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Intrinsic thermodynamics of protein – ligand binding by isothermal titration calorimetry as aid to drug design

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Abstract

Isothermal titration calorimetry (ITC) is one of the main techniques to determine specific interactions between molecules dissolved in aqueous solution. This technique is commonly used in drug development programmes when low molecular weight molecules are sought that bind tightly and specifically to a protein (disease target) molecule. The method allows a complete thermodynamic characterization of an interaction, i.e. ITC enables direct determination of the model-independent *observed* interaction change in enthalpy (ΔH) and a model-dependent *observed* interaction affinity (change in Gibbs free energy, ΔG) in a single experiment. The product of temperature and change in entropy (TΔS) can be obtained by the subtraction of ΔG from ΔH and the change in heat capacity (ΔCp) can be determined as a slope of the temperature dependence of the binding ΔH.

Despite the apparent value of ITC in characterization of interactions, it is often forgotten that many protein-ligand binding reactions are linked to protonation-deprotonation reactions or various conformational changes. In such cases, it is important to determine the linked-reaction contributions and obtain the *intrinsic* values of the changes in Gibbs energy (affinity), enthalpy, and entropy. These energy values can then be used in various SAR-type structure-thermodynamics and combined with structure-kinetics correlations in drug design, when searching for small molecules that would bind the protein target molecule. This manuscript provides a detailed protocol how to determine the intrinsic values of protein-ligand binding thermodynamics by ITC.

Key Words

Isothermal titration calorimetry; ITC; enthalpy of binding; Gibbs energy of binding; drug design; intrinsic thermodynamics of binding.

1. Introduction

1.1. General understanding of the isothermal titration calorimetry method

High-sensitivity isothermal titration calorimetry is often a method of choice when protein-ligand systems are studied. The method has been extensively reviewed [1–5]. ITC works by titrating one binding reagent (titrant, usually chemical compound, ligand) into a second (titrand, usually protein) at a constant temperature. After each injection, the heat, absorbed or released in the sample cell, is measured with respect to a reference cell. The molar ratio between the molecules increase with each injection, and in the middle of an experiment, the titrand gets saturated with the bound molecules of the titrant.

In a properly designed, typical ITC experiment, the binding affinity (dissociation constant, K_d or binding constant, K_b (= 1/ K_d)), stoichiometry (n), and the enthalpy (ΔH) at a constant temperature (T) are measured. From these data, the Gibbs energy change (ΔG) and entropy change (ΔS) may be calculated. If one protein molecule (M) binds one ligand (L), then the 1:1-binding model is applied and:

$$\mathrm{M} + \mathrm{L} \leftrightarrow \mathrm{ML} \tag{1}$$

$$\Delta G = \Delta G^0 - RT \ln\left(\frac{[\mathrm{ML}]}{[\mathrm{M}][\mathrm{L}]}\right) \tag{2}$$

At chemical equilibrium, under standard conditions, when $\Delta G^0 = 0$

$$\Delta G = -RT\left(\frac{[\mathrm{ML}]}{[\mathrm{M}][\mathrm{L}]}\right) = -RT \ln K_b = RT \ln K_d \tag{3}$$

where R is the universal ideal gas constant.

The entropy change is:

$$\Delta S = \frac{\Delta G - \Delta H}{T} \tag{4}$$

ITC can determine three thermodynamic parameters (ΔG, ΔH, and $T\Delta S$) in a single experiment, but only if an empirical condition that $5 < c < 500$ is fulfilled. The c-factor (Wiseman parameter[6]) represents the steepness of the ITC curve. If the curve is too steep or too shallow (Figure 1, left panel A), the binding curve cannot be fit accurately (Figure 1, middle panel B). In situations where the c value is not appropriate the ITC experiment can be modified. For example, one can perform a

displacement ITC experiment where the protein, pre-saturated with a weakly binding ligand, is titrated with a strongly binding ligand (Figure 1, right panel C) as previously explained elsewhere[7,8,9].

1.2. Linked protonation reactions occurring upon ligand binding

ITC yields only the *observed* thermodynamic parameters of the protein-ligand interaction. The term 'observed' indicates that the data is dependent on the prevailing conditions. This is well exemplified in an experiment that was carried out where a typical ligand and protein were titrated in three buffers, sodium phosphate, HEPES, and TRIS (Figure 2A) and characterized by ITC. The three experiments yielded three completely different enthalpy values: +7 kJ/mol in phosphate, -8 kJ/mol in HEPES, and -32 kJ/mol in TRIS. Such a result strongly implicates that the binding reaction is linked to a protonation event associated with the ligand or the protein. The values should arrange linearly if plotted as a function of the buffer deprotonation enthalpy (inset in Figure 1A). Figure 2B shows the relative contributions of enthalpy and entropy to the ΔG of binding. It is obvious that the contributions associated with various buffers are different. Using these data we can determine the *intrinsic* thermodynamic parameters which are corrected for the heats of protonation. Without the analysis of such data to calculate the *intrinsic* enthalpy, the observed enthalpies and other thermodynamic parameters represent data in which more than one equilibrium is occurring and hence the data are not strictly appropriate in describing the interaction between ligand and protein.

Furthermore, the ITC experiments were performed in one buffer at a series of pHs. Both the enthalpy (Figure 2C) and the affinity (Figure 2E) were highly dependent on pH and it is impossible to say what the values are without the analysis of the linked reactions. For example, at pH 5.0, the ΔH is approximately equal to -15 kJ/mol, at pH 7.0 ΔH = -40 kJ/mol, and at pH 9.0 ΔH = -60 kJ/mol. From the first glance, we do not know which is the "true" value. A similar situation is evident with the ΔG, where at pH 7.0 the energy is -45 kJ/mol ($K_b = 8\times10^7$ M^{-1}, $K_d = 1.3\times10^{-8}$ M) while at pH 10.0 ΔG = -37 kJ/mol ($K_b = 3\times10^6$ M^{-1}, $K_d = 3.3\times10^{-7}$ M). Again, it is impossible to say which is the "true" affinity.

It has been shown by Baker and Murphy[10,11] that it is important to dissect any *observed* protein-ligand binding reaction into contributing energetic parts. It is quite convenient to do this analysis using ITC data because the enthalpies vary to a greater extent than affinities as a result of buffer pH. The term *intrinsic* has been coined by Ladbury-Connelly in 1997, to describe the reaction that most correctly describes an actual reaction of a protein-ligand binding. However, it should be kept in mind that there are many more weak interactions formed or broken during the protein-ligand binding event and it is difficult to draw the line as to what is truly intrinsic. For example, the bonds between water molecules and ligand/protein have to be broken before the binding reaction could occur.

In a typical protein-ligand binding reaction, there are several possibilities where the linked protonation-deprotonation reactions could occur. First, the protein may undergo a (de)protonation of an amino acid positioned near the ligand binding site. It could be for example an aspartic acid residue that is negatively charged (deprotonated) in a free protein, but must be protonated in the ligand-bound protein.

Second, the ligand may exist in a different protonation state while free in solution as compared to the protonation state when bound to the protein. There are also possibilities when no linked reactions occur upon binding or there could be complex cases when numerous linked reactions occur.

When the linked protonation reactions occur, there are four general possible mechanisms (Figure 3) for how the protein may bind the ligand.

Analysing the case when only the deprotonated form of the ligand binds only to the protonated form of the protein the *intrinsic* thermodynamic parameters of binding can be calculated as follows:

K_{b_intr} is the *intrinsic* (pH – independent) binding constant.

$$K_{b\_intr} = \frac{K_{b\_obs}}{f_L f_M} \tag{5}$$

$\Delta_b G_{intr}$ can be calculated from (3) and (5)

f_L and f_M are the fractions of the deprotonated ligand and the protonated protein, respectively.

$$f_L = \frac{[\mathrm{L}]}{[\mathrm{L}]+[\mathrm{L}-\mathrm{H}^+]} = \frac{10^{pH-pK_{a\_L}}}{1+10^{pH-pK_{a\_L}}} \tag{6}$$

$$f_{M-H^+} = \frac{[\mathrm{M}-\mathrm{H}^+]}{[\mathrm{M}-\mathrm{H}^+]+[\mathrm{M}]} = 1-\frac{10^{pH-pK_{a\_M-H^+}}}{1+10^{pH-pK_{a\_M-H^+}}} \tag{7}$$

The fractions can be calculated if both pK_a values are known. The pK_a value of ligand pK_{a_L} can be determined from the absorbance spectra at different pH values (Figure 1. Panel on the right), and the value of protein $pK_{a\_M\text{-}H^+}$ is estimated after data fitting.

$\Delta_b H_{intr}$ is the intrinsic enthalpy of binding.

$$\Delta_b H_{intr} = \Delta_b H_{obs} - n_L \Delta_L H - n_M \Delta_M H + n_B \Delta_B H \tag{8}$$

$\Delta_b H_{obs}$ is the observed binding enthalpy, $\Delta_L H$ - the enthalpy of ligand protonation, $\Delta_M H$ - the enthalpy of protein protonation, $\Delta_B H$- the buffer protonation enthalpy.

$$n_L = f_L - 1 \tag{9}$$

$$n_M = 1 - f_M \tag{10}$$

n_L, n_M are the numbers of protons binding to the ligand and protein, respectively.

$$n_B = n_L + n_M \tag{11}$$

n_B is the net sum of uptaken or released protons.

Several examples of the linked protonation effects are provided in Figure 2. Panels D and F show the analysis of the changes in enthalpy and Gibbs energy of the contributing protonation reactions. The ΔH

values in various buffers (Figure 2D) are modeled by Eq. (8) and the binding constants by Eq. (5). The intrinsic values are independent of pH and are shown as horizontal solid black lines.

In Figure 2, panels G and H show the analysis of a protein-ligand binding reaction when only one binding-linked protonation event occurs. In this case, the ligand has to undergo a protonation reaction in order to bind the protein. The equations may be obtained from the above analysis by assuming that the fraction of binding-ready protein is always equal to 1.

1.3. Structure – thermodynamics analysis of the intrinsic binding reactions

When the thermodynamics of binding of a series of ligands to a set of proteins is being studied, it is important to distinguish the intrinsic contributions from the observed contributions. As mentioned above, all experimental techniques will provide only the observed parameters. These observed values cannot inform on the structural detail because they potentially include the effects of different equilibria (e.g. protonation effects described above). Compound structure-thermodynamic correlation analysis requires the parsing out of the different equilibria which are incorporated in the binding event [12]. It is important that the intrinsic values should be calculated and used in such analysis.

Figure 5 illustrates how important it is to obtain the intrinsic dissociation constants in order to understand the reaction of the shown compound binding to recombinant human carbonic anhydrase I. Two ligands were compared; non-fluorinated and tetrafluorinated. The non-fluorinated ligand appeared to bind approximately 100-fold weaker than the fluorinated ligand. It could appear that the fluorine atoms recognize the protein surface and somehow make stronger interaction with the protein. However, after the analysis of the protonation reactions, the intrinsic K_ds differed less than 2 fold. The conclusion was that since the fluorines are strong electron withdrawing groups, they diminished the pK_a of the sulfonamide group and the fraction of the negatively charged sulfonamide increased 100-fold resulting in the increase in affinity shown in the observed binding constant.

Figure 6 compares the observed and intrinsic binding thermodynamics of these ligands to three carbonic anhydrase isoforms, CA I, CA XII, and CA XIII. The observed affinity difference ($\Delta\Delta_bG_{obs}$ for binding to CA I) for the fluorination of the ligand was equal to -12.9 kJ/mol, while the intrinsic difference ($\Delta\Delta_bG_{intr}$) was equal to only -0.9 kJ/mol. Even more pronounced was the difference between the observed and intrinsic enthalpies of the ligand binding to CA I: the observed value in phosphate buffer at pH 7.0 and 37 ºC was -40.6 kJ/mol while the intrinsic value in any buffer at any pH was -11.5 kJ/mol. Without the proper analysis of the linked reactions, one could obtain highly misleading information on the compound chemical structure-thermodynamic correlation and misunderstand the underlying contributions of various chemical groups to the binding affinity, and changes in enthalpy, and entropy.

2. Materials

2.1. Equipment and instruments

- Isothermal titration calorimeter. Two manufacturers currently produce commercially available high sensitivity calorimeters suitable for studying biomolecular interactions: Malvern Instruments (Malvern, UK, formerly MicroCal, Inc., owned by GE-Healthcare, Northampton, MA, USA) and TA Instruments Inc. (New Castle, Delaware, USA)
- Data analysis software. The software is recommended and provided by the manufacturer (Malvern Instruments – Origin software, TA Instruments – NanoAnalyze software). In addition, NITPIC and SEDPHAT software may be used for improved baseline selection and data fitting[13].
- Hamilton syringe. For the sample cell loading.
- Test tubes, Pipettes and Pipette Tips. For sample preparation.
- Vacuum pump. To degas the samples.
- pH meter. To determine the pH of solutions.

2.2. Reagents

- a purified protein
- ligand solution
- various types of buffers at different pH values (e.g. TRIS and NaPi in the range between pH 5 and 9). It is important to determine the pH accurately and pay special attention to cases whe the buffering capacity is low. For example, it is difficult to make TRIS buffer of pH 5.0 when the pKa of TRIS is 8.1.

3. Methods. Procedure for determination of intrinsic parameters of binding

These protocols are for guidance only and it is important to read and follow instructional guides provided by the manufacturers, because the above information is only a basic description.

3.1. Sample preparation

3.1.1. Concentrations

For the purpose of obtaining a sigmoidal isotherm (1:1 model), the molar ratio of titrant to titrand should be equal to 2 at the end of titration. The solution in the syringe should be approximately 10 fold more concentrated than in the cell. Some macromolecules are poorly soluble and aggregate at higher concentrations. For this reason, most often the solution of protein is loaded into the sample cell.

The optimal macromolecule concentration is determined from Wiseman parameter (also called sigmoidality factor or *c* value)

$$c = K_b \times [\mathrm{M}] \times n \tag{12}$$

where K_b - binding constant, [M] - concentration of the protein (macromolecule), n - stoichiometric coefficient (or could mean the purity of the protein preparation).

Optimal values range from approximately 5 to 500.

3.1.2. Buffers

Buffers of both low and high enthalpy of protonation should be prepared. Phosphate has low protonation enthalpy while TRIS has high protonation enthalpy. Good compilation of the values is provided here [14]. Typically prepare 10-50 mM buffer, 100 mM salt, usually but not always NaCl (e.g. sodium phosphate buffer (50 mM NaP_i, 100 mM NaCl), pH 7.0). For intrinsic parameters the reaction should be performed in as wide pH range as possible, usually pH 5.0-9.0.

3.1.3. Composition

Both the cell and syringe samples must be in as closely matched solutions as possible. Difference in composition causes large heats of dilution and masks the desired observation. Solvent matching is best achieved when the macromolecule solution is dialyzed exhaustively against the buffer and then using the final dialysis buffer to make up the ligand solution in the syringe.

There are some special cases of sample preparation. For example, some small molecules may need organic solvents (e.g. DMSO) in order to be soluble or proteins may need reductants to maintain reduced cysteines. It is important to add the same concentrations of each additive to both solutions, considering stability (many proteins are stable up to 2 - 5% of DMSO).

A typical experiment will contain the following components in the ITC syringe and cell:

Sample solution in the cell –

- $\frac{5 < c < 500}{K_b \times n}$ μM protein
- Up to 2 % DMSO
- Buffer, salt

In the syringe –

- $\frac{5 < c < 500}{K_b \times n} \times 10$ μM ligand in DMSO
- Up to 2 % DMSO (only required for insoluble compounds)
- Buffer, salt

3.1.4. Other aspects of solution preparation

Centrifuge the solutions to eliminate possible precipitates.

Degas the prepared samples to avoid spikes caused by bubbles of air released from solution during titration. Centrifugation also helps remove bubbles.

3.2.The experimental parameters

It may take several titrations to optimize the conditions that are the best for system.

3.2.1. Number of injections and injection volumes.

For systems that have strong heat signals - a large number (e.g. 50) of low volume (1 ul) injections.

For binding systems with weak heat signals - a small number (e.g. 20) of large volume (10 ul) injections. The volumes depend on the equipment.

3.2.2. Timing (the time spacing between each injection). After each injection of titrant, the system is given time to return the heat signal to baseline before the next injection occurs. For most systems, five minutes spacing should be adequate. Shorter times may be used if demonstrated that the enthalpy is not diminished.

3.2.3. The temperature of the experiment. 25 ºC being the most common. However, the operating temperature range from 2 to 80 ºC is possible.

3.2.4. The stirring speed of the syringe. Stirring is necessary for adequate mixing, but should be set at a relatively low rate, because some macromolecules may be denatured by stirring.

3.3. Steps

3.3.1. The reference cell must be filled with distilled water or buffer. Usually distilled water can be used as the reference solution. However, it is not entirely correct to compare distilled water to buffers with high ionic strengths. In this case, it is better to use the buffer as a reference solution.
3.3.2. Make sure the sample cell and injection syringe are cleaned according to the manufacturer's protocol.
3.3.3. Rinse the sample cell several times with buffer or sample solution. It is not necessary, but can improve data quality.
3.3.4. Load the sample cell with the solution slowly to avoid bubble formation. The volume should be greater than the active volume of the cell. For example, VP-ITC calorimeter has the active cell volume of approximately 1.4 ml, but about 2.0 ml should be added to the cell so that the stem above the cell is fully filled. Remove any excess volume from the sample cell if overfilled the stem during loading.

3.3.5. Rinse the injection syringe with a buffer or titrant solution. It is not necessary, but can improve the data quality.
3.3.6. Fill the syringe with solution being careful to avoid bubble formation.
3.3.7. Place the injection syringe into the sample cell.
3.3.8. Run the experiment.
3.3.9. Run a control experiment, where the ligand is titrated into buffer in the sample cell to determine the heat of dilution for the ligand.

3.4.Data analysis

3.4.1. Adjust the baseline to eliminate the effect of the baseline.
3.4.2. Integrate the peaks
3.4.3. Subtract the blank experiment to correct for the heat of dilution. The last points of the sigmoid curve (the enthalpy at saturation) should be approximated to zero.

4. Notes

Description of the most common cases when ITC experiments may fail and suggestions how to solve the issues to determine the intrinsic parameters as accurately as possible.

Problem	**Reason**	**Solution**	**Comment**
Observed enthalpy does not match the model at low and high pH	Denaturation of protein	Such pH range may be too far and should not be used.	
pH of TRIS may not be accurate around pH 5 and pH of Pi may not be accurate at pH 10	Buffering capacity is too low	The pH may be necessary to measure in the presence of protein.	The pH could also be measured before and after the titration experiment to confirm its constancy.
pH slope of $\Delta_b G_{obs}$ does not follow the model curve	The model may incorrect or the data is not sufficiently accurate	Check the model and ensure pH accuracy	
Concentrations	Affinity is not known	Perform alternative experiment to get idea of affinity	

	Affinity is approximately known	Perform a range-finding experiment using *c* factor	
Affinity	The binding is very tight	-Use a small c value - displacement [15]	
	The binding is very weak	-use an optimal c value -displacement	
Sample preparation	Reductants are needed to maintain reduced cysteines	Use low concentrations of a reducing agent (β-mercaptoethanol,TCEP or dithiothreitol) in both solutions	To avoid protein aggregation and to minimize artifacts
	Some small molecule may need the organic solvent (e.g. DMSO) to increase solubility.	Calculate the final percentage of DMSO in the ligand solution and add DMSO to the protein solution in order to have the same DMSO percentage in both solutions.	many proteins are stable up to 2 % of DMSO
Errors in the stoichiometry, enthalpy and binding affinity	Incorrect concentration	Check the concentrations (e.g. using spectrophotometer)	Unfolded or inactive components reduce an effective concentration
Non S shape	Too little heat change Never reach saturation Rapidly reach saturation Stoichiometry far from 1	Incorrect concentration Buffer mismatch Other source of heat No binding More than one binding event	
Baseline drift	Air bubbles	Degas the samples and avoid bubbles when filling the cell	

	Cell cleanliness	Wash the system	

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Figures

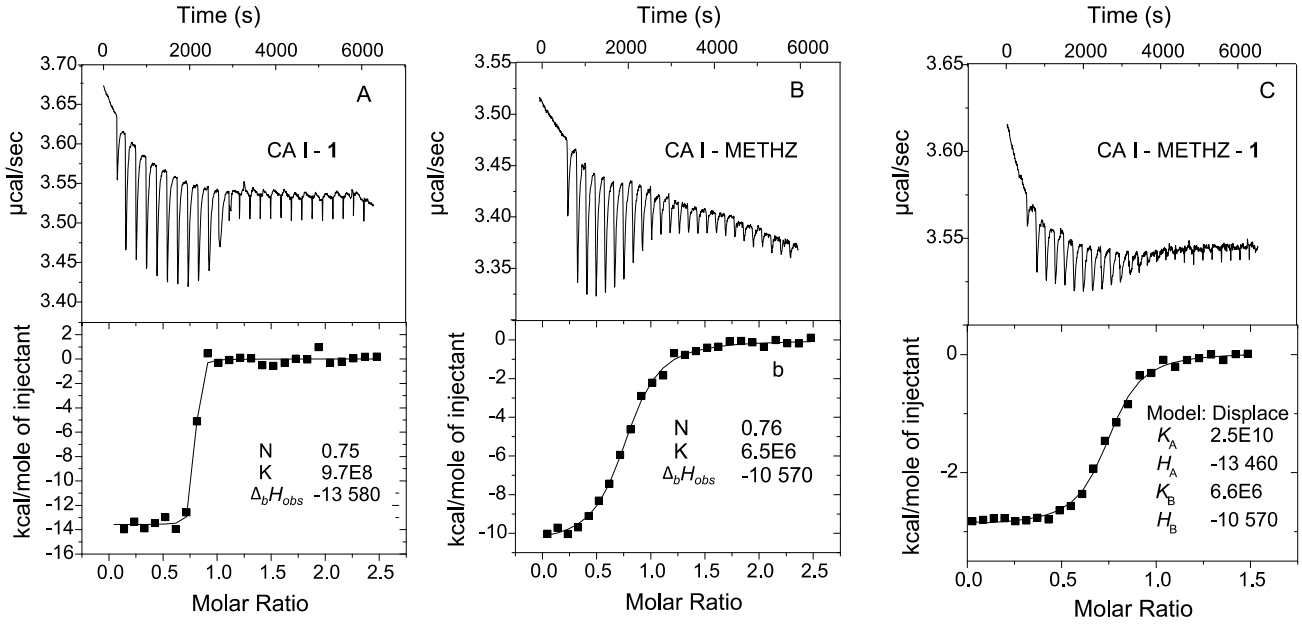

Figure 2**.** Isothermal titration calorimetry experimental curves. Upper panels show raw curves while lower panels show the integrated curves. (a) Direct ITC titration of CA I with the tightly binding compound **1**. (b) Direct ITC titrations of CA I with the weakly binding METHZ. (c) Displacement ITC titration of the CA I pre-saturated with the weakly binding methazolamide (METHZ) by the strongly binding compound **1**. All three titrations were performed in 50 mM sodium phosphate, 100 mM NaCl, 1% DMSO, pH 7.0, 37 °C. Data taken from [9].

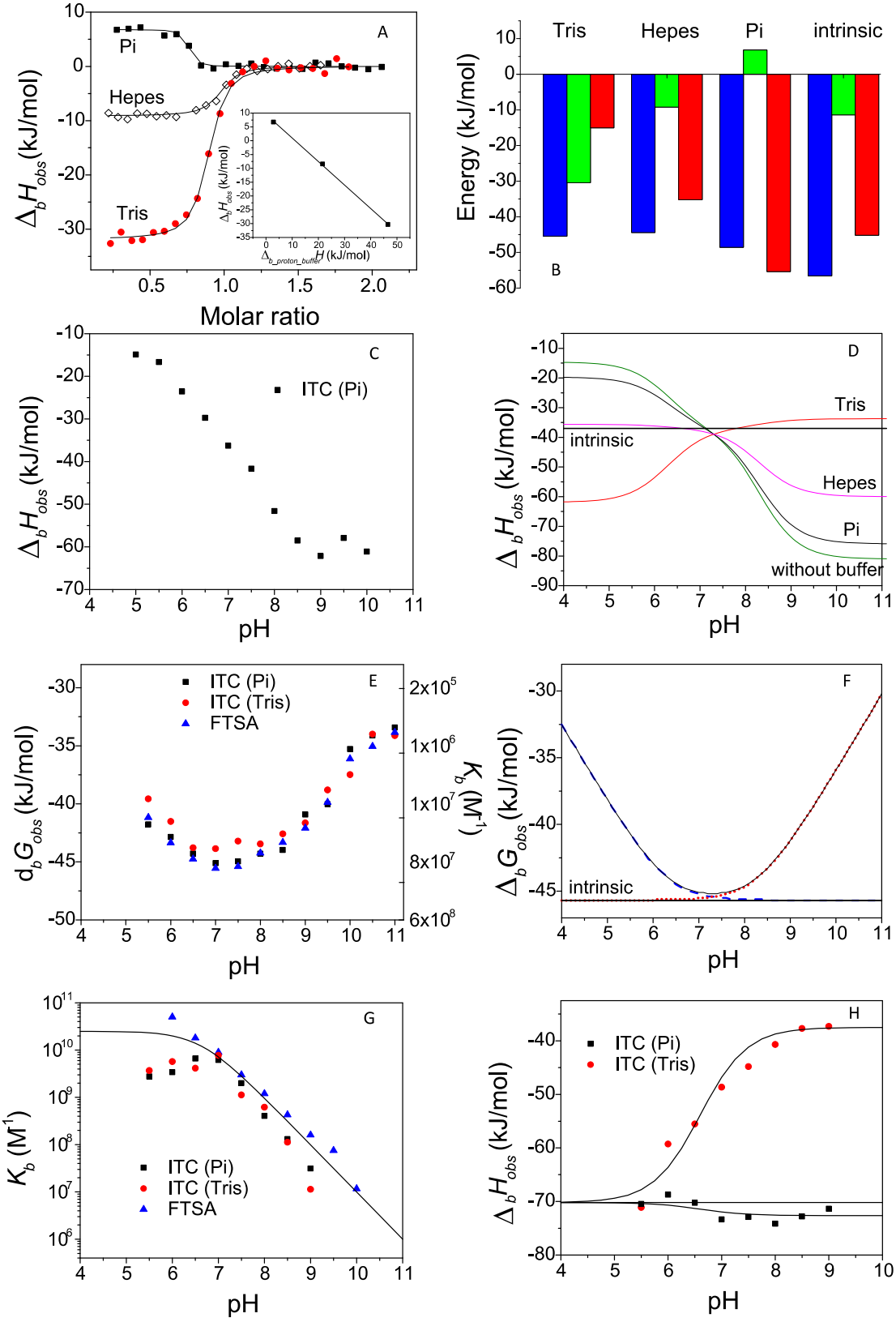
A
Pi
Hepes
Tris
$\Delta_b H_{obs}$ (kJ/mol)
Molar ratio
$\Delta_{b\_proton\_buffer} H$ (kJ/mol)
B
Tris
Hepes
Pi
intrinsic
Energy (kJ/mol)
C
ITC (Pi)
pH
D
Tris
intrinsic
Hepes
Pi
without buffer
E
ITC (Pi)
ITC (Tris)
FTSA
$d_b G_{obs}$ (kJ/mol)
K_b (M^{-1})
F
intrinsic
$\Delta_b G_{obs}$ (kJ/mol)
G
ITC (Pi)
ITC (Tris)
FTSA
K_b (M^{-1})
H
ITC (Pi)
ITC (Tris)

Figure 3. Determination of the intrinsic binding thermodynamics by ITC. Panel A shows the integrated ITC curves of a protein titration with a ligand at pH 7.0 in three different buffers: sodium phosphate (filled black squares), HEPES (open diamonds), and TRIS (filled red circles). The inset shows the observed enthalpy dependence on the buffer of varying enthalpy of protonation. Data taken from . Panel B compares the enthalpy (green bars) and entropy (-TΔS, red bars) contribution to the Gibbs energy (blue bars) of binding in various buffers with the intrinsic values. Panel C shows the observed enthalpy dependence on pH. Panel D shows the modeled calculated enthalpies in various buffers dependence on pH explaining the observation shown in Panel C. Panel E shows the Gibbs energy (affinity) dependence on pH observed by ITC both in sodium phosphate (Pi, filled black squares) and in TRIS (filled red circles) and also by FTSA. Panel F shows the model explanation of the observed data in Panel E. The U-shape line shows the observed ΔG dependence on pH, the dashed blue line shows the contribution of the lack of deprotonated ligand to the affinity, the dotted red line shows the contribution of the lack of protonated protein to the affinity, and the solid horizontal line shows the pH-independent intrinsic affinity. Data taken from [16]. Panel G shows the pH dependence of the affinitydetermined both by ITC and FTSA in a single binding-linked protonation event (ligand protonation). Panel H shows the observed (in TRIS – red filled circles, and in Pi – black filled squares) and intrinsic (horizontal line) enthalpies as a function of pH. The data is taken from [17].

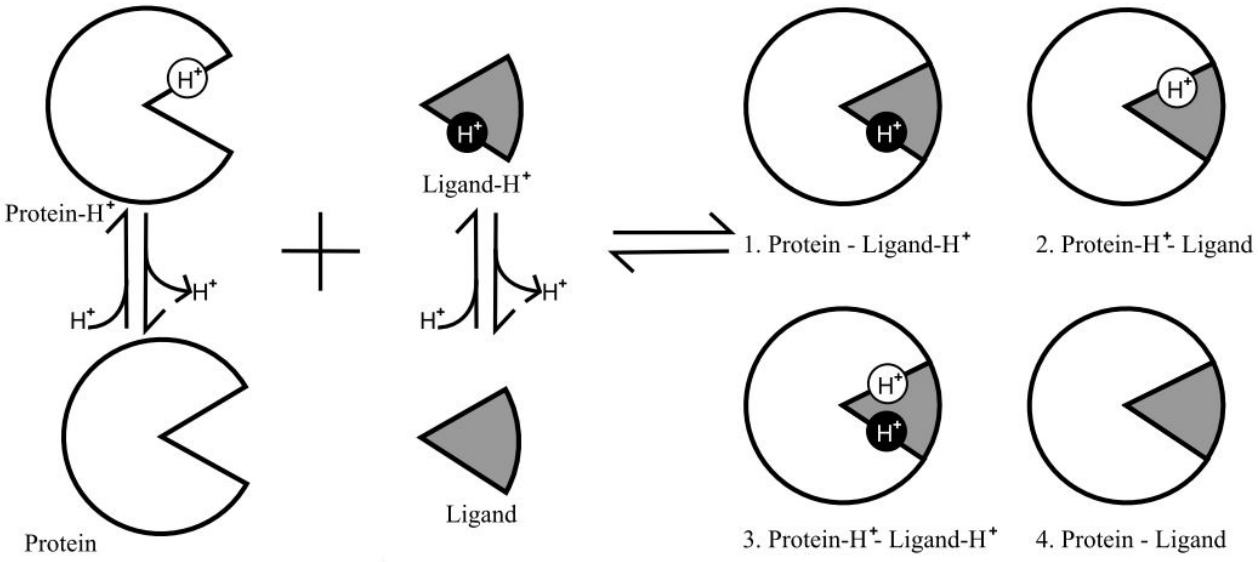

Figure 3. Schematic representation of the possible protonation-deprotonation reactions, both of the protein and ligand, which may occur upon the protein-ligand binding. If there is one linked reaction of the protein and one of the ligand, then there are four possibilities for the protein and ligand binding. However, only one path most likely will occur and the goal of the analysis is to determine this mechanism.

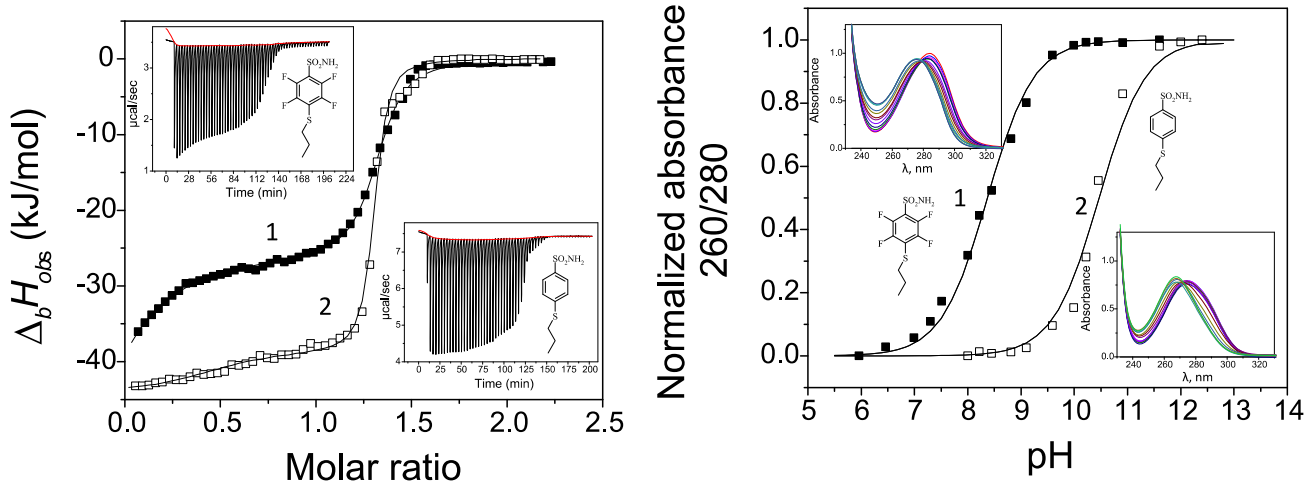

Figure 4. Determination of the pK_a and enthalpy of ligand protonation. Panel A. The enthalpy of sulfonamide protonation could be determined by ITC by titration of compounds **1** and **2** containing 1.3 equivalents of NaOH with HNO_3 at 37°C. The enthalpy of the first transition belongs to water formation from acid and base while the second transition represents the enthalpy of sulfonamide protonation. The insets show raw ITC data with non-flattened baseline. Panel B. Determination of the sulfonamide protonation pK_a may be obtained by UV-VIS spectrophotometry for compounds **1** (■) and **2** (□). The insets show the spectra at various pHs for both compounds. Data taken from [9].

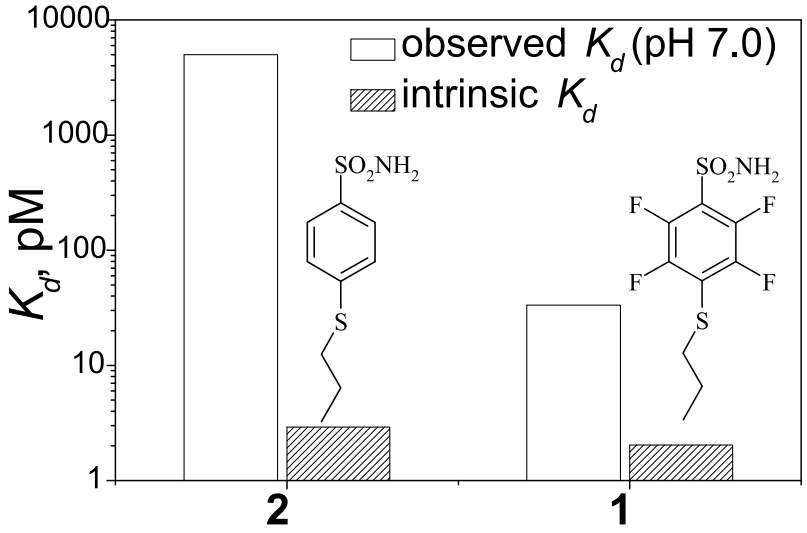

Figure 5. Importance of the determination of intrinsic affinities. The observed affinities of compounds **1** and **2** binding to a protein differed by approximately 100 fold. However, the intrinsic affinities were practically indistinguishable. Therefore, the effect of fluorine was not in the recognition of the protein surface but, as an electron withdrawing group, it reduced the pK_a of the ligand and increased the fraction of the binding-ready component, thus not affecting the intrinsic affinity.

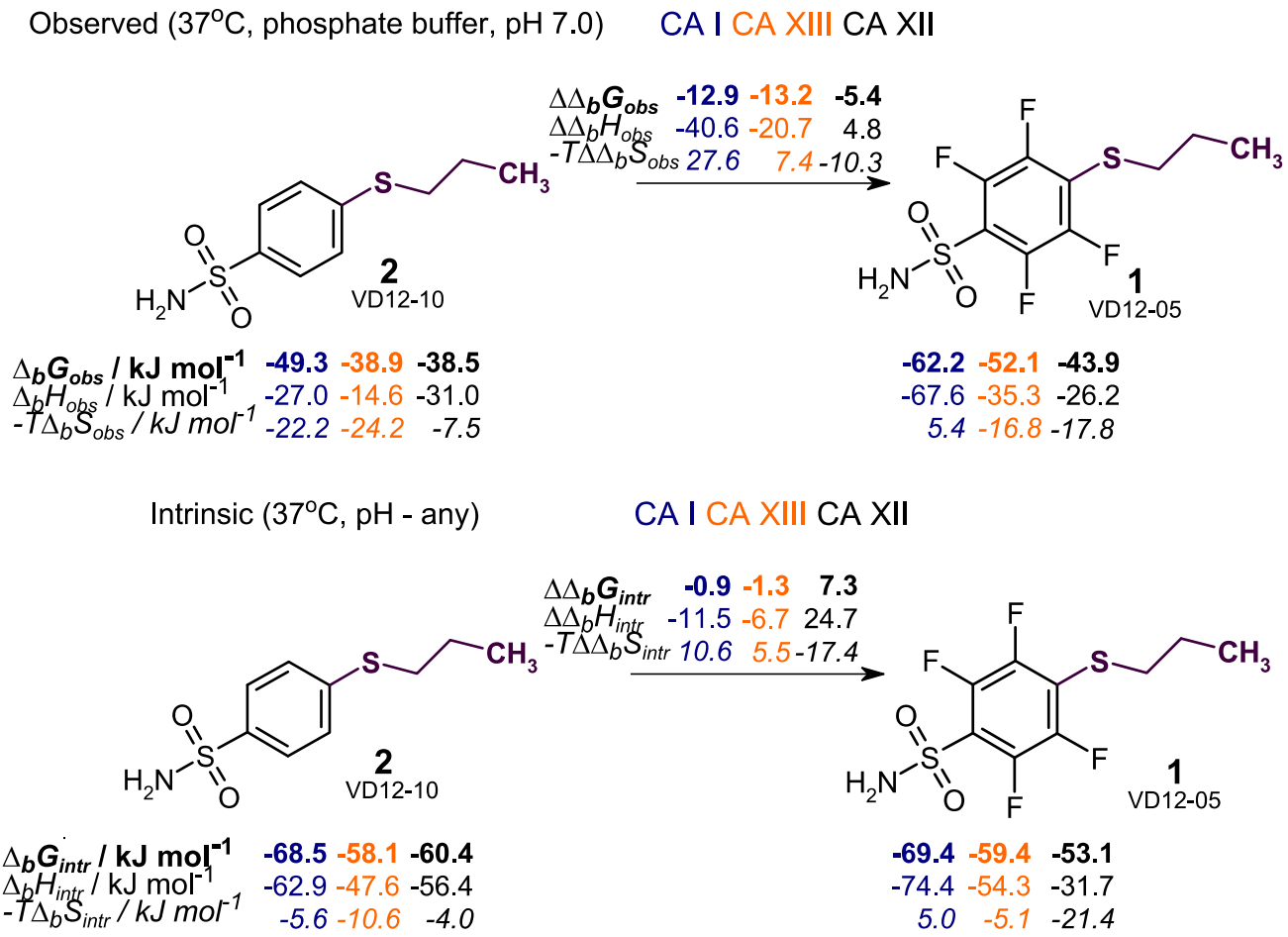

Figure 6. The observed and intrinsic enthalpies, entropies and Gibbs energies of compounds **1** and **2** binding to three proteins, namely, carbonic anhydrase isoforms CA I, CA XII, and CA XIII. The observed thermodynamics (upper half) is significantly different from the intrinsic values and only the intrinsic values should be used in the structure-thermodynamics correlations. Data taken from [9].