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PHYSICAL AND BIOPHYSICAL CHEMISTRY DIVISION\*

# RECOMMENDATIONS ON MEASUREMENT AND ANALYSIS OF RESULTS OBTAINED ON BIOLOGICAL SUBSTANCES USING ISOTHERMAL TITRATION CALORIMETRY

(IUPAC Technical Report)

*Prepared for publication by*

FREDERICK P. SCHWARZ<sup>1,‡</sup>, TIMM REINISCH<sup>2</sup>, HANS-JÜRGEN HINZ<sup>2</sup>,  
AND AVADHESHA SUROLIA<sup>3</sup>

<sup>1</sup>Center for Advanced Research in Biotechnology/National Institute of Standards and Technology,  
9600 Gudelsky Drive, Rockville, Maryland 20850, USA; <sup>2</sup>Institut für Physikalische Chemie,  
Westfälische Wilhelms-Universität, Schlossplatz 4/7 D-48149, Münster, Germany; <sup>3</sup>National Institute  
of Immunology, Aruna Asaf Ali Marg, New Delhi, India-110067

\*Membership of the Physical and Biophysical Chemistry Division Committee during the final preparation of this report was as follows:

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<sup>‡</sup>Corresponding author: E-mail: frederick.schwarz@nist.gov

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# Recommendations on measurement and analysis of results obtained on biological substances using isothermal titration calorimetry

## (IUPAC Technical Report)

*Abstract:* Isothermal titration calorimetry (ITC) is widely used to determine the thermodynamics of biological interactions including protein–protein, small molecule–protein, protein–DNA, small molecule–DNA, and antigen–antibody interactions. An ITC measurement consists of monitoring the transfer of heat between an analyte solution in a sample vessel and a reference solution in a reference vessel upon injection of a small aliquot of titrant solution into the sample vessel at a fixed ITC operating temperature. A binding isotherm is generated from the heat-transferred-per-injection data and values for the binding constants, the apparent binding enthalpies, and the apparent ratio of the amount of titrant to analyte for the binding reaction are then determined from fits of a binding model, whether it is a single site, identical multi-site, or an interacting multi-site binding model, to the binding isotherm. Prior to the fitting procedure, corrections should be made for contributions from extraneous heat of mixing determined separately from injections of the titrant into just the dialysate/buffer solution. Ultra-high binding constants, which cannot be directly determined from an ITC measurement, can be determined by a displacement ITC method where injections of the tight-binding titrant into a solution of a weaker-binding titrant-analyte complex displaces the weaker-binding titrant from the complex. The Michaelis and catalytic constants can be determined for an enzyme reaction from injections of a substrate or enzyme titrant into an enzyme or substrate analyte solution. Several binding reactions are suggested to check the operating performance of the ITC. The reporting of ITC results must be specific with regard to the composition of the titrant and the analyte solutions, the temperature, and the model used in the analysis.

*Keywords:* isothermal titration calorimetry; recommendations; biological substances; measurements; analysis; binding thermodynamics; binding constants; proteins; ligands; enzyme kinetics; IUPAC Physical and Biophysical Chemistry Division.

## CONTENTS

1. INTRODUCTION
2. RECOMMENDATIONS FOR ITC MEASUREMENTS
  - 2.1 Experimental design
  - 2.2 ITC measurement procedures
  - 2.3 ITC calibration and test procedures
3. RECOMMENDATIONS FOR THE PRESENTATION OF ITC DATA
  - 3.1 Introduction
  - 3.2 Correction for the apparent titrant to analyte amounts in the sample vessel

- 3.3 Corrections for extraneous heats of mixing, heats of dilution, and for heats of protonation [3]
  - 3.4 Conversion of the ITC data to a binding isotherm
  - 3.5 Conversion of the ITC kinetics data to an initial reaction rate plot
  4. RECOMMENDATIONS FOR THE ANALYSIS OF ITC DATA
    - 4.1 Introduction
    - 4.2 Non-interactive multiple-site binding model
    - 4.3 Interactive multiple-site binding model
    - 4.4 Ultra-tight binding analysis by displacement ITC.
    - 4.5 Enzymatic conversion of substrate to product
  5. RECOMMENDATIONS ON THE REPORTING OF RESULTS
- LIST OF SYMBOLS  
ACKNOWLEDGMENT  
REFERENCES

## 1. INTRODUCTION

Isothermal titration calorimetry (ITC) is widely used to study the thermodynamics of biological interactions in solution. The range of interactions studied by ITC is very general and covers, although not exclusively, metal ion–protein, sugar–protein, small molecule–protein, DNA–protein, small molecule–DNA, lipid–protein, antigen–antibody, and protein–protein interactions. Basically, an ITC instrument, as shown in Fig. 1, consists of a sample vessel containing the analyte solution and a vessel containing a reference solution (e.g., buffer solution) within an isothermal enclosure. A syringe is positioned above the sample vessel so that small aliquots of a titrant can be titrated into and mixed with the analyte in the sample vessel [1,2]. Upon an injection of the titrant into the analyte solution, a transfer of heat occurs within the isothermal enclosure that is monitored as a function of the number of injections or the amount of titrant added to the analyte solution. The transfer of heat is monitored either through the temperature differences between the reference vessel and a constant temperature block and the sample vessel and the constant temperature block or through power compensation into the sample vessel to maintain it at the same temperature as the reference vessel relative to the temperature of the jacket. The transfer of power per injection is converted to a transfer of heat by integration of the transfer of power pulse over the time. The titration is continued until the transfer of heat reaches a constant minimum value. The ITC binding data are then converted to a binding isotherm where the transfer of heat per amount of titrant injected, corrected for extraneous heat effects, is plotted against the number of injections or the ratio of the amounts of titrant to analyte. The thermodynamic parameters determined from fitting a binding model to the binding isotherm data are the binding constant ( $K$ ), the apparent ratio of the amount of titrant to the amount of analyte, and the change in the standard enthalpy ( $\Delta_r H^\circ$ ) per amount of titrant in the binding reaction or the reaction enthalpy. The binding enthalpy may also include heat contributions from heats of protonation of the buffer if proton transfer occurs in the binding reaction and/or heat contributions from conformational changes occurring in the analyte and/or titrant upon binding. (The more familiar term “protonation” is used instead of the preferred IUPAC term “hydronation” for reaction with the  $H^+$  cation without regard for the nuclear mass of the hydrogen element in its natural abundance or as a specific isotope of hydrogen [3].) The change in the standard entropy of the reaction or binding entropy can then be determined from the fundamental equation of thermodynamics

$$\Delta_r G^\circ = \Delta_r H^\circ - T\Delta_r S^\circ \quad (1)$$

To increase the rate of data acquisition in an ITC measurement, an alternative ITC method consisting of a single slow continuous injection of titrant has been developed in place of the more conventional incremental ITC method just described [4].

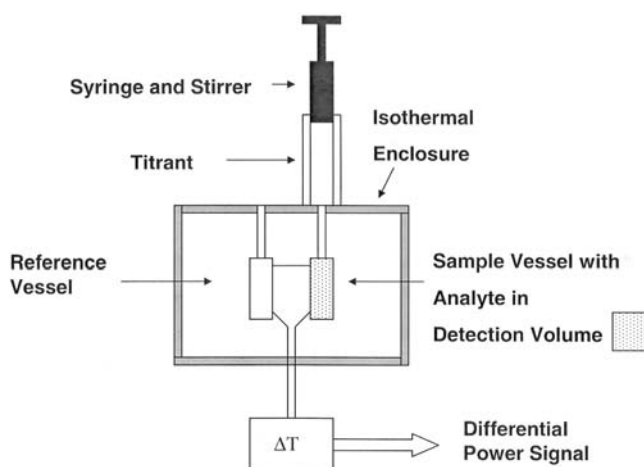


Fig. 1

There are several advantages of ITC compared to other methods for determining the thermodynamics of biological interactions. ITC is an in situ method universally applicable to a wide range of biological interactions. Since ITC is based on the assumption of equilibrium in the sample vessel, it provides the change of thermodynamic-state functions in a single binding reaction and is amenable to determining the dependencies of the change of state functions on temperature. The advantages of ITC were recognized early, and publications describing this method appeared in 1966 [5] and in the 1970s [6,7]. More recently, ITC has been employed to determine the Michaelis constant ( $K_m$ ) and the catalytic rate constant ( $k_{cat}$ ) for enzymatic reactions following the Michaelis–Menten model [8,9]. The kinetic parameters are determined from the dependence of the transfer of power upon mixing the substrate with the enzyme in the sample vessel as a function of the concentration of substrate. This may involve a single injection of the enzyme or substrate titrant [8] or multiple injections of the substrate titrant into the enzyme analyte in the sample vessel [9]. For both the ITC binding and enzyme kinetics measurements, there are several factors involved in the operation and analysis of the ITC data that have to be addressed to ensure universal uniformity in the results and in the reporting of the results. To optimize the scientific value of studies that employ ITC measurements on biological systems, a set of recommendations on the measurement procedures and on the analysis of ITC results are presented in this report. This is particularly important because of the exponential growth in the number of publications on ITC measurements since the late 1980s when commercial ITC instruments with small sample vessel volume sizes on the order of  $\text{cm}^3$  first became available.

A number of reviews on the application of ITC to the study of molecular interactions in biological systems have appeared over the past two decades. Reviews on general applications of ITC in studies of biological systems have appeared in *Analytical Chemistry* [10], *Methods in Enzymology* [11,12], *Methods in Molecular Biology* [13], and on an annual basis in the *Journal of Molecular Recognition* [14]. Specific reviews on drug–DNA interactions [15,16], on lectin–carbohydrate interactions [17], on membrane interactions [18], and on antigen–antibody interactions [19] are also available in the literature. More recent reviews on ITC applications have appeared on protein–protein interactions [20], protein–small molecule interactions [21,22], and drug discovery [23,24]. In addition, several studies have appeared that verify the accuracy of the binding thermodynamic parameters determined from ITC measurements by comparison with the binding thermodynamic parameters determined by other methods [25–27]. Although most reactions studied by ITC are simple one-to-one binding interactions, a number of multiple-binding site interactions that involve cooperativity between the sites and allostery have also been investigated by ITC [28]. These latter interactions exhibit more complex ITC bind-

ing isotherms and, thus, rely on the use of interactive binding models in the analysis of ITC data. ITC has also been employed to determine the effects of various ion concentrations in the solvent, pH, solvent composition, and temperature on the thermodynamics of the binding interactions and how these interactions are linked to structure [29,30]. The involvement of proton transfer in the reactions has also been demonstrated by the contributions of the heats of protonation of different buffers to the reaction enthalpies in ITC measurements [31]. Some progress has been made in relating the thermodynamic binding parameters to structural information based on changes in the solvent-accessible surface area of the analyte determined from X-ray crystal structures [32]. For example, ITC measurements on the binding of the lysozyme antigen to a heterodimer consisting of only the light and heavy chain variable domains of an antibody as a function of single mutations at the binding interface have shown how changes in the binding affinity and enthalpy can be correlated with changes in the solvent-accessible surface at the binding interface [33]. This correlation was based on the earlier development of empirical equations that correlate changes in the solvent-accessible surface areas of the nonpolar and polar residues at the binding interface with changes in the binding enthalpy and the binding heat capacity changes [34]. The widespread use of ITC has led to the development of a database for storing raw ITC data from different laboratories in order to make it accessible for analysis by different binding models [35]. Although ITC is applicable to the measurement of binding affinities below  $10^9 \text{ mol}^{-1} \text{ dm}^3$ , a method which involves displacement of a weak binding ligand at a binding site by a high-affinity ligand has been developed to extend the determinations of binding constants to as high as  $10^{12} \text{ mol}^{-1} \text{ dm}^3$ , in ITC measurements [36].

## 2. RECOMMENDATIONS FOR ITC MEASUREMENTS

### 2.1 Experimental design

A recent study of the results from 14 research laboratories that performed ITC measurements on the binding of the inhibitor 4-carboxybenzenesulfonamide (CBS) (4-sulfamoylbenzoic acid) to the enzyme bovine carbonic anhydrase II (CAII) indicated several important sources of uncertainty in the determination of the thermodynamic binding parameters by ITC [27]. Participants in the study were sent the materials with information only on the magnitude of the CBS binding affinity and were allowed to determine their own experimental design for the ITC measurements. Isothermal titration calorimeters from different manufacturers including different software programs for analysis of the results were employed in the study. It was shown that the results were critically dependent on the following factors [27]:

- i. accurate determination of the inhibitor titrant concentration in the syringe;
- ii. range of CAII solution concentrations so that the product of  $K$  [CAII] is from 10 to 1000; and
- iii. the method of correcting the heats of binding for the extraneous heats of mixing and of dilution generated by injecting the more concentrated CBS titrant into the CAII analyte solution.

Heats of mixing typically arise from differences in the pH and ion concentrations between the titrant and the analyte solution while heats of dilution arise from dilution of the titrant in the sample vessel. In the study, the corrections for the heat of mixing and any heats of dilution were either ignored or taken into account in a number of ways [27]. Factors i to iii thus have to be taken into consideration in the design of any ITC experiment.

When planning the preparation of the titrant and analyte solutions for an ITC measurement, the accurate determination of the titrant and analyte concentrations should be carefully considered as well as the correct range of concentrations of the analyte solution. To minimize the heat of mixing from differences in the solvent composition including pH and ion concentration, the titrant and analyte solutions are dialyzed in the same buffer. For a titrant solute with a low molecular mass, where this is not possi-

ble, the analyte solution is dialyzed in the buffer solution and the resulting dialysate is then used to prepare the titrant solution by weighing the solute into the dialysate.

The concentration range of the analyte solution for binding reactions is limited by the values of  $K$  and  $\Delta_r H^\circ$  such that the concentration is high enough to detect an exchange of heat but low enough so that the product of  $K[A]$  is in the range of 10–1000. (The equilibrium constants,  $K$ , are based on the amount-of-substance concentrations used.) The analyte concentration is also limited by the detection of heat from reactions with low values of  $\Delta_r H^\circ$ . For example, if for an ITC with a detection volume of the analyte in the sample vessel of 1.5 cm<sup>3</sup> and a detection limit of 0.1  $\mu$ J, then for a binding reaction with  $\Delta_r H^\circ = 1$  kJ mol<sup>-1</sup>,  $[A] > 0.7$   $\mu$ mol dm<sup>-3</sup> to detect a transfer of heat at 10  $\times$  detection level. However, a precise value for a  $K > 1.4 \times 10^9$  mol<sup>-1</sup> dm<sup>3</sup>, can only be determined at  $[A] < 0.7$   $\mu$ mol dm<sup>-3</sup>. For reactions with larger binding enthalpies, higher values of  $K$  can be determined using lower analyte concentrations provided that  $K[A]$  is in the range of 10–1000. A range of 10–1000 is specified since an almost horizontal binding isotherm is obtained for  $K[A] < 10$  while an almost vertical change occurs in the binding isotherm at saturation for  $K[A] > 1000$ , both of which are unable to yield precise values of  $K$ . In special cases, however, where the ratio of the amount of titrant to analyte is well known for a binding reaction, values for  $K$  can be determined for  $K[A] < 1$  by employing high concentrations of titrant [36]. Simulation programs are also available that calculate binding isotherms from given  $K$  and  $\Delta_r H^\circ$  values for given titrant and analyte concentrations to aid in the design of an ITC binding measurement [1]. The ratio of the titrant to analyte concentrations should be such that a number of injections can be continued beyond saturation of the binding sites of the analyte solution to ensure that saturation of the binding sites has gone to completion. More details on the optimization of an ITC experiment for a one-to-one binding reaction to obtain precise values of  $K$  and  $\Delta_r H^\circ$  are discussed in ref. [38]. The ITC may also be employed to determine exclusively  $\Delta_r H^\circ$  for those binding reactions with a binding enthalpy that requires a high concentration of the analyte solution although the product  $K[A] > 1000$ .

ITC displacement binding analysis has been employed to determine binding constants higher than those capable of being determined directly from the binding isotherm of an ultra-tight binding reaction [36]. Basically, a weak-binding titrant ( $T_1$ ) with a  $\Delta_r H^\circ$  different than the  $\Delta_r H^\circ$  for the tight-binding titrant is first titrated into the analyte solution and the binding constant and enthalpy are determined from a fit of a binding model to the resulting binding isotherm. Then the tight-binding titrant ( $T_2$ ) is titrated into the weak titrant-analyte complex solution from the first titration to displace the weak-binding titrant by the tighter binding titrant. In an ITC displacement binding experiment, it is recommended that the tight-binding constant ( $K_2$ ) range from two to six orders of magnitude greater than the weak-binding constant ( $K_1$ ) [39]. Since the *observed* binding constant in the second titration is the tight-binding constant,  $K_2$ , reduced by a factor of  $(1 + K_1[T_1])$ , it is recommended that approximately  $10^5$  dm<sup>3</sup> mol<sup>-1</sup>  $< K_2/(K_1[T_1]) < 10^8$  dm<sup>3</sup> mol<sup>-1</sup> to obtain precise values of  $K_2$  from the second displacement titration.

ITC has been utilized for enzyme reactions following the classical Menten–Michaelis model where values for  $K_m$  and  $k_{cat}$  are determined from the dependence of the initial reaction rate on the initial substrate concentration [8,9]. Analysis of the initial rate measurements requires negligible product production, and this is achieved by  $[S]_0 \gg [E]_0$  where  $[S]_0$  and  $[E]_0$  are, respectively, the initial substrate and enzyme amount concentrations. The initial reaction rate in an ITC measurement is measured as the transfer of power between the sample and reference vessels upon mixing the substrate with the enzyme in the sample vessel. If, for example, the volume of the analyte solution in the sample vessel is 1.5 cm<sup>3</sup> and the power detection limit is 0.1  $\mu$ W for an enzyme reaction with  $\Delta_r H^\circ = 1.0$  kJ mol<sup>-1</sup>, then the minimal detectable initial reaction rate is 0.07  $\mu$ mol dm<sup>-3</sup> s<sup>-1</sup>. In the experimental design, the initial reaction rate can be increased by increasing the initial substrate or/and enzyme concentration provided that  $[S]_0 \gg [E]_0$ . Values for  $\Delta_r H^\circ$  are determined from the complete conversion of the substrate to product which is achieved at high enzyme and substrate concentrations provided that there is no product inhibition in the enzyme reaction.

The mixing of two different titrant and analyte solutions potentially involves a heat of mixing that may obscure the heat of reaction. In addition, a heat of dilution, particularly of a titrant usually at a concentration at least 10 times greater than the concentration of the analyte, may also obscure the heat of reaction. The total transfer of heat from mixing and from dilution of the titrant can be determined from a blank titration where the titrant is titrated into the dialysate in the sample vessel. Heats of mixing and dilution can also be observed in the binding isotherm as the difference between zero and the constant heat observed after the binding isotherm has reached a saturation level. Alternatively, the injection of the titrant can also cause a heat of dilution of the analyte solution which can be checked by a blank titration where identical volumes of just the dialysate are injected into the analyte solution. The mixing of an enzyme with its substrate may also produce heats of mixing and of dilution in a single injection of the enzyme titrant into the substrate analyte solution. If for some enzyme reactions  $\Delta_r H^\circ$  is small, then the heat of mixing and the heat of dilution of the titrant may have to be considered in the analysis. Heats of mixing and of dilution, however, do not affect the displacement of the ITC power baseline upon subsequent injections of the substrate titrant into the enzyme analyte solution and thus can be neglected. In both the binding and kinetics ITC measurements, if proton exchange occurs in the reaction, then the values for  $\Delta_r H^\circ$  may also contain a heat of protonation and  $\Delta_r H$  must be corrected for this contribution.

## 2.2 ITC measurement procedures

To optimize the acquisition of ITC thermodynamic data, the following experimental protocol is recommended:

### *Instrument preparation*

The syringe, sample vessel, and reference vessel are rinsed with the dialysate and the reference vessel is filled with the dialysate. For a series of titrations using the same buffer, the buffer solution in the reference vessel is usually not replaced for every titration. Solutions susceptible to oxidation processes by dissolved air may be degassed by stirring and pumping or by purging of the solutions with argon to displace the oxygen before loading into the ITC. Care must be taken to ensure that the concentrations are not altered during this procedure. In addition, filling of the empty sample vessel with the analyte solution may result in some re-aeration of the degassed analyte solution.

### *Blank titration*

A titration of the titrant into just the dialysate solution is performed for about 10 injections to determine if there is any heat from the heat of mixing and of dilution. Any unusual noise in the baseline may be caused by the presence of aggregates in the dialysate solution and should be removed by filtration of the dialysate and the titrant solution. Usually, a stirring rate of 250–300 revolutions per minute is sufficient for a sample vessel of 1.5 cm<sup>3</sup> to ensure complete mixing of the titrant with the dialysate solution. If a heat of mixing in the blank titration exhibits a change in the transfer of heat with successive injections, then the blank titration should be continued for as many injections as planned for the analyte titration. A recording of the change of heat transfer per injection in the blank titration can then be subtracted from the transfer of heat in the subsequent analyte titration. If necessary, the effect of analyte dilution can also be taken into account in a second blank titration where the dialysate solution is used as the titrant for injection into the analyte solution.

### *Analyte solution titration*

The dialysate solution is then removed from the sample vessel and the sample vessel is rinsed by the dialysate several times to remove any residual titrant. The analyte solution is then introduced slowly via a syringe (not the ITC syringe shown in Fig. 1) into the sample vessel, taking care to minimize the formation of trapped air bubbles. After temperature equilibration, the next titration is performed with the remainder of the titrant in the syringe. Changes in the baseline during the titration may also be caused by titrant-initiated aggregation or dissociation of the analyte upon successive injections of the titrant. If,

after a number of injections of the titrant into the analyte solution, there is very little difference between the amount of heat transfer in this titration and the amount of heat transfer in the previous blank titration, the ITC measurements are discontinued. A particular binding reaction may exhibit a  $\Delta_r H^\circ = 0$  at a certain temperature, but if there is a heat capacity change for the binding reaction, then a change of the temperature might result in a detectable  $\Delta_r H^\circ$ . For reactions exhibiting  $\Delta_r H^\circ = 0$  over a range of temperatures, the binding constant  $K$  may be determined by ITC displacement binding as described in Section 4.4 where a titrant with known values of  $\Delta_r H^\circ$  and  $K$  is employed to displace the titrant with  $\Delta_r H^\circ = 0$  complexed to the analyte in the second titration.

#### *Cleaning of ITC vessels and syringe*

After completion of a planned series of titrations, the ITC is cleaned by the addition of a detergent solution into the sample vessel followed by drawing enough deionized water through this vessel to ensure complete removal of the detergent. The reference vessel is usually cleaned by rinsing the vessel several times with deionized water. The stirrer syringe is cleaned by drawing up a detergent solution followed by subsequent rinsing of the syringe with at least 100 cm<sup>3</sup> of deionized water. When not in use, the syringe, reference vessel, and sample vessel are filled with deionized water.

### **2.3 ITC calibration and test procedures**

Most ITC instruments are controlled by computer programs that contain instructions for the power calibration of the instrument. The power calibration is usually performed by the use of small calibration heaters built into the instrument that supply pulses of a known quantity of power to the sample vessel filled with water. This known quantity of power is determined from the current  $\times$  voltage where the voltage is traceable by the manufacturer to a standard reference voltage. The known power to the sample vessel is compared to the ITC power reading, and any discrepancy between these two values is corrected by the manufacturer's ITC software program. It is recommended that the power calibrations be performed at least once a year.

It is recommended that a test solution system consisting of a titrant and a biological analyte solution with well-characterized values for the binding affinity and the binding enthalpy be used to evaluate the operation of the ITC at least once a year. The concentrations of the titrant solution and the analyte solution should be accurately determined by an analytical method such as UV absorption measurements. A test solution system is the binding reaction of the inhibitor CBS (mole fraction 97 % purity) to the enzyme CAII in 20 mmol dm<sup>-3</sup> sodium phosphate buffer with 0.150 mol dm<sup>-3</sup> sodium chloride at pH = 7.4, which has been performed by a total of 73 laboratories with 14 laboratories employing ITC and 59 laboratories employing surface plasmon resonance [27]. The concentration of CAII is determined from its calculated molar decadic absorption coefficient of  $5.01 \times 10^4$  dm<sup>3</sup> mol<sup>-1</sup> cm<sup>-1</sup> at 280 nm and the CBS titrant solution was prepared by weighing using the molar mass of CBS = 201.2 g mol<sup>-1</sup>. The concentrations of CAII ranged from 7 to 71  $\mu$ mol dm<sup>-3</sup> and the CBS concentrations ranged from 0.1 to 1.14 mmol dm<sup>-3</sup> [27]. The ITC results at 298.15 K averaged  $n = 0.94 \pm 0.15$ ,  $K = (1.00 \pm 0.22) \times 10^6$  dm<sup>3</sup> mol<sup>-1</sup>, and  $\Delta_r H^\circ = -(44 \pm 11)$  kJ mol<sup>-1</sup> in close agreement with the surface plasmon resonance results of  $K = (1.10 \pm 0.22) \times 10^6$  dm<sup>3</sup> mol<sup>-1</sup>, and a  $\Delta_r H^\circ = -(44.4 \pm 5.9)$  kJ mol<sup>-1</sup>. The  $\pm$  error in the reported values is the standard deviation of the average value from the participating laboratories. The average molar binding enthalpy value from the surface plasmon resonance results were determined by a van't Hoff analysis of  $K$  as a function of temperature from 5 to 35 °C; the temperature data are from measurements by only 6 of the 59 participating laboratories [27].



### 3. RECOMMENDATIONS FOR THE PRESENTATION OF ITC DATA

#### 3.1 Introduction

To ensure correct analysis of the data, it is important to precisely determine the integrated amount of heat transferred per injection, the amount of titrant and analyte detected in the sample vessel, and to ascertain that the observed integrated transfer of heat is exhibited exclusively by the binding interaction and does not contain any extraneous heats of mixing, heats of dilution, and heats of protonation [3]. It is recommended that for the integration of each power titration peak over time, the baseline be extrapolated linearly from the pre- and post-peak baselines instead of relying on a fixed average baseline from the entire titration. The analysis of ITC binding data basically involves fitting the parameters of a binding model to the experimentally determined binding isotherm. Similarly, analysis of ITC kinetics data involves the determination of the parameters of  $K_m$  and  $k_{cat}$  from a plot of the initial reaction rate against the initial substrate concentration. Values for  $\Delta_r H^\circ$  employed for the conversion of the transfer of power to the initial reaction velocity are determined from the total amount of heat transferred upon a single injection of either the substrate or enzyme into the analyte enzyme or substrate in the sample volume.

#### 3.2 Correction for the apparent titrant to analyte amounts in the sample vessel

Although the determination of the amount of heat transfer is straightforward, the determination of the amounts of titrant that contribute to this transfer of heat is more complex. This results from the construction of the ITC where the ITC sample vessel has a fixed detection volume, as shown in Fig. 1, and a reservoir volume in the sample vessel access tube to take up the excess solution from successive additions of the titrant. Only the amount of injected titrant and the binding sites in the detection volume of the solution vessel contribute to the observed transfer of heat. Successive injections of the titrant into the analyte solution in the sample vessel dilute the analyte and titrant solutions so that the concentrations of titrant and analyte following each injection depend on the number of injections and the injection volume. In addition, only those concentrations of titrant and analyte are monitored calorimetrically in the detection volume of the sample vessel. The successive dilution of the titrant and analyte concentrations in the detection volume depend on the design of the ITC and should be included in the manufacturer's software data analysis programs.

#### 3.3 Corrections for extraneous heats of mixing, heats of dilution, and for heats of protonation [3]

As described in Section 2.1 above, any transfer of heat extraneous to the heat of reaction and the heat of dilution of the titrant can be determined from a titration of the titrant into just the dialysate of the sample solution plus a second blank titration of dialysis buffer into the analyte solution. The latter titration usually results in negligible heat effects but need not in the case of dilution-sensitive association or dissociation phenomena in the analyte. If the total amount of heat transfer from mixing and dilution of the titrant is constant for 10 injections in the blank titration, then it is recommended that an average value be used for subtraction from the amount of heat transferred upon titration of the titrant into the analyte solution. If there is a change in the heat transfer in the blank titration with each successive injection for a binding or kinetics reaction involving successive injections, then it is recommended that the transfer of heat in the blank titration be subtracted from the transfer of heat of reaction corresponding to each injection from the analyte solution titration.

The heats of reaction also have to be corrected for any proton transfer reactions occurring upon titrant binding and enzyme reactions if the buffer exhibits a heat of protonation [8,31]. A number of commonly used buffers exhibit appreciable heats of protonation [3,40] and if proton transfer occurs, the apparent value for  $\Delta_r H^\circ$  will depend on the buffer. For example, the heat of protonation of phosphate buffer is  $3.6 \text{ kJ mol}^{-1}$  at  $\text{pH} = 7.2$  whereas the heat of protonation of tris buffer is  $47.45 \text{ kJ mol}^{-1}$  at

pH = 8.1 [40] and if one proton is transferred from the solution to the product of the reaction, there would be a difference in the apparent  $\Delta_r H^\circ = 39.45 \text{ kJ mol}^{-1}$ . It is recommended that if a proton transfer is known to occur in a reaction, the reaction should be observed in buffers with different heats of protonation in order to convert the ITC  $\Delta_r H^\circ$  value to the  $\Delta_r H^\circ$  for the binding/enzyme reaction. If it is questionable if proton transfer occurs in a reaction, then the reaction should be performed in buffers with different heats of protonation.

### 3.4 Conversion of the ITC data to a binding isotherm

The ITC binding data are converted to a binding isotherm where the total integrated amount of heat per amount of titrant injected is plotted against the ratio of the amounts of titrant to analyte in the sample vessel. It is recommended that the binding isotherms have the correct amounts of each heat-transfer data point corrected by subtraction of any heat of mixing and heat of dilution of the titrant for each data point. It is recommended that the binding isotherm must incorporate only those amounts of titrant and analyte in the detection volume following each injection of titrant into the sample vessel.

### 3.5 Conversion of the ITC kinetics data to an initial reaction rate plot

The initial rate of an enzyme reaction is derived from the transfer of power between the reference vessel and the enzyme-substrate mixture in the sample vessel. For the single injection analysis, the power transferred is displayed as a function of time and it is recommended that the single injection profile be corrected for the time response of the ITC by the Tian equation as described in [8,41]. Values for the transfer of power can be observed at any time within a single injection pulse. For successive injections of the substrate titrant into the enzyme analyte in the sample vessel, the injection of the substrate results in successive displacement of the power transfer baseline and this displacement is converted to the initial reaction rate. For either experimental approach, the amount of power transferred,  $dQ/dt$ , is converted to an initial reaction rate in terms of  $(d[S]/dt)_{t=0}$  as follows,

$$(d[S]/dt)_{t=0} = -(V_{\text{det}} \Delta_r H^\circ)^{-1} dQ/dt \quad (2)$$

where  $V_{\text{det}}$  is the detection volume of the sample vessel. A value of  $\Delta_r H^\circ$  is determined from the total area of an injection pulse at high enzyme and substrate concentrations per known amount substrate in the sample vessel. In the successive injection approach, values of  $[S]_0$  are simply the total substrate concentration of substrate in the sample vessel following each titrant injection. It is recommended that the initial reaction rates determined from either the single or multiple injection method be plotted against the total substrate concentration for analysis of the enzyme reaction data.

## 4. RECOMMENDATIONS FOR THE ANALYSIS OF ITC DATA

### 4.1 Introduction

Values for the binding enthalpy, binding constant, and ratio of the amount of titrant to analyte for the reaction are then obtained from the best fit of the thermodynamic parameters in a binding model. The analytical expressions for the binding models follow from the Law of Mass Action based on the assumption of equilibrium in ITC and the conservation of mass as described below. With the more complex binding models involving multiple sites, the fitting procedure may yield in some cases unrealistic ratios of the amounts of titrant to analyte, binding constants, and binding enthalpies so it would be best to fix some of the known values of these parameters derived from other analytical measurement methods. This would favorably reduce the number of adjustable parameters and render the results of the fitting procedure more reliable.

For binding to multiple sites on an analyte, there are two types of binding constants to consider: a binding-site constant and a macroscopic (analyte) binding constant. For example, the binding constant of a titrant to two sets of identical sites on an analyte can be expressed as a single microscopic binding-site constant,  $k$ , to both sites or as a macroscopic binding constant,  $K_1$ , to either of the two empty sites on the analyte so that  $K_1 = 2k$ . The binding to the second set of identical sites on the analyte yields a second macroscopic binding constant,  $K_2$ , so that  $K_2 = k/2$  since binding is now available to only one set of the two sets on the analyte. The fitting program employed to determine the binding constants in a multiple-site system should state if the binding constant output is a site binding constant or a macroscopic binding constant to the analyte. There are two basic models to consider in binding to multiple sites in biological systems, an independent binding-site model and an interactive binding-site model. All the binding constants described in the two basic models are the macroscopic binding constants. It should be emphasized that additional more complicated binding models are continually being developed and that the following descriptions of binding models are not all inclusive. For the analysis of the kinetics of an enzyme reaction, the kinetic parameters may be determined from a plot of the reciprocal of the initial reaction rate against the reciprocal of the initial substrate concentration.

#### 4.2 Non-interactive multiple-site binding model

In the general case for  $n$  non-interacting sites on the analyte, A, interacting with titrant, T, in the sample vessel, the independent equilibria are as follows



.....



represented by the following binding constants and binding enthalpies:

$$K_1 = [A_1 \cdot T]/([A_1][T]) \text{ and } \Delta_{r1}H^\circ \quad (4a)$$

$$K_2 = [A_2 \cdot T]/([A_2][T]) \text{ and } \Delta_{r2}H^\circ \quad (4b)$$

.....

$$K_n = [A_n \cdot T]/([A_n][T]) \text{ and } \Delta_{rn}H^\circ \quad (4c)$$

The total observed heat of reaction,  $Q$ , at a specific concentration of titrant solution in the sample solution vessel is given by the following:

$$Q = [A]_0 V_{\text{det}} \{ \Delta_{r1}H^\circ K_1[T]/(1 + K_1[T]) + \Delta_{r2}H^\circ K_2[T]/(1 + K_2[T]) \dots + \Delta_{rn}H^\circ K_n[T]/(1 + K_n[T]) \} \quad (5)$$

where  $[A]_0$  is the initial analyte concentration and  $V_{\text{det}}$  is the detection volume of the solution vessel.

For the case of two independent non-interacting sites,

$$Q = [A]_0 V_{\text{det}} \{ \Delta_{r1}H^\circ K_1[T]/(1 + K_1[T]) + \Delta_{r2}H^\circ K_2[T]/(1 + K_2[T]) \} \quad (6a)$$

and again the concentration of T,  $[T]$ , can be determined as the difference between the initial total concentration of titrant,  $[T]_0$ , and the concentration of T complexed with the corrected total binding site concentration of the sample solution

$$[T] = [T]_0 - [A]_0 \{ K_1[T]/(1 + K_1[T]) + K_2[T]/(1 + K_2[T]) \} \quad (6b)$$

where  $[T]$  is one of the two real roots of a cubic equation [41].

For the simplest case of only a single binding site,

$$Q = [A]_0 V_{\text{det}} \Delta_{r1} H^\circ K_1 [T] / (1 + K_1 [T]) \quad (7a)$$

and for  $n$  equivalent sites,

$$Q = n [A]_0 V_{\text{det}} \Delta_{r1} H^\circ K_1 [T] / (1 + K_1 [T]) \quad (7b)$$

Values for the free titrant concentration  $[T]$ , which is the difference between the initial total titrant concentration,  $[T]_0$  and the bound concentration  $[A \cdot T]$ ,

$$[T] = [T]_0 - [A \cdot T] \quad (7c)$$

can be determined from calculated values of  $[A \cdot T]$ , the solutions of a quadratic equation as described in ref. [1].

### 4.3 Interactive multiple-site binding model

The binding model for two or more identical interacting sites consisting of the following equilibria:



.....



represented by the following binding constants:

$$K_1 = [A \cdot T] / ([A][T]) \quad (9a)$$

$$K_2 = [A \cdot T_2] / ([A \cdot T][T]) \quad (9b)$$

.....

$$K_n = [A \cdot T_n] / ([A \cdot T_{n-1}][T]) \quad (9c)$$

yielding the following equation for the observed heat of binding for the binding isotherm:

$$Q = [A]_0 V_{\text{det}} [F_1 \Delta_{r1} H^\circ + F_2 (\Delta_{r1} H^\circ + \Delta_{r2} H^\circ) + \dots + F_n (\Delta_{r1} H^\circ + \dots + \Delta_{rn} H^\circ)] \quad (10a)$$

where  $V_{\text{det}}$  is the detection volume of the sample vessel,  $\Delta_{rn} H^\circ$  is the total enthalpy for binding from  $T$  up to  $T_n$  and the fractional contribution,  $F_n$ , of each binding event to the total heat is

$$F_n = K_1 K_2 \dots K_n [T]^n / (1 + K_1 [T] + K_1 K_2 [T]^2 + \dots + K_1 K_2 \dots K_n [T]^n) \quad (10b)$$

Numerical methods can then be employed to solve eqs. 10 [42].

### 4.4 Ultra-tight binding analysis by displacement ITC

The analysis of displacement ITC data involves two subsequent binding titrations. The first titration consists of  $T_1$  titrated into the analyte solution. After completion of this titration, the tight-binding titrant ( $T_2$ ) is titrated into the  $T_1$ -analyte complex solution to displace  $T_1$ . In the analysis of the results from the second competitive binding titration, there are, thus, two binding equilibria to consider



The analysis of a displacement binding isotherm is similar to the analysis of a two non-interacting site model described in Section 4.2 where the titrant binds independently to two different sites on the analyte. In the displacement binding model described by eqs. 11, two different titrants bind competitively

to the same protein binding site. In the second, displacement titration, the free analyte concentration  $[A]$  is determined from the difference between the initial analyte concentration and the analyte concentration in the bound state

$$[A]_0 = [A] + [A \cdot T_1] + [A \cdot T_2] \quad (12)$$

The initial concentration of the analyte for the second titration of  $T_2$  into the sample vessel is determined from the number and volume of  $T_1$  solution added in the first titration. The amount of free analyte is then one of the two real roots of a cubic equation in  $[A]$  derived from eq. 12. The heat exchange for deriving the binding isotherm is

$$Q = [A]_0 V_{\text{det}} \{ \Delta_{rT1} H^\circ K_{T1} [T_1] / (1 + K_{T1} [T_1]) + \Delta_{rT2} H^\circ K_{T2} [T_2] / (1 + K_{T2} [T_2]) \} \quad (13)$$

Values for  $K_{T1}$  and  $\Delta_{rT1} H^\circ$  are already determined in the first titration of  $T_1$  into the analyte solution. A value for  $\Delta_{rT2} H^\circ$  can be determined from a separate titration of  $T_2$  into A as described in Section 2.2. A value for the total concentration of  $T_1$  is determined as an average from the final total concentration of  $T_1$  added to the analyte solution after completion of the first titration and from the final diluted  $T_1$  after completion of the second titration. Software is available upon request for the analysis of the ITC results from a competitive binding reaction [36].

#### 4.5 Enzymatic conversion of substrate to product

Many enzyme-substrate reactions follow the classical Michaelis–Menten model where a steady-state approximation is utilized in the analysis of kinetics data to determine  $K_m$  and  $k_{\text{cat}}$  from plots of the initial reaction rate against the initial substrate concentration at known initial enzyme concentrations. Equation 2 is used to convert the power transferred,  $dQ/dt$ , to the initial rate of reaction,  $(d[S]/dt)_{t=0}$ . One type of commonly utilized initial rate plot is

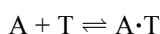
$$-(dt/d[S])_{t=0} = 1/k_{\text{cat}}[E]_0 + K_m/(k_{\text{cat}}[E]_0[S]_0) \quad (14)$$

If product inhibition occurs, then the calculated values for  $K_m$  and  $k_{\text{cat}}$  will decrease with successive injections of either the substrate or enzyme into the reaction mixture in the sample vessel. It is thus recommended to determine if values for  $K_m$  and  $k_{\text{cat}}$  depend on successive injections of the enzyme or substrate in the reaction mixture.

### 5. RECOMMENDATIONS ON THE REPORTING OF RESULTS

It is recommended that the reporting of results should include the following:

- information on the composition of the solutions including the concentration of the solute, buffer components, salt, and any additional substances such as glycerol and/or dithiothreitol, the pH, and whether the solutions were degassed or deaerated;
- information on how the titrant and analyte concentrations were determined such as by mass or by UV spectroscopy, and the UV absorption coefficients, molecular masses of the components as well as the number of binding sites on the analyte and the specific activity of the enzyme in the case of an enzymatic reaction;
- temperature of the reaction;
- type of ITC employed and how it was calibrated;
- binding reaction under study:



or in the case of an enzymatic reaction,



- if there is a heat of mixing and of dilution contribution, how this was determined and if the observed heat transfer values were corrected for contributions from the heats of mixing and of dilution;
- type of binding model used in the analysis for ITC binding data to determine the thermodynamic binding parameters, preferably with the key analytical equations of the binding model. It is also recommended that new binding models developed for use with available software programs be made available for use in the literature.
- type of initial reaction rate vs. initial substrate concentration plot for the analysis of the ITC kinetic data if the reaction follows the Michaelis–Mentel model, if the enzyme reaction undergoes product inhibition and how the power transfer data are corrected for the time response of the ITC;
- values for the binding enthalpy, the binding constant, and the ratio of the amount of titrant to analyte for completion of the binding reaction be identified with an index to specify the binding site if there are different sites for binding and report the values of the binding constant in terms of the macroscopic binding constant; and
- uncertainties in the reported quantities. The uncertainties should be based on a valid statistical method for treating data as described in ref. [43].

### LIST OF SYMBOLS

Symbol	Name	Unit
[A]	analyte amount concentration in the detection volume	mol dm <sup>-3</sup>
[A] <sub>0</sub>	initial analyte amount concentration	mol dm <sup>-3</sup>
[E] <sub>0</sub>	initial enzyme amount concentration	mol dm <sup>-3</sup>
$\Delta_r G^\circ$	standard molar Gibbs energy change of binding	J mol <sup>-1</sup>
$\Delta_r H^\circ$	standard molar binding enthalpy	J mol <sup>-1</sup>
$K$	equilibrium or binding constant, based on amount concentrations	mol <sup>-1</sup> dm <sup>3</sup>
$K_m$	Michaelis constant	mol dm <sup>-3</sup>
$k_{\text{cat}}$	catalytic constant	s <sup>-1</sup>
$n$	stoichiometry of the binding reaction	
$\Delta_r S^\circ$	standard molar binding entropy	J mol <sup>-1</sup> K <sup>-1</sup>
$T$	thermodynamic temperature	K
$Q$	quantity of heat	J
[S]	free substrate amount concentration in the detection volume	mol dm <sup>-3</sup>
[S] <sub>0</sub>	initial substrate amount concentration	mol dm <sup>-3</sup>
[T]	free titrant amount concentration in the detection volume	mol dm <sup>-3</sup>
[T] <sub>0</sub>	initial titrant amount concentration	mol dm <sup>-3</sup>
$t$	time	s

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