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Isothermal titration calorimetry for characterization of recombinant proteins

Lina Baranauskiene[1], Tai-Chih Kuo[2], Wen-Yih Chen[3] and Daumantas Matulis[1]

Isothermal titration calorimetry is widely used to measure the affinities and enthalpies of interaction between proteins and/or small molecules. The quantitative nature of the technique is especially useful in the characterization of recombinant proteins while determining the fraction of protein capable of binding a specific ligand and thus the protein purity. The revealed thermodynamic information sheds light on the binding mechanism, important for the targeted drug design of the biologics. Here we show examples how, together with the thermal shift assay, combination of both techniques enables characterization of protein stability and ligand binding. Furthermore, the binding-linked reactions that strongly affect the observed thermodynamic parameters and must be dissected to obtain the intrinsic parameters that are necessary for the structure-based rational drug design are being demonstrated using inhibitors of Hsp90, an anticancer target protein.

Addresses
[1] Department of Biothermodynamics and Drug Design, Institute of Biotechnology, Life Sciences Center, Vilnius University, Saulėtekio 7, 10257 Vilnius, Lithuania
[2] Department of Biochemistry, Taipei Medical University, Taipei, Taiwan
[3] Department of Chemical and Materials Engineering, National Central University, Jhong-Li, Taoyuan City, Taiwan

Corresponding author: Chen, Wen-Yih (wychen@ncu.edu.tw)

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Introduction

Isothermal titration calorimetry (ITC) is a biophysical technique widely used to determine the thermodynamics of interaction between soluble proteins and/or small molecular weight ligands that specifically bind them [1]. ITC is the only technique that directly determines the enthalpy change occurring upon molecular interactions under isothermal well-controlled [2•]. The quantitative aspect of the titration feature can be used to determine the purity of the recombinantly produced protein if a specific ligand is available. The technique also confirms that the protein is of sufficient solubility and stability in aqueous solution. The demonstration that 80, 95, or even 99% of the produced protein is capable of binding a specific ligand confirms the quality of the preparation to a greatest extent among hour-long experimental techniques. In this review we focus on the applicability of ITC for recombinant protein quality control describing the main strengths and limitations of the method and presenting examples of application . Determination of the intrinsic thermodynamic parameters, namely, parameters that are independent of binding-linked reactions, is an important and often overlooked characteristic of the interaction [3•].

Significance of protein quality

Protein quality is of critical importance both for basic research and industry applications. While reading a scientific manuscript one concentrates on the results and assumes that the proteins were of the best possible quality, that is, chemically pure, fully active, of precise concentration, and thus all the observed phenomena and effects are valid. However, this is not always the case and the protein quality highly influences the repeatability of the results. Therefore, the scientific community promotes more thorough description of protein quality analysis in scientific manuscripts [4,5]. In the pharmaceutical industry, the protein quality is of utmost importance for protein-based therapeutics [6–8]. The fraction of biopharmaceuticals is increasing among drugs. Biopharmaceuticals are defined as substances used for therapeutic or diagnostic purposes and produced by biotechnological means or extracted from biological sources. Recombinantly produced proteins make the majority of all biopharmaceuticals, including antibodies, growth factors, hormones, enzymes, and other proteins [7,9].

Oliveira and Domingues recently drew guidelines for the preparation of high-quality proteins [10]. They distinguished three most important aspects of protein quality: purity, homogeneity and structural conformity. Protein purity is usually evaluated by SDS-PAGE/capillary electrophoresis methods while the homogeneity is determined by dynamic light scattering/size exclusion chromatography. The structural conformity requires more extensive experimental evidence. Preliminary protein secondary structure tests can be performed using UV/VIS absorbance,

fluorescence, CD, FT-IR, and other spectroscopic techniques. The detailed structure of the protein may be determined by nuclear magnetic resonance and X-ray crystallography, but these techniques are too expensive and consume too much time for routine analysis.

The demonstration of protein biological activity is the most direct proof that the protein is of correct 3D structure [[12•],11]. Functional activity of clinically relevant enzymes can be measured by quantifying their catalytic activity. However, reactions, substrates, and products are specific for each enzyme, and many proteins do not possess catalytic activity and therefore other biological assays, including cell-based and animal-based methods are used. Instead, proper protein structure can be demonstrated by showing interaction with a biological partner or a synthetic small-molecule ligand. The binding may be demonstrated using various methods, such as ELISA, surface plasmon resonance, thermal-shift assays (differential scanning calorimetry, fluorescence-based thermal shift assay (FTSA), cellular thermal shift assay [12•]), FRET, AlphaScreen, and numerous other techniques. One of them — ITC is often called the 'golden standard' in the binding analysis. Its direct observable, the heat, is a universal signal because heat is absorbed or emitted in practically every reaction.

Advantages and limitations of ITC

ITC is a titration-based method for binding interaction analysis, performed in solution at constant temperature where only the thermal effects are measured. Heat is the universal signal — practically every reaction is accompanied by absorbed or released heat. Therefore this method can be applied to observe interaction of any type: protein with a small molecule or with another protein. ITC is a straightforward and noninvasive method — no additional steps for signal detection are required. It has no molecular weight limitations or constraints of optical properties. No labeling, immobilization or any other chemical modification is required.

The analysis of ITC curve gives these thermodynamic parameters: the change in enthalpy ΔH, binding constant K_b, and the fractional saturation parameter n. The Gibbs energy of binding can be calculated $\Delta G = -RT \ln K_b$, where R is the universal gas constant, and T is temperature in K. The change in entropy can be determined from the relationship $\Delta G = \Delta H - T\Delta S$. There are useful new tools for the analysis of ITC data that help the user including SEDPHAT [13], NitPic [14•], and Affinimeter [15].

We also highly support presentation of raw data with nonstraightened baselines as emphasized here [16]. Biophysical description of antibodies using ITC was also recently described in great detail [17••].

The greatest advantage of ITC is its ability to provide a comprehensive thermodynamic picture of an interacting system in a single experiment (including ΔH, K_b or K_d, ΔG, and ΔS), and it also determines the stoichiometry of interaction and the fraction of binding-capable protein. When binding model is established (e.g. 1:1), the parameter n can be used to calculate the concentration of active protein. The precision of protein and ligand concentration determination is crucial since they highly influence the results. Furthermore, ITC is highly versatile — it can be performed in various formulations, including the presence of other proteins or even in blood plasma [18]. Instrumentation is simple and straightforward to use, with low maintenance costs. Fast results could be obtained in an hour with modern ITC instruments. Automated sample analysis is possible since there are automated versions of instruments that enable higher throughput.

One of the major disadvantages limiting the use of ITC is that quite large amount of interacting materials is required. Both the protein and the ligand have to be in pure form. Both the protein and the ligand have to be sufficiently soluble in water (at least 10–100 μM) and stable in solution at studied temperature for an hour under stirring. Nevertheless, the unstability of the protein in solution can be observed while following the heat signal change as function of time. There should be no interfering reactions occurring during binding such as possible oxidation–reduction if, for example, oxygen reacts with the sulfhydryl groups of reducing agents such as dithiothreitol.

Application of ITC for protein characterization through interactions

ITC is not expected to fully replace biological tests. For example, while different glycosylation of a protein may have no or little effect on its ligand binding ability, but may alter the stability properties *in vivo*, immunogenicity and affect the drug clearance time (bioavailability and bioequilibrium BA, BE). However there are many reasons for prioritizing simple *in vitro* protein activity assays since they are much faster and less expensive compared to animal-based assays. Functional activity and specific ligand binding tests are also important confirmation when an affinity-tag is used in purification and not cleaved before other experiments.

Biosimilars among biotechnological products resemble generics in pharmaceutical drugs. However, due to protein structure complexity their production may be difficult to repeat under identical conditions and the proteins can be made by different production methods resulting in different posttranslational modifications. Their similarity to the original product is strictly regulated and must be demonstrated considering structural and functional properties, including safety and effectiveness [19,20]. Lopez-Moralez *et al.* extensively characterized biosimilar