Van't Hoff global analyses of variable temperature isothermal titration calorimetry data

Lee A. Freiburger¹, Karine Auclair¹, and Anthony K. Mittermaier^{1,*}

¹Department of Chemistry, McGill University, 801 Sherbrooke Street West, Montréal, Québec, Canada, H3A 2K6

Abstract

Isothermal titration calorimetry (ITC) can provide detailed information on the thermodynamics of biomolecular interactions in the form of equilibrium constants, K_A , and enthalpy changes, H_A . A powerful application of this technique involves analyzing the temperature dependences of ITC-derived K_A and H_A values to gain insight into thermodynamic linkage between binding and additional equilibria, such as protein folding. We recently developed a general method for global analysis of variable temperature ITC data that significantly improves the accuracy of extracted thermodynamic parameters and requires no prior knowledge of the coupled equilibria. Here we report detailed validation of this method using Monte Carlo simulations and an application to study coupled folding and binding in an aminoglycoside acetyltransferase enzyme.

Keywords

Aminoglycoside acetyltransferase; Coupled equilibria; Protein folding; Enthalpy; Heat capacity

1. Introduction

Isothermal titration calorimetry (ITC) characterizes the thermodynamics of biological interactions by measuring the heat released or absorbed as a ligand is incrementally titrated into a solution of its binding partner [1]. ITC data can be analyzed using a variety of approaches to yield detailed information on events that accompany ligand binding at the molecular-level. There are two main classes of binding model, which we will refer to as phenomenological models and mechanistic models. Phenomenological models depend only on the stoichiometry of the interaction and make no assumptions regarding the underlying physical mechanism of binding. According to the theory of binding polynomials, the phenomenological model for an interaction involving n identical ligands contains exactly $n + 1$ thermodynamic states (0-bound, 1-bound, 2-bound, ..., n -bound) and $2n$ thermodynamic parameters (n enthalpy and n affinity constants) [2]. Thus in the case of a 1:1 stoichiometry, ITC data can always be described with two thermodynamic parameters, the affinity K_A , and

*Corresponding Author: Phone: (514) 398-3085, Fax: (514) 398-3797, anthony.mittermaier@mcgill.ca.

Electronic Supplementary Material

Tables of individually fit and globally fit H_A and K_A values and coupled folding/binding parameters. Histograms of H_A and K_A values extracted from Monte Carlo simulated data sets. Plots of H_A and K_A values extracted from Monte Carlo data sets with 5% and 10% simulated errors in protein and ligand concentrations.

the enthalpy change H_A . The advantage of a phenomenological model is that it is rigorously correct for all systems interacting with a given stoichiometry. However, the phenomenological model parameters do not explicitly address how binding actually occurs at a microscopic level. In contrast, mechanistic models represent specific hypotheses regarding the physical processes underlying a binding reaction. They can involve arbitrarily large numbers of thermodynamic states and contain microscopic thermodynamic parameters describing differences in enthalpy, free energy, and heat capacity, as illustrated by the coupled folding/binding model below. Mechanistic model parameters relate directly to the actual thermodynamic transitions involved in binding, such as conformational changes or ionization reactions, and are thus of greater interest than the phenomenological parameters. However, there are a variety of different and mutually exclusive mechanistic models that can be applied to interactions of a given stoichiometry, and it can be challenging to determine which of these models is correct.

ITC results are typically interpreted using a two-step process [2]. The raw ITC isotherms are first fitted using a phenomenological model, yielding values of H_A and K_A . A mechanistic model is then selected and the corresponding model-specific thermodynamic parameters fitted to the phenomenological H_A and K_A values. In this regard, a great deal of insight can be gained by performing ITC experiments over a range of temperatures. Mechanistic models can be fitted to sets of H_A and K_A values obtained at different temperatures, shedding light on the relationship between ligand binding and conformational changes in the protein or additional coupled equilibria [3–5]. For example, in a simple two-state system (free and bound), H_A varies linearly with temperature, provided that the difference in heat capacity between the free and bound states is constant [6]. If a protein undergoes thermal denaturation within the temperature range studied, the dependence of H_A on temperature can be strongly curvilinear [3]. If multiple binding-competent states exist, H_A values can exhibit fairly complex temperature profiles [7]. Linkage to ionization equilibria can also influence the temperature dependence of binding parameters [8]. Thus inspection of the temperature dependences of phenomenological binding parameters provides key information for selecting an appropriate mechanistic binding model and elucidating the physical processes underlying a molecular interaction. This approach is most effective when the experimental binding data are precisely defined. Even fairly modest experimental uncertainties in H_A and K_A can make it difficult to draw quantitative conclusions regarding the linkage between binding and any additional processes. A number of different situations can produce elevated errors in H_A and K_A parameters, such as when affinities are low [9], enthalpy changes are small, or when macromolecules contain multiple non-equivalent binding sites [10].

The accuracy of extracted thermodynamic parameters can be improved by fitting multiple ITC isotherms simultaneously [11–18]. Thus global fitting methods are potentially very useful in situations where binding parameters derived from individual ITC isotherms are prone to error. In order to perform global fitting on variable temperature datasets, all ITC isotherms must be related mathematically. The standard phenomenological binding models described above do not directly consider temperature variation and therefore have not been used in such global applications. Mechanistic models can be fitted directly to multiple ITC isotherms obtained over a range of temperatures [14–16]. However, this approach requires a

priori knowledge of the binding mechanism which may not be readily available, particularly in situations where H_A and K_A are not well defined by fits to individual ITC isotherms.

We recently developed a general approach for global analyses of variable temperature ITC data that employs phenomenological rather than mechanistic binding models [19]. Prior information on the binding process is therefore not required, beyond knowledge of the binding stoichiometry. In other words, it is not necessary to select a specific mechanistic binding model in order to perform the global analysis. The method is based on a simultaneous analysis of raw ITC isotherms, using an integrated form of the van 't Hoff equation to link the phenomenological binding parameters extracted at different temperatures. It yields sets of H_A and K_A values with improved accuracy compared to those obtained from fits of individual ITC isotherms, which can be used to design and test mechanistic binding models. The approach was applied to the interaction between the antibiotic resistance-causing enzyme aminoglycoside 6'-N-acetyltransferase-Ii (AAC(6')-Ii) with one of its substrates, acetyl coenzyme A (AcCoA) [19]. AAC(6')-Ii is a homodimeric enzyme from *Enterococcus faecium* that transfers the acetyl group from AcCoA to a range of aminoglycosides, conferring resistance to these antibiotics. Using the van 't Hoff (VH) global analysis of variable-T ITC data together with NMR and circular dichroism (CD) spectroscopy, we showed that homotropic allostery between the two active sites of homodimeric AAC(6')-Ii is modulated by opposing mechanisms. One follows a classical KNF paradigm [20] while the other follows a recently-proposed mechanism in which partial unfolding of the subunits is coupled to ligand binding [21].

In this study, we validate the VH global fitting procedure using Monte Carlo simulations corresponding to several different binding scenarios. The true binding parameters are faithfully reproduced in each test, confirming our confidence in the method. The Monte Carlo simulations show that the VH global analysis significantly improves the accuracy of extracted H_A and K_A values compared to fits of data obtained at individual temperatures. We apply the method to the monomeric W164A variant of AAC(6')-Ii (AAC-W164A), in order to gain further insight into coupled folding and binding of this enzyme. Trp164 is located at the dimer interface, and its substitution with Ala produces a monomeric protein with about 10% of the wild-type activity [22]. ITC data for binding of AcCoA to monomeric AAC-W164 can be fitted with a simple one-site binding model, supporting the idea that allostery in the wild-type protein involves communication between the two subunits of the dimer. Interestingly, AAC-W164A undergoes coupled folding and binding similar to that of the wild-type protein, shedding new light on the underlying physical mechanism of this process.

2. Experimental

2.1 Materials and Methods

The plasmid encoding the AAC-W164A gene was kindly provided by Dr. Gerard D. Wright, Department of Biochemistry and Biomedical Sciences, McMaster University, Canada. The buffer and cell broth reagents were obtained from Fischer Scientific (Whitby, ON, Canada). The acetyl coenzyme A (AcCoA), and protease inhibitors were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). The Q-sepharose and Superdex 75

chromatography resins were obtained from GE Healthcare. The activated affinity Affi-Gel 15 was purchased from Bio-Rad (Mississauga, ON). The *Escherichia coli* strain BL21(DE3) competent cells were obtained from Invitrogen (Carlsbad, CA).

AAC-W164A was expressed and purified by anion exchange, gel filtration and affinity chromatography as previously described[23] with the following modifications. The affinity purification step employed a paromomycin affinity resin. Size exclusion chromatography was performed with a hand-packed XK16 Superdex 75 column on an AKTA FPLC system and an elution buffer consisting of HEPES (25 mM, pH 7.5), EDTA (2 mM) and NaCl (1 M). The purification was monitored through UV absorbance and two major peaks were detected. The peak corresponding to the correct molecular weight was collected and further purified by affinity chromatography. Protein concentrations were determined by spectroscopic absorbance measurements using a theoretical extinction coefficient of 33,920 M⁻¹ cm⁻¹ at 280 nm (ExpASY Proteomics), and Lowry-Bradford assays.

ITC measurements were performed on a MicroCal VP-ITC isothermal titration calorimeter from MicroCal (North Hampton, MA, USA). Solutions of AAC-W164A were dialyzed against a buffer solution of 25 mM HEPES and 2 mM EDTA at pH 7.5, and the resulting dialysis solution was used to dissolve the AcCoA. All runs were performed using a solution of AAC-W164A (85–200 μ M) in the sample cell (1.4 mL) and a solution of AcCoA (0.85–2 mM) in the injection syringe. Titrations were performed in duplicate at 10, 15, 20, 25, 30, 34, 37, and 40°C. Each experiment comprised an initial delay of 60 s, a first injection of 2 μ L and a delay of 150 s, 26 injections of 10 μ L each followed by 330 s delays, and a final injection of 10 μ L followed by a delay of 600 s. Identical control titrations in which AcCoA solutions were injected into buffer in the absence of AAC-W64A were performed. The heats thus obtained were subtracted from the ITC binding data. The first data point was removed from all isotherms.

CD measurements were performed on a Jasco J-810 spectrometer, using a 0.2 mm path length cuvette. Samples contained 25–30 μ M protein with 25 mM HEPES and 2 mM EDTA at pH 7.5, for both the W164A mutant and wild-type enzymes. Data were collected at temperatures between 0.1 to 70°C and wavelengths from 200 to 300 nm at scan rates of 50–200 nm/min in 0.5 nm increments with bandwidths of 1 nm. Each scan was performed three times then averaged. The final analysis of AAC-W164A data was performed with two sets of thermal unfolding experiments, using data from 0.1 to 50°C. The first employed 1°C intervals with four minute equilibration times, and the second employed 3–5°C intervals with 10 minute equilibration times. The two traces were completely superimposable up to 47°C.

2.2 Coupled Folding and Binding

We employed a coupled folding/binding model in which the folded form of the protein (F) is in equilibrium with both the bound state (FL) and a partially or fully unfolded form (U) that cannot bind ligand, according to the following scheme [4, 28],

where H_F , H_B and $C_{p,F}$, $C_{p,B}$ are the changes in enthalpy and heat capacity for the folding and binding steps, while $K_F = [F]/[U]$ and $K_B = [FL]/([F][L])$ are equilibrium constants. The transition from U to F is exothermic ($H_F < 0$) therefore at low temperatures the protein is predominantly folded and the macroscopic binding affinity and enthalpy are simply given by K_B and H_B , respectively. At higher temperatures, an appreciable fraction of the free protein exists in the unfolded state. ITC measurements therefore involve transitions from the U to FL states and the heat absorbed or released contains contributions from both folding and binding reactions. The phenomenological association constant and enthalpy are given by [3]:

$$K_A(T) = \frac{K_F(T)}{1 + K_F(T)} K_B(T) \quad (2)$$

$$\Delta H_A(T) = \Delta H_B(T) + \frac{1}{1 + K_F(T)} \Delta H_F, \quad (3)$$

where

$$K_X(T) = K_X(T_0) \times \exp \left\{ \frac{\Delta H_X(T_0)}{R} \left(\frac{1}{T_0} - \frac{1}{T} \right) + \frac{\Delta C_{p,X}}{R} \left(\ln \left\{ \frac{T}{T_0} \right\} + \frac{T_0}{T} - 1 \right) \right\}, \quad (4)$$

$$\Delta H_X(T) = \Delta H_X(T_0) + \Delta C_{p,X}(T - T_0), \quad (5)$$

X = F or B, and T_0 is an arbitrary reference temperature, in this case 283.15 K.

In principle, it is possible to extract the values of $K_F(T_0)$, $K_B(T_0)$, $H_F(T_0)$, $H_B(T_0)$, $C_{p,F}$ and $C_{p,B}$ from sets of K_A and H_A values determined by ITC over a range of temperatures. In practice, however there is a high degree of covariance among these parameters, leading to unreliable estimates of their values based on ITC data alone. It has recently been shown that this issue can be resolved by simultaneously analyzing variable-T ITC and CD melt data [3]. CD measurements are sensitive to the secondary structure content of the protein, which differs in U and F states, and therefore provide independent measures of the folding equilibrium constant, K_F . In addition, comparisons of ITC and CD data provide an avenue for validating the VH global fitting procedure in this study.

We modeled the temperature dependence of the molar ellipticity at 222 nm, $[\theta]$, with the following expression [3],

$$[\theta](T) = \frac{K_F(T)}{1+K_F(T)} \left([\theta]_F + m_F(T-T_0) \right) + \frac{1}{1+K_F(T)} \left([\theta]_U + m_U(T-T_0) \right), \quad (6)$$

which assumes that the overall signal is the population-weighted average of the folded and unfolded signals, and that both baselines are linear functions of temperature. Variables $[\theta]_F$ and $[\theta]_U$ are the molar ellipticities of the folded and unfolded states at the reference temperature, and m_F and m_U are the corresponding baseline slopes. Since CD melts can only be extended partway through the unfolding transition, the unfolded baseline slope, m_U , is not well defined by the data. CD studies of protein thermal denaturation typically yield approximately horizontal unfolded baselines [3, 29–31]. Consequently, we performed the analysis by optimizing $[\theta]_U$ while fixing m_U near zero. The results were insensitive to the precise choice of m_U , and in all reported analyses, $m_U = 0$.

2.3 ITC Data Analysis

ITC isotherms were calculated using a heat function, Q , defined such that the heat released or absorbed during the i th injection is given by the expression

$$\Delta Q(i) = Q(i) - Q(i-1) \left(1 - \frac{V_i}{V_c} \right), \quad (7)$$

which takes into account the sample displaced from the working volume of the cell by the injection [1]. V_i is the volume of the i th injection, V_c is the working volume of the sample cell, and $Q(i)$ is the value of the heat function following the i th injection. Heat functions were calculated according to [1]

$$Q = \frac{\Delta H_A V_c}{2} \left(P_T + X_T + \frac{1}{K_A} - \sqrt{\left(P_T + X_T + \frac{1}{K_A} \right)^2 - 4 P_T X_T} \right). \quad (8)$$

The total concentrations of ligand, $[X]_T$, and protein, $[P]_T$, present in the working volume of the cell after n injections may be calculated according to the following expressions[1]

$$[X]_T = [X]_0 \left(1 - \left(1 - \frac{\Delta V}{V_0} \right)^n \right) \quad (9)$$

$$[P]_T = [P]_0 \left(1 - \frac{\Delta V}{V_0}\right)^n, \quad (10)$$

which correct for sample dilution throughout the titration. $[X]_0$ is the concentration of ligand in the syringe and $[P]_0$ is the initial protein concentration.

In both the individual and VH global analyses of ITC data, binding parameters were adjusted to minimize the sum of residual squared differences (RSS) between experimental data points and those calculated using equation (7),

$$\text{RSS} = \sum_i \left(\Delta Q(i)_{\text{calc}} - \Delta Q(i)_{\text{exp}} \right)^2, \quad (11)$$

where $Q(i)_{\text{exp}}$ is the experimentally determined heat from injection i , $Q(i)_{\text{calc}}$ is the injection heat calculated using equation (7), and the sum runs over all data points in a single ITC titration for the individual fits, or over all sixteen ITC samples for the VH global fits. In the individual fitting, three parameters were optimized for each experiment: the association constant (K_A), binding enthalpy (H_A), and the initial protein concentration ($[P]_0$). In the VH global fitting, $[P]_0$ was optimized for each experiment, H_A was optimized for each temperature, and only a single binding constant was fitted (K_0). The binding constants at all other temperatures were determined by trapezoidal integration of the van 't Hoff equation which is given by the expression,

$$\ln K_j = \ln K_0 + \sum_{i=1}^j \frac{(T_i - T_{i-1})}{2R} \left(\frac{\Delta H_{i-1}}{(T_{i-1})^2} + \frac{\Delta H_i}{(T_i)^2} \right), \quad (12)$$

where K_0 is the binding constant at the reference temperature (T_0), and the i th set of ITC experiments are performed at a temperature T_i and fitted with a binding enthalpy H_i . Equation (12) can be recast in exponential form according to:

$$K_j = K_0 \times \left(\prod_{i=1}^j \exp \left\{ \frac{(T_i - T_{i-1})}{2R} \left(\frac{\Delta H_{i-1}}{(T_{i-1})^2} + \frac{\Delta H_i}{(T_i)^2} \right) \right\} \right) \quad (13)$$

Note that when multiple isotherms are collected at the same temperature, this approach yields a single H_i value for all replicates. Fitting each ITC isotherm individually involves optimizing 3 parameters, H_A , K_A , and $[P]_0$. Therefore the total number of adjustable parameters for individual fits of all 16 ITC experiments is 48, with 384 degrees of freedom. There are about half as many adjustable parameters in the VH global analysis of ITC data, 25, and the number of degrees of freedom is 407.

For an ITC dataset of N_R replicate experiments collected at each of N_T different temperatures, a VH global fit has $N_T \cdot N_R$ adjustable $[P]_0$ values (one for each isotherm), N_T adjustable H_A values (one for each temperature), and a single adjustable equilibrium constant (K_θ). Global fitting then comprises the following steps:

1. Starting with the initial values of the parameter set listed above, calculate the remaining ($N_T - 1$) equilibrium constants using K_θ and the set of N_T H_A values, according to Equation (12).
2. For each of the $N_R \cdot N_T$ isotherms, calculate the heat function, Q , using the K_A and H_A value for the corresponding temperature together with the protein concentration ($[P]_0$) for the corresponding experiment, according to Equations (8) to (10).
3. Calculate each isotherm according to Equation (7).
4. Repeat steps 1–4, varying the adjustable parameters in order to minimize the RSS given by Equation (11), using a standard least-squares minimization algorithm.

The sets of $K_A(i)_{\text{exp}}$ and $H_A(i)_{\text{exp}}$ values obtained from either individual fits of ITC isotherms, or from VH global analysis of all ITC isotherms, were fit together with CD-derived molar ellipticities using a coupled folding/binding model by minimizing the target function

$$\text{RSS} = \sum_i \left(\left(\frac{\Delta H_A(i)_{\text{calc}} - \Delta H_A(i)_{\text{exp}}}{\Delta H_A(i)_{\text{exp}}} \right)^2 + \left(\frac{\ln K_A(i)_{\text{calc}} - \ln K_A(i)_{\text{exp}}}{\ln K_A(i)_{\text{exp}}} \right)^2 \right) + \sum_j \left(\left(\frac{[\Theta](j)_{\text{calc}} - [\Theta](j)_{\text{exp}}}{[\Theta](j)_{\text{exp}}} \right)^2 \right), \quad (14)$$

where the sums run over all data points at all temperatures. The values of $H_A(i)_{\text{calc}}$, $K_A(i)_{\text{calc}}$, and $[\Theta](i)$ were computed in terms of the equilibrium constants, $K_F(T_\theta)$ and $K_B(T_\theta)$, enthalpies $H_F(T_\theta)$ and $H_B(T_\theta)$, and heat capacity changes, $C_{p,F}$ and $C_{p,B}$, for folding and binding, respectively, using Eq.(2)–(6), as described in the text. The denominators in this equation ensure roughly equal weighting for the enthalpy, affinity, and CD contributions to the RSS.

2.4 Monte Carlo Simulations

For each simulation, a series of “true” H_A and K_A values were generated according to Equations (2)–(5), or a similar model. These thermodynamic parameters were then used to generate 313 synthetic ITC datasets, each comprising 16 isotherms of 28 injections, according to Equations (7)–(10). Experimental errors were simulated by adding to each heat increment a random value drawn from a population with a mean of zero and a standard deviation equal to the estimated experimental error, σ . The value of σ for each experiment

was calculated from the residual-sums-of-squared deviations of the individual fits of experimental data according to [24]

$$\sigma = \sqrt{\frac{\text{RSS}}{\nu}}, \quad (15)$$

where ν is the number of degrees of freedom of the individual fit, and RSS is given by Eq. (11): $\sigma = 14, 16, 17, 19, 21, 22, 24, 25 \text{ cal mol}^{-1}$ at 10, 15, 20, 25, 30, 34, 37, and 40°C. In addition $[P]_0$ and $[L]_0$ were both randomly varied by 2% in each iteration. Very similar results were obtained when $[P]_0$ and $[L]_0$ were both randomly varied by 5% and 10% (See Supplemental Figures S1 and S2). Each temperature series of simulated ITC isotherms was subjected to both individual and global analyses, as described in the text. Errors in coupled folding/binding analyses were calculated as the standard deviations of parameters extracted from fits of Equations (2) to (6) to each Monte Carlo set of H_A and K_A values. A system with low heats of binding was simulated using Equations (2) to (5), with $H_B(T_0) = -0.5 \text{ kcal mol}^{-1}$, $K_B(T_0) = 25900 \text{ M}^{-1}$, $C_{p,B} = -10 \text{ cal mol}^{-1} \text{ K}^{-1}$, $H_F(T_0) = -10 \text{ kcal mol}^{-1}$, $K_F(T_0) = 85$, $C_{p,F} = 0 \text{ kcal mol}^{-1} \text{ K}^{-1}$, and $T_0 = 283.15 \text{ K}$. A system with two non-equivalent binding sites [12] was simulated with $H_1(T_0) = -3 \text{ kcal mol}^{-1}$, $K_1(T_0) = 43000 \text{ M}^{-1}$, $C_{p,1} = -670 \text{ cal mol}^{-1} \text{ K}^{-1}$, $H_2(T_0) = -5.06 \text{ kcal mol}^{-1}$, $K_2(T_0) = 42500 \text{ M}^{-1}$, $C_{p,2} = -920 \text{ cal mol}^{-1} \text{ K}^{-1}$, and $T_0 = 283.15 \text{ K}$.

3. Results

3.1 Isothermal Titration Calorimetry

ITC experiments were performed on AAC-W164A at 10, 15, 20, 25, 30, 34, 37, and 40°C, and the resulting isotherms are shown in Figure 1. Data were initially fitted on an individual basis, yielding the phenomenological parameters H_A and K_A for each experiment at each temperature (Figure 2A,B). The values are somewhat scattered relative to their overall variation with temperature, particularly in the case of K_A . This is likely due to the low affinity of AAC-W164A for AcCoA ($K_A \approx 3.2 \cdot 10^3 \text{ M}^{-1}$ at 40°C), which makes it challenging to achieve high c -values and to fully saturate the protein by the end of the titrations ($c = [P]_0 K_A$, where $[P]_0$ is the initial protein concentration) [9]. Nevertheless, general trends are apparent. In particular, H_A varies linearly with temperature between about 10 and 30°C. Values decrease markedly above this point, corresponding to a sharp increase in the heat released by binding. This behavior is characteristic of coupling between the binding reaction and an additional conformational transition, such as protein folding [4]. CD spectroscopic measurements (discussed below) indicate that the protein undergoes partial or complete unfolding over the same temperature range, which helps to confirm that ligand binding is coupled to protein conformational changes under these conditions.

3.2 Circular Dichroism

CD spectra of AAC-W164A were measured from 0.1 to 70°C in increments of 1°C. At low temperatures, the molar ellipticity in the 220 to 230 nm region is on the order of -9000 deg

$\text{cm}^2 \text{dmol}^{-1}$, which is indicative of alpha-helical and/or beta-sheet structure (Figure 2C) [25, 26]. In contrast, the molar ellipticities of unstructured polypeptide chains are close to zero or slightly positive at wavelengths of 220 to 230 nm [25–27]. This region of the AAC-W164A spectrum sigmoidally approaches zero as the temperature is raised, suggesting that the protein undergoes partial or complete thermal unfolding. The process is completely reversible up to about 50°C. Above this temperature, the CD signal is progressively lost, likely due to slow protein aggregation. Thus only data from 0.1 to 47°C were used in the following analysis.

3.3 Coupled Folding and Binding

The CD and ITC data suggest that the appropriate mechanistic binding model to describe the interaction of AAC-W164A with AcCoA is one in which ligand binding is coupled to protein folding. As described in the Experimental section, the simplest coupled folding/binding model comprises the folded form of the protein (F) in equilibrium with both the bound state (FL) and an unfolded form that cannot bind ligand (U). The phenomenological ITC-derived H_A and K_A thermodynamic parameters are explained in terms of mechanistic parameters comprising the equilibrium constant, enthalpy change and heat capacity change of protein folding ($U \rightarrow F$: K_F , H_F and $C_{p,F}$ respectively) as well as the affinity constant, enthalpy change, and heat capacity change of ligand binding to the folded protein ($F \rightarrow FL$: K_B , H_B , and $C_{p,B}$ respectively). We fitted the CD and ITC data simultaneously, using the coupled folding/binding model given by Equations (2)–(6), as shown in Figure 2A–C. The overall agreement is reasonable. However the scatter in the ITC data points makes it somewhat difficult to judge the quality of the coupled-equilibrium fit. Furthermore, there are small but systematic deviations between the calculated and experimental CD data throughout the temperature range. Below, we show that these discrepancies are not due to the coupled folding/binding model being incorrect. Instead, the relatively low affinity of the AAC-W164A/AcCoA interaction leads to a level of uncertainty in extracted ITC parameters that obscures their agreement with CD data. Simultaneous analysis of all ITC isotherms using the VH global fitting method significantly increases the accuracy of the extracted H_A and K_A values and improves the agreement with CD data.

3.4 Van 't Hoff (VH) global fitting of ITC isotherms

We have developed a global analysis that makes no assumptions regarding the underlying linkage of binding to other thermodynamic equilibria, and produces temperature series of phenomenological binding parameters with increased accuracy [19]. These improved sets of H_A and K_A values can then be used to select and test mechanistic binding models, such as the coupled folding/binding model used here. In this approach, the van 't Hoff expression,

$$\frac{\partial \ln \{K_A\}}{\partial T} = \frac{\Delta H_A}{RT^2}, \quad (16)$$

is used to constrain the values of H_A and K_A within the global fits. The binding enthalpy, $H_A(T_n)$, is optimized at each experimental temperature, T_n , but only a single association

constant is fitted. Specifically, the value of K_A is optimized at the first temperature of the series, $K_A(T_0)$, and the remaining K_A values are obtained from the set of $H_A(T_n)$ and the integrated form of Equation (16).

$$\ln \{K_A(T_n)\} = \ln \{K_A(T_0)\} + \frac{1}{R} \int_{T_0}^{T_n} \frac{\Delta H_A(T)}{T^2} dT. \quad (17)$$

The last term in Equation (17) is evaluated by trapezoidal numerical integration, in which the function $H_A(T)/T^2$ is approximated by a series of straight lines connecting the experimental $H_A(T_n)/T_n^2$ points (Figure 3). This significantly reduces the number of adjustable variables in the fits and improves the accuracy of the extracted parameters, as discussed below. Importantly, this approach makes no assumptions regarding the underlying physical process, beyond that the numerical integral of $H_A(T)/T^2$ can be evaluated accurately.

3.5 Application to AAC-W164A

Experimental ITC isotherms for AAC-W164A/AcCoA binding at all temperatures were fitted simultaneously using the VH global approach described above, to yield a value of $H_A(\text{global})$ and $K_A(\text{global})$ at each temperature. The agreement between predicted and experimental isotherms is good, as shown in Figure 1, and the residual sum-of-squares (RSS) increases by only a factor of 2.5, relative to the individual fits. Qualitatively, $H_A(\text{global})$ and $K_A(\text{global})$ values exhibit smoother temperature dependences than do $H_A(\text{indiv})$ and $K_A(\text{indiv})$. At many temperatures, the globally-fit and individually-fit values are similar. However at higher temperatures there are sizeable deviations, particularly in H_A . For instance, at 40°C, $H_A(\text{indiv})$ and $H_A(\text{global})$ differ by more than 20 kcal mol⁻¹.

$H_A(\text{global})$ and $K_A(\text{global})$ were analyzed together with the CD data, using the coupled folding/binding model (Equations (2)-(6)) as shown in Figure 4. Excellent agreement was obtained between CD and globally-fit ITC data, confirming that coupled folding/binding is the appropriate mechanistic model. This level of agreement is far better than that obtained with the individually-fit ITC isotherms (Figure 2). The residual-sum-of-squared-deviations for the coupled folding/binding (ITC + CD) analysis is 10-fold lower with $H_A(\text{global})$ and $K_A(\text{global})$ values than that obtained with $H_A(\text{indiv})$ and $K_A(\text{indiv})$. This is consistent with Monte Carlo analyses, described below, that show up to 3-fold reductions in the uncertainties of extracted phenomenological model parameters using the VH global fits for the simulated AAC-W164A data. The coupled folding/binding parameters extracted for the globally-fit and individually-fit ITC datasets differ, particularly those describing the folded/unfolded equilibrium, as listed in Supplemental Table S2. The significantly better fits obtained with $H_A(\text{global})$ and $K_A(\text{global})$ strongly suggest that these provide a clearer picture of the interaction between AAC-W164A and AcCoA.

The binding thermodynamics of the W164A mutant are fairly similar to those of the wild-type apo-enzyme interacting with a single molecule of AcCoA [12]. The binding to AAC-W164A is slightly less exothermic, and the affinity is decreased roughly four-fold: H_A

$W164A(20^{\circ}C) = -8.5 \text{ kcal mol}^{-1}$ and $K_D^{W164A}(20^{\circ}C) = 74 \text{ }\mu\text{M}$, versus $H_A^{WT1}(20^{\circ}C) = -9.3 \text{ kcal mol}^{-1}$ and $K_D^{WT1}(20^{\circ}C) = 17 \text{ }\mu\text{M}$ (for the first binding site on the homodimer). Binding is cooperative for the wild-type protein at this temperature, such that its interaction with the second molecule of AcCoA is tighter and more exothermic than its interaction with the first. Thus AAC-W164A appears to share similarities with monomers comprising the unbound form of the wild-type enzyme. The model predicts that at $42^{\circ}C$, AAC-W164A populates a partially or completely unfolded state to a level of 50%, with $C_{p,F} = -2.1 \text{ kcal mol}^{-1} \text{ K}^{-1}$ and $H_F = -52.9 \text{ kcal mol}^{-1}$ at this temperature. These thermodynamic values are consistent with large-scale unfolding of a protein the size of AAC-W164A (182 aa). It has been shown for a database of globular proteins that C_p can be well approximated by the relation $-N_{\text{res}}.14 \text{ cal mol}^{-1} \text{ K}^{-1}$, while H (extrapolated to $100^{\circ}C$) is given by $-N_{\text{res}}.1.26 \text{ kcal mol}^{-1}$, where N_{res} is the number of residues [32]. The experimental values, $C_{p,F} = -2.1 \text{ kcal mol}^{-1} \text{ K}^{-1}$ and $H_F(100^{\circ}C) = -176 \text{ kcal mol}^{-1}$ are comparable, but smaller than those predicted by the empirical relationships, $C_p^{\text{pred}} = -2.5 \text{ kcal mol}^{-1} \text{ K}^{-1}$ and $H^{\text{pred}} = -229 \text{ kcal/mol}$.

We have previously shown that the wild-type AAC(6')-II enzyme populates at least three distinct thermodynamic states: a well-structured bound form, a more conformationally dynamic free form, and an even more disordered partially unfolded form. The partial thermal unfolding of the free subunits in the wild-type enzyme occurs over the same temperature range as that of the AAC-W164A mutant, and the enzyme remains dimeric in the partially melted state [19]. The free subunits of the wild-type dimer unfold with a temperature midpoint of $41^{\circ}C$ and folding enthalpy of $-82 \text{ kcal mol}^{-1}$ (at $42^{\circ}C$). Binding of a single molecule of AcCoA leads to structural rigidification of the bound subunit, slightly lowers the melting temperature of the remaining free subunit to $39^{\circ}C$, and elevates its folding enthalpy to $-104 \text{ kcal mol}^{-1}$ (at $42^{\circ}C$). Interestingly, a trend emerges when data for AAC-W164A are compared with results for the wild-type enzyme: The monomeric (AAC-W164A) protein is most stable ($T_m = 42^{\circ}C$). The next most stable is the unbound wild-type (AAC(6')-II) enzyme ($T_m = 41^{\circ}C$), i.e. subunits that are adjacent to unbound subunits, which are relatively structurally dynamic. The unbound subunit of the singly-bound enzyme is least stable ($T_m = 39^{\circ}C$), i.e. free subunits that are adjacent to bound subunits, which are relatively structurally rigid. Similarly, folding of the monomeric protein is least exothermic, and least entropically costly ($H_F = -53 \text{ kcal mol}^{-1}$, $S_F = -169 \text{ cal mol}^{-1} \text{ K}^{-1}$), followed by a wild-type subunit adjacent to free subunit ($H_F = -82 \text{ kcal mol}^{-1}$, $S_F = -264 \text{ cal mol}^{-1} \text{ K}^{-1}$), while partial unfolding of a free subunit adjacent to a bound subunit is the most exothermic and also the most entropically unfavorable ($H_F = -104 \text{ kcal mol}^{-1}$, $S_F = -334 \text{ cal mol}^{-1} \text{ K}^{-1}$). One possible explanation for these observations is that the partially unfolded state of a subunit could be stabilized by the presence of an adjacent subunit, with concomitant shielding of non-polar groups from the solvent that leads to a reduction in entropy. However, more study is needed to judge whether this hypothesis is correct.

3.6 Monte Carlo Error Analysis

In order to compare the accuracy of individual and the VH global fitting procedures, we investigated their performance on simulated Monte Carlo (MC) ITC datasets. We used the coupled folding/binding mechanistic model parameters obtained with the globally-fit ITC

data (Supplemental Table S2) to calculate theoretical H_A and K_A values at 10, 15, 20, 25, 30, 34, 37, and 40°C, which are shown with solid lines in Figure 5A–D. From these values, we generated about 300 simulated variable-T ITC datasets, each comprising sixteen isotherms with random experimental errors (two replicate isotherms at each of eight temperatures). The Monte Carlo isotherms were subjected to both individual and global fitting. Each set of individual fits yielded two H_A and K_A values at each temperature (since the MC data sets contained duplicate isotherms), which were averaged prior to further analysis. In what follows, we will refer to these pair-wise averaged parameters as $H_A^{\text{MC(indiv)}}$ and $K_A^{\text{MC(indiv)}}$. Each global fit yielded a single value of $H_A^{\text{MC(global)}}$ and $K_A^{\text{MC(global)}}$ at each temperature. Thus the two fitting procedures each yielded about 300 Monte Carlo H_A and K_A temperature series, which are plotted as points in Figure 5A,B (individually-fit) and Figure 5C,D (globally-fit).

Individual fits of the Monte Carlo ITC datasets produce fairly broad distributions of $H_A^{\text{MC(indiv)}}$ and $K_A^{\text{MC(indiv)}}$ values (points) that deviate significantly from the true values (lines), particularly at higher temperatures. This is consistent with the scattered profiles obtained for individual fits of experimental AAC-W164A data (Figure 2). The relative errors of the $H_A^{\text{MC(indiv)}}$ and $K_A^{\text{MC(indiv)}}$ distributions are plotted in Figure 6, and range from about 2% at 10°C to 13% at 40°C. Much of the uncertainty in these parameters is due to the weakness of the interaction which makes it difficult to fully saturate the protein with ligand. The maximum injectant concentration used in the experiments described above was 2 mM. Even with this relatively high concentration of AcCoA, only about 55% saturation was achieved by the final injection of ligand at 40°C, whereas greater than 90% saturation is required for optimal accuracy in the fitted parameters [33]. Low levels of saturation are a common problem when performing ITC experiments on weakly binding systems, since high (mM to M) concentrations of ligand are required for full saturation. Ligand solubility and aggregation, as well as availability and cost can place restrictions on the maximum concentrations that are experimentally accessible. In this case, we have performed additional Monte Carlo simulations with 30-fold greater concentrations of AcCoA and verified that uncertainties in H_A and K_A are reduced. In practice it would be prohibitively expensive to carry out experiments under these conditions. The interaction of AAC-W164A and AcCoA thus represents a good model for weakly-binding systems in which it is difficult to fully saturate the protein during the titration. As shown below, VH global fitting of the variable-T ITC data significantly improves the accuracy of the extracted phenomenological thermodynamic parameters.

When the same set of Monte Carlo ITC isotherms are fit simultaneously using the VH global approach, the extracted $H_A^{\text{MC(global)}}$ and $K_A^{\text{MC(global)}}$ values (points) are clustered more closely around the true values (lines) over the full temperature range. Importantly, the points are distributed evenly about the true values. This indicates that the global fitting protocol does not introduce bias into the extracted thermodynamic parameters and that numerical integration of H_A/T^2 in Equation (17) does not lead to systematic errors. The relative errors associated with H_A and K_A are plotted in Figure 6 and range from about 1% at 10°C to 6% at 40°C. This represents a roughly two-fold improvement in accuracy over the individually-fitted H_A and K_A values, i.e. $H_A^{\text{MC(global)}}$ and $K_A^{\text{MC(global)}}$ exhibit half the standard deviation of $H_A^{\text{MC(indiv)}}$ and $K_A^{\text{MC(indiv)}}$. The result is significant since it

implies that global fitting produces the accuracy that would be expected for standard analyses of an ITC dataset four times larger than the one at hand (i.e 8 rather than 2 replicates at each temperature). This improvement occurs primarily because the global constraint greatly reduces the parameter-space of the fits; the reduced space is guaranteed to include the true values, since the set of H_A and K_A produced by any physical model must obey the van 't Hoff relationship.

The binding of AcCoA by AAC-W164A is representative of systems where low affinity impedes the saturation of the macromolecule with ligand during the ITC titration, leading to greater uncertainty in extracted H_A and K_A values. We have shown that in such cases, global analyses of variable-T ITC datasets using van 't Hoff constraints lead to significant improvements in accuracy over fits performed on an individual-isotherm basis. There are other situations in which it is challenging to extract well-defined H_A and K_A values. We have tested the applicability of this method to two such scenarios: 1) systems with low heats of binding that lead to poor signal-to-noise ratios in ITC data and consequently poorly defined H_A and K_A values; and 2) systems with multiple non-equivalent binding sites, for which it is difficult to separate the overall heat signals into contributions from the different sites [10, 19]. The results of the simulations are shown in Figure 7. For the hypothetical low-enthalpy interaction, we observe roughly 2-fold decreases in the standard deviations of both H_A and K_A values extracted from the fits. For the scenario involving two non-identical binding sites, we see up to 1.5-fold improvements in the accuracy of H_A and 4-fold improvements in the accuracy of K_A values. Notably, this last calculation helps to validate our application of VH global fitting the interaction between the wild-type AAC(6')-II enzyme and AcCoA [19].

4. Discussion

ITC data collected over a range of temperatures can provide uniquely detailed information on how ligand binding occurs at a molecular level. However, the interpretation of variable temperature ITC data sets is not always straightforward. Ideally, each ITC isotherm can be analyzed using a phenomenological binding model to yield values of H_A and K_A . These, in turn, help to indicate which mechanistic model best corresponds to the actual ligand binding process. Analysis of the phenomenological H_A and K_A values using the selected mechanistic model yields thermodynamic parameters that explicitly describe molecular events associated with binding. However there are situations in which this approach becomes problematic. When binding is weak, saturation can be difficult to achieve during the titration experiment and errors in the extracted parameters are elevated [9]. When binding enthalpies are low, the amount of heat released or absorbed during the experiment is small and the signal-to-noise ratio is reduced. When the binding stoichiometry is greater than 1:1, phenomenological binding models contain larger numbers of adjustable parameters that can suffer from covariation [10]. In addition, analyte impurities or incorrect estimates of the injectant concentration or the can lead to errors in H_A and K_A [34]. Deviations in extracted H_A and K_A values due to any of these factors can make it difficult to determine which mechanistic binding model best describes the system under study.

The VH global fitting method employed here is useful for such challenging systems. It has several advantages over previous global analysis schemes, in which specific mechanistic binding models were fitted directly to ITC isotherms. Unlike the prior studies, this approach does not require any assumptions regarding thermodynamic equilibria that may be coupled to binding. It can be applied to any set of variable temperature ITC data, even in cases where the underlying physics of the interaction are not known, and yields sets of accurate H_A and K_A parameters that can be used to design and test mechanistic binding models. These sets of globally-determined H_A and K_A values have additional significance. Phenomenological binding constants associated with any system must obey the van 't Hoff equation, regardless of the complexity of the underlying coupled equilibria. Conversely, any set of H_A and K_A values that do obey the van 't Hoff equation can in principle be explained by a physical model. The VH global method uses the van 't Hoff equation as a fitting constraint. Therefore any set of H_A and K_A values obtained using the global method is physically realizable. Since no other constraints are applied in the analysis, this implies that VH global fitting provides the best agreement with ITC data that can be achieved by any physical model. This information is useful when interpreting variable temperature ITC datasets. For instance, it can be concluded that any residual deviations between the experimental data and the global fit reflect experimental errors, and do not result from choosing an incorrect mechanistic model, since the VH global fit provides the best agreement achievable with any physical model. It also means that if a mechanistic model closely reproduces the globally fit H_A and K_A values, then no significant improvements in fit to the experimental data can be expected with different physical models.

We have tested the performance of the VH global analysis method using Monte Carlo simulations. We find that it does provide significant improvements in the accuracy of extracted H_A and K_A values, compared to fits performed on individual ITC isotherms. Additionally, the simulations show that the numerical integration procedure that we have used to implement the van 't Hoff constraint does not introduce detectable bias into the results. We applied the method to study the interaction of AAC-W164A with one of its substrates, AcCoA. Compared to the binding parameters extracted from individual isotherms, the globally fit H_A and K_A values are qualitatively less scattered, correspond more closely to the circular dichroism data, and give a better agreement with the coupled folding/binding mechanistic model, with a 10-fold reduction in the residual sum of squared deviations (Figure 2 and Figure 4). These are all indications that the van 't Hoff constraint improves the accuracy of phenomenological binding parameters. As discussed above, any deviations between the experimental data and the global fit (for example in Figure 1E) are due to experimental errors and not due to any incompatibility of the model. The excellent agreement obtained between the set of $H_A(\text{global})$ and $K_A(\text{global})$ values and those predicted by the mechanistic model strongly indicates that AAC-W164A undergoes coupled folding/binding when it interacts with AcCoA in a similar fashion to the individual subunits of the dimeric wild-type enzyme. Interestingly, AAC-W164A is slightly more thermostable than the wild-type enzyme, yet its unfolding is associated with a smaller absolute enthalpy change. This suggests that the presence of an adjacent subunit promotes subunit unfolding in an entropy-driven manner. The results obtained here demonstrate the utility of VH global

analyses of variable-temperature ITC data, and highlight the potential of this approach to shed light on physical processes underlying biomolecular interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors would like to thank Prof. G. D. Wright (McMaster University, Canada) for providing the AAC-W164A expression construct. This research was funded by the Canadian Institutes of Health Research (to KA and AKM). LAF was supported by scholarships from the Chemical Biology Strategic Training and the Drug Development Training Initiatives of CIHR.

References

1. Velazquez-Campoy A, Ohtaka H, Nezami A, Muzammil S, Freire E. *Curr Prot Cell Biol.* 2004; 23:17.18.01–17.18.24.
2. Freire E, Schon A, Velazquez-Campoy A. *Methods Enzymol.* 2009; 455:127–155. [PubMed: 19289205]
3. Cliff MJ, Williams MA, Brooke-Smith J, Barford D, Ladbury JE. *J Mol Biol.* 2005; 346:717–732. [PubMed: 15713458]
4. Eftink MR, Anusiem AC, Biltonen RL. *Biochemistry.* 1983; 22:3884–3896. [PubMed: 6615806]
5. Ha JH, Spolar RS, Record MT. *J Mol Biol.* 1989; 209:801–816. [PubMed: 2585510]
6. Prabhu NV, Sharp KA. *Annu Rev Phys Chem.* 2005; 56:521–548. [PubMed: 15796710]
7. Herman P, Lee JC. *Biochemistry.* 2009; 48:9448–9455. [PubMed: 19719244]
8. Baker BM, Murphy KP. *Biophys J.* 1996; 71:2049–2055. [PubMed: 8889179]
9. Tellinghuisen J. *Anal Biochem.* 2008; 373:395–397. [PubMed: 17920027]
10. Tochtrop GP, Richter K, Tang CG, Toner JJ, Covey DF, Cistola DP. *Proc Natl Acad Sci U S A.* 2002; 99:1847–1852. [PubMed: 11854486]
11. Henzl MT, Larson JD, Agah S. *Anal Biochem.* 2003; 319:216–233. [PubMed: 12871715]
12. Freiburger LA, Auclair K, Mittermaier AK. *ChemBioChem.* 2009; 10:2871–2873. [PubMed: 19856370]
13. Schon A, Freire E. *Biochemistry.* 1989; 28:5019–5024. [PubMed: 2765522]
14. Baker BM, Murphy KP. *J Mol Biol.* 1997; 268:557–569. [PubMed: 9159490]
15. Armstrong KM, Baker BM. *Biophys J.* 2007; 93:597–609. [PubMed: 17449678]
16. Turner DC, Straume M, Kasimova MR, Gaber BP. *Biochemistry.* 1995; 34:9517–9525. [PubMed: 7626621]
17. Bjelic S, Jelesarov I. *J Mol Recognit.* 2008; 21:289–312. [PubMed: 18729242]
18. Houtman JCD, Brown PH, Bowden B, Yamaguchi H, Appella E, Samelson LE, Schuck P. *Protein Sci.* 2007; 16:30–42. [PubMed: 17192587]
19. Freiburger L, Baettig O, Sprules T, Berghuis AM, Auclair K, Mittermaier AK. *Nat Struct Mol Biol.* 2011 in press.
20. Koshland DE, Némethy G, Filmer D. *Biochemistry.* 1966; 5:365–385. [PubMed: 5938952]
21. Hilser VJ, Thompson EB. *Proc Natl Acad Sci U S A.* 2007; 104:8311–8315. [PubMed: 17494761]
22. Draker K, Northrop DB, Wright GD. *Biochemistry.* 2003; 42:6565–6574. [PubMed: 12767240]
23. Wright GD, Ladak P. *Antimicrob Agents Chemother.* 1997; 41:956–960. [PubMed: 9145851]
24. Tellinghuisen J. *J Phys Chem.* 2000; 104:2834–2844.
25. Adler AJ, Greenfield NJ, Fasman GD. *Methods Enzymol.* 1973; 27:675–735. [PubMed: 4797940]
26. Saxena VP, Wetlaue DB. *Proc Natl Acad Sci U S A.* 1971; 68:969–972. [PubMed: 5280530]
27. Uversky VN. *Protein Sci.* 2002; 11:739–756. [PubMed: 11910019]

28. Fisher HF, Colen AH, Medary RT. *Nature*. 1981; 292:271–272. [PubMed: 7254321]
29. Elwell M, Schellman J. *Biochim Biophys Acta*. 1975; 386:309–323. [PubMed: 236026]
30. Kuhlman B, Boice JA, Fairman R, Raleigh DP. *Biochemistry*. 1998; 37:1025–1032. [PubMed: 9454593]
31. Beadle BM, Baase WA, Wilson DB, Gilkes NR, Shoichet BK. *Biochemistry*. 1999; 38:2570–2576. [PubMed: 10029552]
32. Robertson AD, Murphy KP. *Chemical Reviews*. 1997; 97:1251–1268. [PubMed: 11851450]
33. Tellinghuisen J. *The Journal of Physical Chemistry B*. 2007; 111:11531–11537. [PubMed: 17850136]
34. Tellinghuisen J. *Journal of Physical Chemistry B*. 2005; 109:20027–20035.

Figure 1.

Isothermal titration calorimetry data collected at (A) 10, (B) 15, (C) 20, (D) 25, (E) 30, (F) 34, (G) 37, and (H) 40 °C for the interaction of the W164A variant of the aminoglycoside 6'-*N*-acetyltransferase-Ii (AAC-W164A) with acetyl-coenzyme A (AcCoA) (gray circles). Independent fits to each isotherm are indicated with dashed lines while fits using the VH global method are shown with solid lines.

Figure 2. Coupled folding/binding analysis of (A) H_A , and (B) K_A , values obtained from fits of individual ITC isotherms, together with (C) circular dichroism spectroscopic data (222 nm). In A and B, open circles indicate the values obtained for individual replicate experiments (two per temperature), while the solid circles are the pair-wise averages ($H_A(\text{indiv})$ and $K_A(\text{indiv})$). In (C), the upper and lower dashed lines correspond to the unfolded and folded ellipticity baselines, respectively.

Figure 3.

Schematic representation of trapezoidal integration of H_A/RT^2 . The points represent the fitted values of H_A , divided by the corresponding values of RT^2 . The difference in binding affinity between temperatures T_i and T_{ii} corresponds to the shaded area, i.e. $K_A(ii) = K_A(i) + \text{shaded area}$. Thus the H_A/RT^2 profile together with the single value of $K_A(i)$ specifies the affinity constants at all other temperatures.

Figure 4. Coupled folding/binding analysis of (A) H_A (global) and (B) K_A (global) values obtained from a simultaneous fit of all 16 ITC isotherms, together with (C) circular dichroism spectroscopic data (222 nm). In (C), the upper and lower dashed lines correspond to the unfolded and folded ellipticity baselines, respectively.

Figure 5.

Plots of H_A^{MC} and K_A^{MC} values extracted from fits of Monte-Carlo ITC datasets generated using the coupled folding/binding parameters obtained for AAC-W164A and AcCoA. In all panels, the fitted parameters are indicated by points, while the true values are shown as lines. (A,B) Results for individual analyses, where the points correspond to fitted values of (A) $H_A^{\text{MC}}(\text{indiv})$ and (B) $K_A^{\text{MC}}(\text{indiv})$. (C,D) Results for VH global analyses, where points correspond to fitted values of (C) $H_A^{\text{MC}}(\text{global})$ and (D) $K_A^{\text{MC}}(\text{global})$. Histograms of the extracted H_A^{MC} and K_A^{MC} values are shown in Supplemental Figures S3 and S4.

Figure 6. Relative errors (standard deviation/true value, 100%) for (A) H_A and (B) K_A values extracted from Monte Carlo ITC data sets by fitting pairs of individual isotherms (dashed line) or globally fitting all 16 synthetic isotherms (solid line).

Figure 7.

Plots of H_A and K_A values extracted from fits of Monte-Carlo ITC datasets generated for (A–D) a system with a low binding enthalpy, and (E–H) a molecule with two non-equivalent binding sites.[12] Lines indicate the true values, while points indicate the parameters obtained from (A,B,E,F) fits of individual isotherms and (C,D,G,H) global fits of isotherms over the temperature range. In (E–H), the dashed and solid lines correspond to binding of the first and second ligand molecules. Histograms of the extracted H_A and K_A values are shown in Supplemental Figures S5 to S9.