

# Determination of the thermodynamics of carbonic anhydrase acid-unfolding by titration calorimetry

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## Abstract

The enthalpy of unfolding ( $\Delta_u H$ ) of carbonic anhydrase II was determined by titrating the protein with acid and measuring the heat using isothermal titration calorimetry (ITC) in the temperature range of 5 to 59 °C. By combining the ITC results with our previous findings by differential scanning calorimetry (DSC) in the temperature range of 39 to 72 °C, the  $\Delta_u H$  dependence over a wide temperature range was obtained. The temperature dependence of the enthalpy displays significant curvature indicating that the heat capacity of unfolding ( $\Delta_u C_p$ ) is dependent on temperature. The  $T$ -derivative of  $\Delta_u C_p$  was equal to  $100 \pm 30 \text{ J}/(\text{mol} \times \text{K}^2)$ , with the result that the  $\Delta_u C_p$  is equal to 15.8 kJ/(mol × K) at 5 °C, 19.0 kJ/(mol × K) at 37 °C and 21.8 kJ/(mol × K) at 64 °C. The enthalpy of unfolding is zero at 17 °C. At lower temperatures, the  $\Delta_u H$  becomes exothermic.

This method of determining protein unfolding thermodynamics using acid-ITC, significantly widens the accessible  $T$ -range, provides direct estimate of the thermodynamic parameters at physiological temperature, and gives further insight into the third  $T$ -derivative of the Gibbs free energy of unfolding.

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## 1. Introduction

The thermodynamic parameters associated with protein unfolding and their relation to protein structure is an important and disputed subject area in protein science [1,2]. Accurate prediction of stability and unfolding thermodynamics from amino acid sequence alone, is not possible for any protein. Therefore, further investigations and development of the methods to determine and analyze the thermodynamic parameters associated with protein unfolding, including the enthalpy, entropy, Gibbs free energy, the heat capacity, and

even the  $T$ -derivative of the heat capacity, are important for the future development of protein science.

The heat capacity of protein unfolding was recently reviewed by Sharp [3]. Protein unfolding has a positive  $\Delta_u C_p$  that produces a maximum in stability and which thermodynamically explains the phenomenon of cold denaturation. In most studies of aqueous protein solutions, the heat capacity of unfolding is assumed to be  $T$ -independent [2,4]. This assumption is usually correct within the error of experimental technique, primarily differential scanning calorimetry (DSC).

DSC is the most common method to determine the enthalpy of unfolding [5–8]. However, it was shown for lysozyme, and later for other proteins, that the enthalpy of denaturation may be determined either by heating at constant pH (DSC) or by titrating with acid at constant temperature (ITC) [9].

In our earlier study of carbonic anhydrase unfolding by DSC [10] we determined the enthalpy of unfolding in the temperature range of 39 to 72 °C by carrying out DSC experiments at various pHs. Here we extend the temperature range down to

*Abbreviations:* bCAII, bovine carbonic anhydrase II; DSC, differential scanning calorimetry;  $\Delta_u H_{\text{DSC}}$ , enthalpy of unfolding determined by DSC;  $\Delta_u H_{\text{ITC}}$ , enthalpy of unfolding determined by ITC;  $\Delta_u C_p$ , heat capacity of unfolding;  $\Delta_u S$ , entropy of unfolding;  $\Delta_u G$ , Gibbs free energy of unfolding; ITC, isothermal titration calorimetry;  $T_m$ , melting temperature.

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5 °C by titrating the protein with acid at constant temperature in ITC. The two methods give essentially the same result at about 40 °C. Furthermore, the enthalpy of unfolding falls on the same temperature trend. As expected, the heat capacity of unfolding is positive. Unexpectedly, however, the  $\Delta_u C_p$  is found to increase with temperature. In a previous study of the temperature dependence of  $\Delta_u C_p$ , the specific heat capacity increments of protein unfolding were found to decrease with increased temperature [1].

It is important to determine the enthalpy of protein unfolding at physiological temperature in pharmaceutical industry when carrying out ligand binding screening using any type of thermal shift assay, such as the ThermoFluor<sup>®</sup> assay [11,12]. This assay uses unfolding enthalpy ( $\Delta_u H$ ) at 37 °C to determine the ligand binding constant ( $K_b$ ) at 37 °C. The  $K_b$  strongly depends on the accuracy of  $\Delta_u H$  determination, yet it is often difficult or impossible to find appropriate thermal denaturation conditions that afford accurate direct determination of unfolding parameters for most proteins at such a low temperature. Here we suggest that the protein acid-unfolding reaction as followed by ITC can be used as a general method to determine the  $\Delta_u H$  at 37 °C.

## 2. Materials and methods

### 2.1. Materials

Carbonic anhydrase II (from bovine, bCAII) was purchased from Sigma (St. Louis, MO) and used without further purification. Concentration of the protein was determined spectrophotometrically using  $\epsilon_{280} = 52,000 \text{ M}^{-1} \text{ cm}^{-1}$ . Hydrochloric acid and NaCl were reagent grade chemicals. MiliQ (Millipore) deionized water was used in the study.

### 2.2. Isothermal titration calorimetry

Carbonic anhydrase was dissolved in 100 mM NaCl aqueous solution of pH 6–7 to the final concentration of 0.5–2 (usually 1.0) mg/ml. The protein solution was loaded into the Nano ITC-III isothermal titration calorimeter (Calorimetry Sciences Corporation) cell (about 1.2 ml, cell volume 1.025 ml). The titration syringe (250  $\mu\text{l}$  volume) was filled with 5 to 30 (usually 10) mM HCl containing 70 to 95 (usually 90) mM NaCl. Titrations were carried out using 25 injections of 10  $\mu\text{l}$  each, injected at 3 to 15 minute intervals. Stirring speed was 150 rpm. Titrations were carried out at constant temperature in the 5–60 °C temperature range.

## 3. Results

The acid-unfolding of carbonic anhydrase was studied using isothermal titration calorimetry (ITC). Hydrochloric acid was injected into the unbuffered protein solution in the presence of NaCl. Fig. 1 shows an unfolding titration carried out at 37 °C, and shows the control titration to determine the heat of acid dilution in the absence of protein at 37 °C. Fig. 2 shows several ITC raw data curves obtained at 5, 15, 25, and 37 °C. The titration profiles obtained at different temperatures varied

greatly. Peaks were positive (exothermic, note that Calorimetry Sciences Corporation calorimeters use opposite sign standard than MicroCal ITC) at 5 °C and negative (endothermic) at 37 °C.

Since the unfolding reaction is slow, we did not wait for complete heat evolution and added next HCl portion before the completion of relaxation to baseline. Since the heat continued to evolve and the baseline was not reached, a linear baseline was drawn to integrate an entire area. In Fig. 2, one can see that the endothermic peaks are larger at higher temperatures because the enthalpy of unfolding is more endothermic at higher temperatures.

Fig. 3 shows integrated isothermal titration calorimetry curves. At higher temperatures, the endothermic heats were larger and the unfolding reaction was faster. However, between 55 °C and 59 °C there was a steep drop in the observed heat. DSC data show that the midpoint ( $T_m$ ) of thermal denaturation of the protein is about 64 °C, and since there is a large preequilibration period (about 1 h) in ITC, the protein is partially unfolded at 59 °C even before the titration with acid begins. Fig. 4 shows the ITC data obtained at lower temperatures. The observed reaction changes sign and becomes exothermic at temperatures below 17 °C.

By summing up the enthalpies during the course of titration, we obtain the integral molar enthalpies of carbonic anhydrase unfolding as a function of temperature. The black squares in Fig. 5 show the enthalpies ( $\Delta_u H_{\text{ITC}}$ ) obtained by integrating the acid titration curves in Figs. 3 and 4. Open triangles show calorimetric enthalpies of unfolding measured by DSC ( $\Delta_u H_{\text{DSC}}$ ). The DSC experiments were described earlier [10]. The different protein melting temperatures in DSC experiments were obtained by altering the pH.

Fig. 5 shows that there is a clearly non-linear increase in  $\Delta_u H$  as a function of temperature. Both ITC and DSC datasets seem to fall on the same trend. There is also quite good congruence

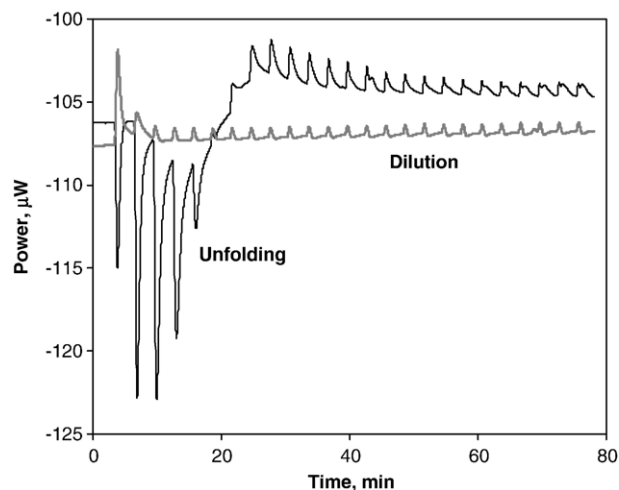


Fig. 1. Isothermal titration calorimetry raw data—titration of carbonic anhydrase with HCl (black line, *Unfolding*) and of the same titration in the absence of protein (grey line, *Dilution*). Endothermic heat evolved upon protein unfolding is visualized by large peaks pointing downwards. Heat of HCl dilution is relatively small. Both shown titrations were carried out at 37 °C.

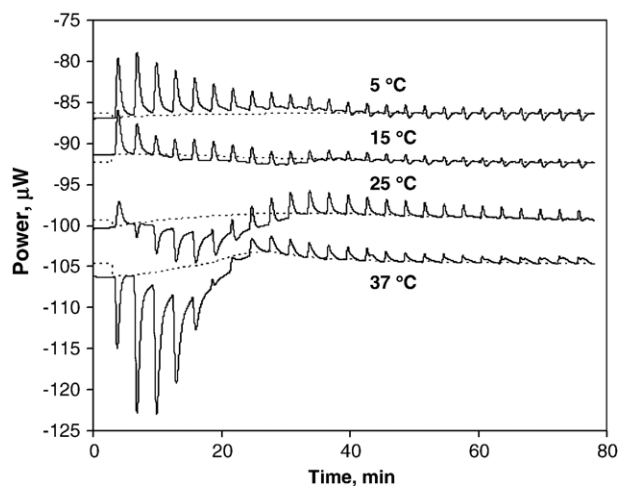


Fig. 2. ITC raw data at various temperatures: 5, 15, 25, and 37 °C. Since the unfolding reaction is not complete in 4 min, the baseline does not reach full relaxation and the reaction continues into the next peak. The reaction is exothermic at 5 °C and endothermic at 37 °C.

between the ITC and the DSC data around 40–45 °C. Above 45 °C, the enthalpy measured by ITC begins to drop due to the long preequilibration in the calorimeter at these higher temperatures (i.e. the three ITC points at 49, 55, and 59 °C). Therefore, at temperatures where thermal unfolding begins, acid-ITC data is no longer reliably comparable to the DSC values. On the other hand, DSC data could not be obtained at temperatures lower than 40 °C. Only the combination of these two methods provided direct calorimetric data on the enthalpy of unfolding for such a wide temperature range.

Each dataset on its own seems to follow an almost straight line without any apparent curvature. However, the overall dependence displays obvious curvature indicating that the heat capacity of unfolding is dependent on temperature.

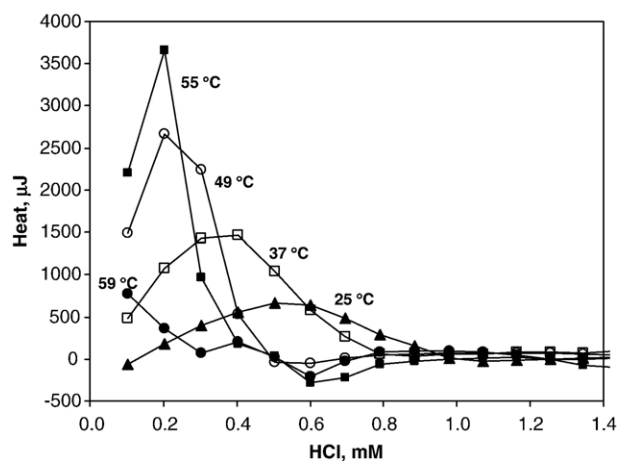


Fig. 3. Integrated curves of the ITC data at high temperatures. The endothermic heats are larger and evolve faster at higher temperatures until between 55 and 59 °C where there is a steep drop because the protein unfolds at this temperature during the preequilibration in ITC.

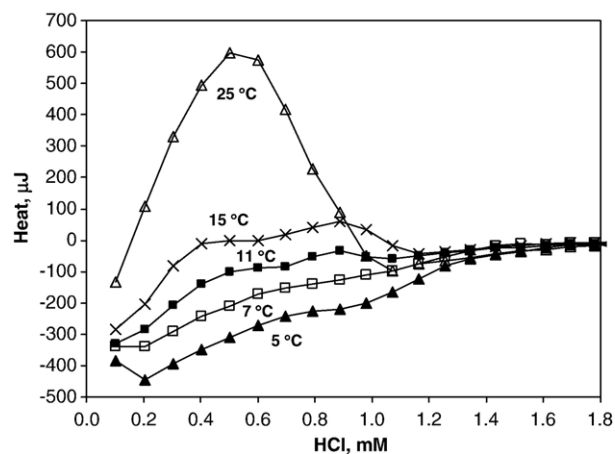


Fig. 4. Integrated curves of the ITC data at low temperatures. The enthalpy of the reaction is exothermic at low temperatures and changes sign at about 17 °C.

### 3.1. Heat capacity of unfolding dependence on temperature

The combined DSC and ITC (5–72 °C) data in Fig. 5 were regressed to a curved line. We assumed that there is a dependence of the heat capacity on temperature, such that:

$$\Delta_u C_p = \Delta_u C_{pT_m} + \Delta \Delta_u C_p (T - T_m) \quad (1)$$

where  $\Delta \Delta_u C_p$  is a constant ( $T$ -independent) coefficient of the heat capacity dependence on temperature. In Fig. 5, the curved line was obtained using  $\Delta \Delta_u C_p = 100 \pm 30 \text{ J}/(\text{mol} \times \text{K}^2)$ . The heat capacity of unfolding, equal to the tangential slope of the line at any specific temperature in Fig. 5, was 15.8 kJ/(mol  $\times$  K) at 5 °C, 19.0 kJ/(mol  $\times$  K) at 37 °C, and 21.8 kJ/(mol  $\times$  K) at the melting temperature ( $T_m = 64$  °C).

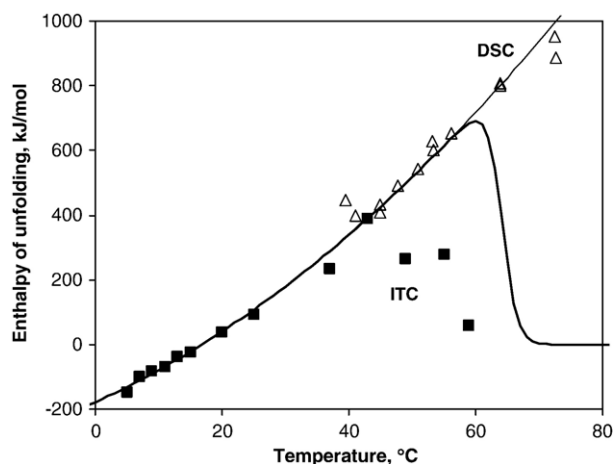


Fig. 5. Integral enthalpies of carbonic anhydrase unfolding as a function of temperature. The black squares show  $\Delta_u H_{ITC}$  values obtained by the titration of the protein with acid as described in the text. The open triangles show the calorimetric enthalpies of unfolding measured by DSC, ( $\Delta_u H_{DSC}$ ) as described previously [10]. Both datasets follow the same trend and there is good agreement at about 40 °C. Above 45 °C the  $\Delta_u H_{ITC}$  drops since the protein unfolds during the long preequilibration period. Bold line shows the best fit under assumption that the  $\Delta_u C_p$  is dependent on temperature as described in the text (Eq. (8)). Narrow line shows the fit of the DSC and ITC data according to Eq. (2).

The enthalpy dependence on temperature was calculated by:

$$\Delta_u H = \Delta_u H_{T_m} + (\Delta_u C_{pT_m} + \Delta \Delta_u C_p (T - T_m))(T - T_m) \quad (2)$$

where  $\Delta_u H_{T_m}$  is the enthalpy of unfolding at melting temperature. It was determined by regression of the experimental data (Fig. 5). The best fit yielded  $\Delta_u H_{T_m} = 803$  kJ/mol.

Other thermodynamic parameters could also be estimated using the same value of  $\Delta \Delta_u C_p$ . The entropy of unfolding was calculated by:

$$\Delta_u S = \Delta_u S_{T_m} + (\Delta_u C_{pT_m} + \Delta \Delta_u C_p (T - T_m)) \ln\left(\frac{T}{T_m}\right) \quad (3)$$

where  $\Delta_u S_{T_m}$  is the entropy of unfolding at the melting temperature obtained by:

$$\Delta_u S_{T_m} = \frac{\Delta_u H}{T_m} \quad (4)$$

since the Gibbs free energy of unfolding at  $T_m$  is equal to zero:

$$\Delta_u G_{T_m} = 0 \quad (5)$$

The Gibbs free energy was computed at various temperatures by:

$$\Delta_u G = \Delta_u H - T \Delta_u S \quad (6)$$

The probability of the protein to remain folded at various temperatures was calculated by:

$$P_{\text{folded}} = \frac{1}{1 + e^{-\Delta_u G/RT}} \quad (7)$$

The probability multiplied by the enthalpy of unfolding gives the enthalpy that could be observed by ITC and predicts the steep drop of the acid-ITC enthalpy (bold line in Fig. 5):

$$\Delta_u H_{\text{max-ITC}} = \Delta_u H \times P_{\text{folded}} \quad (8)$$

This maximum of the enthalpy of unfolding that could be observed by ITC was estimated to be 691 kJ/mol at 60 °C.

#### 4. Discussion

It is common practice to determine the enthalpy of protein unfolding by differential scanning calorimetry (DSC). This approach limits the available data to higher temperatures where most proteins denature, ranging from about 50 to 70 °C. Proteins have to be artificially destabilized to reduce their melting temperature. One of the most common means to destabilize a protein is to reduce the pH. It was shown using lysozyme as a model protein that the enthalpy of unfolding determined by DSC at various pHs is equal to the enthalpy of unfolding determined by isothermal calorimetric titration of the protein with acid [9].

Here we use the same approach to determine the enthalpy of carbonic anhydrase unfolding by isothermal titration calorimetry (ITC), by carrying out denaturation with acid and comparing the observed enthalpy ( $\Delta_u H_{\text{ITC}}$ ) with the enthalpy obtained by

DSC ( $\Delta_u H_{\text{DSC}}$ ) in our earlier study [10]. Such an approach significantly expands the accessible temperature range (down to nearly 0 °C) and measures enthalpies at constant temperature. Furthermore, it provides more detailed information about the  $T$ -dependence of the heat capacity of unfolding.

During any acid titration one observes the sum of processes such as dilution of the acid, dilution of the protein solution, protonation of the ionic groups of the protein (aspartic acid, glutamic acid, and histidine residues), aggregation of the protein, and anion binding to the positively charged protein at low pH. Analysis of the data may be further complicated if the unfolding reaction is not fully reversible, or if unfolding intermediates are present, such as the molten globule that was reported for carbonic anhydrase [13]. Our previous DSC analysis at low pH indicated that the process is reversible and could be described by a simple two-state model at any tested pH [10]. While the possible contributions of anion binding and molten globule formation to the unfolding energetics were not determined in this study, it is remarkable that DSC and ITC approaches yielded essentially the same enthalpy of unfolding at about 40 °C, which is a strong indication that the enthalpies obtained by both methods are accurate and consistent.

The concave-up curvature of  $\Delta_u H$  versus temperature shown in Fig. 5 indicates that the heat capacity of unfolding is becoming more positive as the temperature increases. From the curvature of the  $\Delta_u H$  dependence on temperature (Fig. 5) we have determined the second derivative to be about  $\Delta \Delta_u C_p = 100 \pm 30$  J/(mol  $\times$  K<sup>2</sup>). This is inconsistent with the previous calculations and observations reviewed by [1] since their results indicated that  $\Delta_u C_p$  decreases as the temperature increases. Biophysical community is in general agreement that the  $\Delta \Delta_u C_p$  of protein unfolding should be negative.

However, our value may be explainable by the behaviour of alkanes in water by comparing the reaction of protein refolding with the reaction of alkane aggregation in water. The heat capacity change of linear alkane aggregation expressed per mole of methylene group (CH<sub>2</sub>) per degree is equal to  $-0.24$  J/(mol CH<sub>2</sub>  $\times$  K<sup>2</sup>) [14,15]. The  $T$ -derivative of the heat capacity of carbonic anhydrase unfolding was measured here to be 100 J/(mol CH<sub>2</sub>  $\times$  K<sup>2</sup>), thus for the process of refolding the value is  $-100$  J/(mol CH<sub>2</sub>  $\times$  K<sup>2</sup>). Dividing this value for the entire protein by the value for the methylene group, we obtain that there could be about 420 methylene groups that are hidden from water during the folding reaction.

From the bCAII amino acid sequence and structure, we calculate that there are 530 methylene, methyl, and aromatic carbon atoms in the side chains of amino acids that are hidden inside the folded protein molecule and representing the maximum number of small hydrophobic groups that may become exposed to water if complete unfolding of the protein occurred.

The number estimated from the energetic measurements, 420, is similar to the number of hydrophobic groups calculated from the protein sequence and structure, 530. This could be just a fortuitous coincidence or it may have a physical basis. The difference (~20%) could be accounted for by the fact that not all hydrophobic groups are fully exposed to water upon unfolding. Furthermore, the aromatic and aliphatic groups are probably

hydrated somewhat differently. However, the agreement is interesting, and it suggests that the same physical forces may be behind the heat capacity dependence on temperature in both protein folding and alkane aggregation.

It is important to note that the  $\Delta\Delta_u C_p$  of protein unfolding is considered to be negative by the biophysical community [1]. Our  $\Delta\Delta_u C_p$  value is uncorrected for a number of energetically linked phenomena including protonation, ion binding, and the formation of partially folded intermediates.

Subtracting the heats of glutamic and aspartic acid protonation that get protonated upon the titration with acid from the observed enthalpy of protein unfolding would shift the ITC part of the curve in Fig. 5 upwards increasing the unexpected curvature of the enthalpy dependence on temperature. Therefore, the heats of protonation cannot explain the curvature. Anion binding to the positively charged protein may contribute significantly to the energetics. For example, the enthalpy of iodide binding to the first site of deionized bovine serum albumin is equal to  $-72.8$  kJ/mol [16], while the enthalpy of non-specific anion binding to proteins in the presence of salt and buffer is usually followed by a relatively small exothermic or near zero enthalpy change [17]. Therefore it is unlikely that anion binding could account for the curvature.

The protein denaturation by acid followed by ITC is worth testing on proteins other than carbonic anhydrase to make a general statement that above conclusions apply to other proteins.

## 5. Simplified description of the method

Acid-unfolding measured by ITC is a technique that complements DSC well, and both methods may be used to determine thermodynamics of protein unfolding. The precision of the data obtained by both techniques is comparable. ITC enabled the determination of the enthalpy of unfolding at temperatures far lower than room temperature and under isothermal conditions, while DSC is constrained to protein melting temperatures. Combination of DSC and ITC data gives the dependence of the enthalpy of unfolding on temperature in a wide  $T$ -range — a necessary condition to determine the non-linearity of the enthalpy dependence on temperature. The temperature derivative of the heat capacity of the unfolding of

the protein may be quantitatively accounted for by the thermodynamics of hydrophobic group exposure upon unfolding of the protein.

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