A universal method for fishing target proteins from mixtures of biomolecules using isothermal titration calorimetry

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Abstract

The most challenging tasks in biology include the identification of (1) the orphan receptor for a ligand, (2) the ligand for an orphan receptor protein, and (3) the target protein(s) for a given drug or a lead compound that are critical for the pharmacological or side effects. At present, several approaches are available, including cell- or animal-based assays, affinity labeling, solid-phase binding assays, surface plasmon resonance, and nuclear magnetic resonance. Most of these techniques are not easy to apply when the target protein is unknown and the compound is not amenable to labeling, chemical modification, or immobilization. Here we demonstrate a new universal method for fishing orphan target proteins from a complex mixture of biomolecules using isothermal titration calorimetry (ITC) as a tracking tool. We took snake venom, a crude mixture of several hundred proteins/peptides, as a model to demonstrate our proposed ITC method in tracking the isolation and purification of two distinct target proteins, a major component and a minor component. Identities of fished out target proteins were confirmed by amino acid sequencing and inhibition assays. This method has the potential to make a significant advancement in the area of identifying orphan target proteins and inhibitor screening in drug discovery and characterization.

Keywords: orphan ligand; orphan receptor; ITC; drug development; tracking method

A tracking method is always crucial for efficiently isolating and identifying the target protein from a complex mixture. Usually such a target tracking system can be customized in a variety of ways based on the properties of the target protein. For instance, if the target protein is an enzyme, an enzymatic assay can be designed to track

and isolate the target protein. Or, if the target protein has the ability to inhibit the tumor invasion in vivo, an animal model can be set up to direct the isolation of the target protein. Alternatively, the detection of specific proteins in complex mixtures such as cell or tissue lysate is carried out by immunological methods involving a protein-specific antibody. With recent advances in tandem mass spectrometry (Biemann 1990; Pasquarello et al. 2004), peptides derived from protein mixtures of considerable complexity can now be routinely identified and characterized in depth. For example, MALDI combined with ion-trap mass spectrometry has been recently used to detect peptide pheromones of known amino acid sequence secreted from microorganisms (Kalkum et al. 2003).

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For tracking known target proteins in vitro, besides the above mentioned methods several alternative methods such as affinity chromatography (Knockaert et al. 2000, 2002; Knockaert and Meijer 2002; Freije and Bischoff 2003; Szardenings et al. 2004), surface plasmon resonance (SPR) (Zhukov et al. 2004), and nuclear magnetic resonance (NMR) (Hajduk and Burns 2002; Hajduk et al. 2002; Fejzo et al. 2003) techniques are available. However, the existing methods most often have several drawbacks. For instance, if the target protein of our search is located on the plasma membrane, labeling or immobilization of this compound (e.g., substrate/inhibitor) might not be ideal and, in addition, this process may affect the activity of the target. Moreover, it is impossible to design an ideal biological assay to detect the target because of the lack of knowledge of the properties of the target protein. To fish out such an orphan target, an efficient and feasible method is needed. Isothermal titration calorimetry (ITC) is a thermodynamic technique for monitoring change in the energy levels of the mixture of components that is initiated by the addition of a binding component at a fixed temperature. When substances interact, the binding energy in the form of heat is either absorbed (endothermic) or released (exothermic). The measurement of this heat allows accurate determination of binding constants (K), reaction stoichiometry (N), enthalpy (ΔH), and entropy (ΔS), thereby providing a complete thermodynamic profile of the molecular interaction in a single experiment (Fisher and Singh 1995; Indyk and Fisher 1998). ITC has become a method widely used for characterizing biomolecular interactions such as protein–protein and protein–ligand interactions. Here we have demonstrated ITC as a tracking tool in the isolation and purification of target proteins. We have used crude snake venom as a model system to illustrate ITC as a potential tracking tool. Snake venoms contain several hundred proteins/peptides or toxins. We have used two specific inhibitors—suramin, a myotoxic inhibitor, and tacrine, an acetylcholinesterase inhibitor. Suramin interacts with a major protein Ecarpholin S (Ser-PLA₂) in *Echis carinatus* snake venom (Zhou et al. 2008), whereas tacrine interacts with a minor protein acetylcholinesterase in *Bungarus fasciatus* venom (Cousin et al. 1996; Frobert et al. 1997). Further, these compounds exhibit high and low affinity to their respective target proteins. We demonstrate these target proteins can be easily isolated and purified from their respective venoms using ITC as a tracking tool throughout the purification strategies. The purified target protein is characterized for their respective enzymatic activities and inhibition. This universal approach could be used to track the isolation and purification of any orphan compound (receptors, ligands, or complementary compounds) from any complex mixture of biomolecules. This method not only helps to track

and isolate the target from the crude mixture when the target is unknown, but also does it efficiently in native conditions, avoiding the need for labeling and other modifications.

Results

ITC measures energy changes in the form of heat during protein–protein and protein–ligand interactions. Since the change in the energy levels is independent of whether the binding interaction occurs between two purified individual components or when they are in a complex mixture, we extrapolated the ITC technique to track the isolation and purification of a target protein molecule. In this study, using ITC we fished out the target proteins for two chosen compounds, suramin and tacrine. To our knowledge, this is the first report of the use of ITC to study crude sample–ligand interactions.

Identification of the target protein for suramin

Suramin is an inhibitor of snake venom PLA₂ enzymes (Zhou et al. 2008). To determine whether suramin interacts with any of the components of *Echis carinatus sochureki* crude venom, we titrated 2 mM suramin against the 2 mg/mL crude venom (Fig. 1A). At each injection, heat is exothermic and the heat change gradually decreases to the basal dilution level. The control (titration of 2 mM suramin against buffer without crude venom) titration pattern is flat (Fig. 1A) and showed no interaction. Thus suramin shows clear interactions with crude venom. *E. carinatus sochureki* crude venom was separated using gel-filtration chromatography into six major protein fractions (Fig. 1B). To determine which of these peaks contain protein(s) that interact with suramin, we performed ITC experiments to determine the affinity of suramin for the different fractions. Similar to the binding of suramin against the crude venom, only peak IV showed a binding isotherm. Titration curves with all other protein peaks were flat, similar to the control. These ITC results clearly indicated that the target protein is located in peak IV. To further isolate the target protein, we used a cation-exchange chromatography to separate the proteins in peak IV into three peaks. ITC experiments showed that only peak B bound to suramin (Fig. 1C). SDS-PAGE was performed to check the purity of peak B, which showed a single band in the gel with an apparent molecular weight of 14 kDa (Fig. 1C, inset). Using ESI-MS, its accurate molecular weight was determined to be 13,806.4. N-terminal sequencing showed that SVVELGKMIHQETGK were the first 15 N-terminal residues. Protein database searches identified this protein to be ecarpholin S, a myotoxin Ser49-PLA₂. We have further characterized the

Figure 1. Identification of the target protein for suramin. (A) Isothermal titration calorimetry (ITC) titration of *E. carinatus sochureki* crude venom with suramin. We titrated 2 mg/mL crude venom with 5 μ L injections of 2 mM suramin. For the blank, 2 mM suramin was titrated into 50 mM Tris-HCl (pH 7.0) without crude venom. (B, upper panel) The first step chromatography of *E. carinatus sochureki* snake venom. We loaded 0.2 g crude venom in 50 mM Tris-HCl (pH 7.0) into Superdex 200 (1.6 $\times$ 60 cm) pre-equilibrated with the same buffer. (Lower panel) Two mM suramin were titrated into each above peak (2 mg/mL). Peak IV showed binding with suramin using the ITC Tracking tool. (C, upper panel) The second step chromatography of *E. carinatus sochureki* snake venom. Peak IV was loaded into HP-SP (5 mL) cation-exchange column pre-equilibrated with 50 mM Tris-HCl (pH 7.0) buffer. Protein was eluted with 0.8 M NaCl at a flow rate of 1 mL/min. (Inset) SDS-PAGE pattern of peak B, left lane is a protein maker; the lowest band is 14 kD. The right lane shows peak B. (Lower panel) Two mM suramin were titrated into each above peak. Only peak B showed binding with suramin. After characterization, peak B is ecarpholin S. The thermodynamic parameters for suramin binding with ecarpholin S were obtained according to the integrated heat plot curve fitting using a fully cooperative two-state model. Conditions: 0.03 mM ecarpholin S titrated with 5 μ L injections of 0.5 mM suramin. Both protein and suramin solutions are in 50 mM Tris-HCl (pH 7.0) and 30°C.

interaction between suramin and ecarpholin S and determined the three-dimensional structure of their complex using X-ray crystallography (Zhou et al. 2008). According to the ITC curve fitting analysis using a fully cooperative two-state model, the thermodynamic parameters of the molecular interaction between ecarpholin S and suramin were obtained. $K = (1.9 \pm 0.3) \times 10^{14}$ (M^{-3}), $\Delta H = -26.7 \pm 0.8$ kcal/mol, $N = 1.09 \pm 0.05$ (Zhou et al. 2008).

Identification of the target protein for tacrine

Tacrine is a competitive inhibitor of acetylcholinesterase (Cousin et al. 1996; Frobert et al. 1997). To determine whether tacrine interacts with any of the components of *B. fasciatus* crude venom, we titrated 0.1 mM tacrine to a solution containing 20 mg/mL of the crude venom (Fig. 2A). Compared with the control, tacrine showed a clear binding to crude venom. The crude venom was first separated into

seven peaks using gel filtration (Fig. 2B). ITC measurements showed that only proteins in peak II interacted with tacrine (Fig. 2B). Peak II was further subjected to anion exchange chromatography (HP Q, 5 mL) and fractionated into four peaks (Fig. 2C). Of these peaks, only the second peak showed binding with tacrine. Subsequently, the second peak was further purified by anion exchange chromatography

(Source Q) to obtain a major peak with shoulder of contaminants (Fig. 2D). ITC clearly showed that this major protein bound to tacrine (Fig. 2D). It showed a single protein band on SDS-PAGE. The identity of this peak was confirmed to be acetylcholinesterase by the enzyme assay. Its activity was 720,000 Ellman units/g. Tacrine inhibits acetylcholinesterase with an IC_{50} value of 5.2×10^{-8} M. These values are

Figure 2. (Legend on next page)

similar to the previous report (Frobert et al. 1997). According to the ITC curve fitting analysis using one set of binding model, the thermodynamic parameters of the molecular interaction between acetylcholinesterase and tacrine were obtained with $N = 0.9 \pm 0.03$, $K = (1.2 \pm 0.1) \times 10^6 \text{ M}^{-1}$, $\Delta H = -5.0 \pm 0.2 \text{ kcal/mol}$, $\Delta S = 10.9 \text{ (cal/mol/K)}$.

Discussion

So far, ITC has been used to characterize specific interactions and complex formation between purified components. Here we have demonstrated that the universal nature of heat release or absorption during binding interactions can be exploited to track and isolate unknown target proteins from complex mixtures of biomolecules using the ITC technique. We verified our strategy using two simple examples from crude snake venom, one with a high content of target protein (>10%) and the other with a low content of target protein (<0.2%). Although target proteins ecarpholin S and acetylcholinesterase were known, we treated them as orphan proteins during the whole procedure and tracked their purification only through ITC. In the end, we confirmed the target proteins using properties such as N-terminal amino acid sequencing and enzyme activity. A basic strategy of our method is outlined in Figure 3. Here we will discuss the advantages, limitations, and applications of this method.

Advantages

Compared to traditional target tracking strategies, the method of tracking ITC has several advantages.

- (1) Universal application: The method is universal. Other traditional methods require different assay systems for tracking. However, the ITC method depends only on the universal phenomenon of heat absorption or release that invariably occurs when two molecules interact; thus, it requires only a calorimeter;

- (2) no modification required: Unlike other methods, it does not require any chemical modification, labeling, or immobilization. At times, these modifications lead to loss of conformation and/or function;
- (3) ease and simplicity for sample preparation: The ITC method can be performed in various buffer conditions, including variation in pH, salt, detergent, and organic solvents, as long as the conditions do not interfere with the main interaction. In other methods, one should also make certain that the conditions do not interfere in the assay measurements;
- (4) high selectivity and sensitivity. The ITC method depends completely on highly precise biological specificity. In other methods, there are possibilities of interference by contaminants in assay conditions. The method is suitable for tracking the purification of low levels of target proteins in the crude mixture of biomolecules;
- (5) rapid and cost effective: The method is fairly fast with new advances. Costs of instrumentation (a single instrument is sufficient for tracking) and reagents are minimal;
- (6) quantitative interaction data: This method can provide quantitative interaction results, leading to the thermodynamic information such as the binding constant (K), reaction stoichiometry (N), and entropy (ΔS), which can be useful in design and development of better ligands (in the case of drug leads).

Limitations

Despite the wide application of the ITC method, it has some inherent limitations.

- (1) Larger volumes: Sample volumes (1.5 mL) needed are relatively large;
- (2) sensitivity: ITC measures the heat released or absorbed at a constant temperature. Our ability to measure this small change is currently limited;

Figure 2. Identification of the target protein for tacrine. (A) Isothermal titration calorimetric titration of *B. fasciatus* crude venom with tacrine. We titrated 20 mg/mL crude venom with 5 μL injections of 0.1 mM tacrine. Both crude venom and tacrine solutions are in 50 mM Tris-HCl (pH 7.0) and 30°C. For the blank, we titrated 0.1 mM tacrine into 50 mM Tris-HCl (pH 7.0) without crude venom. (B, upper panel) The first step chromatography of *B. fasciatus* snake venom. We loaded 0.25 g crude venom in 50 mM Tris-HCl (pH 8.0) into Superdex 75 ($1.6 \times 60 \text{ cm}$) pre-equilibrated with the same buffer. (Lower panel) One mM tacrine titration into the above peaks. Only Peak II showed binding with tacrine using the ITC tracking tool. (C, upper panel) The second step chromatography of *B. fasciatus* snake venom. Peak II from gel filtration was loaded into HP-Q (5 mL) anion-exchange column pre-equilibrated with 50 mM Tris-HCl (pH 8.0) buffer using the AKTA FPLC system. Protein was eluted with 0.8 M NaCl. (Lower panel) One mM tacrine titration into above peaks. Only peak B showed binding. (D, left panel) The third step chromatography of *B. fasciatus* snake venom. Peak B from Q column was loaded into Source Q anion-exchange column pre-equilibrated with 50 mM Tris-HCl (pH 8.0) buffer. Protein was eluted with 0.8 M NaCl. One main peak was obtained. (Inset) SDS-PAGE pattern of the main peak; left lane is protein maker, the right lane shows the main peak. (Right panel) Tacrine titration into the main peak. The main peak showed binding with tacrine. After characterization, the main peak is AChE. The parameter constants for tacrine binding with AChE were obtained according to the integrated heat plot curve fitting using one set of binding model. Conditions: 4 μM AChE titrated with 10- μL injections of 0.2 mM tacrine. Both protein and tacrine solutions are in 50 mM Tris-HCl (pH 8.0) and 30°C.

Figure 3. A schematic representation of the ITC method for fishing orphan target proteins. When we initially titrate a mixture of biomolecules with ligand, if it shows binding, subsequently it will be fractionated and tracked using ITC. After several cycles, the target protein can be identified.

- (3) speed: Until recently, each ITC titration needed 2–3 h;
- (4) lack of automation: Currently available ITC instruments do not have automation. However, some of the commercial companies are keen to overcome these limitations. Recently, a new model, auto-iTC200, (http://www.eventbuilder.com/files/ebe/24078/File/auto-itc200_insert.pdf) claims to use only 200 μ L of sample (5–10 μ g of protein) for each experiment. Furthermore, it can handle 50 samples per day with a capacity to run 384 samples unattended;
- (5) for highly complex systems, such as human blood and cell/tissue lysate, the components are far more complicated than snake venom; the application of this current method has to be approached cautiously, particularly when some of the matrix proteins may nonspecifically bind to the ligand. In such complex mixtures, the ligand may interact with more than one protein. In such cases, each of the target proteins should be purified, and further interactions with individual proteins should be validated carefully, keeping in view various factors such as their compartmentalization and accessibility;

- (6) trial and error: The first round ITC test is the key part for this method. One has to vary the ITC running parameters (especially the sample concentration) to obtain the final conclusion. For instance, in this study, the high concentration of (20 mg/mL) *B. fasciatus* venom shows binding with the inhibitor, and the low concentration shows the lack of significant binding (data not shown) as the amount of AChE in the venom is fairly low. However this could be avoided with the improved sensitivity of new ITC machines.

Applications

Based on its property, the ITC method has several applications in the area of drug discovery and development as well as basic sciences.

- (1) Drug discovery: The ITC tracking strategy can be exploited particularly in the area of drug discovery and isolation of interacting ligands from a crude mixture when the target protein (receptor/ion channel/enzyme) is known;
- (2) drug mechanism: Compounds often selected for further development and clinical testing are based on their positive biological responses with little or no knowledge of their biological targets. The lack of knowledge of its mechanism of action can severely impact the likelihood of its usefulness. In these cases, the ITC method may be useful in tracking the purification and identification of the target protein;
- (3) side effects profile: The issue of toxicity can often arise during the development of drug candidates. Such undesired effects often occur through interactions of the drug with proteins other than the desired efficacy target(s). The identification of these off-target proteins using ITC method may be a first step toward the improvement of drug or lead compounds;
- (4) hunt for orphan receptors or ligands: In the post-genomic era, understanding the function of protein products is of primary importance. This is achieved by newer methods including yeast two hybrid systems and interactomes. The ITC method as a powerful tool can contribute significantly in the identification of orphan target receptors or ligands and help us solve many biological problems.

Materials and Methods

Materials

Lyophilized *E. carinatus sochureki* (saw-scaled) snake venom was bought from Latoxan. Lyophilized *B. fasciatus* crude venom was obtained from Venom Supplies Pty Ltd. Suramin (8, 80-[carbonylbis [imino-3,1-phenylenecarbonylimino (4-methyl-3,1-phenylene) carbonylimino]] bis-1,3,5-naphthalenetrisulfonic

acid hexasodium salt) and tacrine (1,2,3,4-tetrahydroacridin-9-amine) were obtained from Sigma Chemical Co. All other reagents were of analytical grade.

Isothermal titration calorimetry (ITC) and purification of target proteins

Crude venom or fractions titration with suramin were performed in a VP-ITC titration calorimetric system (MicroCal, LLC) at 30°C. In the first step, ITC was used to study the binding pattern of suramin titration into crude venom. Solutions of 2 mg/mL *E. carinatus sochureki* venom in 50 mM Tris-HCl (pH 7.0) were titrated with 2 mM suramin in the same buffer. The reaction cell contained 1.5 mL of the crude venom solution. Suramin was added in 5-μL increments at 500-sec intervals. Blank injections of suramin solution into same buffer in the absence of venom were used to account for the heats of mixing and dilution. Crude venom was later separated into several peaks using a Superdex 200 gel-filtration column (1.6 × 60 cm; Amersham Biosciences), and each peak was tracked using ITC to determine whether the peak(s) shows any interaction with suramin or not. The next step was to further isolate the tracked peak to get the pure proteins using ion-exchange (HP-SP, 5 mL; Amersham Biosciences). The last step is to determine which pure protein can interact with suramin using ITC. In this step the binding constants were estimated from the obtained isotherms using the calorimetric analysis program Origin (Todd and Gomez 2001). A similar procedure was followed to identify the target protein of tacrine from *B. fasciatus* crude venom, using ITC as a tracking tool and chromatography for isolating the target protein.

Acetylcholinesterase enzyme assay

AChE activity was measured by means of the colorimetric method of Ellman et al. (1961) using acetylthiocholine iodide as substrate. One Ellman unit was defined as the amount of enzyme producing an absorbance increase of 1 unit (412 nm) at 25°C, in 1 min, in 1 mL of medium and for an optical path length of 1 cm. The inhibitory assay was carried out as described by Frobert et al. (1997).

Electrospray ionization mass spectrometry

The homogeneity and mass of the target proteins were determined by electrospray ionization mass spectrometry using a PerkinElmer Life Sciences API-300 liquid chromatography/tandem mass spectrometry system. Ion spray, orifice, and ring voltages were set at 4600, 50, and 350 V, respectively. Nitrogen was used as a nebulizer and curtain gas. A Shimadzu LC-10AD pump was used for solvent delivery (40% acetonitrile in 0.1% trifluoroacetic acid) at a flow rate of 50 μL/min. BioMultiview software (PerkinElmer Life Sciences) was used to analyze and deconvolute raw mass spectra.

N-terminal sequencing

N-terminal sequencing of the target proteins was performed by automated Edman degradation using a PerkinElmer Life Sciences Model 494 pulsed liquid-phase sequencer (Procise)

with an on-line Model 785A phenylthiohydantoin-derivative analyzer.

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