PARAMETERS OF INTERACTION BETWEEN PROTEINS AND THEIR SPECIFIC LIGANDS, DEDUCED BY ISOTHERMAL TITRATION CALORIMETRY*

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The interactions between proteins and their specific ligands can be studied by different biophysical methods (e.g. NMR, CD, fluorescence, etc). But the only method, at the same time, very sensitive, rapid and versatile is the isothermal titration calorimetry (ITC). By this method, a ligand is successively injected, in small amounts, in a measure cell containing the protein solution in a much smaller concentration. The interaction between the two molecules is accompanied either by the liberation (exothermic reaction) or absorption (endothermic reaction) of heat. The peaks of heat involved during the successive injections are recorded by a precision microcalorimeter. By integrating each heat peak and by fitting the experimental points, with the aid of the ORIGIN sofwtare the following parameters are obtained: the affinity constant (K_a), the variation of the reaction enthalpy (ΔH), and the stoichiometry (n). The variations of entropy (ΔS) and of Gibbs free energy (ΔG) can be then computed, indicating if the reaction is enthalpically or entropically driven. The present work is reviewing the theoretical and practical ITC principles along with some ITC biological applications, giving as examples some original experimental data of the authors.

INTRODUCTION

The molecular recognition is a very complex phenomenon, fundamental for the molecular organization of living matter. Specific binding between large and flexible molecules is a very complicated process. The calorimetric methods allow direct determination of the reaction enthalpy. The isothermal titration calorimetry (ITC) is a particular calorimetric method specially designed to measure small amounts of heat, generally associated to any process, be it exothermic or endothermic one, as it is the case of the physico-chemical interaction between two reactants, usually in a liquid solution. Measuring the minutes heats involved in

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successive series of interactions between the same partners (i.e. protein and ligand) one can determine their affinity constant (K_a), and the associated thermodynamics parameters: the variation of the reaction enthalpy (ΔH), the variations of entropy (ΔS) and of Gibbs free energy (ΔG) and, also, the stoichiometric index (n) [Brandts *et al.*, 1990], [Bundle and Sigurskjold, 1994]. If the ITC experiments are performed at different temperatures one can obtain the heat capacity variation, (ΔC_p), too.

ITC is widely used for studying interactions in liquid solutions (protein-ligand, protein-protein, protein-nucleic acids and/or small molecules) [Cooper, 1999], [Pierce *et al.,* 1999], [Weber *et al.,* 2003]. ITC is the only technique that is universally applicable to all reactions regardless the chemical nature or size of the interacting components, which does not require sample reloading between each concentration used to define the binding isotherm curve, and has a short equilibration and analysis time [Brandts *et al,* 1990].

In a recent work [Markova and Hallen, 2004], a new calorimeter procedure, called cITC, is developed. By cITC the ligand is continuously titrated in the microcalorimeter cell, thus shortening the time of the experiment. The benefit of cITC with regard to binding model selection and the range of attainable equilibrium constant is discussed. Nakamura and Kidokoro propose another new method, called isothermal acid-titration calorimetry (IATC), used for evaluating the enthalpy of protein molecules as a function of pH [Nakamura and Kidokoro, 2003]. By this method, the acid denaturation of bovine ribonuclease A is analyzed.

INSTRUMENT FEATURES

A very high-sensitivity computerized titration calorimeter can facilitate the characterization of molecular interactions, which exhibit binding constants between 10^2 M^{-1} and 10^9 M^{-1} [Cooper *et al.,* 2001]. The simplified schema of an ITC calorimeter is given in the Figure 1. As one can see, this type of calorimeter consists essentially in two cells: a) a measure cell (MC), usually containing the reactant solution, which is the macromolecule, M (i.e. the protein) and b) the reference cell (RC), containing distilled water or buffer solution as a thermal reference [Pierce et al., 1999], [Jelesarov, 1999], [Harvey *et al.,* 1995], [Fisher and Singh, 1995].

The other reactants, henceforth called the ligand (L), is injected, in small quantities (5-15 μL), by the aid of a rotating syringe (RS) the plunge of which is driven by a stepping motor according to a pre-established programme. ITC can perform the power (heat flux) compensation only above the ambient temperature, without an additional refrigerating device. The heat flux (μcal/s) produced or absorbed, as a consequence of the interaction between M and L is measured for each injection, as a function of time. Therefore, its temporal integration will conduct to the integral heat associated to each injection.

The ITC is functioning in the *"power compensation mode"*. This means that, the thermal effects, accompanying the reactions are actively balanced by a feedback acting on the MC, which thus maintains both MC and RC at quite the same temperature: $\Delta T_1 = T_M - T_R \approx 0$.

This temperature difference is very precisely measured and it is this non zero signal, called *cell feedback signal* (*CFB*) that is acting on the electronic net of the calorimeter injecting heat into MC, in order to assure $\Delta T_1 \approx 0$.

RC is continuously heated by the aid of an electrical resistance (RRC) giving a miniature *reference output* (RO) of only 20 μW. For this reason, the increase of temperature is too small: 0.03-0.06 K/h, so that the process (e.g. the coupling *M-L*) is considered isothermal, during the entire experiment that, beginning with the first injection, is lasting 1-2 hours. In the same manner, the MC is heated by another resistance (RMC) but, as we have already mentioned, only if the difference, ΔT_1, is negative.

The *CFB* signal, elicited by the MC, is recorded by a computer. It is this signal that in the standby state of the ITC, is giving the baseline of the device. During the standby state, baseline is maintained at approximately the same level, in spite of the small fluctuations, so that: 1. *CFB* is following the heat flux permanently injected into RC, decreasing ΔT_1 to zero. 2. *CFB* is activated (i.e. it is heat injected into MC) only if $\Delta T_1 < 0$.

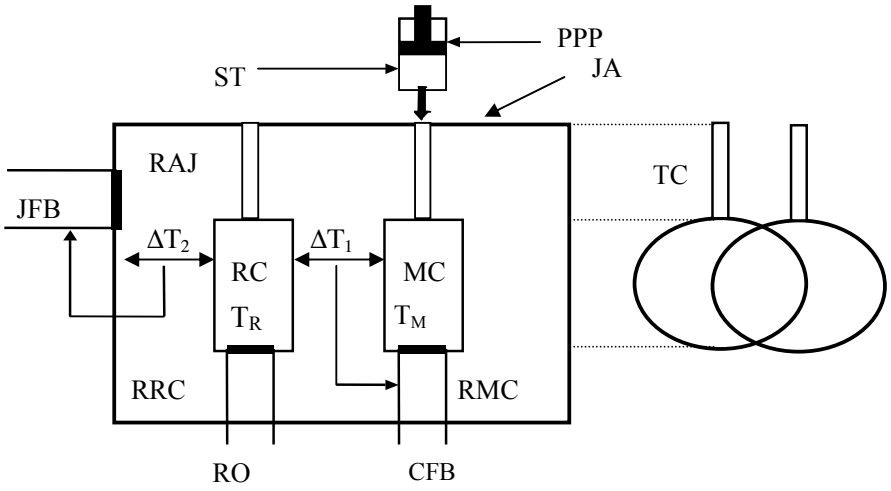

Fig. 1. The simplified schema of ITC calorimeter.

Therefore, supposing that an exothermic process is taking place in MC ($\Delta T_1 > 0$), the heat production by *RMC* is diminished or even stopped, resulting thus a negative CFB, that is a negative (downward) deviation of the baseline. On the

contrary, if an endothermic process is taking place into *MC* ($\Delta T_1 < 0$), then the heat production will be intensified, resulting thus a positive *CFB*, that is a positive (upward) deviation of the baseline. It results that, during the acquisition of the heat flux, injected into *MC*, one can record every absorbed or liberated heat peak. The entire series of recorded peaks (i.e. heat fluxes versus time) is representing a thermograme. By time integration of the thermograme peaks one obtains the total heats involved in each ligand injection *i*, as a function of the molar fraction, $[L_{ti}]/[M]$, ($[L_{ti}]$ is representing the total concentration of the ligand after the injection, *i*). That is, one obtains the so called interaction isotherms.

By fitting these interaction isotherms, by the aid, for instance, of ORIGIN software, one can obtain: n, ΔH and K_a, in the frame of each model proposed for the fit (i.e. one interaction site, two independent sites, many dependent identical sites, etc.).

The two cells (i.e. *MC* and *RC*) are thermally isolated by the exterior environment by an adiabatic jacket (*AJ*) that, in its turn, is heated by a third electrical resistance (*RAJ*) controlled also by feedback mechanism (JFB) in such a way that it is activated by the signal $\Delta T_2 = T_R - T_J > 0$. This signal must be very small (usually, this difference is between 0.0004°C and 0.0007°C). The jacket temperature is maintained in the range of the average temperature of the MC and RC, in order to prevent the heat leakage from these ones. All the experimental setup is thermally isolated in order to minimize the heat exchange with the exterior. For this reason the ITC enceinte is considered to be an adiabatic one.

THEORY

Generally, ITC allows the characterization of any type of physico-chemical interactions, which release ($Q > 0$) or absorb ($Q < 0$) a heat. ITC is based on the state functions (internal energy, *U*, entropy, *S*, Gibbs free energy, *G* and enthalpy, *H*) and on the first and second thermodynamic principles. From the first principle, $\Delta U = Q - L$, one can deduce that at constant pressure and volume, $\Delta U = Q = \Delta H$. From the second principle it results that $\Delta S > 0$, $\Delta G < 0$, and $\Delta H > 0$ or $\Delta H < 0$.

The biomolecular interactions may be *endergonic,* if $\Delta G > 0$ or *exergonic* if $\Delta G < 0$. Only the *exergonic* interactions are spontaneous, while the *endergonic* interactions must be driven by a coupled *exergonic* reaction. Therefore, *in vitro*, that is inside MC of the calorimeter, only the case $\Delta G < 0$ is considered, a situation compatible both with $\Delta H < 0$ and $\Delta H > 0$. Measurement of the heat involved into an interaction, allows determination of the affinity constant, stoichiometric index, variation of the enthalpy and variation of the entropy.

The most commonly used models for analysis of protein-ligand binding data are the one-site and two-site models. For the one-site binding model, the common feature of these methods is that they rearrange an original non-linear equation into a linear form [Wang and Jiang, 1996].

We shall analyze the equilibrium of particular reaction between the macromolecule (M) and the ligand (L) in the case of 1:1 stoichiometry (n = 1) [Indyk and Fisher, 1998]. Chemically, one can write the chemically reversible reaction:

$$M + L \underset{k_2}{\overset{k_1}{\Longleftrightarrow}} ML \tag{1}$$

where k_1 and k_2 are the rate constants of the reactions.

This reaction is characterized by the equilibrium affinity constant K_a (the inverse of the dissociation constant K_d)

$$K_a = \frac{k_1}{k_2} = [ML] \ / \ [M] \ [L], \quad [Ka] = \mathrm{M}^{-1} \tag{2}$$

A very important parameter in describing the biomolecular equilibrium is the variation of Gibbs free energy (ΔG). This parameter is indicating the evolution of the process direction, be it an endothermic or an exothermic one. For any process, at constant pressure, the modification of the Gibbs free energy is given by the well-known relation:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{3}$$

where ΔG^0, ΔH^0 and ΔS^0 represent the standard variation of G, H and S.

At molecular level, this equation reflects two fundamental processes: the trend to decrease the energy (the binding formation, $\Delta H^0 < 0$) and the thermal movement (the breaking of different bindings, $\Delta S^0 > 0$). The Gibbs free energy modification is also computed from the relation:

$$\Delta G^0 = -RT \ln K_a \quad \text{(kcal/mole)} \tag{4}$$

where R is the gas universal constant, T is the absolute temperature and K_a is the equilibrium constant.

The ORIGIN software allows the determination of both ΔG^0 and ΔS^0 and one can decide whether the reaction is entropically, enthalpically or both driven. For the spontaneous reactions, $\Delta G^0 < 0$ (*exergonic* reactions), this condition is imposed both in exothermic and endothermic reactions:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 < 0 \tag{5}$$

or

$$T\Delta S^0 > \Delta H^0 = Q \tag{6}$$

The standard free energy is composed by two terms: a caloric one (the standard enthalpy variation, ΔH^0) and an entropic one (the standard entropy variation, ΔS^0), as one can see from the next fundamental equation:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{7}$$

From this relation one can calculate the standard variation of the entropy:

$$\Delta S^0 = \frac{\Delta H^0 + RTlnK_a}{T} \quad \text{(kcal/mole K)} \tag{8}$$

In the case of an *endothermic process,* for every process at constant pressure and volume, from the first thermodynamic principle it results that $L = 0$, that is:

$$\Delta H_{p,V=ct} = Q > 0\,, \quad T\Delta S > 0\,, \quad \Delta S > 0\,. \tag{9}$$

In this case, the system entropy increases, the reaction being characterized as *entropically driven*. As the interaction is progressing, the number of the freedom degrees decreases, the consequence being an entropy decrease ($\Delta S_{assoc} < 0$), meaning that the order of the system increases, by the association of the two partners. So, the global increase of the entropy $\Delta S > 0$ is due to the compensatory molecules disorganization of the environment (e.g. water) after the complex formation, $\Delta S_{water} > 0$. Therefore the total entropy variation of the system is composed by two terms:

$$\Delta S = \Delta S_{assoc} + \Delta S_{water} > 0\,, \quad \text{where } \Delta S_{assoc} < 0 \text{ and } \Delta S_{water} > 0 \tag{10}$$

From (10) it results that:

$$\Delta S_{water} > \left|\Delta S_{assoc}\right| \tag{11}$$

For an *exothermic process* $\Delta H_{p,V=ct} = Q < 0$ three cases are possible:

1. $T\Delta S < 0$, when ΔG has a minimum negative value, and the reaction is enthalpically driven. In this case, the environmental molecules are not so much perturbed.
2. $T\Delta S = 0$, when $\Delta G = \Delta H > 0$, and the reaction is enthalpically driven.
3. $T\Delta S > 0$. In this case it results that ΔG has a maximum negative value, and the reaction is both enthalpically and entropically favourable.

If the total concentration of the macromolecule is $[M_T]$, and the total concentration of the ligand is $[L_T]$, the concentration of the free macromolecule is $[M]$ and that of the free ligand is $[L]$, one can write the following equations:

$$[M_T] = [M] + [ML] \tag{12}$$

$$[L_T] = [L] + [ML] \tag{13}$$

where $[ML]$ is the concentration of the complex.

From these relations result that:

$$[M]=[M_T]-[ML] \text{ and } [L]=[L_T]-[ML] \tag{14}$$

From these last two equations and equation (2) it results:

$$K_a=\frac{[ML]}{([L_T]-[ML])\ \ ([M_T]-[ML])} \tag{15}$$

Making some rearrangements in equation (15) one can obtain:

$$[ML]^2+\left[-([M_T]+[L_T]+1/K_a)\right]\ [ML]+[L_T][M_T]=0 \tag{16}$$

The physically admitted solution is the following:

$$[ML]=\frac{[M_T]+[L_T]+1/K_a}{2}-\frac{\sqrt{([M_T]+[L_T]+1/K_a)^2-4[L_T][M_T]}}{2} \tag{17}$$

In an ITC experiment, small "discrete" quantities of ligand are introduced into the MC, so that $[L_T]$ is a variable and so is $[ML]$, which depends on the $[L_T]$. The experimental parameter determined in an isothermal titration experiment, is $dq/d[L_T]$. This parameter does not depend on the absolute value of the $[M_T]$, but only of the K_a and $[L_T]$ values.

From (17) one can compute the first derivative, *d[ML] / d[L$_T$],* resulting:

$$\frac{d[ML]}{d[L_T]}=\frac{1}{2}+\frac{2-(1+[L_T]/[M_T]+1/K_a[M_T])}{2\left[(1+[L_T]/[M_T]+1/K_a[M_T])^2-4[L_T]/[M_T]\right]^{1/2}} \tag{18}$$

This equation is easy to solve, using the following notations:

$$c=1/r=K_a[M_T] \tag{19}$$

$$X=[L_T]/[M_T] \tag{20}$$

where r is a dimensionless constant specific to the system and X represents the molar ratio between the total concentrations of the ligand and of macromolecule.

Using the notations (19) and (20) in equation (18) it results:

$$\frac{d[ML]}{d[L_T]}=\frac{1}{2}+\frac{1-(1+r)/2-X/2}{[(1+r)^2+X^2+2X(1-r)]^{1/2}} \tag{21}$$

At constant pressure and volume, it results that the molar heat is equal to molar enthalpy:

$$q=\frac{dQ}{d\nu}=\Delta H^0 \quad \text{(kcal/mole)} \tag{23}$$

where ν is the number of moles.

As $[ML] = \frac{\nu_{ML}}{V_{CM}}$, it results that $d\nu_{ML} = V_{MC} d[ML]$, so that (24)

$$dQ = \Delta H^0 d\nu = \Delta H^0 V_{MC} d[ML] \tag{25}$$

$$\frac{1}{V_{MC}} \frac{dQ}{d[L_T]} = \Delta H^0 \frac{d[ML]}{d[L_T]} \tag{26}$$

From equations (21) and (26), one can obtain:

$$\frac{1}{V_{MC}} \frac{dQ}{d[L_T]} = \Delta H^0 \left\{ \frac{1}{2} + \frac{1 - (1+r)/2 - X/2}{\sqrt{(1+r)^2 + X^2 - 2X(1-r)}} \right\} \tag{27}$$

We shall make the notation:

$$\frac{1}{V_{CM}} \frac{dQ}{d[L_T]} = y \tag{28}$$

From (19) and (27) it results that y depends on X and r parameters, the variation of the enthalpy, and indirectly, via r parameter, on the affinity constant

$$y = f\left(X, r(K_a), \Delta H^0\right) \tag{29}$$

The binding isotherms (29) can be generated by the ORIGIN software, for every selected values of parameters, as one can see in Figure 2.

Two cases are more interesting:

1. r = 0 $(c \to \infty)$

$$y = f(X, \Delta H^0) = \Delta H^0 \left(\frac{1}{2} + \frac{1 - 1/2 - X/2}{\sqrt{1 + X^2 - 2X}} \right) \text{ or} \tag{30}$$

$$y = f(X) = \Delta H^0 \left(\frac{1}{2} + \frac{1/2 - X/2}{\sqrt{1 + X^2 - 2X}} \right) = \Delta H^0 \left(\frac{1}{2} + \frac{1/2 - X/2}{|1 - X|} \right) \tag{31}$$

We shall emphasize three situations:

a) If $[L_T] = 0$, then $X = 0$ and $y(0) = \Delta H^0 \left(\frac{1}{2} + \frac{1}{2} \right) = \Delta H^0$ (32)

Therefore the binding isotherm gives the same ΔH^0 value (Figure 2).

b) If $[L_T] \to \infty$, then $X \to \infty$ and $y = 0$.

Therefore the binding isotherm reaches zero value asymptotically (Figure 2).

$$y(\infty) = \Delta H^0 \left(\frac{1}{2} - \frac{1}{2} \lim_{X \to \infty} \frac{X}{|1 - X|} \right) = \Delta H^0 \left(\frac{1}{2} - \frac{1}{2} \right) = 0 \tag{33}$$

c) If $[L_T] = [M_T]$, then $X = 1$ and

$$y\ (1) = \Delta H^0 \left(\frac{1}{2} + \frac{1/2 - 1/2}{|1 - 1/2|} \right) = \frac{\Delta H^0}{2} \tag{34}$$

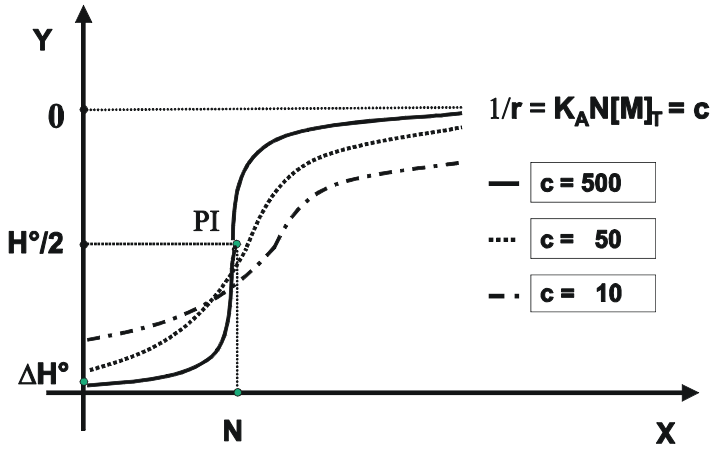

Fig. 2. Binding isotherms for different values of *c* parameter.

This situation corresponds to the inflexion point of the binding isotherms (Figure 2).

In the case of very strong binding (r = 0 or $c \to \infty$), all the added ligand quantity will be bound to the macromolecule, and the saturation phenomenon will occur at the stoichiometry index n. The curve obtained, in this case, is a rectangular one and is characterised by a large value of the enthalpy ΔH^0 [Brandt *et al.,* 1990]. Therefore the reactions with large affinity constants must be studied at low concentration of macromolecule and the reactions with small affinity constants must be studied at high concentration of macromolecule. For reactions with very large K_a, the response will eventually be reached where the detection limit of the calorimeter precludes studying the reaction at low enough concentration to fall within the so called *"K window"*. In this case, only n and ΔH^0 may be determined

and not K_a [Wiserman *et al.,* 1989], [Turnbull and Daranas, 2003]. The ITC calorimeter allows studies of interactions, which are characterized by affinity constants of 10^9 M^{-1}, for 10 Kcal/mol value of ΔH^0 or greater. The inflexion point of the binding isotherm allows the determination of the stoichiometry index, as one can see from the Figure 2.

2. $c = 0 \qquad (r = \infty)$

$$y = f(X,\ r,\ \Delta H^0) = \Delta \mathrm{H}^0 \left(\frac{1}{2} - \frac{1}{2} \sqrt{\frac{r^2}{r^2 + 2r(1+X)+1}} \right) = \Delta \mathrm{H}^0 \left(\frac{1}{2} - \frac{1}{2} \right) = 0 \quad (35)$$

For very weak bindings ($c \to 0$), one can obtain a horizontal trace, which, as in the very strong binding, leads to an incorrect value of the K_a constant. Although ΔH^0 values may be easily obtained, even at the largest c values, accurate estimates of the binding constant, K_a, require that the c value be between 1 and 1000. For the *"K windows"*, one can write:

$$1 < c = [M_T] < 1000. \quad (36)$$

For moderately values of the c parameter between 1 and 1000, the shape of the binding isotherms is very sensitive to small modifications of this parameter. The intercept of this curves on the ordinate, is no longer exactly ΔH^0, but this parameter, as well as the binding constant is easily obtained from curve fitting [Brandt *et al.,* 1990].

As we already know, many biomolecular interactions are characterized by affinities in the range of $10^3 - 10^8$ M^{-1}, but a large number of important reactions occur with binding constants of significant larger or smaller magnitude. This kind of binding is studied by displacement techniques, as displacement titration calorimetry [Sigurskjod, 2000].

By repeating titrations, at different temperatures, one can obtain the heat capacity variation (ΔC_p) associated to the binding of two molecules:

$$\Delta C_p = dH / dT \quad \text{(kcal/mole K)} \quad (37)$$

In all ITC experiments the data errors are very important. In ITC, there are two main sources of statistical errors, associated with the extraction of the heat from the measured temperature changes and of the delivery of metered volumes of titrated ligand. The standard errors in the analysis of ITC data are the equilibrium constant and the enthalpy. The parameter precisions depend strongly of the assumption on the random error in the data, especially of the titrated volume error [Tellinghuisen, 2003].

Protonation effect. Most of the reactions between a ligand and a protein are coupled with the processes of proton absorption or release. Only in those cases where the binding reaction follows a lock-and-key or rigid-body mechanism, that

can be described by a two-state transition between free and complexed molecules, and in which there is no change in the protonation state of *L* and/or *M* nor in the hydration state of the interface, ΔH is equal to the 'true' binding enthalpy attributable to non-covalent bonds in the complex [Jelesarov, 1999].

Generally, the reaction is dependent on the pH, and the binding enthalpy depends on the ionization enthalpy of the buffer solution in which the reaction occurs [Leavitt and Freire, 2001], [Ortiz and Salmeron, 2001]. ITC allows the study of the absorption or release of protons. For such a reactions, the measured enthalpy (ΔH) is composed by the binding reaction enthalpy (ΔH_{bin}) and the buffer ionisation enthalpy (ΔH_{ion}):

$$\Delta H = \Delta H_{bin} + \Delta n_i \quad \Delta H_{ion} \tag{38}$$

The binding reaction enthalpy is not dependent on the buffer solution, but is pH dependent. The ionization enthalpy of the buffer solution is multiplied by the number of the released protons (if $\Delta n_i < 0$) or absorbed protons (if $\Delta n_i > 0$) to the complex, after binding reaction. In present work, ITC is applicable for understanding the mechanism of the complex formation and for the determination of the binding thermodynamic parameters of protein-ligand interaction.

INFORMATIONAL CONTENT OF ITC DATA

As we have already mentioned, small aliquots of ligand are introduced and mixed with a solution containing the protein, in a stepwise manner, and the resulting heat flux is measured and converted to an integrated enthalpy at each step. Because the heat is measured in a cumulative way, a complete enthalpic titration curve can be obtained in a single ITC experiment [Fisher and Singh, 1995]. In order to see what kind of information is extracted from ITC experiments, we shall present some of our unpublished data on the titration of EDTA molecules with Ca^{2+} ions.

EDTA is known for its capacity to bind Ca^{2+} with large affinity constant. Characterization of the interaction of this chelating agent with Ca^{2+} serves as model to gain insight the mechanisms and also the specificity of the process of interaction of metals with the specific metal binding site [Griko, 1999]. After curve t fitting and some computations we can obtain both the entropic and the Gibbs free energy term, as one can see from the Table 1. In order to obtain the real enthalpy for the binding of Ca^{2+} to EDTA solution, which is not dependent on the buffer solution, one can make the linear regression of the observed enthalpy (ΔH_{obs}) by Ca^{2+} titration in EDTA solution, versus the buffer ionization enthalpy (ΔH_{ion}), at pH 7.4 and 30 ºC (Figure 4).

Table 1

ITC parameters of 0.5 μM EDTA titration with 2 mM–5 mM $CaCl_2$ solution, in different buffer solutions, at 7.4 and 30 °C.

Buffer Solution	*n*	$K_a \times 10^7$ (M^{-1})	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
MOPS/KCl	0.96	2.7 (± 1.0)	-4.4 (± 0.1)	-5.91	-10.31
HEPES/KCl	1.05	1.7 (± 0.1)	-4.0 (± 0.1)	-6.03	-10.03
Glycerophosphate/KCl	0.52	1.3 (± 0.3)	-1.0 (± 0.1)	-8.87	-9.7
PIPES/KCl	1.00	1.6 (± 1.0)	-3.0 (± 0.1)	-8.42	-11.42
Tris-HCl/KCl	0.95	2.3 (± 0.3)	-12.8(±0.1)	+1.55	-11.25

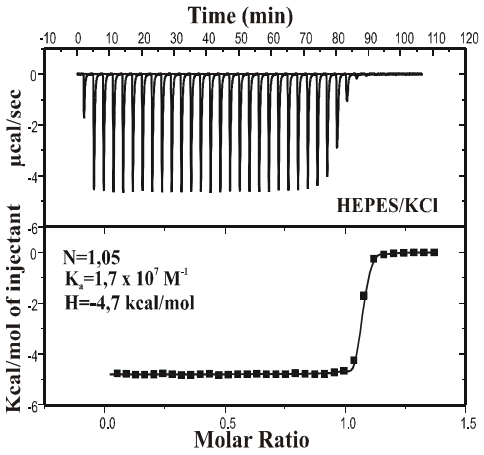

Fig. 3. The thermograme (up) and the titration isotherm (down) of a 5 mM $CaCl_2$ solution in 1.4 ml 0.5 μM EDTA solution, in 50 mM HEPES/100 mM KCl, at pH 7.4 and 30 ºC.

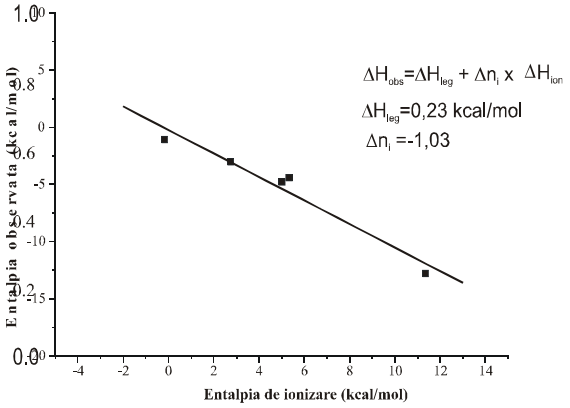

Fig. 4. Linear regression of the ITC observed enthalpy (ΔH_{obs}) by Ca^{2+} titration in EDTA solution as a function of the buffer ionization enthalpy (ΔH_{ion}), at pH 7.4 and 30 ºC.

SOME BIOLOGICAL APPLICATIONS OF ITC

ITC allows the direct investigation of a wide range of systems [Gaisford and Buckton, 2001], offering much potential, but we shall limit only to some biological applications.

Molecular recognition. Characterization of the thermodynamics interactions is important in improving our understanding of biomolecular recognition. This complex and fundamental process is essential for life. Although ITC has facilitated the measurement of the thermodynamics governing binding reactions, interpretation of these parameters in structural terms is still a great challenge.

Due to significant improvements in instrument sensitivity, ITC is becoming a routine method for the generation of thermodynamic data related to biomolecular association [Holgate, 2001], [Popescu *et al.*, 2003]. Binding of a ligand to a protein involves changes in the intramolecular interactions and dynamics of the system components (ligand, protein, water and any additional components that may be present). Signaling ligands with transmembranar receptor interactions, for example, is a very important class of reactions that is difficult to characterize by the conventional binding techniques, because they are localized into cellular membranes. ITC allows the study of such a system [Brandts et all, 1990], [Sabury, 2003].

Measurements of protein-protein interactions or protein-polynucleotide interactions, particularly if molecular weights are high and solubility are low may become more difficult [Brandts et all, 1990].

Antibody-antigen complexes. ITC is a useful tool for the biophysical characterization of antibodies and antibody-related products, which uses heat as a detection medium. It is known that the antigen-antibody complexes are important issues in research, biotechnology and pharmaceutical industry. These complexes are characterized by large values of the affinity constants (more than 10^9 M^{-1}) and for this reason they are crucial for the living matter. A complete thermodynamic analysis, when it is possible, provides a richer source of information, which can be used to interpret the molecular forces at work between the antibody and antigen. Further steps are concentrated on the building of strong and more potent antibodies for use in cancer therapy [Livingstone, 1996].

Calorimetry and drug design. Because the ITC data are rich in thermodynamic information, it is anticipated that this technique will play an expanding role in the molecular characterization of binding mechanism and rational drug design [Doyle, 1997]. The determination of ITC data is a fundamental step in the drug design process [Labury, 2001]. As more information is acquired from the ITC data experiments, the process will become more honed and the success rate in structure-based drug design and optimization will increase. Many proteins studied are targets for pharmaceutical drug development, underscoring the

essential role of ITC in drug design. Reactions involving the binding of drugs or inhibitors to the active site of small proteins can be easily characterized since the stoichiometry is well defined [Brandts et al., 1990], [Leavitt and Freire, 2001], [Weber and Salemme, 2003].

CONCLUSION

ITC is a reliable and rapid method for study of the (bio)chemical interactions. A great amount of information about the association of biological macromolecules can be determined from ITC, as it results from the present minireview. This technique can provide the user with a complete thermodynamic characterization of the system under study. It does not require immobilization of the binding components or chemical tagging. ITC is much faster than other alternative analytical methods, like ultracentrifugation. A typical ITC experiment requires only about 30-90 minutes, with only a few minutes of "hand-on time".

ITC experiments are non-destructive and non-invasive and can be applied over a broad range of solution conditions, including turbid solution. ITC can, in a single experiment, yield the binding constant, stoichiometry and thermodynamic parameters of binding of a ligand to a protein (that is, "the fingerprint" of the reaction). A disadvantage of this method is its limitation to the determination of the binding constant, if the binding is too strong ($K_a > 10^9$ M^{-1}) or too weak ($K_a < 10^3$ M^{-1}). However, if K_a is too strong, it is possible to determine K_a, by using another competitive ligand to the same site, but with a measurable constant affinity.

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