



In The Mix: Simultaneous Affinity Determination for Isomers and Enantiomers

Application Note

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This application note describes a method to determine simultaneously the affinities of two ligands which bind competitively to a single target, using one straightforward ITC experiment. Fitting routines for analysing such isotherms have been made freely available and can be downloaded from the MicroCal website at <http://www.microcalorimetry.com>. The relevant equilibrium binding models are described and have been validated by comparing measurements of the affinities of ligand-receptor interactions, using single ligands and ligand mixtures.

1. Introduction

A number of drug discovery strategies are currently employed in the pharmaceutical industry, ranging from the use of high-throughput screening assays to identify compounds which modulate the activity of a target enzyme or receptor at concentrations of a few micromolar^{1,2}, to medium-throughput biophysical assays which are configured to detect ligands which bind to the target at millimolar ligand concentrations^{3,4,5}. Once the initial hits have been identified, a typical drug discovery program will involve the synthesis and testing of between a hundred and several hundreds of compounds, during which time the affinity, selectivity and physico-chemical properties of the compounds are altered to be more drug-like^{6,7}.

With improvements in instrumentation and methodologies, particularly low C-value analysis⁸, ITC (Isothermal Titration Calorimetry) can now be applied to the study of molecular interactions with dissociation constants up to 1mM. In drug discovery, this allows ITC to be used at earlier stages of the process, from characterisation of the initial screening hits to the development of lead compounds^{9,10}, in addition to its traditional role in the determination of affinity during the final stages of pre-clinical development. Thus many more compounds may be considered for characterisation of their binding by ITC and thermodynamic information is available at an earlier stage in many drug discovery projects. This application note considers one of the consequences of this change.

During the hits-to-leads process, a significant number of compounds are synthesised as mixtures of enantiomers, diastereoisomers or regioisomers. Knowledge of the relative affinities of different isomers of a lead compound, when combined with information on the three-dimensional structure of the protein-ligand complex or a reliable computational model, allows informed decisions to be made concerning the design of further compounds. In turn this can greatly reduce the number of compounds that must be synthesised and tested, thus significantly improving the efficiency of the drug-discovery programme.

In many cases, compound mixtures can be separated using preparative HPLC and one or more isomers will be tested separately. However in some cases, particularly those involving

mixtures of enantiomers, separation may be difficult and time consuming and in these cases the mixture must be tested directly. The practical consequence of testing mixtures of ligands depends on the nature of the enzyme assay. For example, a bioassay may rely on the detection of substrate turnover using sensitive fluorimetric or radiometric techniques to monitor the rate of reaction. The enzyme is usually present at very low concentrations (0.1nM to 10nM, depending on its catalytic efficiency) compared to the dissociation constants to be measured, while the other components of the assay are present at concentrations around their K_m values or in saturating amounts (*e.g.* 1 μ M-100 μ M for substrates and cofactors in a typical kinase assay).

When an enzyme is titrated with an approximately equimolar mixture of ligands under these conditions, its activity is sensitive only to the most potent inhibitor in the mixture. At the start of the titration, the ligand concentration is too low to allow the weaker components of the mixture to bind while, later in the titration, insufficient enzyme activity remains to detect the effects of binding to low-affinity sites. Thus a bioassay provides information on the tightest binding component only in a mixture of ligands and this analysis remains true whether the ligands bind to different inhibitory sites or compete for the same binding site. Information on weak binding events is lost and cannot be used to inform structure-activity relationships (SAR) for the protein-ligand interaction.

In contrast to a bioassay, ITC experiments are carried out at much higher protein concentrations, typically where the concentration of protein equals or exceeds the expected dissociation constant of the ligand ($C \geq 1$). Fokkens and Klebe¹¹ have demonstrated that, under typical ITC conditions, both strong and weak binding events can influence the shape of the binding isotherm. The authors show experimental isotherms which are clearly non-sigmoidal, for titrations of thrombin and trypsin with racemic ligands. It is the purpose of this application note to explore the effects of ligand affinity and binding enthalpy on the shapes of these isotherms and to provide a mathematical model to allow deconvolution of the isotherms in the case of a two-component ligand mixture. It will also be demonstrated that, under some circumstances, mixtures of ligands can generate deceptively simple isotherms whose analysis using a simple 'one-site'

model will not reveal a tight binding event. Experimental tests for this situation will be discussed and strategies that may allow detection of both binding events will be proposed.

ITC titrations have been performed using EDTA and a mixture of two metal ions, as a model system to simulate the effects of a mixture of competitive ligands binding to a protein receptor. Barium (Ba^{2+}) and calcium (Ca^{2+}) were chosen since their EDTA complexes have significantly different dissociation constants and enthalpies [$K_{11}/K_{12} \sim 500$ and $\Delta\Delta H \sim 1.4 \text{ kcal mol}^{-1}$]. These thermodynamic values are within the range of those encountered in protein-ligand interactions. The use of a metal-ion/EDTA system also allows the ratio of metal ions in the mixture to be varied freely so that ligand mixtures other than racemates can be simulated easily.

2. Materials & Methods

ITC experiments were carried out using a VP-ITC instrument (MicroCal Northampton, MA). All reagents were obtained from Sigma-Aldrich (Poole, Dorset). The samples for each titration were prepared at the desired concentration in a buffer containing 200mM Hepes at pH 7.2. Experiments were conducted by injecting mixtures of BaCl_2 and CaCl_2 from the VP-ITC syringe into solutions of EDTA, hereafter referred to as forward titrations, or by injecting EDTA from the syringe into mixtures of BaCl_2 and CaCl_2 , referred to as reverse titrations. Molar ratios have been calculated as the ratio of total metal ion ($\text{Ba}^{2+} + \text{Ca}^{2+}$) to EDTA. A typical experiment consisted of a preliminary 3 μl injection followed by a number of subsequent 8 μl injections and the data were collected using a time constant of 2 seconds. The temperature was 25°C for all experiments and all reported ITC data are based on measurements from one single experiment using the stated concentrations of EDTA, BaCl_2 and CaCl_2 .

3. Experimental Results:

Figure 1:

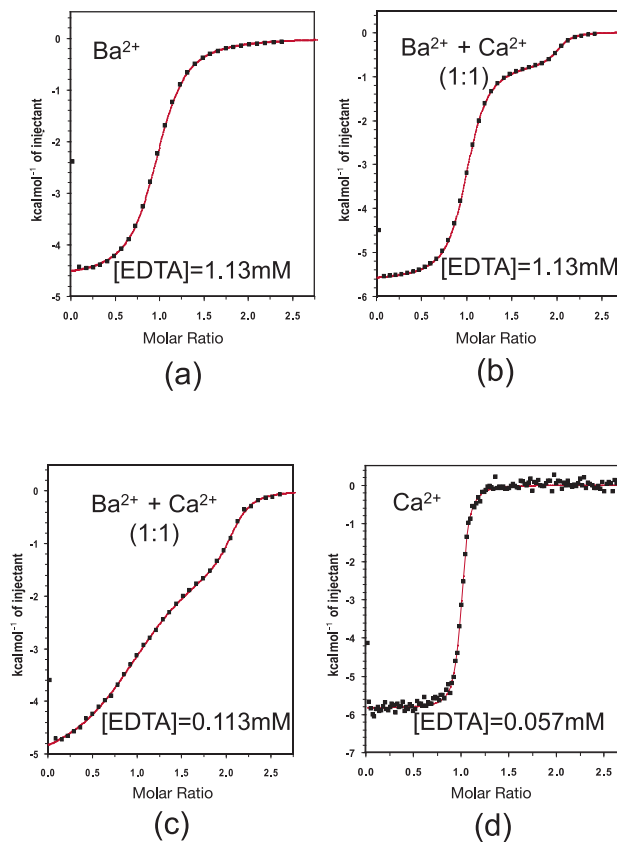


Figure 1: Representative isotherms for titrations of EDTA with Ba^{2+} , Ca^{2+} and an equimolar mixture of metal-ions.

Fitted curves (red lines) have been generated for single ion titrations (a, d) using the 'one site' model and the parameters given in Table 1. Titrations involving mixtures of metal ions (b, c) have been fitted using the model described in Appendix 1 and the parameters given in Table 2.

Representative titrations of EDTA with Ba^{2+} and Ca^{2+} alone are shown in Figure 1 (a) and 1 (d). Fitting of these data using a one-site model allows estimation of the thermodynamic parameters for chelation of Ba^{2+} and Ca^{2+} by EDTA. Three titrations were performed for each metal ion and the results are summarised in Table 1. These values agree well with those reported by Christensen *et al*¹².

Table 1: Summary of best-fit ('one-site') parameters from single ion titrations.

Titrant mM	Titrand mM	K_{d1} μM	K_{d2} μM	ΔH_1 kcal mol^{-1}	ΔH_2 kcal mol^{-1}	n
12.7 BaCl ₂	1.13 EDTA	33.3	-	-4.64	-	1.01
12.7 BaCl ₂	1.13 EDTA	34.4	-	-4.74	-	0.98
12.7 BaCl ₂	1.13 EDTA	33.2	-	-5.02	-	0.99
0.73 CaCl ₂	0.057 EDTA	-	0.097*	-	-5.84	1.00
1.45 CaCl ₂	0.113 EDTA	-	0.065*	-	-6.53	0.94
1.45 CaCl ₂	0.113 EDTA	-	0.074*	-	-6.67	0.92
Averages:		33.6 ± 0.8	0.079 ± 0.018	-4.81 ± 0.22	-6.34 ± 0.50	

* These affinities are obtained from curves for which the C value is 500 or above and thus are associated with higher uncertainties.

Isotherms generated by the titration of EDTA with an equimolar mixture of Ba²⁺ and Ca²⁺ at two concentrations of EDTA are shown in Figure 1(b) and 1(c). Two distinct phases of the titration are apparent and the overall shapes of the isotherms are consistent with those observed by Fokkens and Klebe¹¹. At an EDTA concentration of 1.13mM both ions have C-values greater than 30 and the isotherm displays a central plateau. When the EDTA concentration is reduced to 0.113mM (C-values of 3.4 [Ba²⁺] and 1.4x10³ [Ca²⁺]) the plateau is lost, although the additional inflection points are still clearly visible and the curve cannot be fitted to a simple 'one-site' model.

Figure 2 shows isotherms generated by the titration of EDTA with a mixture containing a 3-fold excess of Ba²⁺ or Ca²⁺. When the tighter-binding ion (in this case Ca²⁺), is the minor component in the mixture, care must be taken to extend the titration until its concentration exceeds that of EDTA.

Figure 2:

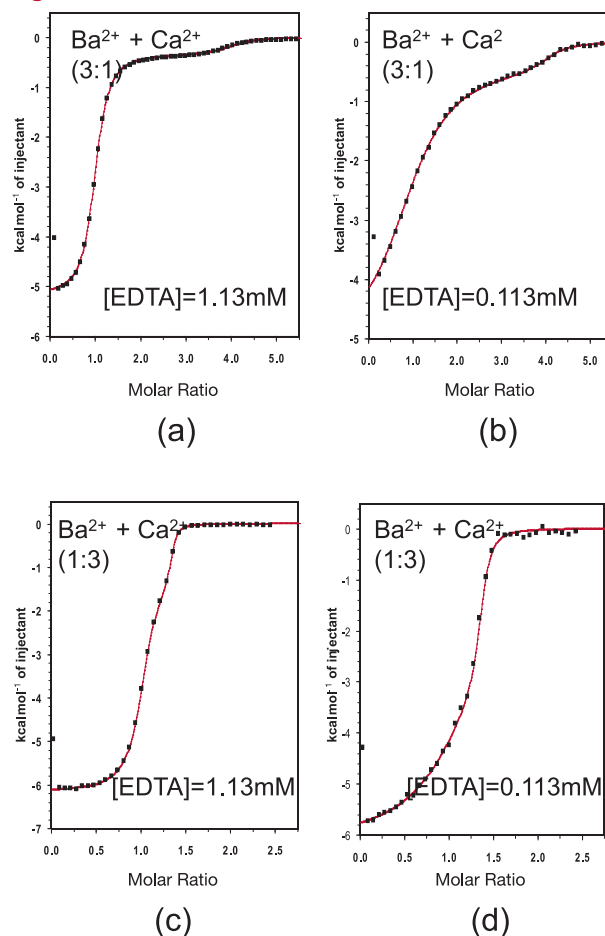


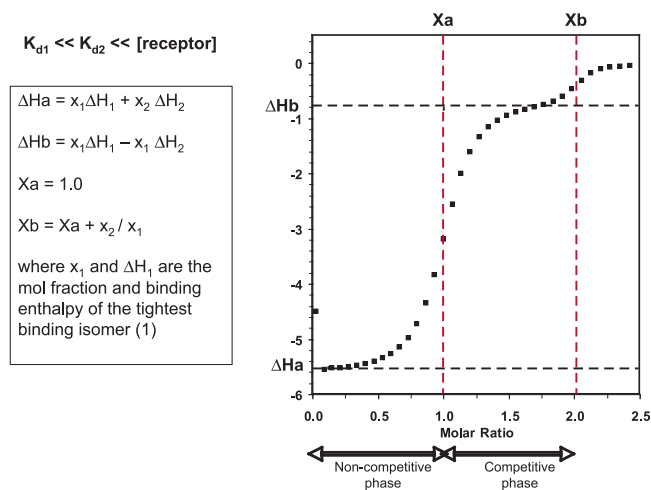
Figure 2: Isotherms for the titration of EDTA with 3:1 and 1:3 mixtures of Ba²⁺ and Ca²⁺.

Fitted curves have been generated in Excel using the model described in Appendix 1 and the parameters given in Table 2. All parameters were obtained by least squares minimisation using the program Mathematica (see section 4).

The two phases of the titration apparent in Figure 1(b) can be simply understood. Consider the titration of a mixture of ligands with K_{d1} and K_{d2} , where $K_{d1} \ll K_{d2}$. When this mixture is first titrated into the ITC cell containing a receptor (EDTA) whose concentration exceeds both K_{d1} and K_{d2} , the measured heat change will reflect the sum of their binding enthalpies weighted appropriately by their mol fractions, x_1 and x_2 . At this stage the receptor is present in excess over the total amount of ligand and

hence the ligands bind essentially independently. This is the non-competitive phase of the titration. If both ligands bind at independent sites on the receptor then, as the ligand concentration is increased, the binding isotherm will simply represent a sum of two individual titration curves. However if both ligands compete for the same site on the receptor, then, as the titration proceeds and the concentration of free protein decreases below K_{d2} , ligand 2 will dissociate while ligand 1 continues to bind. During this competitive phase of the titration, the measured heat output will reflect the difference between the binding enthalpies of ligands 1 and 2. Finally, once the displacement of ligand 2 by ligand 1 is complete and the receptor binding site is fully saturated, the heat output of the reaction will decrease rapidly to zero. For the 1:1 interaction described, the change in heat output from $x_1 \Delta H_1 + x_2 \Delta H_2$ to $x_1 \Delta H_1 - x_2 \Delta H_2$ occurs when the molar ratio of protein to total ligand is 1.0 while the change from $x_1 \Delta H_1 - x_2 \Delta H_2$ to zero occurs when the molar ratio of protein to ligand 1, the more potent ligand, is 1.0 (Figure 1).

Figure 3

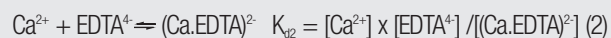
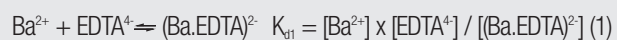


As will be described in more detail below, analysis of the binding isotherm can, in favourable cases, provide the full thermodynamic binding parameters for both ligands in the mixture. In other cases it may be possible to obtain reasonable values for one ligand while placing limits on the affinity of the second: this information is sufficient to allow the data to be incorporated into a structure-activity relationship for the protein-ligand interaction. Finally, in the special case where $\Delta H_1 \sim \Delta H_2$, it will be shown that the ITC data may be (erroneously) fitted to a single binding event with an affinity approximating to that of the weaker-binding component. In this case, comparison of the ITC result with biological assay data or reformatting of the ITC experiment will be necessary to determine if a tight binding event has been overlooked.

4. Data Analysis

Data from experiments involving only one of the two metal ions were analysed using the one-site model within the Origin software accompanying the MicroCal instrument¹³.

In cases involving mixtures of BaCl_2 and CaCl_2 we applied the competitive-binding model of Sigurskjöld¹⁴ to the following equilibria, under conditions where both metal ion concentrations are changing:



Parameters of interest in these experiments include the apparent dissociation constants K_{d1} and K_{d2} for binding of Ba^{2+} and Ca^{2+} respectively to EDTA, the apparent stoichiometry or concentration correction factor n (arbitrarily applied by replacing the total concentration $[\text{EDTA}]_{\text{tot}}$ with $n \times [\text{EDTA}]_{\text{tot}}$ in all equations), the enthalpies ΔH_1 and ΔH_2 for binding of Ba^{2+} and Ca^{2+} respectively to EDTA as well as the fraction f describing the percentage of BaCl_2 in a mixture of BaCl_2 and CaCl_2 :

$$f = 100 \times [\text{Ba}^{2+}] / ([\text{Ca}^{2+}] + [\text{Ba}^{2+}]) = 100 \times [\text{Ba}^{2+}] / [\text{Metal}] \quad (3a)$$

$$100 - f = 100 \times [\text{Ca}^{2+}] / ([\text{Ca}^{2+}] + [\text{Ba}^{2+}]) = 100 \times [\text{Ca}^{2+}] / [\text{Metal}] \quad (3b)$$

Where [Metal] represents the sum of the concentrations of the metal ions.

The experimental isotherms were fitted to this model, as described in Appendices 1 and 2, to obtain estimates of K_{d1} , K_{d2} , ΔH_1 , ΔH_2 , n and f , using the NonlinearRegress function within the Mathematica software (version 3.0, Wolfram Research). This function employs the Levenberg-Marquart method for minimisation of the sum of the squared residuals between experimental data and model data.

Thermodynamic parameters for the binding of Ba^{2+} and Ca^{2+} to EDTA, obtained by fitting data generated from titrations of EDTA with mixtures of metal ions, are given in Table 2. These data include the isotherms shown in Figure 1(b and c) and Figure 2(a-d), as well as those resulting from two titrations in which EDTA was present in the syringe (Figure 6b and data not shown). Excellent agreement was obtained with the parameters derived from single-ion titrations (Table 1) and the model accurately described the shapes of the isotherms giving small, randomly distributed residuals.

In practice, analysis of isotherms that show two clear inflection points *e.g.* Figures 1b and 1c, gives convergent fits that yield accurate values for all six parameters. As indicated in Figure 3, the abscissa of the inflection points contain information on the relative molar amounts of the species present (n and f) and the ordinate values of the plateaus depend on the binding enthalpies (ΔH_1 and ΔH_2) while the curvature of the isotherm on either side of the inflection points is determined by the binding constants, K_{d1} and K_{d2} . Finally, the fitting of some isotherms, notably those which were extended to high molar ratios in order to observe saturation by a tight-binding, minor component (Figures 2a and 2b), was improved by correction of each titration point by the heat of dilution. This was determined experimentally from the residual heat change at the end of the titration ($\sim 0.02 \text{ kcal mol}^{-1}$) and was assumed to be constant throughout the experiment. However, caution should be exercised here: the effect of the heat of dilution on the isotherm is indistinguishable from that of a third tight-binding metal ion present at very low molar ratio, unless the titration can be extended to very high molar ratios.

Table 2: Summary of best-fit parameters from titrations using Ba^{2+}/Ca^{2+} mixtures.

Titrant mM	Titrand mM	K_{d1} μM	K_{d2} μM	ΔH_1 kcal mol^{-1}	ΔH_2 kcal mol^{-1}	n	$f\%$
6.35 $BaCl_2$ 6.35 $CaCl_2$	1.13 EDTA	32.0	0.058	-4.93	-6.36	1.00	51.4
0.635 $BaCl_2$ 0.635 $CaCl_2$	0.113 EDTA	30.4	0.076	-4.21	-6.51	0.937	52.3
30.00 $BaCl_2$ 10.00 $CaCl_2$	1.13 EDTA	39.7*	0.074*	-4.84*	-6.23*	1.02*	75.4*
3.00 $BaCl_2$ 1.00 $CaCl_2$	0.113 EDTA	36.2	0.055	-4.78	-6.37	0.81	76.1
3.18 $BaCl_2$ 9.53 $CaCl_2$	1.13 EDTA	40.1	0.084	-5.05	-6.53	0.993	25.8
0.318 $BaCl_2$ 0.953 $CaCl_2$	0.113 EDTA	39.4	0.106	-4.86	-6.56	0.999	26.8
10 EDTA	0.5 $BaCl_2$ 0.5 $CaCl_2$	36.3	0.062	-4.93	-6.29	0.892	49.7
1 EDTA	0.05 $BaCl_2$ 0.05 $CaCl_2$	39.2	0.075	-4.98	-6.51	0.848	52.0
Averages:		36.7	0.074	-4.82	-6.32		
\pm Std. Dev:		± 3.7	± 0.016	± 0.26	± 0.12		

* Fitting included a correction for the heat of dilution ($0.024 \text{ kcal mol}^{-1}$), measured at high values of the molar excess.

A Mathematica workbook for fitting MicroCal data from similar experiments can be obtained from <http://www.microcalorimetry.com>. The authors have also provided a Microsoft Excel worksheet that describes these calculations and which can be used for the generation of simulated isotherms and speciation curves (see section 5).

5. Simulation of Competitive Binding Isotherms

(a) Effects of enthalpies

Simulated binding isotherms are shown in Figure 4 for the titration of a protein with an equimolar mixture of two

competitive ligands, whose dissociation constants are 0.001 μ M and 1 μ M. The protein concentration was chosen to be 20 μ M so that the C-value is high for both ligands and the molar ratio is defined as $[\text{ligand}]_{\text{tot}}/[\text{protein}]$. The isotherms illustrate the effect of changes in the enthalpies of binding. In each simulation, the average enthalpy of binding has been fixed at -8 kcal mol⁻¹ while the individual enthalpies range from -12 kcal mol⁻¹ to -4 kcal mol⁻¹.

Figure 4

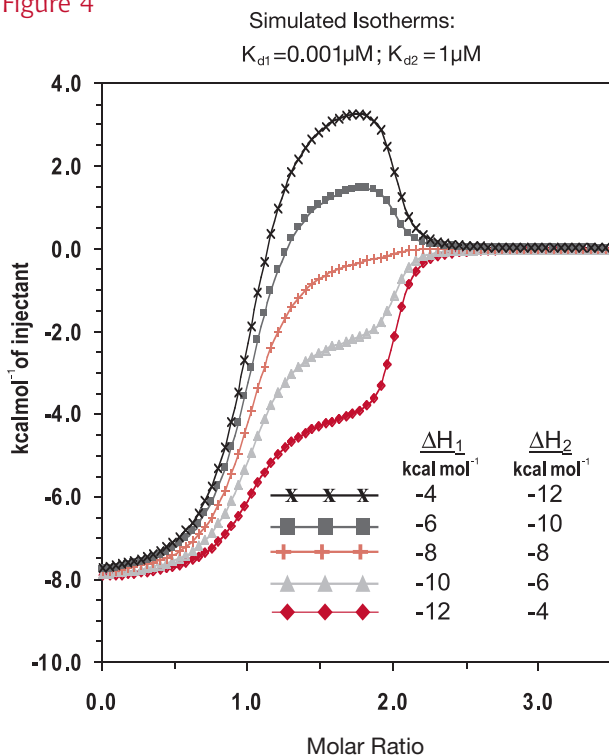


Figure 4: Simulated binding isotherms for competitive ligand mixtures, showing the effects of changes in binding enthalpy. The isotherms were generated in Excel using the mathematical model outlined in the appendices. The Excel worksheet can be downloaded from <http://www.microcalorimetry.com>.

The isotherms illustrate the range of behaviour which is possible. When the more potent ligand also has the more negative enthalpy of binding, a clear two-step titration is observed with a central plateau around an enthalpy value given by $(\Delta H_1 - \Delta H_2)/2$ and with transitions at molar ratios of 1.0 and 2.0 (bold red and light grey curves).

When the more potent ligand, ligand 1, has a less negative enthalpy of binding than the weaker ligand, then the competitive phase of the titration is marked by an endothermic process, even though all binding enthalpies are exothermic (dark grey and black curves).

If $\Delta H_1 \approx \Delta H_2$, the second phase of the titration, during which ligand 1 replaces ligand 2, is almost undetectable. Any heat output arises simply from a small change in the total amount of protein bound to ligand and, since $C \gg 1$ for both ligands, only a few percent of the protein will contribute. Under these conditions, only the first phase of the titration, where both ligands are able to bind independently to the protein, is apparent and the titration curve closely resembles an isotherm generated by a single ligand species (light red curve).

If such a curve is measured for a ligand that is known to be a mixture of two species *e.g.* a racemic mixture, then extreme caution should be applied to the analysis of the results. In the example shown above, one of the ligands binds very tightly ($K_{d1} = 0.001\mu\text{M}$) and so its binding isotherm approximates to a step function. During the non-competitive phase of the titration, this step function is superimposed on a sigmoidal binding isotherm arising from binding of the second ligand ($K_{d2} = 1\mu\text{M}$). The effect of this addition is to stretch the sigmoid in the y-direction while leaving its curvature relatively unchanged. It is easily shown that, under these conditions ($\Delta H_1 \approx \Delta H_2$) the data are fitted reasonably well by a single binding event with $K_d \sim 0.5\mu\text{M}$ and an enthalpy given by the average of ΔH_1 and ΔH_2 ; the tight binding event contributes equally to the observed enthalpy but only marginally to the apparent affinity.

(b) Effects of Binding Constants

A series of isotherms for an equimolar mixture of ligands whose binding enthalpies are -12 kcal mol⁻¹ and -4 kcal mol⁻¹ and whose ratio of dissociation constants varies from 1 to 1000 are shown in figure 5. As above, the simulation has been carried out under conditions where the C-value is ≥ 20 for each ligand in all titrations.

Figure 5

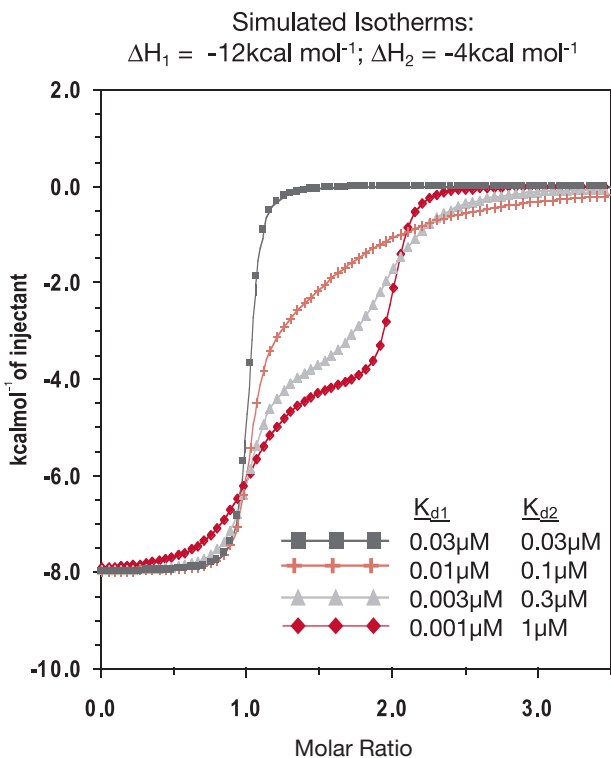


Figure 5: Simulated binding isotherms for competitive ligand mixtures, showing the effects of changes in relative affinity. The isotherms were generated in Excel using the mathematical model outlined in the appendices. The Excel worksheet can be downloaded from <http://www.microcalorimetry.com>.

These four isotherms correspond to ITC titrations in which the concentrations of species in solution are given by the speciation curves of Figure 7. As the dissociation constants become more similar, the central plateau is lost. However, even when the dissociation constants differ only by a factor of 10 (light red curve), the asymmetric nature of the curve demonstrates the presence of a competitive binding event occurring during the second half of the titration. The degree of this asymmetry depends on the enthalpy difference between the ligands; the large enthalpy difference in this simulation makes discrimination between the dissociation constants particularly straightforward.

6. Optimisation of Experiments

Many isotherms can be fitted to a number of different mathematical models. The most important factor in the correct analysis of the data is the selection of physically reasonable models. This in turn will often require additional information, such as the purity or enantiomeric excess of the ligand, the number of binding sites for the ligand on the receptor and an appreciation of any additional possibilities such as changes in the oligomeric states of the receptor caused by complexation.

Analysis should choose the simplest model which successfully describes the data. However this should not be used to hide known complexities in the system. Thus, although titration data involving a racemic mixture can, under some circumstances, be successfully fitted to a model involving a single protein-ligand interaction, this may conceal significant differences in enantiomeric affinity.

An experimental test of any mathematical model is to repeat the titration over a range of receptor concentrations or to exchange the components of the titration. If the best-fit parameters change significantly during this procedure then the model is inadequate.

There are two circumstances in which titrations involving ligand mixtures give deceptively simple curves: these occur when $\Delta H_1 = \Delta H_2$ or $K_{d1} = K_{d2}$. The former case is particularly problematic since it may conceal very large differences in ligand affinity. In this case, fitting the curve to a single interaction reveals only the weaker of the two affinities. Only if the titration can be carried out at a low receptor concentration, such that the C-value for the weaker-binding component is close to 1, will deviations from a simple sigmoidal curve be apparent and can both binding affinities be fitted using the model described here. A more reliable strategy to detect the different binding steps is to reverse the titration such that the receptor is titrated into the ligand.

Experimental and simulated isotherms for forward (EDTA in cell) and reverse (metal ions in cell) titrations of EDTA with a 1:1 mixture of Ba^{2+} and Ca^{2+} are shown in Figure 6a and 6b. Simulated isotherms for forward and reverse titrations, involving a mixture of a hypothetical pair of ions whose affinities are the same as Ba^{2+} and Ca^{2+} but whose binding enthalpies are equal to the average value for Ba^{2+} and Ca^{2+} , are shown in Figure 6c and 6d.

Figure 6

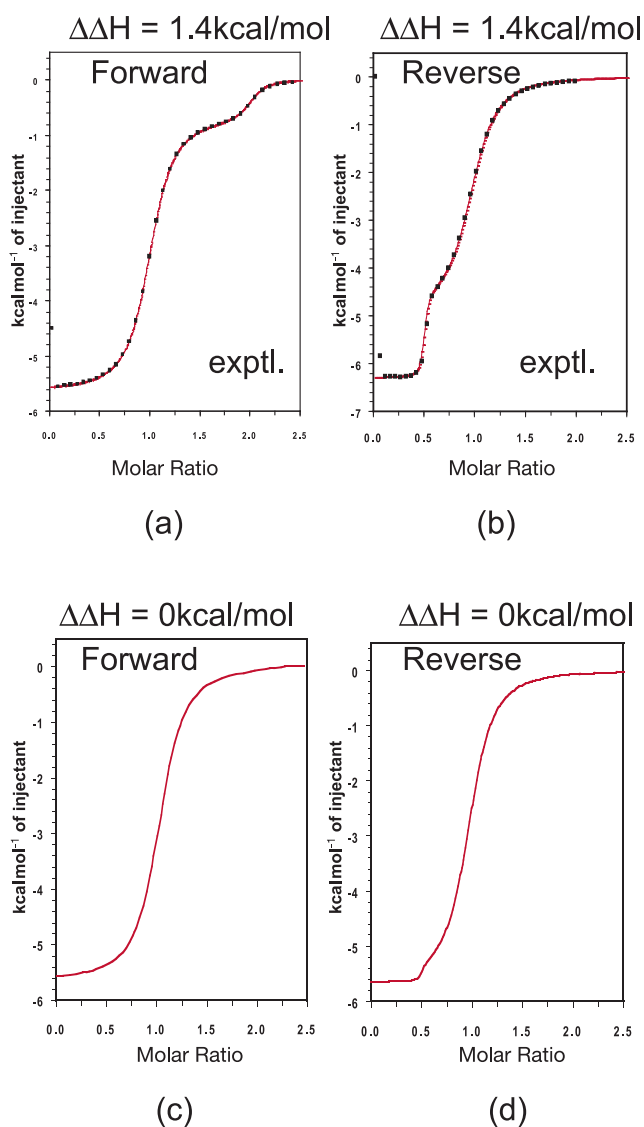


Figure 6: Comparisons of isotherms for forward and reverse titrations of EDTA with an equimolar mixture of metal ions. Experimental data are shown in black and simulated isotherms are in red.

- Titration of EDTA (in cell) with Ba^{2+} and Ca^{2+} (in syringe). $[\text{EDTA}] = 1.13 \text{ mM}$
- Titration of Ba^{2+} and Ca^{2+} (in cell) with EDTA (in syringe). $[\text{Metal}] = 1.13 \text{ mM}$
- Simulated titration of EDTA (in cell) with an equimolar mixture of metal ions (in syringe). The metal-ion dissociation

constants are given by those of Ba^{2+} and Ca^{2+} ($30 \mu\text{M}$ and $0.06 \mu\text{M}$) and their enthalpies are equal to the average value for Ba^{2+} and Ca^{2+} ($-5.6 \text{ kcal mol}^{-1}$). $[\text{EDTA}] = 1.13 \text{ mM}$. Note that the curve resembles a simple sigmoid and that the competitive phase of the titration is not readily detected. Fitting the data using a one-site model gives a value for the apparent dissociation constant of $17 \mu\text{M}$, close to that of the weaker-binding metal ion.

- Simulated titration of an equimolar mixture of metal ions (in cell) with EDTA (in syringe). All thermodynamic parameters are as (c). $[\text{Metal}] = 1.13 \text{ mM}$. Despite the equivalence of their binding enthalpies, the presence of two metal ions in the mixture is apparent from the beginning of the isotherm and this can be fitted to derive the two dissociation constants.

The isotherms demonstrate that either experimental arrangement leads to clear deviations from a simple sigmoid when both the dissociation constants and enthalpies are significantly different. However, when the binding enthalpies are identical, only the reverse titration gives an isotherm that must be fitted using two dissociation constants. The second, competitive phase of the forward titration (displacement of the weaker-binding metal from EDTA by the tighter binding metal) leads to little or no enthalpy change and is not apparent. In contrast, the isotherm of the reverse titration reveals both events. At the start of the reverse titration, when the ligand is in large excess, the curvature of the isotherm is dominated by the tighter binding metal-ion. However when the EDTA concentration exceeds that of the tighter-binding metal ion (molar ratio > 0.5 in Figure 6d) then the lower-affinity component is able to bind and the curvature of the isotherm reflects this.

For protein-ligand systems, reverse titrations may not always be practical. The solubility and availability of the protein and sometimes ligand may be limited and non-specific interactions can contribute to the early part of the isotherm. In such cases, variation of the solution conditions (pH, temperature, ionic strength) may alter the ligand binding enthalpies sufficiently that both binding events can be distinguished when ligand is titrated into protein.

The data presented here are of very high quality. The enthalpy changes are reasonably large and sufficient reagent is available to carry out the titrations at high concentrations where small injections will give rise to readily detected heat changes. Thus the shapes of the isotherms are well-defined and any deviation from a simple sigmoid is apparent. In contrast many protein-ligand titrations are carried out with fewer injections and generate isotherms with lower signal-to-noise (the noise figure is approximately 0.5 μ cal per injection in a typical calorimeter). As such, extreme care should be taken when using this approach to fit binding isotherms with low heats and with poorly resolved 'features'.

In order to validate our approach in a less-ideal system, random noise (generated using a Monte-Carlo method) was added to the metal-ion/EDTA data so that the standard deviation of each titration point was comparable to the signal-to-noise of a typical protein-ligand titration. Provided that deviation from a simple sigmoid was still evident, the isotherms could be successfully fitted using Mathematica and the parameters derived were in good agreement with the values obtained from fitting the unmodified data.

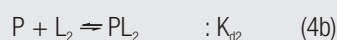
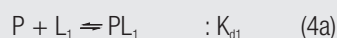
7. Summary

It is clear that ITC is capable of simultaneously measuring the affinity of mixtures of isomers and enantiomers. The excellent agreement between all the thermodynamic data derived from 'traditional' experiments and those with ligand mixtures is very encouraging and clearly demonstrates the applicability of this approach to differentiating the binding properties of isomers, without the need for their separation. Indeed, in some cases, the non-sigmoidal appearance of the isotherm is so apparent that the technique can be used with confidence to identify the presence of ligand mixtures.

Not all systems will be as clearly delineated as this one. Indeed, for ligand mixtures which are characterised by similar thermodynamics of interaction, the individual contributions are hard to dissect out and the shape of the isotherm may conceal the tighter-binding interaction. However with prior knowledge of the presence of a ligand mixture, there are a number of experimental approaches that can be used to optimise the ITC experiment and thus obtain discrete thermodynamic values.

Appendix 1: Competitive Binding Equilibria

Competitive binding of two ligands to a single receptor can be described by two equilibria.



Provided the ligands L_1 and L_2 do not interconvert on the timescale of the binding experiment and that the concentrations of other complexes are negligible, then these equilibria are sufficient to fully describe the system.

The relative affinity of two ligands which bind to a protein target can be investigated by carrying out ITC, NMR or kinetic assay experiments under conditions where the ligands bind competitively. In most cases, the concentration of the macromolecular target and one of the competing ligands is fixed while the concentration of the second ligand is varied^{14,15}. Measurement of the enzyme activity, heat evolved or NMR parameters of the ligands can be used to define the apparent affinity of the second ligand from which its true affinity can be calculated, provided the affinity of the first ligand is known.

This approach has previously been described for precise analysis of ITC displacement data by Sigurskjöld¹⁴ and such data can be fitted using the Competitive.dH binding model in the MicroCal software.

When a titration involves an unresolved mixture of competitive ligands, the situation is somewhat different. In this case, the concentration of ligands cannot be varied independently and both concentrations will change in the course of the titration.

If the two dissociation constants are K_{d1} and K_{d2} then:

$$[P][L_1] = K_{d1} [PL_1] \quad (5a)$$

$$[P][L_2] = K_{d2} [PL_2] \quad (5b)$$

$$[P]_{tot} = [P] + [PL_1] + [PL_2] \quad (6a)$$

$$[L_1]_{tot} = [L_1] + [PL_1] \quad (6b)$$

$$[L_2]_{tot} = [L_2] + [PL_2] \quad (6c)$$

Where the total concentrations of protein and ligands is given by $[P]_{tot}$, $[L_1]_{tot}$ and $[L_2]_{tot}$ and their unbound concentrations are given by $[P]$, $[L_1]$ and $[L_2]$.

Combining these equations to eliminate $[PL_1]$, $[PL_2]$, $[L_1]$ and $[L_2]$ gives:

$$[P]^3 + a[P]^2 + b[P] + c = 0 \quad (7)$$

where

$$a = K_{d1} + K_{d2} + [L_1]_{tot} + [L_2]_{tot} - [P]_{tot}$$

$$b = K_{d1}([L_2]_{tot} - [P]_{tot}) + K_{d2}([L_1]_{tot} - [P]_{tot}) + K_{d1} K_{d2}$$

$$c = -[P]_{tot} K_{d1} K_{d2}$$

Wang¹⁶ has shown that there is only one physically meaningful root of this cubic equation, given by:

$$[P] = 1/3 [-a + 2(a^2 - 3b)^{1/2} \cos(\Delta/3)] \quad (8)$$

where

$$\Delta = \arccos [(-2a^3 + 9ab - 27c) / \{2(a^2 - 3b)^{3/2}\}]$$

Provided the total concentration of protein and ligands is known, in addition to the values of the dissociation constants K_{d1} and K_{d2} , $[P]$ can be calculated. It is then a simple matter to calculate the concentrations of all other species in solution.

Excel spreadsheets that perform these calculations, given suitable input, have been used to generate speciation curves and isotherms for hypothetical titrations of a receptor with a mixture of two ligands. The simulated isotherms are discussed in more detail in section 5.

Speciation curves, showing the amounts of free protein and the two protein-ligand complexes during titrations involving an equimolar mixture of two ligands are shown in Figure 7. In all cases, the protein (titrand) concentration is considerably higher than the dissociation constant of the weakest ligand present. In an ITC titration this would correspond to a C-value greater than 20 for each protein-ligand complex. The dissociation constants have been chosen to show the effects of a variation in the relative ligand affinities, from 1 to 1000, while the geometric mean of the affinities (*i.e.* the average binding free energy) is unchanged. If the ligand mixture was a racemate, this would represent a range of possibilities, from no selectivity by the receptor, to almost complete discrimination between the enantiomers.

Figure 7

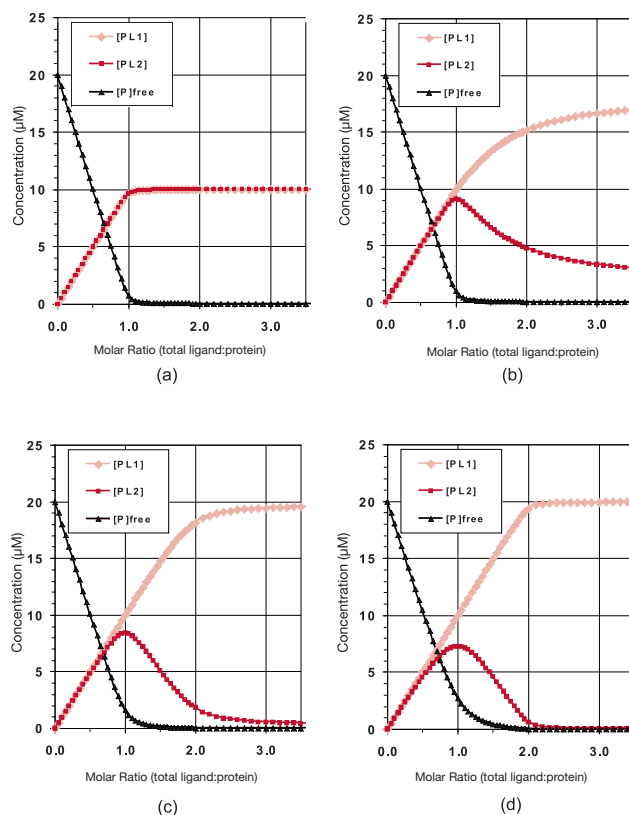


Figure 7: Speciation curves for the titration of a protein with a mixture of two competing ligands, showing the effects of changes in relative affinities.

(a) $K_{d1} = 0.03\mu\text{M}$; $K_{d2} = 0.03\mu\text{M}$

(b) $K_{d1} = 0.01\mu\text{M}$; $K_{d2} = 0.1\mu\text{M}$

(c) $K_{d1} = 0.003\mu\text{M}$; $K_{d2} = 0.3\mu\text{M}$

(d) $K_{d1} = 0.001\mu\text{M}$; $K_{d2} = 1\mu\text{M}$

Inspection of Figure 7(a-d) shows that the variation in the concentration of free protein over the course of the titration does not depend greatly on the relative affinity of the two ligands (black traces). The same conclusion holds for the variation in the concentration of bound protein and the *total* concentrations of free and bound ligands $[L_1] + [L_2]$. Thus any experiment whose outcome depends only on these parameters will be unable to detect clearly any variation in the relative affinities of the two ligands. This includes biological assays which measure enzyme activity as well as NMR experiments which detect only the spectrum of the protein (*e.g.* SAR

by NMR¹⁷) or where the ligands have indistinguishable NMR spectra (e.g. NMR studies of racemates).

The speciation curves of Figure 7 differ most clearly in the relative amounts of ligand 1 and ligand 2 which are bound to protein during the later stages of the titration. Once the molar ratio, defined as the total mols ligand to mols protein, exceeds 1 the less tightly bound ligand (ligand 2) is displaced by the more tightly bound ligand (ligand 1). At high concentrations of the equimolar ligand mixture, the relative amount of the two complexes, PL₁ and PL₂, tends to a value given by the ratio of their dissociation constants. More importantly, the value of the molar ratio at which this limit is approached also depends on the relative affinity of the two ligands.

If the binding enthalpies of ligand 1 and ligand 2 differ significantly, then this phase of the titration will lead to a detectable heat change. Thus, in principle, deconvolution of an ITC binding isotherm in this region could be used to estimate the relative affinities of the two ligands.

Appendix 2: Instrumental Factors

In the experiments described above, the total concentration of all reactants is diluted during the course of the titrations since each injection displaces part of the volume that was in the calorimetric cell prior to the injection. In the forward titrations, the total concentrations of protein (or EDTA) and ligands (L₁ and L₂) in the calorimetric cell can be calculated following each injection, using equations 9 and 10:

$$[P]_{\text{tot}} = [P]_0 \times (1 - V_i/(2 \times V_0))/(1 + V_i/(2 \times V_0)) \quad (9)$$

$$[L]_{\text{tot}} = V_i \times [L]_0 \times (1 - V_i/(2 \times V_0))/V_0 \quad (10a)$$

$$[L_1]_{\text{tot}} = f/100 \times V_i \times [L]_0 \times (1 - V_i/(2 \times V_0))/V_0 \quad (10b)$$

$$[L_2]_{\text{tot}} = (100 - f)/100 \times V_i \times [L]_0 \times (1 - V_i/(2 \times V_0))/V_0 \quad (10c)$$

V_i and V₀ represent the total injected volume and the volume of the calorimetric cell, respectively, whereas [P]₀ and [L]₀ represent the starting concentrations of protein and ligands (L₁ + L₂) in the calorimetric cell and the syringe, respectively. Once the total concentration of each ligand and the protein is known, the concentrations of the complexes, [PL₁] and [PL₂], can be

calculated for any given combination of K_{d1} and K_{d2} as described in Appendix 1. The expected heat exchange (ΔQ_i) resulting from every injection (i) can then be calculated as the difference in total heat content (Q) in the cell between successive injections, with a small correction for volume displacement in the calorimetric cell as described by equations 11 and 12 (see the Appendix, MicroCal VP-ITC manual):

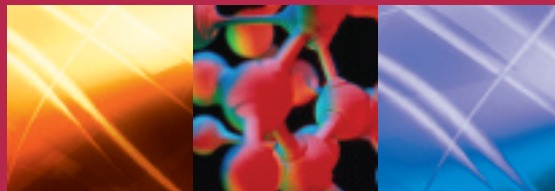
$$Q_i = V_0 \times \Delta H_1 \times [PL_1] + V_0 \times \Delta H_2 \times [PL_2] \quad (11)$$

$$\Delta Q_i = Q_i - Q_{i-1} + dV (Q_i + Q_{i-1}) / (2 V_0) \quad (12)$$

Where dV is the volume of the ith injection. The heat exchange is then normalised by dividing ΔQ_i with the injection volume (dV) and the concentration [L]₀ in the syringe, i.e. kcal per mole of injectant.

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