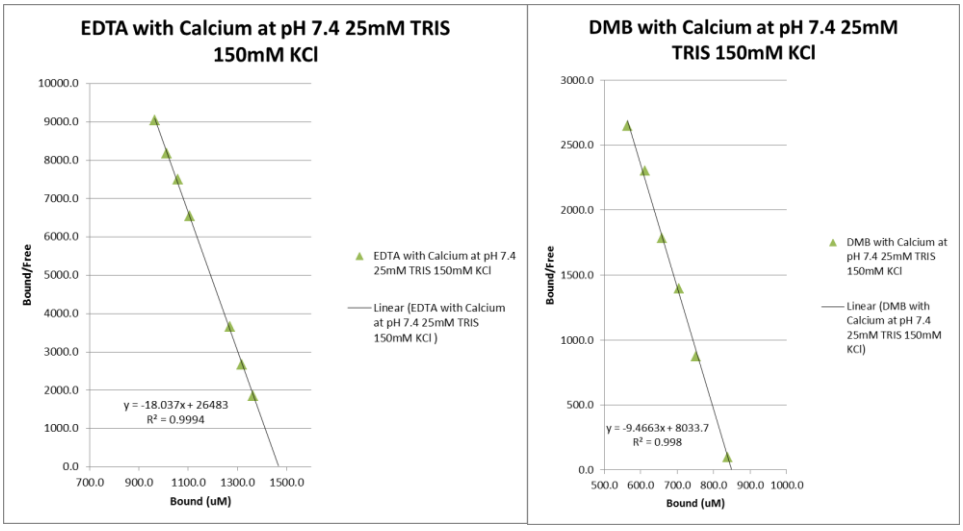
Potentiometric measurements, analytical sample preparation details, and Schatchard plots for EDTA and 5,5'-dimethylBAPTA in pH 7.4 25 mM TRIS 150 mM KCl binding to calcium. Asterisks denote samples used in ITC experiments for data presented in Figures 1 and Supporting Figure S1.

sample number	[5,5' dimethyl BAPTA] mM[1]	5,5' dimethyl BAPTA Vol. (ml)	Stock [5,5' dimethyl BAPTA] mM	[Calcium Standard] mM							Sample vol. (ml)	[Ca]mM	V_{Ca} (mV)	pCa^2	[Ca] free, uM	[Ca] bound, uM	B/F	ITC
			3.814	10.00	2.30	1.20	1.00	0.80	0.10	0.00								
				Volume of Standard Added (ml)														
0	1.122	3		0.00	0.00	0.00	0.00	0.00	0.00	7.20	10.20	0.000	n/a					*
1	1.122	5		0.00	0.00	0.00	0.00	12.00	0.00	0.00	17.00	0.565	-109.4	6.671	0.2132	564.5	2647.7	*
2	1.122	5		0.00	0.00	0.00	4.00	8.00	0.00	0.00	17.00	0.612	-106.6	6.576	0.2656	611.5	2302.5	
3	1.122	5		0.00	0.00	0.00	8.00	4.00	0.00	0.00	17.00	0.659	-102.4	6.433	0.3692	658.5	1783.3	*
4	1.122	5		0.00	0.00	0.00	12.00	0.00	0.00	0.00	17.00	0.706	-98.4	6.296	0.5053	705.4	1395.9	
5	1.122	5		0.00	0.00	4.00	8.00	0.00	0.00	0.00	17.00	0.753	-91.6	6.065	0.8615	752.1	873.0	
6	1.122	5		0.00	0.00	12.00	0.00	0.00	0.00	0.00	17.00	0.847	-62.5	5.073	8.448	838.6	99.27	*
7	1.122	5		0.00	12.00	0.00	0.00	0.00	0.00	0.00	17.00	1.624	-6.7	3.172	672.9	950.6	1.413	
				cal(1)									27.9					
				cal(2)									28					
							cal(1)						-2.1					
							cal(2)						-2.2					
									cal(1)				-30.2					
									cal(2)				-31.3					

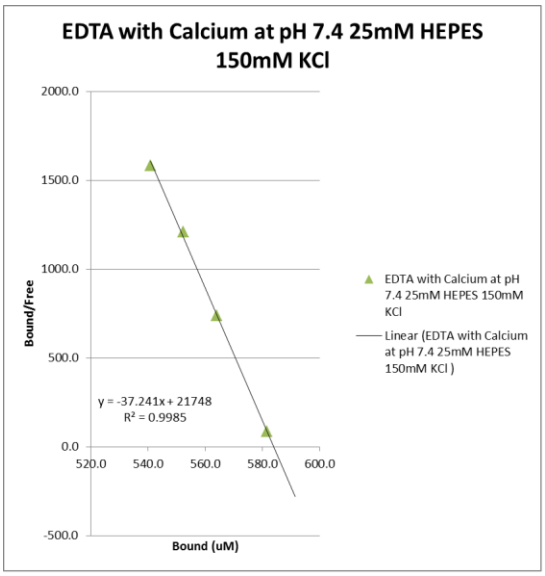
sample number	[EDTA] mM[1]	EDTA Vol. (ml)	Stock [EDTA] mM	[Calcium Standard] mM							Sample vol. (ml)	[Ca]mM	V_{Ca} (mV)	pCa^2	[Ca] free, uM	[Ca] bound, uM	B/F	ITC
			4.975	10.00	2.30	1.20	1.00	0.80	0.10	0.00								
				Volume of Standard Added (ml)														
8	1.463	5		0.00	0.00	0.00	0.00	0.00	0.00	12.00	17.00	0.000	-128.5					*
9	1.463	5		0.00	4.00	0.00	4.00	4.00	0.00	0.00	17.00	0.965	-118.7	6.972	0.1067	965	9043	*
10	1.463	5		0.00	4.00	0.00	8.00	0.00	0.00	0.00	17.00	1.012	-116.8	6.908	0.1237	1012	8176	
11	1.463	5		0.00	4.00	4.00	4.00	0.00	0.00	0.00	17.00	1.059	-115.1	6.850	0.1413	1059	7492	
12	1.463	5		0.00	4.00	8.00	0.00	0.00	0.00	0.00	17.00	1.106	-112.8	6.772	0.1691	1106	6538	*
13	1.463	5		0.00	8.00	0.00	0.00	4.00	0.00	0.00	17.00	1.271	-103.6	6.460	0.3470	1270	3661	
14	1.463	5		0.00	8.00	0.00	4.00	0.00	0.00	0.00	17.00	1.318	-99.1	6.307	0.4931	1317	2671	
15	1.463	5		0.00	8.00	4.00	0.00	0.00	0.00	0.00	17.00	1.365	-93.9	6.131	0.7402	1364	1843	
				cal(1)									26.4					
				cal(2)									27.4					
							cal(1)						-2.3					
							cal(2)						-0.4					
									cal(1)				-33.2					
									cal(2)				-30.5					

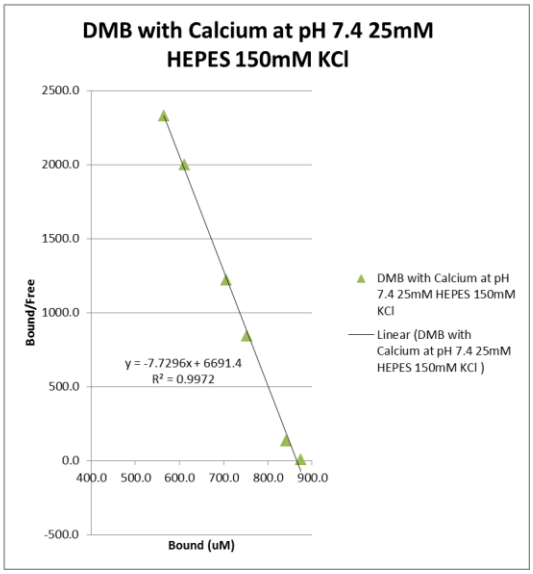


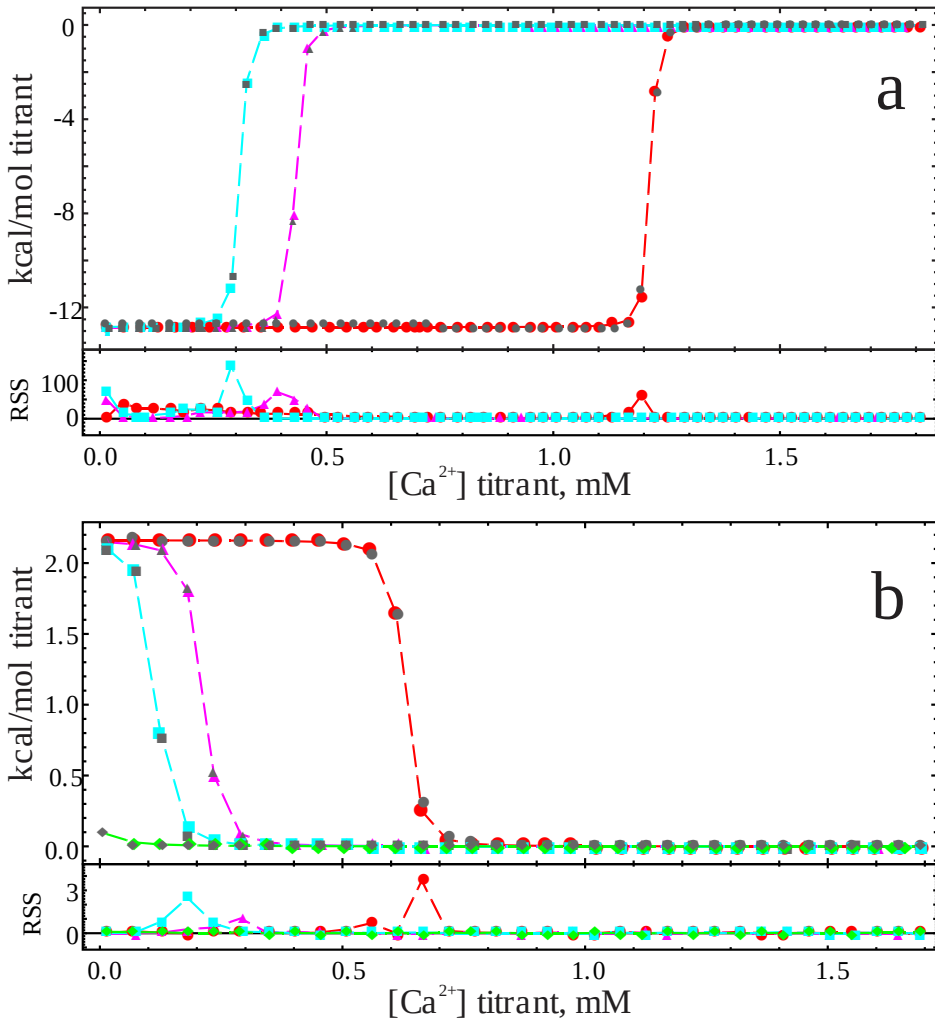
Potentiometric measurements, analytical sample preparation details, and Schatchard plots for EDTA and 5,5'-dimethylBAPTA in pH 7.4 25 mM HEPES 150 mM KCl binding to calcium. Asterisks denote samples used in ITC experiments for data presented in Figure 2.

sample number	[5,5' dimethyl BAPTA] mM[1]	5,5' dimethyl BAPTA Vol. (ml)	Stock [5,5' dimethyl BAPTA] mM	[Calcium Standard] mM													
			3.845	10.00	1.60	1.00	0.40	0.10	0.00	Sample vol. (ml)	[Ca]mM	V_{Ca} (mV)	pCa^2	[Ca] free, uM	[Ca] bound, uM	B/F	ITC
				Volume of Standard Added (ml)													
16	1.131	5		0.00	4.00	0.00	8.00	0.00	0.00	17.00	0.5647	-111.2	6.616	0.2423	564.5	2329.4	*
17	1.131	5		0.00	4.00	4.00	0.00	0.00	4.00	17.00	0.6118	-108.2	6.515	0.3058	611.5	2000	
18	1.131	5		0.00	0.00	12.00	0.00	0.00	0.00	17.00	0.7059	-100	6.238	0.5774	705.3	1221	
19	1.131	5		0.00	8.00	0.00	0.00	0.00	4.00	17.00	0.7529	-94.4	6.050	0.8914	752.0	844	
20	1.131	5		0.00	8.00	0.00	4.00	0.00	0.00	17.00	0.8471	-69.0	5.195	6.387	840.7	131.6	
21	1.131	5		0.00	8.00	4.00	0.00	0.00	0.00	17.00	0.9882	-31.9	3.946	113.4	874.9	7.7	
				cal(1)								26.8					
				cal(2)								25					
						cal(1)						-3					
						cal(2)						-4.7					
								cal(1)				-32.8					
								cal(2)				-34.2					

sample number	[EDTA] mM[1]	EDTA Vol. (ml)	Stock [EDTA] mM	[Calcium Standard] mM													
			2.004	10.00	1.60	1.00	0.40	0.10	0.00	Sample vol. (ml)	[Ca]mM	V_{Ca} (mV)	pCa^2	[Ca] free, uM	[Ca] bound, uM	B/F	ITC
				Volume of Standard Added (ml)													
22	0.5894	10		0.00	0.00	16.00	0.00	8.00	0.00	34.00	0.494	-109.6	6.726	0.1877	493.9	2631	
23	0.5894	10		0.00	4.00	12.00	0.00	0.00	8.00	34.00	0.541	-102	6.467	0.3415	540.8	1584	*
24	0.5894	10		0.00	4.00	12.00	0.00	4.00	4.00	34.00	0.553	-98.3	6.340	0.4569	552	1209	
25	0.5894	10		0.00	0.00	16.00	8.00	0.00	0.00	34.00	0.565	-91.8	6.118	0.7622	564	740	
26	0.5894	10		0.00	4.00	12.00	4.00	0.00	4.00	34.00	0.588	-64.1	5.171	6.7462	581	86	
				cal(1)								28.2					
				cal(2)								28.7					
						cal(1)						-0.4					
						cal(2)						0					
								cal(1)				-30.4					
								cal(2)				-29.7					







Supporting Figure S1: Titration isotherms of calcium binding to solutions of 1.122 mM 5,5'-dimethylBAPTA and 1.463 mM EDTA chelator in pH 7.4 25 mM TRIS 150 mM KCl buffer at 25 °C with various concentrations of residual Ca^{2+} present. Baseline-corrected titration isotherms are depicted by solid grey symbols and their sum-of-squares best-fit synthetic isotherms are depicted below as colored solid symbols with overlaid dashed lines. a) Simultaneously fitted isotherms of EDTA with no added Ca^{2+} (red with solid circles), 965 µM Ca^{2+} (magenta with solid triangles), and 1.106 mM Ca^{2+} (cyan with solid squares) titrated with 10 mM Ca^{2+} using 5 µl injections. b) Simultaneously best-fit isotherms of 5,5'-dimethylBAPTA with no added calcium (red with solid circles), 565 µM Ca^{2+} (magenta with solid triangles), 659 µM Ca^{2+} (cyan with solid squares), and 847 µM Ca^{2+} (orange with solid diamonds) titrated with 8 µl injections of 10 mM Ca^{2+}. All fitted isotherms were best-fit using the ODE approach and a single binding site (EDTA) or a single binding site with a 3.5 % fraction dedicated to a second binding site to represent impurities of 5,5'-dimethylBAPTA using parameters from Table 1. Residual sum-of-squares (cal/mol) for each best-fit isotherm are shown below their respective data set. The isotherms are presented with titrated ligand as the independent variable for direct comparison to experimentally derived isotherms and ease-of-visualization, whereas the concentration of the total ligand used in each simulation would necessarily include the dilution-corrected residual ligand as described by Eq. 22. Parameter estimates for best-fit minimizations were based on Ca^{2+}-selective potentiometric readings of the samples used in the ITC experiments, with electrode calibrations made from Ca^{2+} standards (R^2>0.999), which were also used as titrant.

General differential expression for NDH

ITC involves the stepwise addition of a titrant (L) at a specified concentration (L_0) with injection volume, V_{inj}, into a reaction cell with working volume, V_0, containing a binding partner (M) at an initial concentration, M_0. At the start of each titration, the calorimetric cell of working volume V_0 and communication tube is filled completely with macromolecule solution. The injection of titrant displaces an equal volume of solution (i.e. V_{inj}) into a thermally isolated sample reservoir. An ITC experiment is traditionally conducted with a discrete number of injections (i) of small volumes of titrant and we define the titration progress by the total volume injected ($V_{inj,tot}$), or iV_{inj}. Under the assumption that added titrant reacts only within the working volume (i.e. not the communication tube or overflow reservoir) we calculate a predictable dilution of titrant and binding partner, involving a dilution factor:

$$D = \left(1 - \frac{V_{inj}}{V_0}\right)^i, or \lim_{i\to\infty}\left(1 - \frac{V_{inj,tot}}{iV_0}\right)^i = e^{-\frac{V_{inj,tot}}{V_0}} \quad (1)$$

Upon completion of the the i^{th} injection, the total binding partner concentration, M_t, and the total titrant concentration, L_t, in the cell can be defined (1):

$$M_t = M_0 D; \quad (2)$$

$$L_t = L_0(1 - D). \quad (3)$$

In our analysis, the total ligand concentration is provided from the manufacturer's software as an array of correctly calculated total ligand concentrations after each injection, but could also be calculated using the identical discrete or nearly identical continuous dilution terms of Eq. 1. It is convenient to define an array of analogously diluted total binding partner concentrations, M_t, in terms of L_t:

$$D = 1 - \frac{L_t}{L_0}; \quad (4)$$

$$M_t = M_0\left(1 - \frac{L_t}{L_0}\right). \quad (5)$$

MicroCal defines the total change in heat due to an injection (ΔQ) as the difference in heat from the total volume of the reaction vessel (V_0) between injections plus a small addition from one-half of the total heat in the displaced volume, V_{inj}. We will consider the change in heat in terms of the titration progress (i.e. the sum of the individual injections), or total volume injected, $V_{tot,inj}$. Thus,

$$\frac{dQ}{dV_{tot,inj}} = \frac{dQ_{i,V_0}}{dV_{tot,inj}} + \frac{1}{2}\left(\frac{dQ_{i,V_{inj}}}{dV_{tot,inj}}\right) \quad (6)$$

Using the definition of Q as the molar change in standard state enthalpy ($\Delta H°$) multiplied by the change in bound ligand (L_{bound}) concentration times the working volume of the reaction vessel,

$$\frac{dQ}{dV_{tot,inj}} = \Delta H° V_0\left(\frac{dL_{bound}}{dV_{tot,inj}}\right) + \frac{\Delta H° V_{inj}}{2}\left(\frac{dL_{bound}}{dV_{tot,inj}}\right) \quad (7)$$

$$\frac{dQ}{dV_{tot,inj}} = \Delta H° V_0\left(\frac{dL_{bound}}{dV_{tot,inj}}\right)\left(1 + \frac{V_{inj}}{2V_0}\right) \quad (8)$$

The dependent variable to be analyzed is the "heat per mole of injected titrant (that contributes to the heat)" or NDH:

$$NDH = \left(\frac{dQ}{dV_{tot,inj}}\right)\left({}^{1}/_{L_0}\right) \qquad (9)$$

$$NDH = \Delta H^{\circ}\frac{V_0}{L_0}\left(\frac{dL_{bound}}{dV_{tot,inj}}\right)\left(1+\frac{V_{inj}}{2V_0}\right) \qquad (10)$$

However, we require a differential equation to express an infinitesimal change in the fraction bound (X) due to an infinitesimal change in the concentration of titrated ligand, dX/dL_t (in general, X goes from 0 to N, where N is the degeneracy of the site). We note that the total ligand concentration can also be expressed with an equivalent exponential dilution term:

$$L_t = \left(1 - e^{\frac{-V_{tot,inj}}{V_0}}\right)L_0. \qquad (11)$$

This allows an infinitesimal change in L_t to be related to $V_{tot,inj}$:

$$\frac{dL_t}{dV_{tot,inj}} = \left(\frac{L_0}{V_0}\right)\left(e^{\frac{-V_{tot,inj}}{V_0}}\right). \qquad (12)$$

Using the chain rule for differentiation, this can be substituted into NDH:

$$NDH = \Delta H^{\circ}\frac{V_0}{L_0}\left(\frac{dL_{bound}}{dL_t}\frac{dL_t}{dV_{tot,inj}}\right)\left(1+\frac{V_{inj}}{2V_0}\right), \qquad (13)$$

Noting that $L_{bound} = M_t{*}X$,

$$NDH = \Delta H^{\circ}\frac{V_0}{L_0}\left(M_t\frac{dX}{dL_t}\left(\frac{L_0}{V_0}\right)\left(e^{\frac{-V_{tot,inj}}{V_0}}\right)\right)\left(1+\frac{V_{inj}}{2V_0}\right). \qquad (14)$$

After simplification and substitution of dilution terms and consideration of Eqs. 4 and 5,

$$NDH = \Delta H^{\circ}M_0\frac{dX}{dL_t}D^2\left(1+\frac{V_{inj}}{2V_0}\right). \qquad (15)$$

When there are multiple classes of sites on a single binding partner,

$$NDH = M_0D^2\left(1+\frac{V_{inj}}{2V_0}\right)\sum_n \Delta H_n^{\circ}\frac{dX_n}{dL_t}, \qquad (16)$$

where the dilution terms can be computed directly from L_t and M_t provided from the manufacturer's software or derived independently.

Use of binding polynomials to define dX_n/dL_t

We next turn to a general method for the introduction of binding polynomials for defining dX/dL_t, as originally described by Wyman and Gill(9). We prefer using the "site-specific" formulation described by DiCera, where molar enthalpy is defined for each individual binding site(28) as would be appropriate for binding in multicomponent reactions. Given a binding polynomial, Z, and microscopic binding constant, K_n, the fraction bound at site n (X_n) is defined:

$$X_n = \frac{dZ}{dK_n}\frac{K_n}{Z}. \qquad (17)$$

The binding polynomial (Z) is usually expressed as a function of the free ligand concentration, L_f, which is generally not known. However, using the chain rule for differentiation:

$$\frac{dX_n}{dL_t} = \frac{dX_n}{dL_f}\frac{dL_f}{dL_t}. \qquad (18)$$

The free ligand concentration can be expressed as the difference between total ligand and the sum of all bound ligand:

$$L_f = L_t - \sum_n M_{t,n}X_n, \quad (19)$$

$$\frac{dL_f}{dL_t} = 1 - \sum_n \left(\frac{dM_{t,n}}{dL_t}X_n + M_{t,n}\frac{dX_n}{dL_t}\right). \qquad (20)$$

Considering Eq. 5 and combining provides

$$\frac{dX_n}{dL_t} = \frac{dX_n}{dL_f}\left(1 - \sum_n \left(M_{0,n}\left(1 - \frac{L_t}{L_0}\right)\frac{dX_n}{dL_t} - \frac{M_{0,n}X_n}{L_0}\right)\right). \qquad (21)$$

where, for each binding model, the free ligand concentration (L_f) appearing in X_n and dX_n/dL_f must be substituted according to Eq. 20 to generate the final expression depending only on L_t. Note that for models with multiple sites analogous expressions must be generated for each site and solved either analytically or, more likely, numerically.

Consideration of residually bound ligand

In order to incorporate the residual ligand concentration (L_R) into our expression for dX_n/dL_t, we recognize that the total ligand concentration is a sum of the ligand titrant and the residual ligand, both corrected by the appropriate dilution factor (Eq. 22), with the understanding that the residual ligand concentration would be less than that of the titrant in the addition syringe. Upon rearrangement, we obtain an updated solution for the dilution term (Eq. 23), which leads ultimately to an updated version of Eq. 5 and a new expression for dX_n/dL_t (Eq. 24):

$$L_t = L_0(1 - D) + L_R D, \quad (22)$$

$$D = \frac{L_t - L_0}{L_R - L_0}. \quad (23)$$

$$\frac{dX_n}{dL_t} = \frac{dX_n}{dL_f}\left(1 - \sum_n \left(M_{0,n}\left(\frac{L_t - L_0}{L_R - L_0}\right)\frac{dX_n}{dL_t} + \frac{M_{0,n}X_n}{L_R - L_0}\right)\right). \qquad (24)$$

When Eq. 24 is considered instead of Eq. 12 in the expression for NDH:

$$NDH = M_0\frac{L_0 - L_R}{L_0}\left(\frac{L_t - L_0}{L_R - L_0}\right)^2\left(1 + \frac{V_{inj}}{2V_0}\right)\sum_n \Delta H_n^\circ \frac{dX_n}{dL_t}. \quad (25)$$

Unfortunately, the introduction of a new unknown variable, L_R, prevents proper fitting of the ITC data without the introduction of a new experimental restraint. In our case, we have chosen

the addition of a second binding partner to the reaction cell, which is well characterized thermodynamically with respect to binding the ligand of interest. A Ca^{2+}-selective chelator, such as 5,5'-dimethylBAPTA, can be added to a recombinantly prepared Ca^{2+}-binding protein with an unknown amount of residually bound Ca^{2+} to aid in the proper extraction of thermodynamic parameters.

Models including two or more independent binding partners

Binding models have been described, and the simplest analytically solved, for the simultaneous interaction of two ligands with one binding partner. However, to the best of our knowledge, quantitative analysis of ITC data for the titration of a single ligand into a reaction cell containing two or more independent binding partners in the presence of residual ligand has not been previously reported. The approach reported here readily adapts to the inclusion of two, independent binding partners, which we will illustrate for the case of a macromolecule, M, and a chelator, B, both containing a single class of N binding sites for the ligand, L. We will also include an unknown amount of residual ligand, L_R, present in the system at the start of the titration. The process begins with defining the relevant binding polynomials, using them to create expressions for the corresponding site-specific fraction bound terms, differentiating with respect to L_f, and finally use of the chain rule for differentiation to construct the final dX/dL_t terms.

$$Z_M = \left(1 + K_M L_f\right)^{N_M} \qquad (26)$$

$$Z_B = \left(1 + K_B L_f\right)^{N_B} \qquad (27)$$

$$\frac{dZ_M}{dK_M} = N_M L_f \left(1 + K_M L_f\right)^{N_M - 1} \qquad (28)$$

$$\frac{dZ_B}{dK_B} = N_B L_f \left(1 + K_B L_f\right)^{N_B - 1} \qquad (29)$$

$$Fraction\ Bound\ on\ Macromolecule\ M = X_M = \frac{dZ_M}{dK_M}\frac{K_M}{Z_M} = \frac{N_M K_M L_f}{1 + K_M L_f} \qquad (30)$$

$$Fraction\ Bound\ on\ Macromolecule\ B = X_B = \frac{dZ_B}{dK_B}\frac{K_B}{Z_B} = \frac{N_B K_B L_f}{1 + K_B L_f}; \qquad (31)$$

$$\frac{dX_M}{dL_f} = \frac{N_M K_M}{1 + K_M L_f} - \frac{N_M K_M^2 L_f}{\left(1 + K_M L_f\right)^2} \qquad (32)$$

$$\frac{dX_B}{dL_f} = \frac{N_B K_B}{1 + K_B L_f} - \frac{N_B K_B^2 L_f}{\left(1 + K_B L_f\right)^2} \qquad (33)$$

After substitution into Eq. 24, replacing all L_f terms according to Eq. 19, and using Mathematica™ to simplify, expressions for dX_M/dL_t and dX_B/dL_t are obtained:

$$\frac{dX_M}{dL_t} = \frac{N_M K_M (L_0 - L_R)\left(L_0 - L_R + B_0 X_B + M_0 X_M - (L_0 - L_t)\left(B_0 \, {dX_B}/{dL_t} + M_0 \, {dX_M}/{dL_t}\right)\right)}{\left((L_0 - L_R)(1 + K_M L_t) - K_M (L_0 - L_t)(B_0 X_B + M_0 X_M)\right)^2} \qquad (34)$$

$$\frac{dX_B}{dL_t} = \frac{N_B K_B (L_0 - L_R)\left(L_0 - L_R + B_0 X_B + M_0 X_M - (L_0 - L_t)\left(B_0 \, {}^{dX_B}/_{dL_t} + M_0 \, {}^{dX_M}/_{dL_t}\right)\right)}{\left((L_0 - L_R)(1 + K_B L_t) - K_B (L_0 - L_t)(B_0 X_B + M_0 X_M)\right)^2} \quad (35)$$

Equations 34 and 35 represent a system of implicit, coupled, ordinary differential equations. Instead of seeking an analytical solution, which is likely not possible, we utilize the numerical differential equation solver (NDSolve) employing the iterative method of Runge-Kutta in Mathematica™ to derive numerical solutions for ITC data based on the above model. Computer-aided data fitting is achieved with built-in minimization routines typically employing the principal axis method of Brent, and subsequent confidence intervals derived from the critical value of the F distribution for a *p*-value of 0.05. Minimization routines are often best guided by an initial estimate of parameters, which can be obtained by visually examining the dependence of individual parameters on the simulated isotherms.

Presented below for completeness is an alternate derivation for NDH using a different definition of the dilution factor. Equations 1 and 9 -14 differ from what is presented in the manuscript, but converge to an identical final form for NDH (Equations 15 & 16).

Alternate general differential expression for NDH. ITC involves the stepwise addition of a titrant (L) at a specified concentration (L_0) with injection volume, V_{inj}, into a reaction cell with working volume, V_0, containing a binding partner (M) at an initial concentration, M_0. The injected of titrant displaces an equal volume of solution (i.e. V_{inj}) into a thermally isolated sample reservior, resulting in a predictable dilution of titrant and binding partner, involving a dilution factor:

$$D = \frac{V_0}{V_0 + iV_{inj}}. \quad (1)$$

Upon completion of the the i^{th} injection, the total binding partner concentration, M_t, and the total titrant concentration, L_t, in the cell can be defined:

$$M_t = M_0 D; \quad (2)$$

$$L_t = L_0(1 - D). \quad (3)$$

In our analysis, the total ligand concentration is provided from the manufacturer's software as an array of correctly calculated total ligand concentrations after each injection. It is convenient to define an array of analogously diluted total binding partner concentrations, M_t, in terms of L_t:

$$D = 1 - \frac{L_t}{L_0}; \quad (4)$$

$$M_t = M_0\left(1 - \frac{L_t}{L_0}\right). \quad (5)$$

Microcal defines the total change in heat due to an injection (ΔQ) as difference in total in the cell (V_0) between injections plus one-half of the total heat in the displaced volume, V_{inj}. We will consider the change in heat in terms of the cumulative or total volume injected, $V_{tot,inj}$. Thus,

$$\frac{dQ}{dV_{tot,inj}} = \frac{dQ_{i,V_0}}{dV_{tot,inj}} + \frac{1}{2}\left(\frac{dQ_{i,V_{inj}}}{dV_{tot,inj}}\right) \quad (6)$$

Using the definition of Q as the molar change in standard state enthalpy (ΔH°) multiplied by the change in bound ligand (L_{bound}) concentration times the volume,

$$\frac{dQ}{dV_{tot,inj}} = \Delta H^{\circ} V_0 \left(\frac{dL_{bound}}{dV_{tot,inj}}\right) + \frac{\Delta H^{\circ} V_{inj}}{2}\left(\frac{dL_{bound}}{dV_{tot,inj}}\right) \qquad (7)$$

$$\frac{dQ}{dV_{tot,inj}} = \Delta H^{\circ} V_0 \left(\frac{dL_{bound}}{dV_{tot,inj}}\right)\left(1 + \frac{V_{inj}}{2V_0}\right) \qquad (8)$$

The dependent variable to be analyzed is the "heat per mole of injected titrant (that contributes to the heat)" or NDH:

$$NDH = \frac{dQ/dV_{tot,inj}}{L_0 D} \qquad (9)$$

$$NDH = \Delta H^{\circ} \frac{V_0}{L_0 D}\left(\frac{dL_{bound}}{dV_{tot,inj}}\right)\left(1 + \frac{V_{inj}}{2V_0}\right) \qquad (10)$$

However, we require a differential equation to express an infinitesimal change in the fraction bound (X) due to an infinitesimal change in the concentration of titrated ligand, dX/dL_t (in general, X goes from 0 to N, where N is the degeneracy of the site). We note that the total ligand concentration can also be expressed:

$$L_t = \frac{L_0 V_{tot,inj}}{V_0 + V_{tot,inj}}. \qquad (11)$$

This allows an infinitesimal change in L_t to be related to $V_{tot,inj}$:

$$\frac{dL_t}{dV_{tot,inj}} = \frac{L_0 V_0}{\left(V_0 + V_{tot,inj}\right)^2}. \qquad (12)$$

Using the chain rule for differentiation, this can be substituted into NDH:

$$NDH = \Delta H^{\circ} \frac{V_0}{L_0 D}\left(\frac{dL_{bound}}{dL_t}\frac{dL_t}{dV_{tot,inj}}\right)\left(1 + \frac{V_{inj}}{2V_0}\right), \qquad (13)$$

Noting that $V_{tot,inj} = i*V_{inj}$ and $L_{bound} = M_t*X$,

$$NDH = \Delta H^{\circ} \frac{V_0}{L_0 D}\left(M_t \frac{dX}{dL_t}\frac{L_0 V_0}{\left(V_0 + iV_{inj}\right)^2}\right)\left(1 + \frac{V_{inj}}{2V_0}\right). \qquad (14)$$

After simplification and consideration of Eqns. 4 and 5,

$$NDH = \Delta H^{\circ} M_0 \frac{dX}{dL_t} D^2 \left(1 + \frac{V_{inj}}{2V_0}\right). \qquad (15)$$

When there are multiple classes of sites on a single binding partner,

$$NDH = M_0 D^2 \left(1 + \frac{V_{inj}}{2V_0}\right) \sum_n \Delta H_n^{\circ} \frac{dX_n}{dL_t}, \qquad (16)$$

NDH sensitivity to thermodynamic parameters when total ligand approaches zero for single and multiple binding site models. We are interested in the limiting behavior of NDH as the total ligand concentration approaches zero. First, we consider the case without any residual ligand:

$$NDH = \left(1 - \frac{L_t}{L_0}\right)^2 \left(1 + \frac{V_{inj}}{2V_0}\right) \sum_n M_{0,n} \Delta H_n^\circ \frac{dX_n}{dL_t}$$

$$\lim_{L_t \to 0}(NDH) = \left(1 + \frac{V_{inj}}{2V_0}\right) \sum_n M_{0,n} \Delta H_n^\circ \lim_{L_t \to 0}\left(\frac{dX_n}{dL_t}\right)$$

Where

$$\lim_{L_t \to 0}\left(\frac{dX_n}{dL_t}\right) = \lim_{L_t \to 0}\left(\frac{dX_n}{dL_f}\right) \Big/ \left[1 + \sum_n \left(M_{0,n} \lim_{L_t \to 0}\left(\frac{dX_n}{dL_f}\right)\right)\right]$$

In the absence of residual ligand, as the total ligand approaches zero, so does the free ligand. Regardless of the thermodynamic model for binding, at vanishing free ligand concentrations, only single-site binding reactions matter as there are no previously occupied binding sites to generate multivalent complexes. Hence,

$$\lim_{L_t \to 0}\left(\frac{dX_n}{dL_f}\right) = K_n N_n$$

$$\lim_{L_t \to 0}\left(\frac{dX_n}{dL_t}\right) = K_n N_n \Big/ \left[1 + \sum_n \left(M_{0,n} K_n N_n\right)\right]$$

$$\lim_{L_t \to 0}(NDH) = \left(1 + \frac{V_{inj}}{2V_0}\right) \sum_n \left(\Delta H_n^\circ M_{0,n} K_n N_n \Big/ \left[1 + \sum_n \left(M_{0,n} K_n N_n\right)\right]\right)$$

For a single class of binding sites, this reduces to

$$\lim_{L_t \to 0}(NDH) = \left(1 + \frac{V_{inj}}{2V_0}\right) \Delta H^o \frac{M_0 K N}{1 + M_0 K N}$$

Under "stoichiometric" binding conditions, where $M_0K >> 1$, the limiting values reduces to $\sim \Delta H^o$.

However, in the presence of multiple competing binding reactions, the limiting value depends on both the individual changes in enthalpies and the relative affinities of the sites. For example, for two competing binding sites:

$$\lim_{L_t \to 0}(NDH) = \left(1 + \frac{V_{inj}}{2V_0}\right)\frac{\Delta H_1^{\circ} M_{0,1} K_1 N_1 + \Delta H_2^{\circ} M_{0,2} K_2 N_2}{1 + M_{0,1} K_1 N_1 + M_{0,2} K_2 N_2}$$

It is clear in the above equation that the limiting value depends not only on the two standard state changes in enthalpy but also on the relative binding affinities, concentrations and stoichiometries of the two sites.

In the presence of residual ligand, analogous behavior is observed; however, the limiting value also depends strongly on the residual ligand concentration resulting in complex, non-intuitive expressions. In the presence of residual ligand:

$$NDH = \frac{L_0 - L_R}{L_0}\left(\frac{L_t - L_0}{L_R - L_0}\right)^2 \left(1 + \frac{V_{inj}}{2V_0}\right)\sum_n M_{0,n}\Delta H_n^{\circ}\frac{dX_n}{dL_t}.$$

$$\lim_{L_t \to L_R}(NDH) = \frac{L_0 - L_R}{L_0}\left(1 + \frac{V_{inj}}{2V_0}\right)\sum_n M_{0,n}\Delta H_n^{\circ}\lim_{L_t \to L_R}\left(\frac{dX_n}{dL_t}\right).$$

Where

$$\lim_{L_t \to L_R}\left(\frac{dX_n}{dL_t}\right) = \lim_{L_t \to L_R}\left(\frac{dX_n}{dL_f}\right) \Bigg/ \left[1 + \sum_n \left(M_{0,n}\lim_{L_t \to L_R}\left(\frac{dX_n}{dL_f}\right)\right)\right]$$

Additional fitted parameters and F-test confidence intervals for (simultaneously) simulated isotherms of calcium binding to hPC2-EF and suPC2-EF-x-z1 proteins with and without 5,5'-dimethylBAPTA (P=0.05). Confidence intervals noted by N/A were extremely broad and those labeled "fixed" were unchanged during F-test minimization routines.

Macromolecule (trial #)	5,5'-dimethylBAPTA (fixed parameters)			Purity (%)	ΔH (cal/mol)	K_D (μM)	Residual Ligand (μM)
	Purity (%)	ΔH (cal/mol)	K_D (nM)				
^{15}N hPC2-EF (2)	73	2180	125	86 (N/A)	-12873 (N/A)	434 (N/A)	0(fixed)
^{15}N hPC2-EF (3)	73	2180	125	91 (N/A)	-12400 (N/A)	469 (395-547)	2.36 (1.91-2.83)
^{15}N suPC2-EF-x-z1 (2)	73	2275	125	100 (fixed)	N/A	N/A	0(fixed)
^{15}N suPC2-EF-x-z1 (3)	73	2275	125	110 (97-126)	-18261 (-15935–-20641)	1.98 (1.81-2.25)	11.1 (10.9-11.4)

Notes on the impurity in 5,5' dimethylBAPTA Ca^{2+} chelator A single-site model, using the potentiometry-derived purity and Ca^{2+} affinity, was generally insufficient to properly describe the wide and asymmetric curvature of the binding isotherm for samples containing 5,5'-dimethylBAPTA. Isotherms of samples with 5,5'-dimethylBAPTA were fitted using a two independent single-site binding model, with one binding site represented by the potentiometric values, and a second component comprised of 3.5% of the purity of the major component with much weaker Ca^{2+} affinity. Adding in this Ca^{2+} binding impurity improved the quality of the data fit, as can be observed when adjusting the 'BAP IMP' terms in the associated CDF program "DMB-EDTA-mixture-ODE-ITC". In correspondence with the manufacturer of the 5,5'-dimethylBAPTA chelator, the organic impurities are expected to be trialkylated and dialkylated products owing to incomplete alkylation of the diamino intermediate during its synthesis. These impurities, along with the difficulty of completely removing water from organic salts, likely explain the low purity observed in both the ITC and potentiometric results. The experimental isotherms of this chelator may reflect the presence of these impurities as the evolved heat is that of all species in the mixture, and lower order alkylated products would likely complex with Ca^{2+} and with lower affinity. Moreover, the 5,5'-dimethylBAPTA chelator stocks can be observed to change color over the course of a few days at room temperature, suggesting that sample degradation may further affect analytical measurements with this chelator. Unfortunately, a higher purity product was unavailable to investigate the potential discrepancies caused by impurities being present. It has been the experience in our laboratory that aqueous stocks of this chelator must be used soon after their preparation for consistent results and it should be noted that alternate formulations and BAPTA derivatives may behave more favorably for these types of experiments. For the sake of computational efficiency, in some ITC simulations a single binding site model with weaker Ca^{2+} affinity and slightly higher total purity is used to approximate the combination of 5,5' dimethylBAPTA and its Ca^{2+} binding impurities.

ODE-Based ITC Data Simulator Program A standalone Computable Document Format (CDF) (Wolfram Research, Champaign, IL) program (ITCsim.cdf) based on the ODE implementation is provided for predicting isotherms given a set of experimental conditions before the onset of an experiment to aid in the experimental design of isothermal titration experiments. The flexible range of input parameters can accommodate a variety of experimental sample and commercial instrument configurations. Users can select which model to employ, be it a single site or two-site receptor (macromolecule) model, with or without residual ligand and in the case where residual ligand is selected, an independent chelator (or competing macromolecule, as the model makes no assumption regarding the nature of the chemical interaction between the macromolecule and ligand). A cooperative model is available when a two-site interaction is selected. Notes: In the case where the selection of a box or button press conflicts with a visual slider for input, the corresponding slider and slider value is not employed in the model. When changing cell volume, injection volume, number of injection parameter, the user must select outside of the input box to implement the new value.

ODE-Based ITC Data Fitting Program, EDTA and 5,5'-dimethylBAPTA with Residual Calcium The included program (DMB-EDTA-mixture-ODE-ITC.cdf) is provided in Computable Document Format (CDF) (Wolfram Research, Champaign, IL) and can be executed using the freely available Wolfram CDF Player (http://www.wolfram.com/cdf/), provided for popular Windows, Mac, and Linux platforms. The code to generate the program is provided in Wolfram Mathematica 9.0 (Wolfram Research, Champaign, IL) notebook format "DMB-EDTA-mixture-ODE-ITC.nb", along with model generation examples which require the parent program to be viewed and edited.

The program "DMB-EDTA-mixture-ODE-ITC.cdf" was used to generate Figure 2 of the associated manuscript, and simulates the titration calorimetry of 2.5 mM Ca^{2+} titrated in 8 μL injections against 589.4 μM EDTA with 541 μM residual Ca^{2+} (magenta with triangle symbols) and 1.130 mM 5,5'-dimethylBAPTA with 565 μM residual Ca^{2+} (red with round symbols), both individually and as a 50/50 v/v mixture (cyan with square symbols). The CDF program contains the raw experimental data, reagent concentrations, and models specific to the sample configuration and cannot be altered. The CDF program executes with a slider panel for thermodynamic parameter control, provided with practical limits for the context of the sample composition. The stoichiometry parameter (N_X) for each macromolecule (BAP = 5,5'-dimethylBAPTA and BAP IMP = 5,5'-dimethylBAPTA impurity) can be set from zero to 3 identical, independent binding sites. The effective concentration of each macromolecule is controlled through the purity parameter slider, and the total mixture concentration can be adjusted slightly with the "Total [Macromol] offset" slider. Three simulated isotherms are simultaneously generated, with simulation I corresponding to a sample with 5,5'-dimethylBAPTA and its impurity, simulation II corresponding to a sample with only EDTA, and simulation III corresponding to a 50/50 v/v mixture of the EDTA and 5,5'-dimethylBAPTA containing samples. To the right are a series of output visualization frames, with the uppermost frame titled "Manually fitted isotherms" dedicated to the experimental (grey symbols) and simulated isotherms (colored symbols). The lower frames are dedicated to the total and titration Residual Sum-of-Squares (RSS) error, Fraction Bound of each macromolecule displayed for each sample, and Ligand profile, and are correlated based on the symbol shapes and their color. The frames dedicated to the fraction bound each show two traces, with one trace representing the fraction bound (0 to N sites) for the macromolecule in the single chelator sample and the second

trace representing the macromolecule in the sample of the 50/50 v/v mixture. The EDTA alone is modeled with an N integer stoichiometry independent binding site parameter, with N set to 1 by default ("Model_SingleChelator_residual.nb"). The 5,5' dimethylBAPTA sample is modeled with two independent sites, one dedicated to the bulk form of the macromolecule and one dedicated to the impurity, and each can be modeled with up to three independent binding sites (default stoichiometry N set to 1 for each macromolecule- "Model_ImpureChelator_residual.nb"). The 50/50 v/v EDTA/5,5'-dimethylBAPTA mixture is modeled with three independent macromolecules representing EDTA and 5,5'-dimethylBAPTA and its impurity ("Model_ImpureChelator_SecondChelator_Mixture_residual.nb"), with the concentrations of each macromolecule being half of that in the individual samples and the residual calcium being the average of the Residual I [Ca^{2+}] and Residual II [Ca^{2+}] values.