Anal Bioanal Chem (2016) 408:6413–6422
DOI 10.1007/s00216-016-9759-6

RESEARCH PAPER

HypCal, a general-purpose computer program for the determination of standard reaction enthalpy and binding constant values by means of calorimetry

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Received: 17 March 2016 / Revised: 18 June 2016 / Accepted: 1 July 2016 / Published online: 16 July 2016

Abstract The program HypCal has been developed to provide a means for the simultaneous determination, from data obtained by isothermal titration calorimetry, of both standard enthalpy of reaction and binding constant values. The chemical system is defined in terms of species of given stoichiometry rather than in terms of binding models (e.g., independent or cooperative). The program does not impose any limits on the complexity of the chemical systems that can be treated, including competing ligand systems. Many titration curves may be treated simultaneously. HypCal can also be used as a simulation program when designing experiments. The use of the program is illustrated with data obtained with nicotinic acid (niacin, pyridine-3 carboxylic acid). Preliminary experiments were used to establish the rather different titration conditions for the two sets of titration curves that are needed to determine the parameters for protonation of the carboxylate and amine groups.

Keywords Calorimetric titration · Standard enthalpy of reaction · Binding constant · Experiment design

Electronic supplementary material The online version of this article (doi:10.1007/s00216-016-9759-6) contains supplementary material, which is available to authorized users.

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Introduction

The standard Gibbs energy change (ΔG^0) for a chemical equilibrium reaction and the related equilibrium constant (K) are important entities with which the equilibrium can be quantified. Indeed, these quantities provide useful chemical comparators for stoichiometrically identical reactions under given conditions of temperature and pressure [1]. Further resolution of ΔG^0 into its standard enthalpy (ΔH^0) and entropy (ΔS^0) components provides more information which may be used to characterize the nature of the interaction at the molecular level.

In the past, standard Gibbs energy change and enthalpy terms have been determined by means of different instrumental techniques, such as potentiometry and calorimetry. Methods for the determination of both enthalpy and Gibbs energy in a single experiment were actually proposed more than half a century ago [2–5]. Sensitive titration calorimeters are now commercially available, and they have been used to determine both equilibrium constants and standard enthalpies from a single set of measurements [6, 7].

Although simultaneous determination of the two thermodynamic parameters is now commonplace [7, 8], the software for deriving them from experimental data has been a limiting factor on the complexity of the reactions that may be investigated. The majority of publications deal with the formation of a single species, usually with 1:1 stoichiometry. Some software are concerned with independent or cooperative binding models and often result in values for apparent constants and enthalpies being obtained [9–11].

The majority of the software available imposes limitations on the complexity of equilibrium systems that can be investigated. This was highlighted recently in connection with the complexation of Cu^{2+} with an amyloid-beta peptide in the presence of a competing ligand (glycine) [12]. It was stated that the software then available "treats the equilibrium of Cu^{2+}

with the peptide but ignores all competing equilibrium between the metal and glycine" and thus may lead to an erroneous binding constant [13]: it was necessary in that instance to also include competitive equilibria (including protonation of the ligand) in the chemical model used in the calculations. The need for software capable of handling simultaneous equilibria involving, for example, competing ligand reactions was emphasized in a recent review [14].

In order to accommodate these needs, we have now created HypCal, a new program for obtaining standard enthalpy and binding constant values, specifically designed for the treatment of data obtained from isothermal titration calorimetry (ITC) instruments operating in overfilled mode. The program also includes facilities for treating data obtained with traditional calorimeters operating in partially filled mode (e.g., calorimeters with gas phase). HypCal follows the same principles as HypΔH which was previously developed as a general-purpose program for data obtained with calorimeters in which the volume of the thermally active test solution increases with each batch-wise addition of a reactant [15].

HypCal imposes no limitations on the size or complexity of the systems that may be investigated. The program may be used for the determination of both binding constants and standard reaction enthalpies or just enthalpies using stability constant values that will have been determined separately. It has been extensively tested with new ITC data collected in the overfilled mode but is designed to deal with data obtained by operating in the partially filled mode. It also contains a module that can be used to explore suitable experimental conditions, such as titrate and titrant concentrations, in order for the parameter refinement to succeed. An important feature of the new program is that it can process together data from many titration curves. This is illustrated with data for nicotinic acid (niacin). Though the two protonation steps involved have quite different standard enthalpies, HypCal successfully calculates both the binding constant and the standard enthalpies values, provided experiments are appropriately designed.

Definitions

When a complex is formed in solution from two reagents, A and B, the equilibrium reaction may be expressed as

$$pA + qB \rightleftharpoons A_pB_q \tag{1}$$

In this and the following expressions, any electrical charges on the reagents or on the complexes are omitted for the sake of generality and clarity. The thermodynamic stability constant for the binary system shown in Eq. (1), β^T_{pq}, is defined as

$$\beta^T_{pq} = \frac{\{A_pB_q\}}{\{A\}^p\{B\}^q} = \frac{[A_pB_q]}{[A]^p[B]^q}\Gamma \tag{2}$$

where {} and [] denote the activity and concentration, respectively, and Γ is the quotient of activity coefficients.

Experimentally, measurements are often performed using solutions containing an excess of inert salt, that is, where Γ can be taken to be constant. Under these conditions, stoichiometric stability constant, expressed in terms of concentration quotients, may be used (Eq. (3)). In so doing, it is assumed that the quotient of activity coefficients (Γ) is constant over the conditions used for data collection.

$$\beta_{pq} = \frac{[A_pB_q]}{[A]^p[B]^q} \tag{3}$$

It must be noted that such concentration constants are valid only under the conditions, including ionic strength, at which they were determined [16, 17], whereas thermodynamic constants are dependent only upon temperature and pressure. HypCal can handle systems with more than two reagents. In general

$$\beta_{pqr..} = \frac{[A_pB_qC_r..]}{[A]^p[B]^q[C]^r..} \tag{4}$$

A stability constant is related to the standard Gibbs energy change (ΔG^0) for the equilibrium by

$$\Delta G^0 = -RT \ln K \tag{5}$$

where R is the gas constant and T is the absolute temperature. It must be assumed that temperature (commonly 298 K) and pressure (commonly 1 bar) are constant. The ΔG^0 term is comprised of two components: the standard enthalpy change (ΔH^0) and the standard entropy change (ΔS^0) as shown in Eq. (6)

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \tag{6}$$

Splitting the standard Gibbs energy change term in this way provides information on how factors such as size, shape, and electronic structure of the donor and the acceptor influence the stability of a complex [18]. The split provides an immediate link to experiments [1]. When looking at related systems, it may reveal differences that are not obvious when comparing stability constant values [19–21]. Equilibrium constants, and hence standard Gibbs energy change, are determined using experimental techniques such as potentiometry [22, 23], UV/visible spectrophotometry [24–26], and NMR spectroscopy [27, 28]. When the standard enthalpy is obtained, the standard entropy change may be calculated from Eq. (6).

The enthalpy of a reaction may be determined in two ways: indirectly by measuring the temperature dependence of the equilibrium constant, the so-called "van't Hoff method," and directly using calorimetry. In the van't Hoff method, an expression relating the standard enthalpy to the equilibrium constant for a

reaction is obtained by combining Eqs. (5) and (6) and differentiating with respect to $1/T$

$$\Delta H^0 = -R\frac{d\ln K}{d(1/T)} \tag{7}$$

Equation (7) should be used only if the standard enthalpy is independent of temperature, within the experimental error. Studies on a variety of systems have shown that it may be a poor approximation [6, 29] even in cases where a linear least squares calculation gave a correlation coefficient sufficiently close to unity to appear to validate it [30–32]. If ΔH^0 changes significantly with temperature, implying that the heat capacity change is significantly greater than zero, the integrated form of the van't Hoff method (Eq. (8)) may be used as suggested by Naghibi et al. [6]

$$\ln\frac{K}{K_{\mathrm{ref}}} = \frac{\left(\Delta H_{\mathrm{ref}} - T_{\mathrm{ref}}\Delta C_{\mathrm{p}}\right)}{R}\left(\frac{1}{T_{\mathrm{ref}}} - \frac{1}{T}\right) + \frac{\Delta C_{\mathrm{p}}}{R}\ln\frac{T}{T_{\mathrm{ref}}} \tag{8}$$

where T_{ref} is the arbitrarily chosen reference temperature (in K); K_{ref} and ΔH_{ref} are the initial values of the equilibrium constant and van't Hoff enthalpy at that temperature, respectively, and ΔC_{p} is the van't Hoff-derived heat capacity change. ΔC_{p} is assumed to be non-zero and independent of temperature [6]; the latter assumption is valid due to the narrow temperature interval usually investigated. The accuracy of the ΔH^0 values obtained by means of the van't Hoff method is considerably lower than that of corresponding values obtained by calorimetry [33, 34]. In particular, ΔS^0 values obtained using Eq. (6) and ΔH^0 values from Eq. (7) may be hopelessly inaccurate. For example, Li et al. calculated for the formation of the copper diglycinate complex a ΔS^0 value of −4.2 J mol^{-1} deg^{-1} via polarography [35], whilst a calorimetric investigation gave a value of 125 J mol^{-1} deg^{-1}. The complexation of fluoride with the uranyl ion provides a further example where the values obtained by the two methods were of opposite sign: −8.4 kJ mol^{-1} (van't Hoff) and 2 kJ mol^{-1} (calorimetric) [36].

Calculation of concentrations

The concentrations of the various species in equilibrium are calculated by solving the mass balance equations in the total concentrations

$$C_{\mathrm{A}} = [A] + \Sigma p\beta_{pq..}[A]^p[B]^q \tag{9}$$

$$C_{\mathrm{B}} = [B] + \Sigma q\beta_{pq..}[A]^p[B]^q \tag{10}$$

where C_{A} and C_{B} are the analytical concentrations of the reagents A and B. For this calculation, the stability constants $\beta_{\mathrm{pq..}}$ have fixed values. When one of the reagents is the hydrogen ion, the self-association constant (K_{w}) of water must be included in the equation of mass balance

$$C_{\mathrm{H}} = [H] + \Sigma q\beta_{pq..}[A]^p[H]^q{}_{..} - K_{\mathrm{w}}[H]^{-1} \tag{11}$$

These are simultaneous non-linear equations in the unknown "free" concentrations [A] and [B]. At the first point in a titration curve, these values are completely unknown. Initial estimates are obtained by a trial and error method. These estimates are then refined using the Newton-Raphson method. With two reagents, the equations to solve have the form

$$\begin{pmatrix} [A]\frac{\partial C_{\mathrm{A}}}{\partial [A]} & [B]\frac{\partial C_{\mathrm{A}}}{\partial [B]} \\ [A]\frac{\partial C_{\mathrm{B}}}{\partial [A]} & [B]\frac{\partial C_{\mathrm{B}}}{\partial [B]} \end{pmatrix}\begin{pmatrix} \frac{\delta[A]}{[A]} \\ \frac{\delta[B]}{[B]} \end{pmatrix} = \begin{pmatrix} \Delta C_{\mathrm{A}} \\ \Delta C_{\mathrm{B}} \end{pmatrix} \tag{12}$$

where $[A]$ and $[B]$ are the values of the free concentrations of the reagents A and B. Expressions for the derivatives are simply obtained from the mass balance Eqs. (9) and (10)

$$\begin{aligned} [A]\frac{\partial C_{\mathrm{A}}}{\partial [A]} &= [A] + \sum p^2\left[A_pB_q\right] \\ [B]\frac{\partial C_{\mathrm{A}}}{\partial [B]} &= [A]\frac{\partial C_{\mathrm{B}}}{\partial [A]} = \sum pq\left[A_pB_q\right] \\ [B]\frac{\partial C_{\mathrm{B}}}{\partial [B]} &= [B] + \sum q^2\left[A_pB_q\right] \end{aligned} \tag{13}$$

As can be seen, the values for these expressions are simply obtained from the values of the concentrations of the species. The quantities ΔC_{A} and ΔC_{B} are the differences between actual and calculated concentrations. Solution of these equations provides values for the quantities $\frac{\delta[A]}{[A]}$ and $\frac{\delta[B]}{[B]}$ which are the incremental relative shifts and which are added to the values of the free concentrations at each cycle of refinement until the equations of mass balance are satisfied. At the second and subsequent titration points, initial estimates are taken as the values at the previous titration point. The refinement is protected from divergence.

Calculation of the change of the number of moles in a titration step

The calculated heat change (Q_{calc}) for a single reaction step is obtained, for a given set of stability constants and experimental conditions, from Eq. (14)

$$Q_{\mathrm{calc}} = -\sum_{i=1}^{n}\delta n_i\Delta H_i^0 \tag{14}$$

where δn_i is the change in the number of moles of the ith

active species and ΔH_i^0 is the standard enthalpy change for the reaction involving that species. The treatment of data obtained with calorimeters which operate in partially filled mode is relatively simple, and descriptions may be found in the literature [15, 37, 38]. By contrast, isothermal titration calorimeters commonly operate in overfilled mode; when an aliquot of titrant solution is injected into the test solution, an equal volume of the test solution is expelled from the thermally sensitive region and is collected in a thermally inactive receptacle. This arrangement has the advantage that there is no space above the solution for vaporization or condensation to occur [7, 39].

For the overfilled mode of operation, two schemes have been proposed [40, 41].

1. Injection and expulsion occur instantaneously and are followed by mixing of the contents of the active volume. The expelled solution has the same composition as the solution had before the injection. An equation based on this model has been discussed by both Tellinghuisen [41] and Velazquez-Campoy and Freire [42] and is the basis of the TA software [10].
2. Mixing occurs instantaneously during the injection: the expelled solution will contain material from both the initial solution and the added aliquot [40]. The algorithm employed by the Origin software [9] is consistent with this scheme.

It has been shown that both regimes give virtually the same result [41]. However, the correct way of doing this also depends on the injection and mixing rates, both of which are variable in commercially available calorimeters. We have confirmed this observation: the difference in calculated concentrations between the two models was found to be less than 1 %. Consequently, we decided to use the first regime for calculating the concentrations of the reagents in the active volume. Labeling the reagent in the burette as A and any other reagent as B, the analytical concentrations of the reagents in the active volume after the *i*th addition of titrant are calculated as in Eqs. (15) and (16)

$$C_{\mathrm{A}(i)} = C_{\mathrm{A}(i-1)}\left(1-\frac{v_i}{V_0}\right) + C_{\mathrm{A}}^0\left(\frac{v_i}{V_0}\right) \tag{15}$$

$$C_{\mathrm{B}(i)} = C_{\mathrm{B}(i-1)}\left(1-\frac{v_i}{V_0}\right) \tag{16}$$

where $C_{\mathrm{A}(i)}$ and $C_{\mathrm{B}(i)}$ are the concentrations of the reagents and v_i is the volume of the *i*th injection. C^0 is the initial concentration of the titrant, and V_0 is the volume of the active region. The change of number of moles (δn) of each complex species (X) is calculated as the difference between the number of moles before and after the *i*th addition

$$\delta n(X)_{(i)} = [X]_{(i)}V_0 - [X]_{(i-1)}(V_0 - v_i) \tag{17}$$

The heat developed at the *i*th injection ($Q_{(i)}$) can then be calculated with Eq. (14).

Parameter refinement

The parameters are obtained by optimizing the agreement between observed and calculated reaction heats. The optimization is performed by means of a non-linear least squares process, minimizing the objective function (U) defined as

$$U = \sum (Q_{\mathrm{obs}} - Q_{\mathrm{calc}})^2 \tag{18}$$

where Q_{obs} is the value of the heat change determined experimentally, corrected for all non-chemical energy terms (stirring, dilution of titrant and titrate, etc.). The calculated reaction heat (Q_{calc}) is related to the change (δn_i) in the number of moles of the *i*th chemical species by Eq. (14).

The parameters to be determined are the equilibrium constant value and the standard enthalpy value for each chemical equilibrium. A vector (**p**) of the parameters is subject to iterative refinement by the method of non-linear least squares

$$\mathbf{p}^{i+1} = \mathbf{p}^i + \left(\mathbf{J}^T\mathbf{J}\right)^{-1}\mathbf{J}\Delta\mathbf{Q} \tag{19}$$

where i is the iteration number, $\mathbf{J}$ is the Jacobian matrix, $\mathbf{J}^T\mathbf{J}$ is the normal equations matrix, and $\mathbf{\Delta Q}$ is the vector of residuals, $Q_{\mathrm{obs}} - Q_{\mathrm{calc}}$. Elements of the Jacobian are the partial derivatives $\frac{\partial Q}{\partial \beta}$ and $\frac{\partial Q}{\partial(\Delta H^0)}$. These are calculated as follows. At each *i*th titration point, the change in the number of moles of a given species $A_pB_q..$ produced by adding a volume v_i of the titrant to a solution in the ITC cell of volume V_0 is given by

$$\begin{aligned}\delta n_{pq..} &= \beta_{pq..}\left(V_0[A]^p[B]_{..}^q\right)_2 - \beta_{pq..}\left((V_0 - v_i)[A]^p[B]_{..}^q\right)_1 \\ &= \beta_{pq..}(m_2 - m_1)\end{aligned} \tag{20}$$

The subscripts 1 and 2 refer to the concentrations before and after the addition of titrant.

Partial differentiation in Eq. (14) gives $\frac{\partial Q}{\partial(\Delta H^0)}$ as

$$\frac{\partial Q}{\partial\left(\Delta H^0{}_{pq..}\right)} = -\delta n_{pq..} \tag{21}$$

The calculation of the derivatives $\frac{\partial Q}{\partial \beta}$ from Eqs. (14) and (20) is more complicated. To simplify the notation, the subscript $pq..$ is now omitted from Eq. (22)

$$\begin{aligned}\frac{\partial Q}{\partial \beta} &= -\Delta H^0\left(\frac{\partial(\delta n)}{\partial \beta}\right) = -\Delta H^0\left((m_2 - m_1) + \beta\left(\left(\frac{\partial m_2}{\partial \beta}\right) - \left(\frac{\partial m_1}{\partial \beta}\right)\right)\right) \\ \frac{\partial m}{\partial \beta} &= vp[A]^{p-1}[B]^q\left(\frac{\partial[A]}{\partial \beta}\right) + vq[A]^p[B]^{q-1}\left(\frac{\partial[B]}{\partial \beta}\right)\end{aligned} \tag{22}$$

The derivatives $\frac{\partial[A]}{\partial\beta}$ and $\frac{\partial[B]}{\partial\beta}$ are obtained by implicit differentiation of the equations of mass balance (Eqs. (9) and (10)), using the procedure previously described in detail [43]. When all the derivatives have been calculated, the normal equations are solved for the vector **p**, which contains the iterative corrections for both stability constants and standard enthalpies. The refinement is protected against divergence by means of a shift-cutting procedure.

The chemical model may contain any number of reagents and any number of the complexes that are formed from them. There may be any number of titration curves and any number of titration points in each curve. An example of the simulation screen with two titration curves is shown below.

An application: nicotinic acid

The robustness of the program was tested with a variety of experimental data. In particular, data for the protonation of nicotinic acid (niacin) were subject to a detailed examination. This system was chosen because the nicotinate anion may undergo two protonation steps with substantially different standard reaction enthalpies: values of ca. −12 kJ mol^{-1} and ca. −3 kJ mol^{-1} were obtained previously [44]. This means that their values cannot be determined with data from a single ITC curve since the heat values for the first portion of the curve would differ significantly from that obtained for the second part of the same experiment. The nicotinic acid system demands careful experimental design. Total concentrations must be chosen so as to provide substantial ranges of both species' concentrations and reaction heat values over the course of a titration.

Each step of the process of stability constant and standard enthalpy determination is now described in detail.

Step 1: specify the chemical model

The model is specified by defining the cumulative association constants, β_1 and β_2, in terms of the equilibrium concentrations of the species A^-, AH, and AH_2^+ which are involved in the equilibria.

$$K_1 = \beta_1 = \frac{[AH]}{[A^-][H^+]} \tag{23}$$

$$\beta_2 = \frac{[AH_2^+]}{[A^-][H^+]^2} \tag{24}$$

whence

$$K_2 = \frac{\beta_2}{\beta_1} = \frac{[AH_2^+]}{[AH][H^+]} \tag{25}$$

Estimates of the values of the stability constants can be obtained (in this instance) from the literature as log $\beta_1 = 5$ and log $\beta_2 = 7$ (log $K_1 = 5$, log $K_2 = 2$). A calculation of the concentrations in a titration, using these values, is shown in Fig. 1 [45].

The data with which the diagrams shown in Fig. 1 were constructed are also available in HypCal as tables or numerical values of concentrations and others. Program control can be passed directly to the application HySS [45] and then back again. The HQD data file (see Electronic Supplementary Material (ESM)) contains both the data needed by HypCal and by HySS; indeed, the model specification is stored in the same file for both applications. The speciation diagram shows that the optimum pH range is 1–7, as each species reaches a maximum degree of formation of at least 90 % in that range. It also shows that all three species are present in the pH range 3–4. This occurs because the stepwise constant for the formation of AH has the small value of 10^2; a smaller value would give more overlap.

Step 2: estimate what concentrations are needed

This will depend on the magnitude of the standard reaction enthalpies, the concentrations of the titrant, and the titrant/titrate ratio one wishes to obtain in order to optimize the refinement. Concentrations were chosen on the basis that ΔH^0 for the first reaction step is ca. −12 kJ mol^{-1}. Figure 2 illustrates the result of the first run.

The data for the first protonation step are satisfactory. The magnitude of the heat developed drops to very small values after one equivalent of acid has been added. The heat developed, under these conditions, in the second protonation step is too small to be useful. This has arisen because the standard enthalpy for the second step has a much smaller value for the first protonation step than for the second step. It indicates that a higher concentration of analyte will be needed to get better data for the second step. One way to handle the disparity in

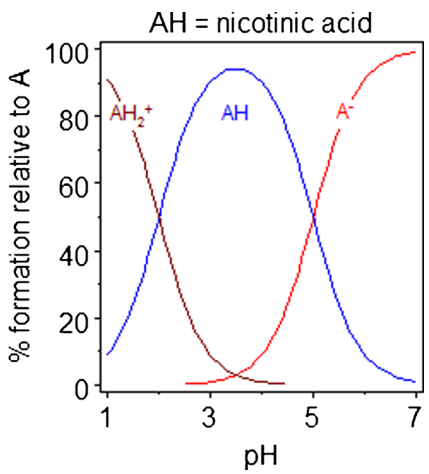

Fig. 1 Speciation calculation for nicotinic acid

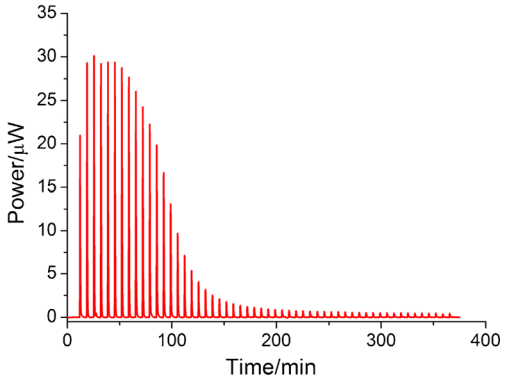

Fig. 2 ITC titration of HNO_3 (20.06 mM) into nicotinic acid (0.995 mM) in water at 25 °C. Injection volumes were 4 μL (except for the first one, 2 μL); 55 injections at 400-s intervals

experimental values would be to assign much lower weights to the data points in the second part of the titration curve. This option of weighting the data has been extensively debated, and there are arguments both for and against it [37].

An alternative procedure is to obtain a second titration curve, using higher concentrations such that the heats generated in the second titration would be comparable to the heats generated in the first titration curve. The two titration curves may then be refined together since the program allows for the simultaneous refinement of many titrations.

HypCal can be used to explore the possible experimental conditions with the aim of optimizing the quantities of heat to be measured. This is achieved by examining the table of quantities calculated, with an estimated value of the standard reaction heat, as a function of reagent concentrations and pH. For example, such a table, relating to the second titration step, is shown in Table 1.

This simulation indicates that these conditions will provide satisfactory experimental values of reaction heat. The use of a second titration with conditions based on this simulation eliminates the need for the data to be weighted. The procedure involving the refinement of more than one titration curve has been extensively tested in studies concerning the encapsulation of ammonium guest into a cluster [20] and the formation of supramolecular capsules [21, 46, 47]; it was found that breaking down the curve into two portions and choosing the concentrations in such a way as to have similar heat values, the residuals tended to have a random distribution. Another advantage of treating the two curves together is that this procedure automatically caters properly for those data points where there are more than two species in equilibria with each other.

It should be noted that HypCal deals with net heat values only; thus, the heat of blanks must always be subtracted from the gross heat values measured for the reaction.

The importance of correctly estimating and subtracting heats of dilution (blank) has been extensively discussed [48].

Table 1 Simulation of a nicotinic acid (AH) titration with HypCal, using log $\beta_1 = 5$, log $\beta_2 = 7$, and $C_{AH} = 1$ mM. The standard heat values for the first ($A^- + H^+ = AH$) and second ($AH + H^+ = AH_2^+$) steps were estimated to be −12 and −3 kJ mol^{-1}, respectively

Titer/μL	pH	delta *n*/μmol		Calculated *Q*/mJ
		AH	AH_2^+	
8	3.713	0.0397	0.0101	0.68
23	3.353	0.0036	0.0237	0.442
38	3.149	−0.0156	0.0237	0.193
53	3.01	−0.0187	0.0223	0.131
68	2.905	−0.0188	0.0209	0.105
83	2.822	−0.0182	0.0195	0.09
98	2.752	−0.0173	0.0182	0.079
113	2.692	−0.0163	0.0170	0.072
128	2.641	−0.0153	0.0158	0.065
143	2.595	−0.0144	0.0148	0.06
158	2.554	−0.0135	0.0138	0.055
173	2.517	−0.0127	0.0130	0.051
188	2.483	−0.0119	0.0121	0.048
203	2.452	−0.0112	0.0114	0.045
218	2.424	−0.0106	0.0107	0.042
233	2.397	−0.0099	0.0100	0.039

The dilution of both titrant and titrate needs to be considered. Dilution of the titrate may well be negligible, though this needs to be checked. On the other hand, the heat of dilution that occurs when a reagent is added from the burette to the solution, containing all chemicals except the titrate, may not be negligible. A procedure routinely adopted in our laboratory to minimize errors when the heat of dilution is small compared to the gross heat has been described recently: a number of blank titrations are performed and the data are fitted to a low-order polynomial (ESM, Figs. S3 and S4). The coefficients of the polynomial are then used to calculate the best heat values for the blank [49].

Step 3: collection of experimental data

Following the preliminary experiments and data analysis, two sets of titrations were performed for the determination of the four parameters: log β_1, log β_2, ΔH^0_1, and ΔH^0_2. Experimental conditions were chosen such that in one set, the principal reaction is the protonation of the nitrogen atom, whilst in the other set it is mainly the carboxylate group that is being protonated.

In the first set of titrations, the cell in the calorimeter was filled with a solution containing nicotinic acid and potassium hydroxide in the molar ratio 1:1. This ensured that the carboxylate group was fully deprotonated at the start of the titration. A set of experimental data is shown in Fig. 3.

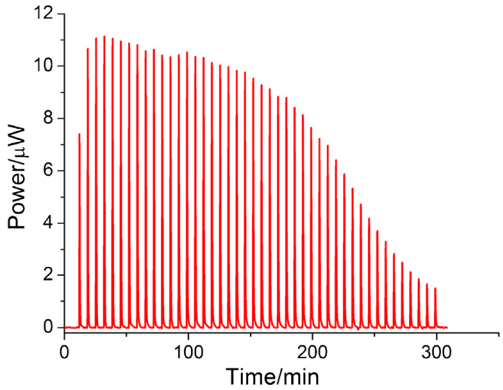

Fig. 3 ITC titration of HNO_3 (6.74 mM) into nicotinic acid (0.998 mM) containing one equivalent of KOH at 25 °C. Injection volumes were 4 μL (except for the first one, 2 μL); 45 injections at 400-s intervals

For the second titration, i.e., the protonation of the carboxylate group, higher concentrations of acid in the burette were used, along with larger injection volumes, in order to obtain heat amounts comparable to those generated in the first set of titrations. An experimental data set is shown in Fig. 4.

All measurements were carried out using aqueous solutions without the addition of an inert electrolyte for ionic strength control. Blank experiments carried out in the presence of 0.1 M KNO_3 were found to be strongly endothermic; consequently, the subtraction of the dilution heats from the raw heats to obtain the net heats of reaction would have lead to large uncertainties resulting from the combination of two opposing and, in the case of the second protonation step, comparable contributions. Titrations were run without any background electrolyte and required only small corrections for heat of dilution.

The experimental power curves were integrated by using the NanoAnalyze software (TA Instruments, USA) to obtain the gross heat for reaction step. Corrections for heat of dilution were applied using the procedure described [49].

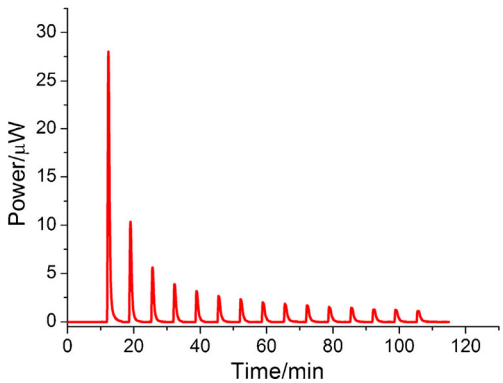

Fig. 4 Second protonation step: ITC titration of HNO_3 (20.06 mM) into nicotinic acid (1.05 mM) at 25 °C. Injection volumes were 15 μL (except for the first one, 2 μL); 16 injections at 400-s intervals

Step 4: refine the parameters

The calorimetric data, comprising a total of 12 titration curves, were processed by HypCal. The fit obtained along with the observed-calculated residuals and the species distribution diagram for typical first and second titration steps are shown in Fig. 5. The accord between experimental and calculated values is shown in ESM, Table S1.

Note that three species are present at the end of the left-hand curve and at the start of the right-hand curve. Small discrepancies observed in the first part of both the curves may be ascribed to the initial backlash effect (i.e., the reduction of the titrant volume in the first injections due to the mechanical properties of the syringe system) that has been previously reported by others [50] and thoroughly discussed by our group [49].

Processing a set of 12 titration curves gave the parameters shown in Table 2. Agreement with previously published values is satisfactory, particularly in view of the fact that they were obtained together rather than individually as was done previously [44, 51–53]. There is a small difference of 1 kJ mol^{-1} between the value we obtained for ΔH^0 and the value reported by Martell et al. [44] for the first protonation step; this value was extracted from a series of values mainly obtained using the van't Hoff method. Among these values, only that obtained by Christensen et al. [51] was determined by means of calorimetry, but its calculation used equilibrium constant values which had been published by others.

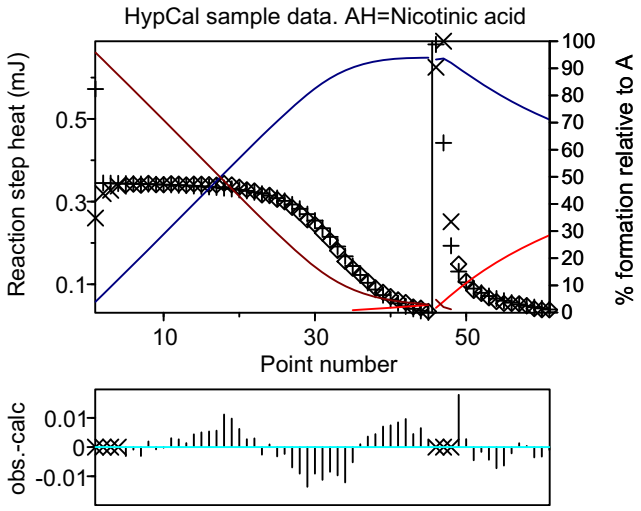

Fig. 5 HypCal output for the first and second protonation step of nicotinic acid: experimental heats, *diamonds*; calculated heats, *crosses*. The species distribution diagram [A^- (*brown*), AH (*blue*), AH_2^+ (*red*)] is also calculated by the software and shown in the *same window*. Residuals (observed - calculated values) are shown *below the curves*. Data for two titration curves are illustrated in this figure. The parameters in Table 2 were obtained by refinement of data from 12 titration curves (ESM, Fig. S2); × *sign* indicates that the specific titration point has been excluded from the refinement

Table 2 Thermodynamic parameters for the protonation reactions of nicotinate (A^- = pyridine-3-carboxylate) in water at 25 °C obtained by HypCal refinement

Reaction	Log β		ΔH^0 (kJ/mol)	
$A^- + H^+ \rightleftharpoons AH$	4.83 (6)	4.83 (5)[a]	−12.81 (1)	−11.7 (8)[a]
$A^- + 2H^+ \rightleftharpoons AH_2^+$	6.9 (2)	6.86 (5)[a]	−15.59 (3)	−14.2 (8)[a]

The figure in parentheses is the standard deviation referred to last significant digit of the value

[a] Smith and Martell [44]

Concluding remarks

HypCal is a powerful program than can be used for the simultaneous determination of both stability constants and standard enthalpies relating to any chemical equilibrium system (see "HypCal: key features" in Electronic Supplementary Material). This has been illustrated with the example of nicotinic acid for which there are some conditions in which three species are in equilibrium with each other in solution. The software imposes no limits on the chemical constitution of the system or on the quantity of experimental data to be analyzed. Typical applications could be in studies of ligand protonation, host-guest reactions, metal-ligand complexation, and competition reactions. HypCal may also be used to process data from traditional, partially filled, calorimeters, replacing the program HypΔH which has been described previously [15].

The numerical algorithms used are based on the well-tested routines that are used in Hyperquad [22] and HypSpec [24], modified and adapted as needed for the specific characteristics of calorimetric data. The parameter refinement procedure is robust and efficient. The program is fully protected against hard failure: when the experimental data are not adequate to fully characterize the parameters, a soft failure will occur. The program can also be used to determine standard enthalpies, using stability constant values that have been obtained by another technique, such as potentiometry, spectrophotometry, NMR, etc.

The criteria for optimum experimental design, when using HypCal to derive thermodynamic parameters, involve both the concentrations of the complexes formed and the heats developed over the course of the titrations. The program HySS, which may be accessed by HypCal, may be used for this purpose. The nicotinic acid example illustrates how preliminary experiments may be performed in order to establish experimental conditions that comply with this criterion.

HypCal deals with chemical models in which stoichiometry is specified explicitly, rather than with binding models. One advantage of this arrangement is that systems with a competing ligand can be processed.

Materials and methods

Materials

Pyridine-3-carboxylic acid (Sigma-Aldrich) was of the highest purity commercially available (≥99.5 %) and was used as received. Solutions of HNO_3 and KOH (Merck, Titrisol Normex) were standardized by titration with tris(hydroxy methyl)aminomethane (TRIS) and potassium hydrogen phthalate, respectively. The titer of HNO_3 was further checked by potentiometric titration of independent TRIS batches. The KOH solution was kept under inert atmosphere and stored in a Metrohm Dosimat 665 dispenser that is equipped with a soda-lime guard tube. High-purity water (Millipore, Milli-Q Element A 10 ultrapure water) and grade A glassware were employed throughout.

Calorimetric titrations

ITC titrations were carried out at 25 °C using a nano-isothermal titration calorimeter Nano-ITC^{2G} (TA Instruments, USA) with an active cell volume of 0.988 mL (overfilled mode) and a 250- or 100-μL injection syringe. The reaction mixture in the sample cell was stirred at 250 rpm during the titration. The calorimetric apparatus was initially calibrated chemically by titrating an HCl solution (1 mM) into a buffered TRIS solution (30 mM containing 10 mM HCl, [TRIS]/[TRISH$^+$] = 2:1) according to the procedure previously described [49]. The calorimeter was also checked by electrical calibration. Prior to each titration experiment, all solutions were degassed with gentle stirring under vacuum for about 15 min. ITC measurements for the first protonation step were carried out by titrating an aqueous solution of HNO_3 (6.74 mM) into a nicotinic acid solution (0.99–1.05 mM) containing one equivalent of KOH. For the second protonation step, a 20.06 mM HNO_3 solution was titrated into nicotinic acid (0.99–1.05 mM) in plain water. The heats of dilution were determined in separate blank experiments by titrating solutions of HNO_3 (either 6.74 or 20.06 mM) into plain water. The net heats of reaction were obtained by subtracting the heat evolved/absorbed in the blank experiments. Typically, six independent experiments were run for each protonation step reaction (Table 3) so as to collect an adequate number of points to achieve a satisfactory fit of both the first and last portion of the curve; the

Table 3 Experimental conditions for the calorimetric titrations

Titration	Cell		Burette		No. of points
	C^0_{AH} (mM)	C^0_{KOH} (mM)	C^0_H (mM)	Injection volume (μL)	
1–6	0.998	0.998	6.74	4	45
7–12	1.047	0	20.06	15	16

two portions of the curve were refined together to yield the final parameter values.

Acknowledgments The University of Catania (FIR 2014, 9DD800, and 018B9A) is gratefully acknowledged for the financial support.

Compliance with ethical standards

Conflict of Interest GA and CS declare that they have no conflict of interest. PG is the sole proprietor of Protonic software.

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