fig. 9.11. It will again open a new window showing different models.

It is generally recommended that if you do not have any prior information regarding the interaction, try to first fit the data in the independent model. This model tries to fit the data considering that there is only one binding site.

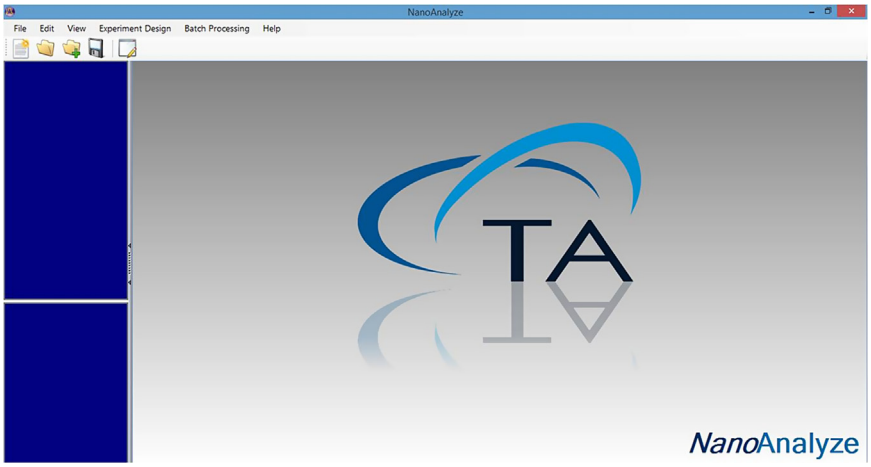


Figure 9.6 Snapshot showing the Nanoanalyze software window.

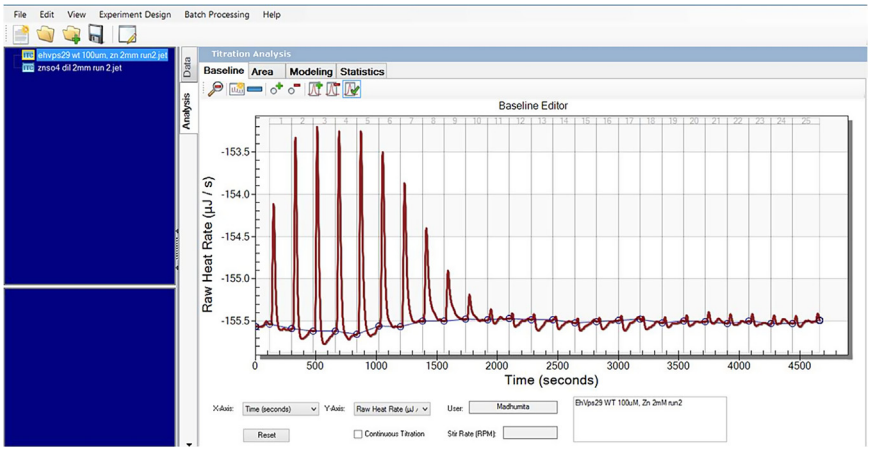


Figure 9.7 Snapshot showing the raw data in Nanoanalyze software window.

Now, data is ready to fit into independent model. Just start the fitting procedure by clicking on the green button in Fit data option. Data fitting is an iterative process that ultimately gives the list of thermodynamic parameters such as enthalpy (ΔH), entropy (ΔS), affinity constant (K_d), and the stoichiometry of the interaction (n) as shown in Fig. 9.12.

Now, after processing the data it is very important to check its statistical reliability. Statistical analysis for thermodynamic parameters can be executed using the program provided with the Nanoanalyze software by keeping the constant value of standard deviation (0.1) and desired confidence level (99%) with 1000 trials as shown in Fig. 9.13.

Finally, when the statistical analysis is complete, it will give the histograms as shown in Fig. 9.14 along with the values of all the thermodynamic parameters with associated standard error. The good Gaussian histogram signifies the good statistics of the data. If this is not good, you can again try to fit the data in different models and check the statistics of the parameters. By comparing the statistics of the thermodynamic

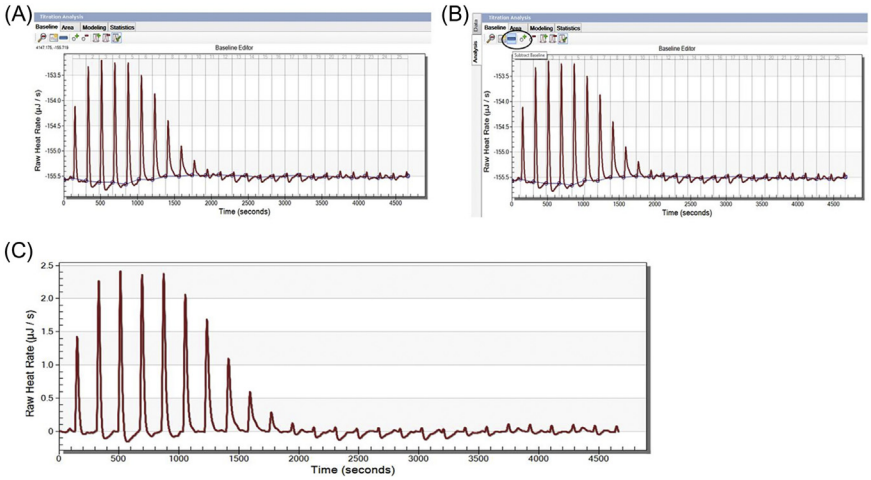
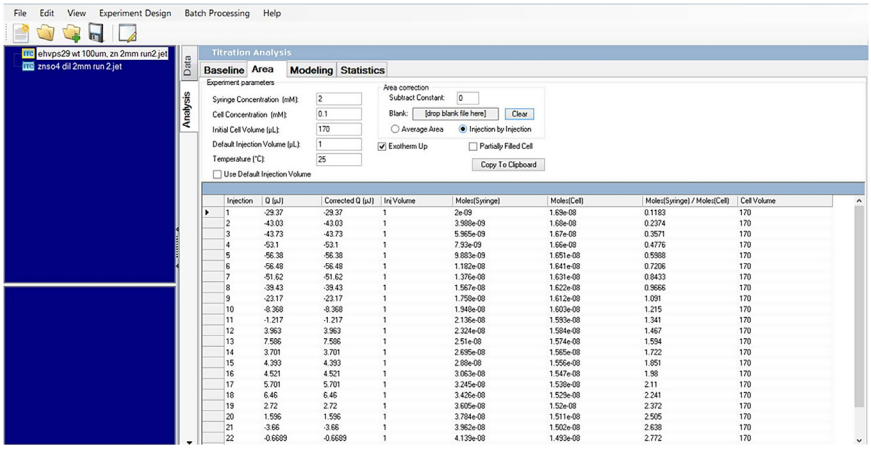


Figure 9.8 Baseline correction. (A) Raw data without baseline correction. (B) For baseline correction click on the option highlighted with black circle. (C) Data after baseline correction.



Injection	Q (µJ)	Corrected Q (µJ)	Inj Volume	Moles(Syringe)	Moles(Cell)	Moles(Syringe) / Moles(Cell)	Cell Volume
1	-29.37	-29.37	1	2e-09	1.69e-08	0.1183	170
2	-43.03	-43.03	1	3.988e-09	1.68e-08	0.2374	170
3	-43.73	-43.73	1	5.965e-09	1.67e-08	0.3571	170
4	-53.1	-53.1	1	7.93e-09	1.66e-08	0.4776	170
5	-56.38	-56.38	1	9.883e-09	1.651e-08	0.5988	170
6	-56.48	-56.48	1	1.182e-08	1.641e-08	0.7206	170
7	-51.62	-51.62	1	1.376e-08	1.631e-08	0.8433	170
8	-39.43	-39.43	1	1.567e-08	1.622e-08	0.9666	170
9	-23.17	-23.17	1	1.758e-08	1.612e-08	1.091	170
10	-8.368	-8.368	1	1.948e-08	1.603e-08	1.215	170
11	-1.217	-1.217	1	2.136e-08	1.593e-08	1.341	170
12	3.963	3.963	1	2.324e-08	1.584e-08	1.467	170
13	7.586	7.586	1	2.51e-08	1.574e-08	1.594	170
14	3.701	3.701	1	2.695e-08	1.565e-08	1.722	170
15	4.393	4.393	1	2.88e-08	1.556e-08	1.851	170
16	4.521	4.521	1	3.063e-08	1.547e-08	1.98	170
17	5.701	5.701	1	3.245e-08	1.538e-08	2.11	170
18	6.46	6.46	1	3.426e-08	1.529e-08	2.241	170
19	2.72	2.72	1	3.605e-08	1.52e-08	2.372	170
20	1.596	1.596	1	3.784e-08	1.511e-08	2.505	170
21	-3.66	-3.66	1	3.962e-08	1.502e-08	2.638	170
22	-0.6689	-0.6689	1	4.139e-08	1.493e-08	2.772	170

Figure 9.9 Snapshot showing the window for area correction to subtract the heat of dilution from interaction data.

parameters in different models of the same data, you can confidently know the correct model for your data. As mentioned in the previous section, the statistical analysis for thermodynamic parameters can be executed by keeping the constant value of standard deviation (0.1) and desired confidence level (99%) with 1000 trials. The values of standard deviation, confidence level %, and the number of trials can be changed. Generally, lower value of standard deviation (0.1−1.0), higher confidence level %, and greater number of trials give the expected good Gaussian shape to distributions of the parameters.

9.4 Examples

Characterization of thermodynamic interactions between the molecules (proteins, nucleic acids, and lipids) is important to understand the functional aspects of the biological systems. Although, recent advances in the field of structure biology have provided the mechanistic view of the interactions at atomic level with a biochemical and functional correlation, still the picture is not complete and requires detailed characterization of the interactions involving estimation of affinity, number of binding sites

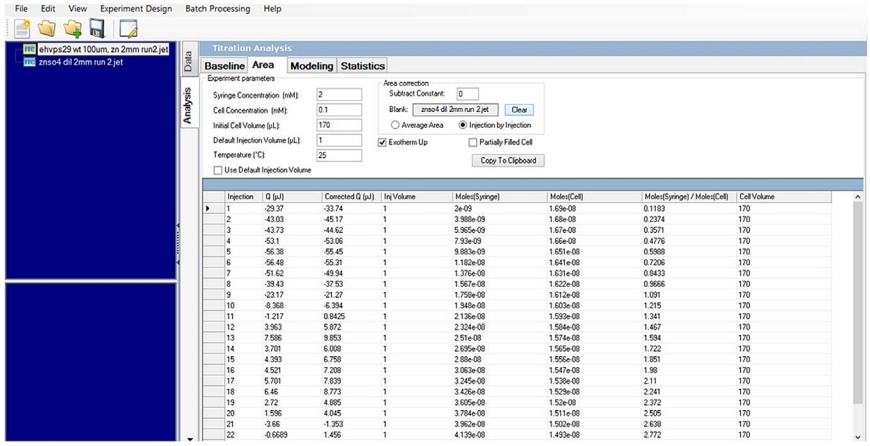


Figure 9.10 Snapshot showing the window for subtracting the heat of dilution from the interaction data.

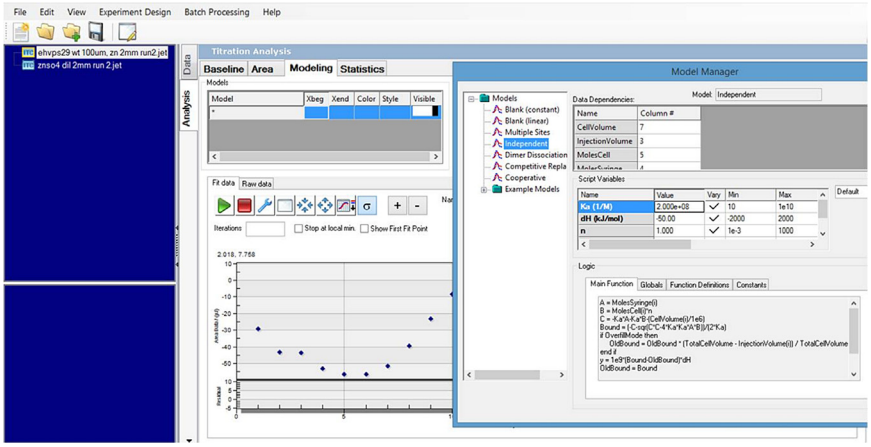


Figure 9.11 Snapshot showing the window for modeling the data in independent model.

and thermodynamic parameters. This helps in describing the function and mechanism at the molecular level.

The ITC technique is used for the thermodynamic characterization of biomolecules in solution and is universally accepted for studying protein–protein interactions, and protein–ligand interactions. In subsequent discussions, with the help of selected examples we will explain the complete ITC data analysis and its interpretation.

9.4.1 Protein–metal interactions

Here we take the example of amoebic protein Vps29 (EhVps29) and its interaction with zinc metal. The choice of control is very important, that is, heat of ligand dilution as mentioned in previous sections. But apart from this, you need to include some negative control, that is, interaction with any nonspecific ligand in your studies for assessing the specificity of the interaction. In a recent study, the author assessed the metal specificity of EhVps29, comprehensive studies on its interaction with several divalent metals (Mg, Mn, Zn, and Ca) using ITC [6]. Interestingly, among several metals, they identified that the binding of vacuolar sorting protein 29 from *Entamoeba histolytica* is preferable to zinc. Table 9.1 represents the summary of all the thermodynamic parameters obtained for the interaction of EhVp29 to zinc.

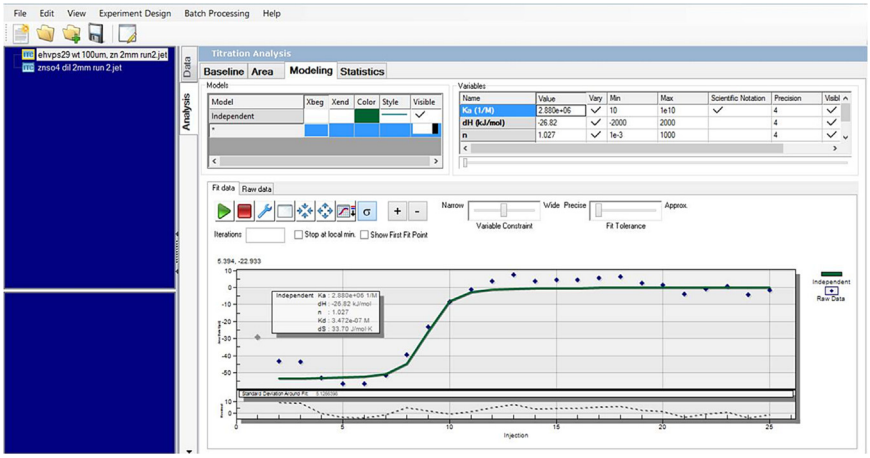


Figure 9.12 Snapshot showing the window for modeled data in independent model with all the thermodynamic parameters.

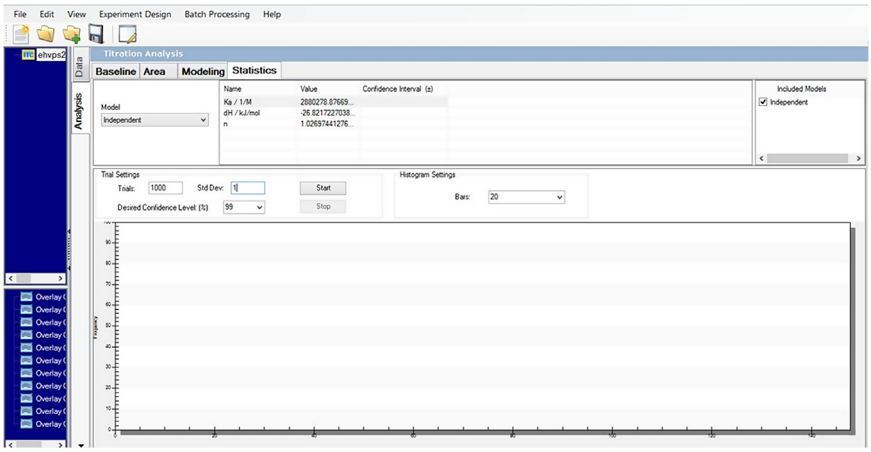


Figure 9.13 Snapshot showing the window for checking the statistics of the modeled data in independent model for all the thermodynamic parameters.

As shown in Fig. 9.15 and Table 9.1, EhVps29 binds to zinc metal with micromolar affinity ($-2.9 \times 10^6\ M^{-1}$). Overall Zn^{++} binding to EhVps29 is found to be spontaneous and both the enthalpic as well as entropic factors contributed towards the feasibility of interactions [6].

In the above discussion, you have seen the example of protein–metal interactions with favorable enthalpy and entropy. Now we will introduce another example of protein–metal interaction, which is spontaneous but with favorable enthalpic contributions and unfavorable entropic contribution (decrease in entropy).

9.4.2 Thermodynamic basis of calcium binding to EhCRD from *Entamoeba histolytica*

To establish EhCRD as a calcium-binding protein, authors have analyzed its interaction with calcium using ITC [8]. As shown in Fig. 9.16, the affinity and stoichiometry of EhCRD to calcium ion found to be in micromolar range (an association constant of $1.0 \times 10^5\ M^{-1}$) and one respectively.

Thermodynamic parameters for the calcium binding to EhCRD are given in Table 9.2. These values of

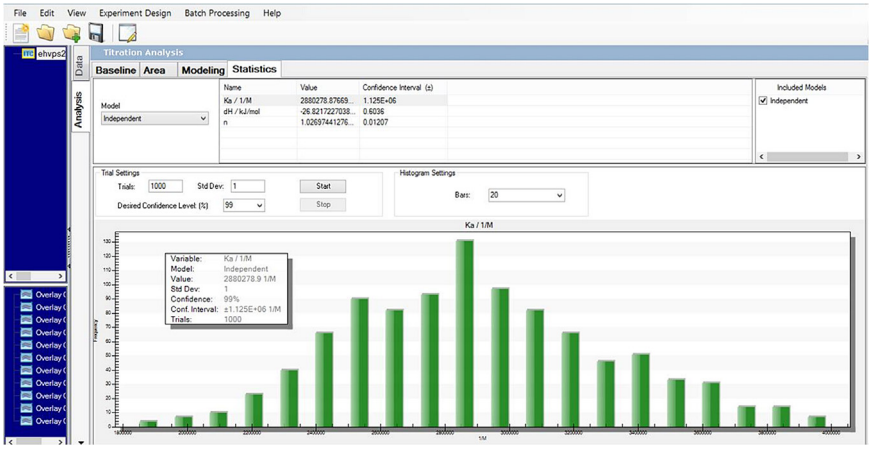


Figure 9.14 Snapshot showing the result of the statistical analysis of the modeled data in independent model for all the thermodynamic parameters.

Table 9.1 Thermodynamic parameters for the interaction of wild type EhVps29 to several metals.

Protein (100 μm)	Ligand (2 mM)	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	$T\Delta S$ (kJ mol^{-1})	ΔG (kJ mol^{-1})	K_a (M^{-1})
EhVps29 wild type	$ZnSO_4$	−26.8	33.7	10.1	−36.8	2.9×106
EhVps29 wild type	$MnCl_2$			No interaction		
EhVps29 wild type	$MgCl_2$			No interaction		
EhVps29 wild type	$CaCl_2$			No interaction		

thermodynamic parameters suggest that the interaction is spontaneous, which is majorly driven by enthalpy with reduction in entropy. Entropic reduction is attributed to loss in conformational degree of freedom of the protein upon binding to calcium ion. Further, to rule out the possibility of its interaction with other metals, authors have also included Mg^{++} ion in their study. As a result, they found that EhCRD does not bind to magnesium. This further confirms the metal specificity of EhCRD for calcium [8].

9.4.3 Protein carbohydrate interactions

Here, we are taking the example of EhCRD, that is, carbohydrate recognition domain of Gal/GalNAc lectin from *E. histolytica* and its interaction with synthetic sugar, Gal-GalNAc [8]. Table 9.3 represents the thermodynamic parameters for interaction of EhCRD to Gal-GalNAC sugar. EhCRD binds to Gal-GalNAc with micromolar affinity (association constant of 2.3×10^6 M^{-1}).

To assess the sugar specificity of EhCRD, the authors have also studied its interaction with maltose as a negative control. As shown in Fig. 9.17, EhCRD did not interact with maltose. Binding was spontaneous and favored by entropic contribution with unfavorable enthalpy. The observed positive enthalpy and entropy for Gal-GalNAc interaction with EhCRD might be because of reorganization of water molecules resulting from the release of structured water molecules upon binding. Literature reports substantiating these findings are not rare. Multiple investigations on multivalent

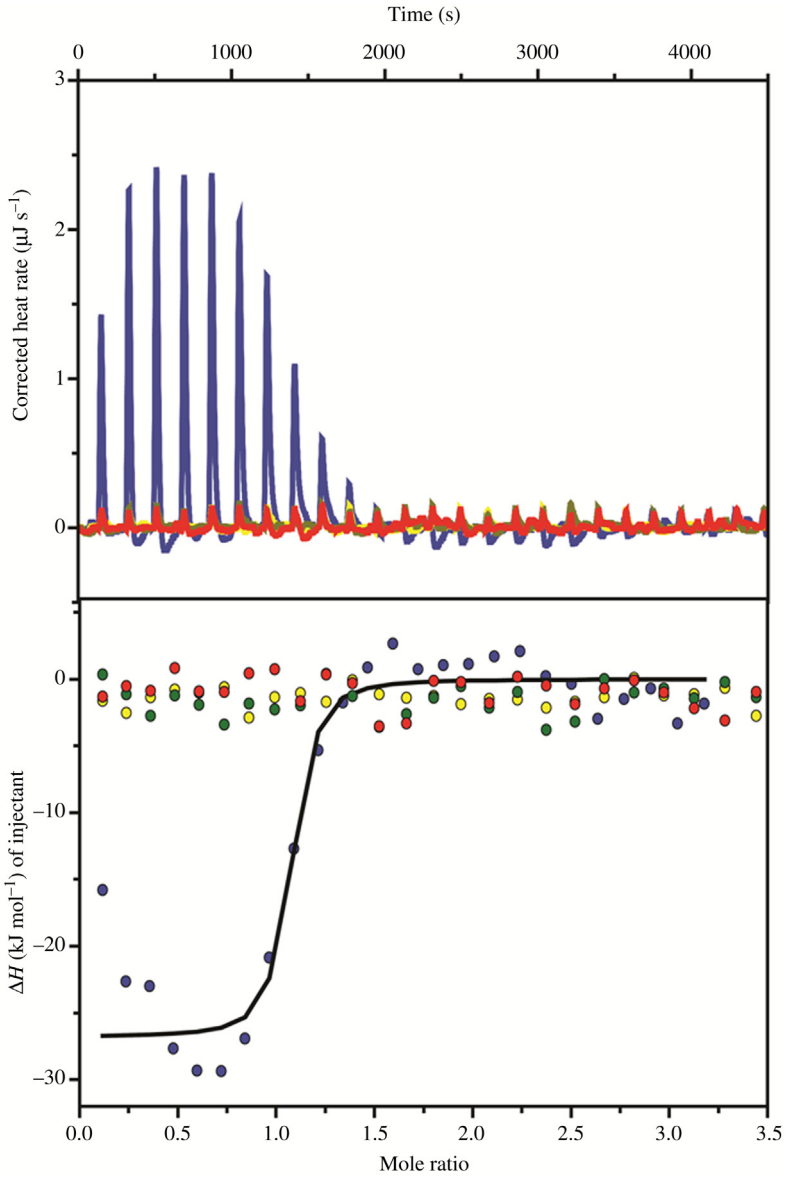


Figure 9.15 Interaction of vacuolar sorting protein 29 form *E. histolytica* with several metals. Upper panel shows the raw data for the binding of EhVps29 to Zn^{++} (*blue*), Mn^{++} (*red*), Mg^{++} (*green*), and Ca^{++} (*yellow*). The lower panel represents the integrated peaks obtained from the raw data for the interaction of EhVps29 with several metals. EhVps29 showed interaction with zinc metal. The other tested metals (Mn^{++}, Mg^{++}, and Ca^{++}) did not show any binding [6].

lectin–carbohydrate interaction reported the importance of positive entropic contributions in stabilizing, making the interaction more feasible and for the increase in affinity for the multivalent carbohydrate compared to monovalent analogs [9–11]. The stoichiometry of EhCRD for the Gal-GalNAc was found to be 0.5. Since EhCRD exists as a dimer in vitro, the fractional value of stoichiometry (0.5) raises the possibility of cross-linking of protein upon carbohydrate binding. Similar phenomenon has also been documented for protein Concanavalin A [12]. The fractional values for stoichiometry (n) for lectin–carbohydrate interactions signify the carbohydrate mediated cross-linking in lectins [13]. Hence, it was proposed that the fractional stoichiometry could be due to the binding of Gal-GalNAc to a cross linked *Eh*CRD.

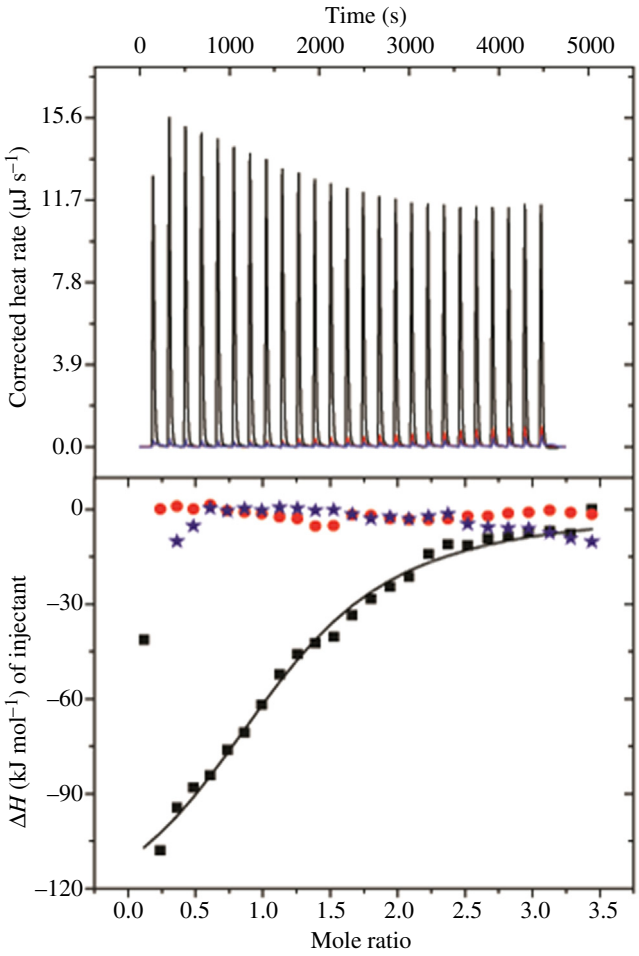


Figure 9.16 Interaction of EhCRD with calcium. Upper panel represents the raw ITC data for the binding of *Eh*CRD to calcium (*black square*) and $MgCl_2$ (*red circle*). The lower panel represents the integrated normalized data fitted into independent model of binding [8].

Table 9.2 Thermodynamic parameters of binding of EhCRD wild type with calcium.

Protein	Ligand	[EhCRD] (μm)	[Ligand] (mM)	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	$T\Delta S$ (kJ mol^{-1})	ΔG (kJ mol^{-1})	K_a (M^{-1})
EhCRD wild type	$CaCl_2$	50	0.5	−136.0	−362.0	−108.0	−27.6	1.0×10^5

Table 9.3 Thermodynamic parameters for the binding of EhCRD to Gal-GalNAC sugar.

Protein	Ligand	[EhCRD] (μm)	[Ligand] (mM)	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	$T\Delta S$ (kJ mol^{-1})	ΔG (kJ mol^{-1})	K_a (M^{-1})
EhCRD wild type	Gal-GalNAc	25	0.1	933.0	3251.0	969.0	−36.0	2.3×10^6

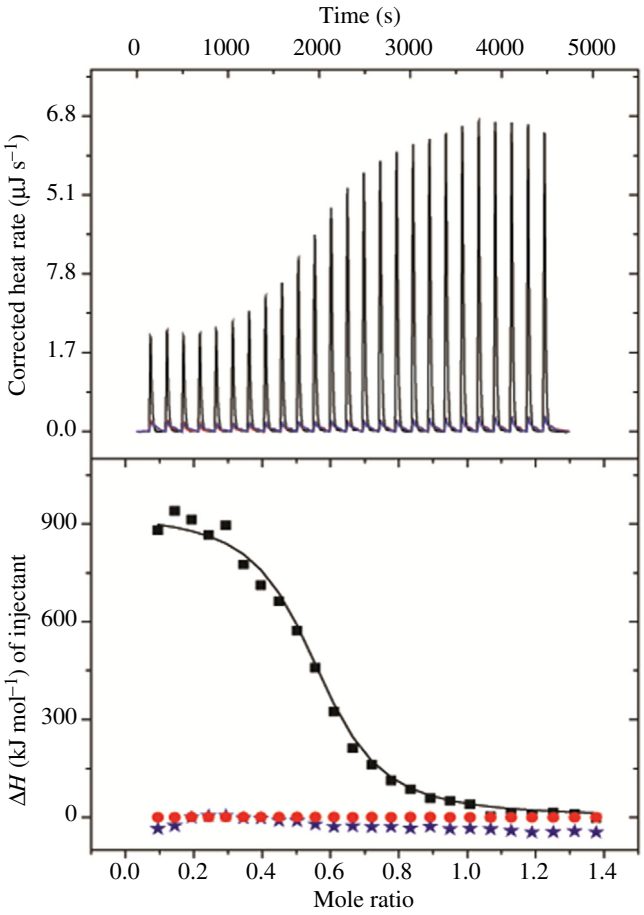


Figure 9.17 Interaction of EhCRD to Gal-GalNAc sugar. Raw data for the interaction of EhCRD to Gal-GalNAc (*black square*) and maltose (*red circle*) is shown in the top panel. The lower panel represents the integrated normalized data fitted into independent model of binding [8].

9.5 Conclusion

The chapter contains the basic knowledge about ITC and how a layperson can perform the experiment to identify the interactions between the two molecules. We have explained the experimental procedure with the help of suitable examples exhibiting exothermic and endothermic reactions, describing in detail the raw data processing to obtain the various thermodynamic parameters. We sincerely hope our efforts will be embraced by students with appreciation and enthusiasm for learning.

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