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Chapter 22

Isothermal Titration Calorimetry to Study Plant Peptide Ligand-Receptor Interactions

Judith Lanooij and Elwira Smakowska-Luzan

Abstract

The field of plant receptor biology has rapidly expanded in the past three decades. However, the demonstration of direct interaction between receptor-ligand pairs remains a challenge. Identifying and quantifying protein-ligand interactions is crucial for understanding how they regulate certain physiological processes. An important aspect is the quantification of different parameters of the interaction, like binding affinity, kinetics, and ligand specificity that drive the formation of signaling complexes. In this chapter, we discuss Isothermal Titration Calorimetry (ITC) as a label-free technique to measure thermodynamic parameters of ligand binding with high accuracy and reproducibility. We provide a detailed guideline how to design, perform, analyze, and interpret ITC measurements using as an example the interaction between the SCHENGEN3/GASSHO1 (SGN3/GSO1) leucine-rich repeat receptor-like kinase and its sulfated peptide ligand CASPARIAN STRIP INTEGRITY FACTOR 2 (CIF2).

Key words Isothermal Titration Calorimetry (ITC), Peptide binding, Protein/peptide interactions, Thermodynamic parameters, Dissociation constant, Binding affinity

1 Introduction

Small, secreted peptides are key components of cell-to-cell communication, allowing plants to defend themselves and regulate their development [1–3]. In general, peptide ligands are perceived via plasma membrane–localized receptor kinases (RKs), which transmit extracellular signals across membranes to induce downstream signaling. RKs perceive a wide range of "self" and "non-self" derived signals, like peptides, hormones, or other bioactive molecules, in the extracellular space [4, 5]. RKs are modular proteins, consisting of a structurally conserved intracellular kinase domain (KD), a single transmembrane domain (TD), and a variable Extracellular Domain (ECD), which facilitates receptor-receptor and receptor-ligand interactions. An enormous number of peptide–receptor pairs are expected to exist given the large number of peptide ligands and RKs in plant genomes, and the possibility that one ligand may

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interact with multiple receptors, and one receptor may recognize multiple ligands. However, to date, only a small portion of those possible pairs have been identified and characterized. Therefore, receptor–ligand pair identification and quantification of their interaction are critical steps toward a system-wide understanding of how peptide ligands, as short- and long-distance signaling molecules, orchestrate plant development and integrate internal cues with external environmental stimuli [3].

A crucial aspect of unraveling the biochemical mechanisms behind cell communication is the assessment of the different parameters characterizing the formation of signaling complexes. There are different techniques available to quantify biomolecular interactions that provide binding affinity (how strong the interaction is between two molecules), kinetics (how fast the interaction happens), and ligand specificity (how specific the interaction is between two molecules). In this book chapter we discuss Isothermal Titration Calorimetry (ITC), a label-free technique for the characterization of macromolecular interactions [6].

ITC directly determines the heat exchange that occurs during complex formation, providing information on the thermodynamics of biomolecular binding processes [7]. ITC measures the heat released (exothermic reaction) or absorbed (endothermic reaction) during the binding reaction and allows the distinction between enthalpic and entropic contributions to the binding mode. It is important to mention that ITC is more reliable for measuring entropy-driven interactions as compared to other methods, like NMR relaxation measurements, that is more appropriate for enthalpy-driven reactions [8, 9].

ITC experiment is performed in a calorimeter that uses a power compensation system to maintain the same temperature between the sample cell (containing the receptor protein) and the reference cell (filled with water or buffer) at each ligand titration (Fig. 1a). During the experiment, a titration system injects precise amounts of ligand to the sample cell causing heat to be released or absorbed (depending on the nature of the reaction), and consequently, a temperature imbalance between the sample and the reference cell will occur. Such imbalance is then rapidly compensated by modulating the feedback power applied to the cell heater [10]. The thermogram generated shows a series of peaks that return to baseline, with the area of each peak corresponding to the heat released or absorbed at each ligand injection (Fig. 1b) [11, 12]. As the receptor-binding site becomes saturated with ligand, the peak area decreases gradually until only dilution heat is observed. The binding curve (Fig. 1c) represents the heat of the reaction per titration/injection as a function of the molar mass ratio between the ligand and the receptor protein. Fitting the binding curve to a specific binding model provides the binding stoichiometry (n); the thermodynamic parameters of the binding reaction (enthalpy, ΔH;

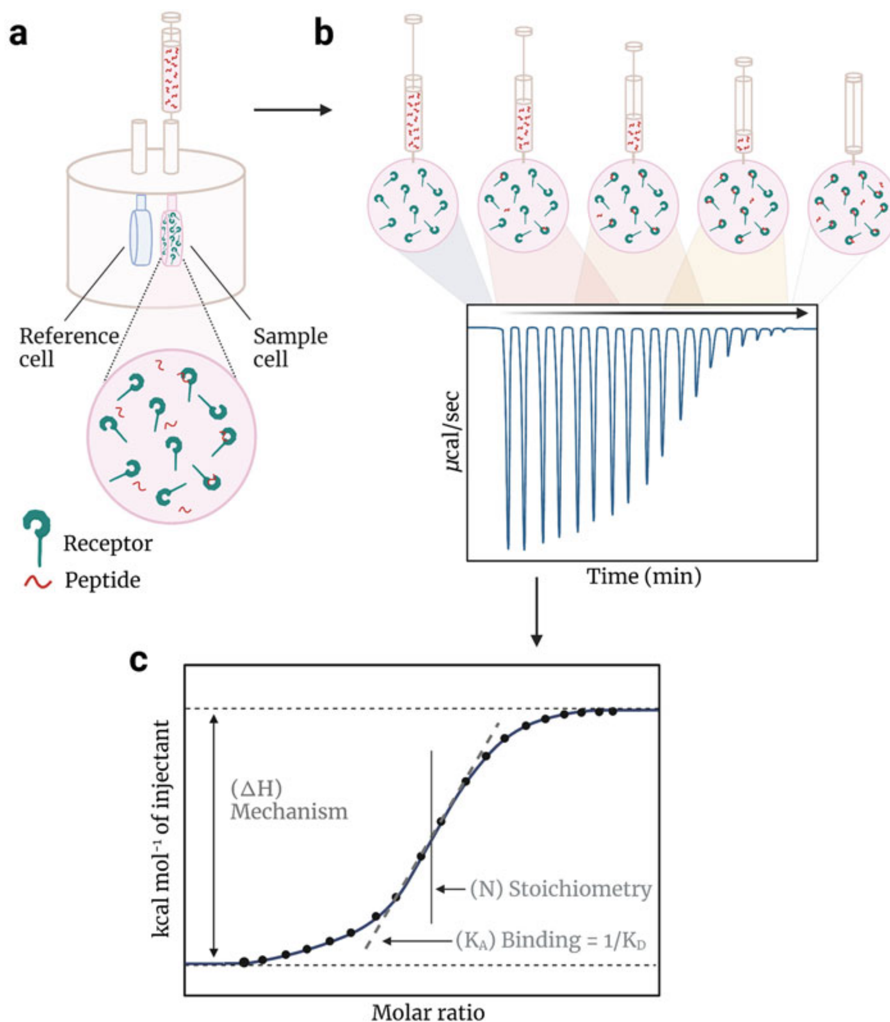

Fig. 1 Basis of Isothermal Titration Calorimetry (ITC). (**a**) The ITC instrument consists of a reference cell (blue) filled with MilliQ water, a sample cell (pink) containing a protein (SGN3 ECD), and an automated injection syringe containing ligand (CIF2 peptide variants) used to titrate the ligand into the sample cell. (**b**) During the ITC measurement, a small volume of the ligand solution, at the defined time intervals, is injected into the cell triggering the binding reaction and producing the characteristic peak sequence in the recorded signal. (**c**) After the measurement is finished, an appropriate software integrates the area under each peak (meaning subtraction of the dilution heat effects and normalization per mol of injected ligand) and the individual heat events are plotted against the molar ratio. Based on this information, using nonlinear regression, it is possible to estimate the thermodynamic parameters N, K_A, and ΔH

entropy, ΔS; and Gibbs free energy, ΔG); the strength of the interaction (the equilibrium association constant K_A, from which the more commonly used equilibrium dissociation constant K_D can be derived) [6, 12].

ITC has two important advantages in comparison to other methods used to assess protein-ligand interactions: (i) the tested

molecules (protein and peptide) are free to move in solution and are not labeled, which ensures a direct characterization of the binding event, unbiased by labeling and/or by limited mobility of the molecules due to their immobilization on a surface; (ii) ITC is the only method that allows a detailed characterization of the binding event by providing not only the binding affinity, but also other critical information including the binding stoichiometry and the thermodynamic parameters [8, 13]. Knowing them can help significantly in the understanding of the molecular mechanism of the binding reaction, even when no structural data are yet available. Furthermore, ITC measurements can be used as complementary data to validate structural results.

The aim of this chapter is to provide detailed know-how on how to set up an ITC experiment, describe the crucial steps and related complications, and provide advice on how to overcome such problems. Moreover, the presented protocol describes the analysis of an ITC experiment measuring the single binding event between the ECD of SGN3 and its peptide ligand CIF2 (sulfated and non-sulfated) [14]. Detailed protocols for the expression and purification of the receptor protein are not included.

2 Materials

2.1 Materials and Reagents

1. Hamilton® syringe, 700 series, fixed needle – different volumes.
2. Disposable borosilicate glass tubes, 0.7 mL.
3. Disposable 5 mL syringe with plastic tubing.
4. 0.22 μm membrane filter.
5. ITC buffer: 50 mM NaH_2PO_4/Na_2HPO_4, pH 7.5, 200 mM NaCl, 5% (v/v) glycerol (*see* **Notes 1** and **2**).
6. MilliQ Water (*see* **Note 3**).
7. Purified receptor protein (*see* **Notes 4–6**): In the experiment described here we used the C-terminally StrepII-9xHis-fused ECD of SGN3. It was produced by secreted expression in baculovirus-infected insect cells and purified by chromatography [14]. Briefly, the ECD of SGN3 was inserted into the baculovirus transfer vectors pMeIBac B1 (Invitrogen) by ligation-independent cloning between the existing Honeybee melittin signal sequence and the C-terminal Strep II-9x histidine tag. The C-terminally tagged SGN3 ECD was harvested from baculovirus-infected High Five insect cells 72 h post-infection. SGN3 ECD was purified by Ni-NTA affinity chromatography (Qiagen) followed by two consecutive runs of gel filtration using a Superdex 200 16/60 column (GE Healthcare) pre-equilibrated with interaction buffer.

Protein purity and identity was checked by SDS-PAGE and anti-His immunoblot (*see* **Note 7**). The protein was subsequently concentrated to 3.5 mg/mL (*see* **Notes 8–10**) via centrifugal filtration with a 20 kDa filter.

8. Peptide ligand: CIF2 (DY(SO_3) GHSSPKPKLVRPPFKLIPN), custom-synthesized in the tyrosine-sulfated and non-sulfated state (*see* **Note 11**). Prepare 10 mM stock solutions of the two peptides in filter-sterilized ITC buffer (*see* **Notes 3, 8, 9,** and **10**).
9. Protein assay: commercial kit for the determination of protein concentration (e.g., the Bradford or Qubit assay systems).

2.2 Equipment

1. Microcalorimeter (we used Malvern Panalytical, model: MicroCal VP-ITC).
2. Vacuum pump (provided together with MicroCal VP-ITC, Malvern Panalytical).
3. Filling syringe (provided together with MicroCal VP-ITC, Malvern Panalytical).
4. 3 mL plastic tubes (provided together with MicroCal VP-ITC, Malvern Panalytical).
5. Benchtop refrigerated centrifuge with rotor.
6. Magnetic stirrer and 7 mm stir bars.
7. UV spectrometer.
8. pH meter.

2.3 Software

1. Software controlling the operation of the VP-ITC calorimeter: VPViewer 2000. Within this software, all ITC experimental parameters are entered, runs are controlled, and data is saved to the hard disk of the computer. When VPViewer 2000 is started it opens a linked copy of a project window in Origin, named VPITCPlot.opj. This project window of Origin is strictly for real-time display of data from VPViewer and is not intended for data analysis or saving data; VPViewer automatically saves all relevant experimental data.
2. Software for data analysis; we used Origin version7 (MicroCal, Malvern Instruments Ltd., RRID: SCR_014212). There is other software like NITPIC for the integration of the ITC data, SEDPHAT for data analysis, and GUSSI which is a program that can illustrate the output of SEDPHAT.

3 Methods

In this section, we first give more general recommendations on how to set up of an ITC experiment (Subheading 3.1). Then we provide the protocol for the characterization of the SGN3-CIF2 interaction by ITC (Subheading 3.2), instructions for the setup of the ITC instrument (Subheading 3.3), and a protocol for data analysis and interpretation (Subheading 3.4).

3.1 General Considerations

1. Decide on how to load sample cell compartment and syringe.

   Typically, the protein is loaded into the sample cell compartment of the calorimeter while the syringe contains the peptide solution (Fig. 1a). This is because it is usually easier to reach high concentrations of the peptide than the protein (*see* **Note 12**).

2. Estimate starting concentrations required for the receptor and the peptide ligand.

   The concentration of the injected ligand should be high enough to reach saturation within the first third to half of the experiment. For a one-site model (when one or several identical sites bind to the same analyte with the same enthalpy and binding affinity), the shape of the titration curve changes according to the product of the K_A, n, and the sample cell concentration (Fig. 2) [12, 15]. The product of these parameters, called the Wiseman "c" parameter, suggests an optimal experimental window where c is between 10 and 500 (for instance for $c = 1$, fitting is not feasible). Figure 2 shows the effect of c on the binding isotherm.

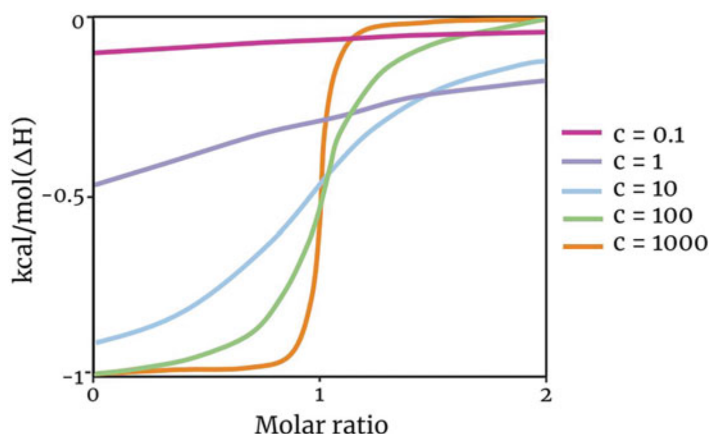

Fig. 2 Simulated ITC titration curves with various c parameters. Each simulated curve has the same ΔH, N, and K_A. Higher c-values result in titration curves that are too steep to resolve K accurately (although n and ΔH are well resolved) because the cell protein concentration is too high relative to K ($c < 100$), whereas lower c-values result in shallow titration curves from which all three parameters (n, K, and ΔH) are poorly resolved ($c = < 10$). Most papers recommend a c-value between 10 and 100

Table 1
Controls and an actual experiment that should be performed to study peptide/protein interactions by ITC

	Run	Syringe	Cell
1	Peptide/Protein	Peptide	Protein
2	Peptide/Buffer	Peptide	Buffer
3	Buffer/Protein	Buffer	Protein
4	Buffer/Buffer	Buffer	Buffer

If the expected stoichiometry of the interaction is 1:1, and assuming that the availability of protein and peptide will not be limiting, a ratio of 10: 1 ([Syringe]: [Cell]) is typically a good starting point (e.g., 100 μM in the sample cell versus 1 mM of the ligand in the syringe) [6, 7]. After obtaining preliminary fit parameters from the first ITC titration, experimental design software can be used to improve the experimental concentrations [6].

3. Choose the proper experimental design.

   Four ITC runs, the experimental run and three controls are required to test how the chosen protein, peptide, and buffers will behave during the ITC assay (Table 1).

   Runs 2 and 3 are important to detect the presence of oligomeric states in the peptide or the protein samples, respectively. For instance, peptide precipitation that may occur at the high concentration used in the syringe will lead to an endothermic response upon dilution into the sample cell filled with the same buffer. Run 4 should not give any signal, unless the two buffers do not match perfectly despite the rigorous preparation of both the syringe and cell solutions (*see* **Note 3**).

3.2 ITC Assay of the SGN3-CIF2 Interaction

To determine the thermodynamic parameters of the SGN3-CIF2 and SGN3-CIF2ns (non-sulfated) interactions, perform the ITC experiment at 25 °C in a microcalorimeter (e.g., VP-ITC Microcalorimeter, Malvern Instruments Ltd).

1. Switch on the computer and the ITC device. Start the VPViewer 2000, software that controls the instrument.
2. Prepare the sulfated (CIF2) and unsulfated (CIF2ns) peptide samples at the final concertation of 12.5 μM in the ITC buffer. The intended volume should be at least 500 μL.
3. Prepare SGN3 ECD at the final concentration of 5 μM in the ITC buffer. The intended volume should be at least 2 mL.
4. Equilibrate all the solutions at room temperature if the experiment is conducted at 20 °C or higher for at least 1 h before

starting the measurement (25 °C for the experiment described here) (*see* **Note 13**).

5. Load the protein and peptide solutions as well as deionized water into the 3 mL plastic tubes together with a 7 mm stir bar and degas them for 5 min using a vacuum pump (*see* **Note 14**).
6. Rinse the reference cell several times with deionized water and the sample cell with the ITC buffer as described by the manufacturer.
7. In the VPViewer 2000 adjust the temperature to 25 °C under the "Thermostat/Calib" tab.
8. Immediately before the experiment, centrifuge the protein and peptide solutions at 2000 × *g* for 2 min to eliminate bubbles.
9. Remove the deionized water from the reference cell and then slowly load the cell with 1600 μL of degassed deionized water (*see* **Note 15**) to allow air bubbles to evacuate through the opening of the cell (*see* **Note 16**).
10. Remove the ITC buffer from the sample cell and then load the cell with 1600 μL of the protein solution (SGN3 ECD) (*see* **Note 16**).
11. Load the injection syringe with 300 μL of the desired peptide solution (CIF2 or CIF2ns). Special attention must be paid to remove any air bubbles from the syringe. The presence of bubbles will lead to unstable baseline and, in consequence, to irreproducible data (*see* **Notes 17** and **18**).
12. Gently wipe the needle with a tissue, and then insert the syringe into the sample cell.
13. Turn on the stirrer at the appropriate speed, typically around 300 rpm, and allow the system to thermally equilibrate until the heat value shown on the calorimeter controller is stable (*see* **Note 19**).

3.3 Data Acquisition

Choosing right ITC run parameters is crucial. In addition to the parameters previously discussed (concentration, stirring speed, cell temperature), pay special attention to the parameters listed below. Use the following settings for:

1. Total Number of Injections: 38. This parameter will be determined by your experimental design and sample concentrations. You will need a minimum of 10–15 injections to define a binding isotherm (*see* **Note 20**).
2. Reference Power: 10 μcal/s. This setting determines the approximate value that the baseline will settle at when the system is equilibrated. Measuring highly exothermic reactions will require a high reference power of about 30 μcal/s, while highly endothermic reactions will require a low reference

power setting of about 2 μcal/s. If you have little information about the expected heats, it will be best to use a reference power setting of 10–15 μcal/s.

3. Volume of injection: 2 μL for the first (*see* **Note 21**), 8 μL for all following injections (*see* **Note 22**). Injection volume is generally between 3 and 15 μL. This range ensures high volumetric accuracy while allowing enough time for the injectant (peptide) to equilibrate to the temperature of the cell before injection. The injectant equilibrates to the cell temperature in the stem prior to reaching the cell. Injections ≥15 μL may result in reduced reproducibility of the injection blank heat. Injection blank heat is the thermal energy associated with the force of the injection and any temperature differences between the cell volume and the injection volume. A water/water and buffer/buffer titration will show the injection blank heat.
4. Duration of the injection: 0.5 μL/s. This is the default value and the slowest rate at which you can inject, which usually does not need to be adjusted. Changes in control peak shape and size that can be obtained by varying the injection duration are very subtle and usually not beneficial.
5. Spacing between injections: 280 s. This parameter is very important. It defines the time interval between two consecutive injections. The spacing needs to be long enough for the baseline to re-establish before the next injection (Fig. 3). Spacing between injections is usually set to 240–360 s (*see* **Note 23**).

Table 2 summarizes the experimental parameters used to estimate affinity of interaction between SGN3 and two versions of its ligand, sulfated CIF2 and non-sulfated CIF2ns (*see* **Note 24**).

3.4 Data Analysis and Interpretation

The most common software for the analysis of calorimetric data is Origin for MicroCal (version7, MicroCal, RRID:SCR_014212). In this section, we provide the protocol for how to process the ITC raw data and for the subsequent analysis. A single-binding model is needed to obtain the binding curve for the SGN3-CIF2 interaction and demonstrate lack of interaction with CIF2ns. Figure 4a shows negative heat changes (μcal/s) during consecutive injections of 8 μL of CIF2 (12.5 μM) into the sample cell containing SGN3 (5 μM). This graph indicates that the SGN3-CIF2 interaction releases heat in the exothermic binding reaction. On the contrary, injections of the non-sulfated CIF2 do not cause any significant heat changes, meaning that there is no interaction. Sulfation is thus required for CIF2 binding to SGN3 ECD (Fig. 4b).

To perform routine ITC Data Analysis and Fitting:

1. Start Origin 7.0 for ITC. The program opens and automatically displays the RawITC plot window.

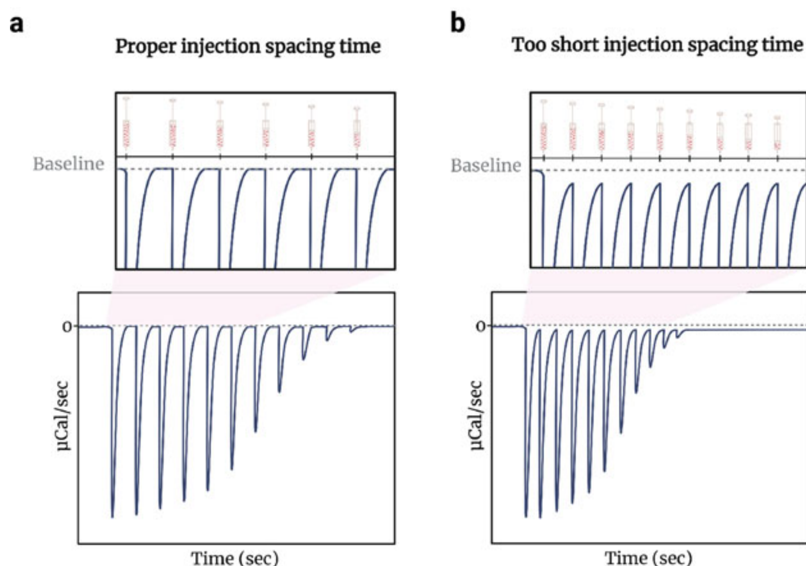

Fig. 3 Spacing between injections: (**a**) an appropriate injection spacing time and (**b**) too short injection spacing time. The heat signal should return to the basal level before proceeding to the next injection. The flat region between two injections will be used to define the baseline, subsequently subtracted from raw data before power integration over time

Table 2
The experimental parameters used to estimate affinity of interaction between SGN3-CIF2 (sulfated) and SGN3-CIF2ns (non-sulfated)

Experimental parameters	CIF2	CIF2ns
Syringe concentration, peptide (µM)	12.5	12.5
Cell concentration, protein (µM)	5	5
Cell temperature (°C)	25	25
Stirring rate (rpm)	300	300
Total number of injections	38	38
Volume, first injection (µL)	2	2
Volume, following injections (µL)	8	8
Injection spacing (s)	280	280
Reference power µcal/s	10	10

2. Click on the *Read Data* button and the dialog box should be open with the *ITC Data* (*.it).
3. Navigate to the *C:\Origin70\Samples* folder, then select *YourExperiment.itc* from the Files list (*see* **Note 25**). After opening

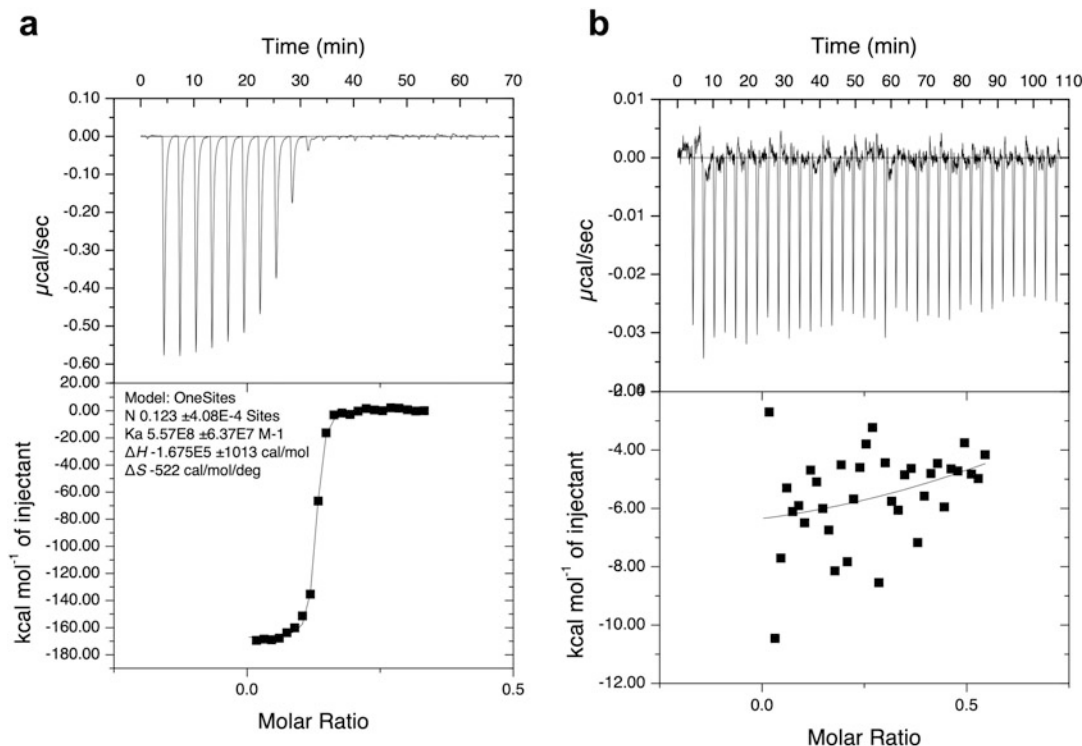

Fig. 4 Determination of CIF2 (**a**) and CIF2ns (**b**) binding affinity with SGN3 extracellular domain by ITC. The association constant (K_A), stoichiometries (N), ΔH, and ΔS are indicated for SGN3-CIF2. No detectable binding dissociation constant was determined for CIF2ns (unsulfated). Similar results were obtained in at least three independent experiments

YourExperiment.itc, this file is read and plotted as a line graph in the *RawITC* window, in units of μcal/second vs. minutes.

4. Manually adjust baseline and integration range for peak integration. Origin performs peak integration automatically. However, two critical integration details, the baseline and the integration range may not be accurate in cases where the signal-to-noise ratio is low. In these cases, both the baseline and the integration range need to be manually adjusted.

5. Origin also automatically opens the DeltaH window and plots the normalized area data rnahhh_ndh, in kcal per mole of injectant versus the molar ratio ligand/macromolecule. The analysis software calculates the area of each peak by integrating the power (μcal/s) over time. Knowing the peptide and protein concentrations, this step results in a plot of heat normalized by the concentration of injectant (leading to the unit of kcal/mol) versus the [peptide]/[protein] molar ratio. The first injection point is systematically discarded to remove the effect of solution diffusion across the syringe tip during the equilibration process (*see* **Note 20**).

6. Select Window: DeltaH to make DeltaH the active window. Before fitting a curve to the data, it is advised to re-check the current concentration values for this experiment.
7. To fit the area data to the "One Set of Sites" model click on the "One Set of Sites" button. A new command menu display bar appears – The Fitting Function Parameters dialog box. Click "1 Iter." to perform a single iteration or "100 Iter." to perform up to 100 iterations. Once you have a good fit, click on the "Done" button and the fitting parameters will be automatically pasted into a text window named "Results Log" and to the DeltaH window in a text label.
8. The fitting procedure yields the association constant (K_A), the stoichiometry ratio (N), and the binding enthalpy (ΔH) and entropy (ΔS).
9. Compare "N" to the expected value of 1.0. "N" is the average number of binding sites per mole of protein in your solution. It is expected to be 1.0 for the one-site non-cooperative binding model of the SGN3 ECD-CIF2 interaction. This expectation is based on the assumptions that all binding sites are identical and independent, you have pure protein (and ligand), you have given the correct protein and ligand concentrations, all your protein is correctly folded and active. This is rarely true in practice! Protein (and ligand) concentration determinations depend on the accuracy of the methods used. Protein extinction coefficients, for example, are rarely known better than ±5%, and are usually worse. Poor measurement techniques, incorrect UV baseline corrections, and attempts to conserve material using "micro" cuvettes, for example, can lead to serious errors. Even if protein concentration is correct, not all the protein may be correctly folded (a common experience with recombinant proteins). Table 3 summarizes potential reasons for N being smaller or higher than 1.

Table 3
Summary of potential reasons for N being smaller or higher than 1

$N < 1$	$N > 1$
Protein concentration is lower than estimated	Protein has multiple binding sites
Protein is impure	Ligand concentration is lower than estimated
Protein is pure but not all correctly folded	Non-cooperative binding model isn't correct
Ligand concentration is higher than estimated	
Non-cooperative binding model isn't correct	

4 Notes

1. Tris-HCl and reducing agent DTT (dithiothreitol) are not recommended in the buffer as they are causing intense heat changes, and this can severely affect the measurements. If reducing agent is required, it is recommended to use β-mercaptoethanol or TCEP (tris(2-carboxyethyl) phosphine) solutions at neutral pH.
2. The buffer used in this protocol was optimized to test the interaction between SGN3 ECD and its ligands. Buffer requirements should be optimized for each individual protein-peptide ligand pair. ITC is compatible with aqueous buffers in the range pH 2–12. However, amine buffers such as Tris should be avoided due to large enthalpy of ionization and substituted with HEPES or phosphate buffer. If there is a need to use glycerol, its final concentration should be kept below 20% (v/v) to avoid bubbles and heat distortions. The presence of organic solvents can cause signal artifacts, so if dimethyl sulfoxide is needed, keep it below 10% (v/v) in all your solutions.
3. Use ultrapure MilliQ water for the preparation of all buffers and solutions.
4. We highly recommend using ITC buffer for storage and dilution of both the ligand and the protein, to avoid buffer mismatch that can greatly contribute to measurement artifacts. Therefore, the last purification step of the protein preparation should also be performed in ITC buffer. All buffers used for protein purification and ITC experiments should be filter-sterilized using a 0.22 μm membrane filter, stored at 4 °C and use within 48 h. Prior to the ITC experiment, degas all buffers and solutions for 5–30 min under vacuum with a stirring rate of about 250 rpm.
5. The expression system and purification procedure will have to be adapted to the protein that is being studied.
6. Try to collect as much information as possible about the protein you want to analyze, particularly with respect to protein synthesis, purification, and characterization. This information will be important for troubleshooting and may be helpful in case you encounter problems with interpreting ITC measurements.
7. Protein identity, purity, and homogeneity can also be assessed by mass spectrometry.
8. The accurate determination of protein and peptide concentrations is essential for determination of high-quality thermodynamic parameters. Ideally, concentration of the protein and

peptide should be determined with an error not larger than 5%. For the determination of protein concentration, several reliable assay systems are commercially available. In these assays, the protein samples are added to the assay reagent, producing a color change (Bradford Assay) or increased fluorescence (Qubit Protein Assay) in proportion to the amount added. Protein concentration is determined by reference to a standard curve generated with known concentrations of a reference protein (e.g., bovine serum albumin). Alternatively, concentration can be determined by absorbance at 280 nm, if the protein or peptide sequence contains at least one aromatic residue, using the molar extinction coefficient that can be accurately calculated from the primary sequence. However, determining the concentration of a polypeptide that does not contain any aromatic residue is more challenging. In that case the absorption of the peptide bond at 205 nm, or 1D 1H quantitative NMR can be used.

9. We advised to perform the ITC experiment immediately after determining protein and peptide concentration to limit the effects of possible changes in sample properties over time.
10. Unless specified otherwise, it is not recommended to freeze the protein and the peptide sample as the physical environment of solution changes dramatically and may impact the protein stability, solubility, and activity. In addition, especially during slow-freezing process, the concentration of the polypeptide remaining in the unfrozen phase is increasing, potentially inducing denaturation and/or precipitation.
11. The choice of peptides depends on the receptor to be studied. CIF2 was chosen here as one of the known ligands of SGN3. Usually, in the ITC experiments synthetic peptides are used. They are obtained by solid-phase peptide synthesis and purified by reverse-phase HPLC. As the ITC is a sensitive and quantitative approach, the synthetic peptide should have the highest quality. A purity grade of >95% is recommended, as contaminants may cause unwanted heat effects.
12. Typically protein concentration ranges from the tens to hundreds in the micromolar range, while the peptide concentration is in the millimolar range. In case that protein and peptide availability are not a limiting factor, preliminary tests should be performed to verify that the protein and the peptide do not precipitate at chosen concentrations and conditions used for ITC. This can be done by increment titration of the peptide into protein solution in a glass vial.
13. Typically, the binding constant and heat of binding will be temperature dependent, but not the stoichiometry of binding.

14. The formation of gas bubbles in the ITC cells during the run needs to be avoided, since the resulting data may become noisy due to bubble-driven liquid displacement effects.
15. It is not necessary to refill the reference cell at every run. However, it is good practice to replace the deionized water at least once a week.
16. To obtain reliable measurements, both reference and sample cells must be precisely and sufficiently filled. Therefore, we recommend using a larger volume (2 mL) compared to the maximum capacity of the cells (1.6 mL). Use a 2.5 mL glass Hamilton® syringe, 700 series and insert it into the cell until you gently touch the bottom with the tip of the syringe. Inject the solution slowly and constantly, while retracting the needle from the cell. In this way, the cell is filled from the bottom to the top, and the operation avoids the formation of bubbles. Position the tip of the syringe on the ledge just below the visible portion of the cell port and draw the liquid to remove any excess of liquid from the cell.
17. Connect the plastic tubing of a filling syringe to the filling port of the injection syringe. Place the peptide ligand in borosilicate glass tubes and insert it into the pipette stand of the machine. Slowly withdraw the plunger of the filling syringe to draw up the solution containing the titrant. Purge the bottom "close fill port" as soon as the titrant solution begins to exit the filling port. Perform a refill step to remove any air bubbles from the injection syringe.
18. To avoid any contaminations, two different syringes should be used to transfer the protein and the peptide solutions, respectively.
19. Faster stirring speeds will increase the baseline noise level but may be necessary if solutions are more viscous than water. Also, when binding is extremely tight, a significant error is observed close to the equivalence point, if the injected ligand solution does not mix completely throughout the entire volume of the sample cell. In those cases, you may obtain better values using a stir speed of 500–600 rpm. Also, in case you are studying particulate suspensions, which tend to settle from gravity, more stirring will be needed to keep a uniform suspension.
20. Do not stop the experiment until your binding is saturated, otherwise the data are harder to fit.
21. The first injection is discarded from the data set to avoid the effect of protein diffusion across the syringe tip during the equilibration process. The volume of this first injection is usually smaller than the following ones to maximize the integrated heat observed within the second injection, which is used to evaluate the binding enthalpy of the reaction.

22. The limiting VP-ITC sensitivity is ~0.1 μcal. For precise measurements, each injection should have an average of at least 3–5 μcal of heat absorbed or evolved. If the heats are too small, then you need to increase either the concentration of the reactants or the injection volume.
23. Sometimes it takes longer for the ligand to find unoccupied binding sites after some (or most) have been filled, so the equilibration time is longer in the middle of an experiment than at the start. 600-s intervals between injections are unusual, but not unheard of.
24. The ITC measurements should be repeated at least three times to have enough data for statistics.
25. Data file names should not begin with a number, nor should they contain any hyphens, periods, or spaces. Also, Origin truncates the filenames to the first 15 characters, therefore when reading in multiple files, the first 15 characters of the filename must be a unique combination to prevent overwriting the data.

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