

Calorimetry

ARNE SCHÖN and ADRIÁN VELÁZQUEZ-CAMPOY

Department of Biology, The Johns Hopkins University, Baltimore, MD

Introduction

Differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) are the main calorimetric techniques used in characterizations of proteins and their reactions with ligands or other macromolecules. The energetics associated with biochemical reactions involves noncovalent interactions, and the measured heat effects can therefore be significantly lower compared to chemical reactions. Due to the technological and methodological achievements of the past three decades, it is now possible to monitor and measure accurately the energetics associated with biological reactions, also when the amount of material is limiting.

DSC is used to characterize the thermal stability of biomolecules (e.g., proteins, nucleic acids, lipids). The direct observable is the heat capacity of the sample as a function of the temperature. It is possible in one experiment to obtain the complete temperature profile of the Gibbs energy change associated with the denaturation process, as well as the enthalpy and entropy changes.

ITC allows the complete thermodynamic characterization of binding processes (e.g., protein-ligand, protein-protein, protein-nucleic acid, etc.). The direct observable is the heat effect related to the binding reaction as a function of the composition of the sample (usually the concentration of titrant reactant or the titrant/titrate molar ratio). ITC is unique in that both the affinity and the enthalpy of binding are determined directly and simultaneously in one experiment. Therefore, unlike other analytical techniques (e.g., spectroscopy), it is possible to obtain the partition of the binding affinity into enthalpic and entropic contributions without any model-related assumptions.

Nowadays, pharmaceutical companies and laboratories are incorporating these two techniques as basic procedures in research and development. The calorimetric techniques and their use in basic

research have thoroughly been reviewed in the literature. This chapter shows how modern high-sensitivity calorimetry can be used as a tool in research and quality control of proteins.

Differential Scanning Calorimetry

Differential scanning calorimetry is a generally accepted technique for the study of the energetics of protein denaturation. It does so by measuring the heat capacity of a sample as a function of temperature. A typical instrument is composed of two cells: a sample cell containing the protein solution and a reference cell containing buffer. During the experiment, a heating element is used to increase the temperature in both cells while the individual temperature of each cell is monitored continuously. Any difference in heat capacity of the sample compared to the reference cell will give rise to a temperature difference that triggers a feedback system to supply additional heat to the cell that has the lower temperature. The feedback power necessary to keep the temperature difference equal to zero between the cells is the measured signal and has the unit $\mu\text{J/s}$ or $\mu\text{cal/s}$ prior to any normalization by the scan rate. For a well-matched system with the two cells containing identical buffers, the only difference being the protein in the sample cell, heating of the cells can be seen as an increase in temperature that is monotonic in both cells until the protein starts to denature. Because the denaturation of a protein is an energy-requiring process, the feedback system will supply the heat necessary to keep the sample cell at the same temperature as the reference cell until all the protein has unfolded. Subtracting a separate scan where both cells are filled with the same buffer eliminates instrumental effects.

Figure 1 shows the result normalized by the scan rate and by the total amount of protein present in the calorimetric sample cell. The midpoint of the denaturation transition takes place at a temperature referred to as T_m , which usually is used to describe the stability of a protein. Moreover, the graph shows the typical shift of the baseline to a higher value upon completion of the transition and corresponds to the change in heat capacity, ΔC_p , for the denaturation. As the hydrophobic groups in the interior of the protein get hydrated, the water molecules get structured with an increase in the heat capacity as a consequence. The positive value for ΔC_p is thus mainly due to the exposure of hydrophobic groups to water. Because a protein denaturation is associated with a considerable value for ΔC_p , the calculation of the area involves subtraction of a sigmoidal baseline indicated in Figure 1. The integrated area under the peak corresponds to the overall enthalpy change, ΔH (at temperature T_m), associated with the denaturation of the protein. By performing a nonlinear least-squares fit to the data, the heat capacity change, ΔC_p , the midpoint temperature, T_m , and the enthalpy change at the midpoint, ΔH_m , can directly be determined. In general, the thermodynamic parameters can be estimated

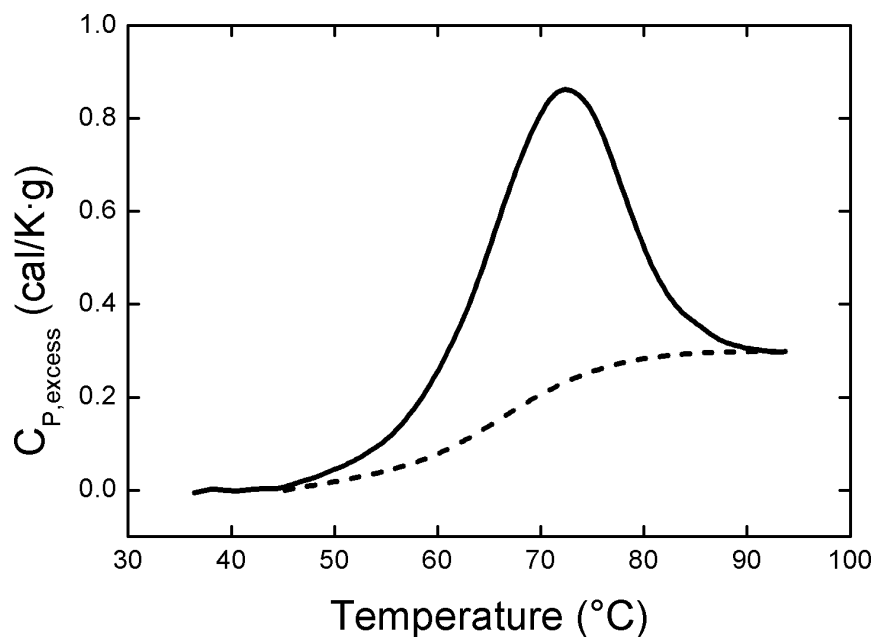


Figure 1 Example of the result from a typical DSC experiment showing the excess heat capacity for a protein (here expressed per gram) in solution as a function of temperature. The enthalpy is calculated by integration of the curve after subtracting the sigmoidal baseline corresponding to the change in heat capacity, ΔC_p , for the reaction.

by direct integration:

$$\Delta H(T) = \int_{T_0}^T \Delta C_p dT \quad (1)$$

$$\Delta S(T) = \int_{T_0}^T \frac{\Delta C_p}{T} dT \quad (2)$$

$$\Delta G(T) = \Delta H(T) - T \Delta S(T) \quad (3)$$

Moreover, for a monomeric (nonassociating) protein that undergoes a two-state transition, the change in Gibbs energy, ΔG , and entropy, ΔS , can be derived from the following relations

$$\begin{aligned} \Delta G(T) = & \Delta H_m + \Delta C_p(T - T_m) \\ & - T[\Delta S_m + \Delta C_p \ln(T/T_m)] \end{aligned} \quad (4)$$

$$\Delta S_m = \Delta H_m/T_m \quad (5)$$

where ΔH_m is the measured enthalpy for the denaturation and T_m is the corresponding midpoint, as discussed above.

The analysis of more complex systems (e.g., non-two-state proteins, oligomeric proteins, irreversible denaturation) can be found in the literature (Freire et al., 1990b; Freire and Murphy, 1991; Freire, 1995b; Johnson et al., 1995; Boudker et al., 1997; Kasimova et al., 1998; Todd et al., 1998).

Before extracting meaningful information from DSC scans of a protein, it is important to assess whether the denaturation is reversible or not. For a protein that has not yet been studied by DSC, two consecutive scans are normally run in order to check the degree of reversibility. The denaturation is considered reversible if the enthalpy obtained for the second scan is equal or close (at least 80%) to that obtained for the first scan. Usual causes for irreversible denaturation are aggregation and chemical modification upon heating. Additionally, it is customary to perform several scans at different temperature scan rates in order to check if there are kinetic effects coupled to the denaturation process.

The recent development of extremely sensitive DSC instruments capable of producing accurate data from samples of less than a tenth of the concentration previously used (less than 1 mg protein per experiment) has made DSC the method of choice for the characterization of proteins. In addition to its fundamental role in protein research, DSC is of direct use in the design of proteins, for optimization of protein formulations, and as a tool in quality control of protein batches. Excellent detailed reviews on the technique including rigorous protocols for the thermodynamic analysis of DSC are available (Privalov and Potekhin, 1986; Freire, 1995a).

Isothermal Titration Calorimetry

Isothermal titration calorimetry is used to measure directly the energetics associated with the interactions between two reactants, for example, the binding of a small ligand to a protein or the association of two proteins, as in the case of binding of a protein drug to its receptor. A typical instrument is composed of two cells: one contains one of the reactants; the other serves as a temperature reference and contains water or buffer. A syringe that also serves as the stirrer adds the other reactant in a stepwise fashion at preset intervals during the course of the experiment. Heat produced or absorbed during the binding reaction is monitored as a change in temperature. Any difference in temperature of the sample compared to the reference cell will activate a feedback system that modulates the thermal power applied in order to keep the temperature difference between both cells as low as possible. The result from a titration is a plot of the recorded power, dQ/dt , as a function of time, as shown in the upper panel of Figure 2. Each peak

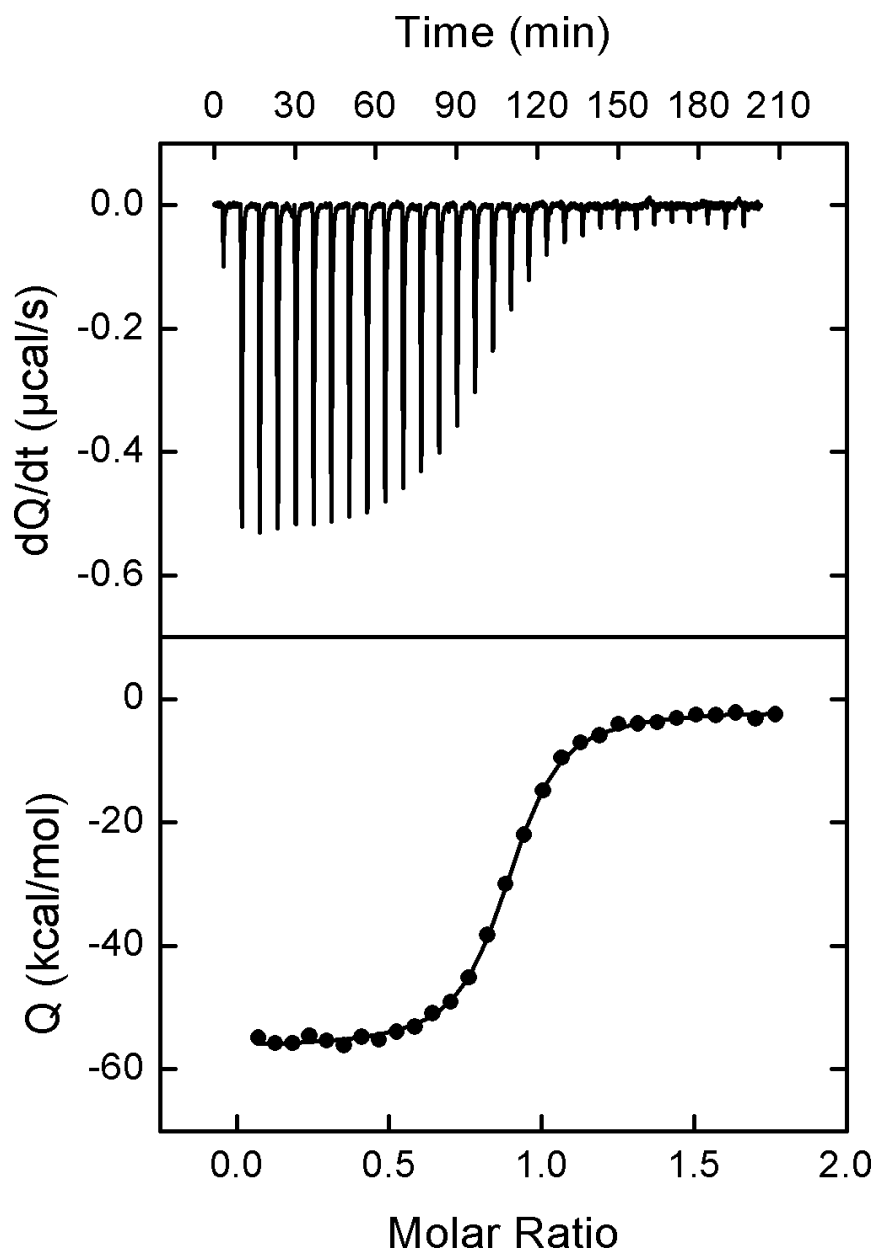


Figure 2 Result from an ITC experiment. The upper panel shows the recorded power, dQ/dt in $\mu\text{cal/s}$ as a function of time. Each peak corresponds to the heat produced upon reaction of injected ligand with the protein in the calorimetric cell. The lower panel shows the heat per injection obtained by integration of the areas as a function of molar ratio.

represents the thermal effect associated with each injection. The lower panel shows the integrated areas as a function of the ligand/protein molar ratio. By performing a nonlinear least-squares fit to the data, the stoichiometry, n , the affinity, K_a , and the enthalpy, ΔH , can be determined directly. The change in Gibbs energy, ΔG , and entropy, ΔS , can be derived from the following relations

$$\Delta G = -RT \ln K_a \quad (6)$$

$$\Delta G = \Delta H - T\Delta S \quad (7)$$

In biological literature, the affinity is usually expressed in terms of the dissociation constant, K_d , which is the inverse of K_a . For details on the technique including the calorimetric setup, see for example Wiseman et al. (1989), Freire et al. (1990a), Leavitt and Freire (2001), and Velazquez-Campoy et al. (2004).

ITC is unique in that the affinity of binding can be understood in terms of the separate contributions to Gibbs energy (i.e., its enthalpic and entropic components). As such, ITC has become an important technique in studies of various biochemical processes including protein-ligand, protein-protein, and DNA-protein binding, which makes it a valuable tool in the process of rational design and drug discovery.

Protein Stability Under Different Conditions

Formulation Stability *versus* Thermodynamic Stability

A very important concern with pharmaceutical proteins is the identification of chemical and physical conditions that maximize the stability of the protein. Instability of a protein can be divided into chemical instability and physical instability. Chemical instability is due to covalent modification as a result of, for example, oxidation or deamidation reactions, whereas physical instability is related to changes in structure of the protein. The physical instability is normally not a problem for small drugs, whereas it is of major concern for pharmaceutical proteins (Manning et al., 1989).

All modifications of the protein or of the excipients in a pharmaceutical formulation are made in order to achieve a formulation where the native conformation of a protein is as physically and chemically stable as possible.

It has been observed that formulation stability is related to thermal stability (Remmele et al., 1998; Remmele and Gombotz, 2000). This can be justified considering that a protein in solution is more susceptible to aggregation and other deleterious effects when the thermodynamic stability is low and other states different from the native one are significantly populated. If this is the case, DSC thermal stability assays can be conducted in order to substitute costly real-time shelf-life experiments.

Physical Stability of a Monomeric Protein

The result from denaturation of a protein studied by DSC provides a complete thermodynamic description of the unfolding of a native protein. The unfolding process can be described as an equilibrium between the denatured (D) and the native form (N) of the protein characterized by an equilibrium constant, K . The stability of the native form can be expressed in terms of the change in Gibbs energy, ΔG , for the process

$$\Delta G = -RT \ln K = -RT \ln \frac{[D]}{[N]} \quad (8)$$

where R is the gas constant [1.987 cal/(K × mol) or 8.314 J/(K × mol)] and T is the absolute temperature in kelvin. The enthalpy, ΔH_m , is obtained directly from the area as described above, and as ΔG is equal zero at T_m , the entropy, ΔS_m , can be determined directly from Equation 5. Consequently, the stability of a protein at a temperature below T_m can be described in terms of ΔG ; the more positive value for ΔG the more stable is the native conformation of the protein. Thermodynamic stability of a protein can be expressed in terms of Gibbs energy for any temperature using Equations 3 or 4.

Figure 3 shows ΔG as a function of temperature for two different proteins having different stability. The curvature observed for ΔG is related to the value for ΔC_p . Sometimes, due to large change in heat capacity compared to the Gibbs energy of stabilization, two values for T_m can be observed (the temperatures where ΔG equals zero), the lower indicating that the protein also can undergo cold denaturation. Although T_m often is used as a measure of stability, Figure 3 shows that an increase in stability may or may not correspond to an increase in T_m for the protein. Thus, it is possible to distinguish between stability in the high- and low-temperature ranges. This fact stresses the importance of obtaining the complete temperature profile of ΔG when comparing proteins.

Assessment of Protein Association

DSC can be used to obtain information on functional quaternary oligomers such as dimeric HIV-1 protease or tetrameric hemoglobin or to assess whether a proteins exists as ordered aggregates in solution at certain conditions. The usefulness of DSC in studies of proteins that exist as oligomers can be explained by Le Chatelier's principle. The dissociation of an oligomeric protein composed of n subunits undergoing a two-state denaturation can be written as



where N_n is the native oligomer and D is the denatured monomer. In contrast to a monomeric protein, the fraction of denatured protein will in this case be determined by both the equilibrium constant and the

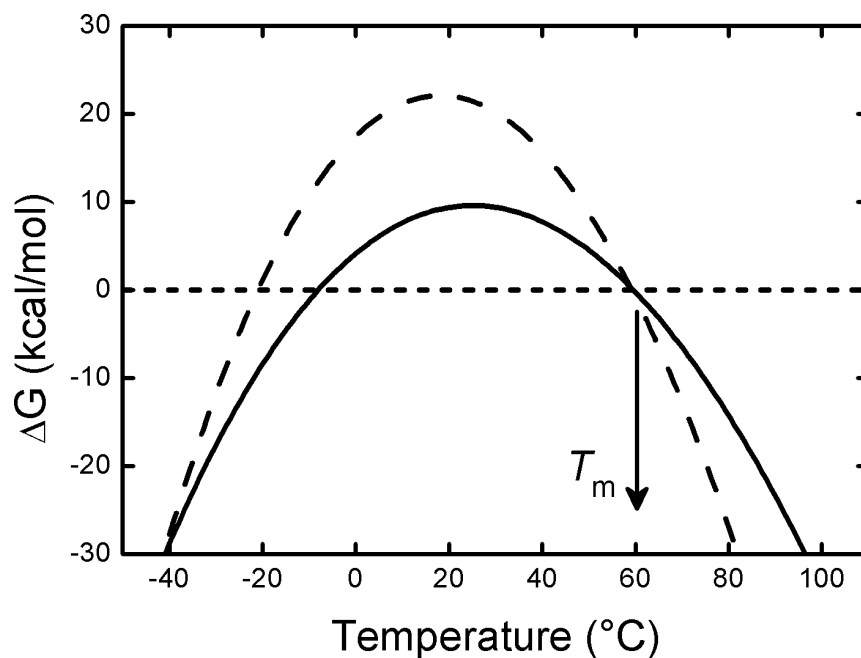


Figure 3 Calculated Gibbs energy of stabilization for two different proteins having the same value for T_m . The solid line represents Gibbs free energy calculated using $\Delta H = 180$ kcal/mol, $\Delta S = 0.54$ kcal/(K·mol), and $\Delta C_p = 5.0$ kcal/(K·mol). Gibbs energy for the more stable protein (dashed line) was calculated using $\Delta H = 350$ kcal/mol, $\Delta S = 1.05$ kcal/(K·mol), and $\Delta C_p = 8.0$ kcal/(K·mol). The T_m value is the same for both proteins (60°C); however, the stability at low temperatures is quite different: the temperature for maximal stability is 25°C and 18°C, and the Gibbs energy of stabilization (or unfolding) at these temperatures is 9.6 and 22.1 kcal/mol, respectively.

concentration of protein. As a consequence, an increase in protein concentration will shift the equilibrium from the denatured toward the native state or, as stated in Le Chatelier's principle, toward the state of lower molecularity. DSC studies of a protein denaturation at different concentrations will show increasing T_m with increasing concentration if the protein exist as oligomers in solution. Figure 4 shows simulated scans of different concentrations of a dimeric protein. Scans of a monomeric protein would have had the same T_m for all concentrations.

Parameters That Affect Protein Stability

A vast number of external factors can stabilize or destabilize the native conformation of a protein. The stabilization energy of the native state of a protein is the result of many energetic contributions associated with inter- and intramolecular interactions, usually classified as enthalpic and entropic interactions, depending on their different nature

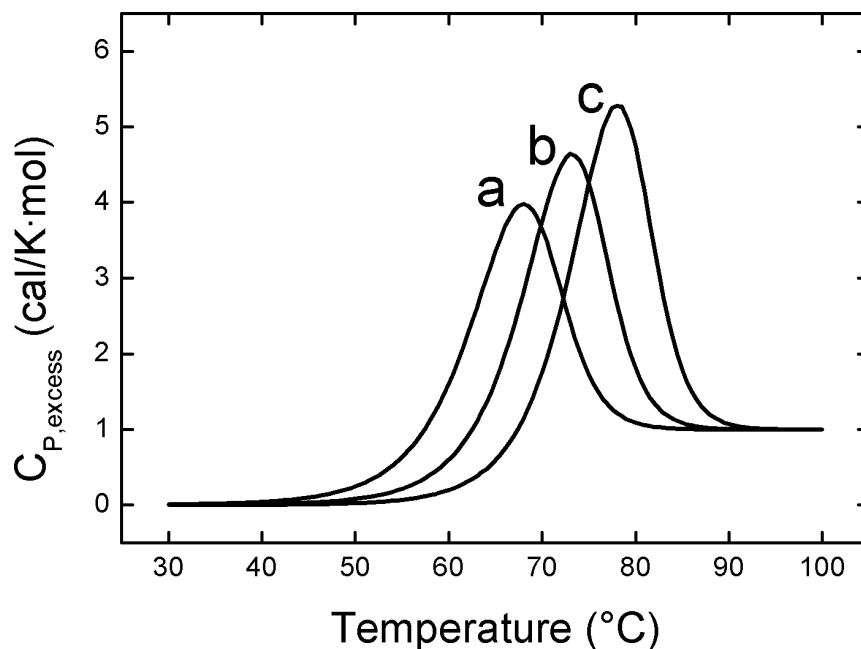


Figure 4 Simulated results for DSC experiments on a dimeric protein at (a) 10, (b) 100, and (c) 1000 μM . The concentration dependence for a dimeric or oligomeric protein can be seen in the value for T_m occurring at higher temperatures for higher protein concentrations. The curves would have been overlapping in the case of a monomeric protein.

(electrostatic and dipolar interactions, van der Waals interactions, hydrogen bonds, hydrophobic interactions, conformational entropy, etc.).

Thermodynamically, the energy difference between the native state and the denatured state reflects their intrinsic character. The compact, ordered native state of a protein is characterized by a high degree of interaction between the individual components (at short and long range) and low conformational entropy. However, the denatured state, being loose and disordered, is characterized by a lower degree of interactions and high conformational entropy. Therefore, the native state is stabilized at low temperature by the noncovalent interaction network established between residues and domains (thus, enthalpically stabilized), counterbalancing the tendency to undergo denaturation driven by the potential gain in conformational entropy. Any factor or environmental parameter affecting any of these interactions will modulate the stability of the protein. Factors that can have an effect on the stability of the native protein and therefore are modified in the optimization of the formulation are pH and ionic strength as well as the presence of different excipients such as sugars, polymers, metal ions, and amino acids that might be added or removed.

Calorimetry and Quality Control of Proteins

Proteins like other macromolecules require a proper conformation for activity. A protein solution can be chemically pure but contain finite amounts of nonactive conformations with lowered effect as a result. The protein might also be chemically pure and fully active at the time of production but deteriorate more or less rapidly into nonactive conformations or conformations that even have adverse effects. Calorimetry can be used in different ways to determine the presence of nonactive conformations in the protein sample. The first one involves the use of DSC and the second ITC.

DSC and the Determination of Active Protein Fraction

The enthalpy of denaturation correlates with the amount of properly folded active protein, and DSC can thus be used to estimate directly the quality of a protein preparation by estimating the fraction of protein molecules in the native state. There are several ways of performing this analysis. The most reliable is by comparing the heat capacity function of a protein preparation with that of a known standard of the same protein. The protein standard is one that has been evaluated by different techniques with respect to specific activity, purity, and so forth. Usually, this analysis is time consuming and involves the use of a variety of techniques, such as NMR, mass spectroscopy, and circular dichroism. Once this procedure is accomplished, the heat capacity function of the protein standard can be measured by DSC and the resulting profile used for the evaluation of unknown samples of the same protein. The active fraction, F_a , of a protein batch of unknown activity is then determined from

$$F_a = \frac{Q}{\Delta H^\circ} \quad (10)$$

where Q is the experimental enthalpy per mole of protein and ΔH° is the enthalpy determined for the standardized protein sample. Figure 5 shows simulated curves for a protein from batches of different activity. The correlation between the active fraction protein and the ratio between the actual and the standardized enthalpy according to Equation 10 is shown in Figure 6.

For a protein where the enthalpy of denaturation has not been determined earlier, an average literature value can serve as a reference. In this case, all enthalpy values must be normalized per gram, as the difference in molecular weight makes the comparison of molar enthalpies meaningless. The mean enthalpy value for denaturation of globular proteins at 60°C is 6.7 ± 1.7 cal/g (Robertson and Murphy, 1997). By using the average heat capacity change of 0.12 ± 0.03 cal/(K × g) for globular proteins, the expected enthalpy of unfolding can be calculated

for the temperature of interest according to

$$\Delta H(T) = (6.7 \pm 1.5) + (0.12 \pm 0.03) \times (T_m - 60) \quad (11)$$

It should be noted, however, that the error increases the further T_m is from the reference temperature of 60°C. In addition, the degree of folding can vary for some proteins and can deviate markedly from that of a standard protein as in the case of gp120, described in the section "Example: Determination of Active Fraction of gp120," below.

Determination of Concentration of Binding Sites by ITC

ITC is very useful for the determination of the active protein concentration as long as the protein has a well-defined site for a ligand, preferably a small-molecular-weight molecule. Such an active site titration requires the ligand concentration to be known with high accuracy. The concentration and purity of a stock ligand solution can accurately be determined by an appropriate analytical method. Once the concentration of the ligand is determined, the binding to the protein from a batch of unknown activity is studied by ITC for determination of the stoichiometry. In general, the apparent stoichiometry, n , is equal to the fraction of active protein, F_a , present in the sample:

$$n = F_a = \frac{[P]_{\text{active}}}{[P]_{\text{tot}}} \quad (12)$$

where $[P]_{\text{active}}$ and $[P]_{\text{tot}}$ are the active and total concentrations of protein in the calorimetric cell, respectively. The resulting apparent stoichiometry is consequently equal to one if the protein batch 100% activity.

As long as the concentration of the ligand in the syringe is known with high accuracy, the enthalpy and affinity are always exactly determined, and the uncertainty in protein concentration due to the presence of an inactive protein fraction will only be reflected in the value for the apparent stoichiometry. Because the enthalpy value obtained from the analysis depends only on the concentration of titrating reactant in the syringe, it can be used as an additional independent control parameter for the reactant in the syringe in the active protein concentration determination assay. In that way, even if the concentration of reactant in the syringe is unknown, if the expected binding enthalpy is known, it is possible to use two independent adjustable parameters, stoichiometry and binding enthalpy, during the analysis of the ITC experiment to determine simultaneously both the concentration of reactant in the syringe (from the estimated enthalpy) and the concentration of reactant in the cell (from the estimated stoichiometry).

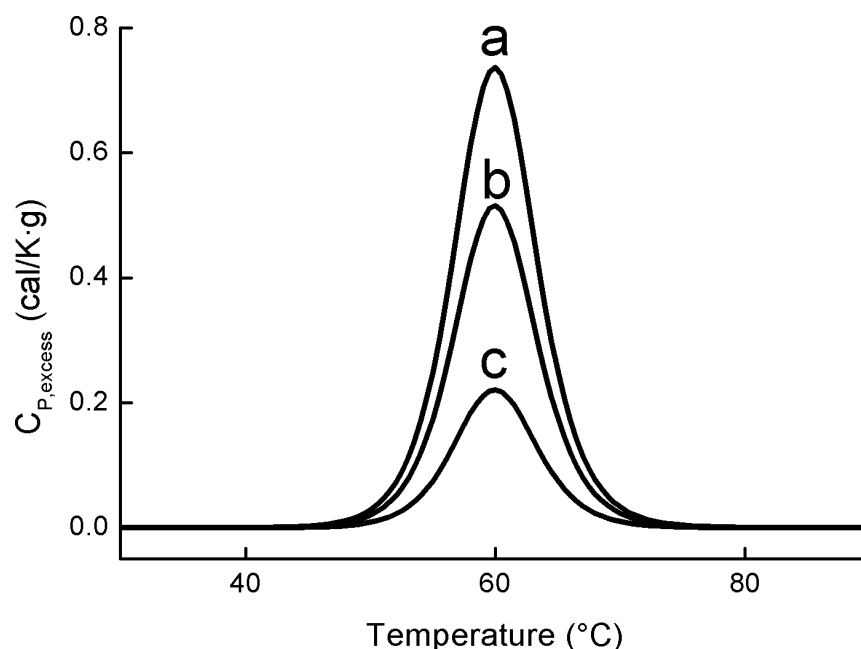


Figure 5 Simulated temperature scans of a protein from a batch of (a) 100%, (b) 70%, and (c) 30% activity. The denaturation enthalpy for the protein of 100% activity was 6.5 cal/g, which serves as the standard enthalpy, ΔH° , in Figure 6.

Example: Determination of Active Fraction of gp120

The presence of inactive protein fractions poses a serious problem in studies where both the reactants are proteins. In the case of proteins with measurable enzymatic activity, the activity can usually be determined by spectrophotometric or fluorometric assays. However, in the absence of enzymatic activity, the active fraction of the reactants must be determined by other means (e.g., as described in this section).

The glycoprotein gp120, found on the surface of the viral envelope of HIV-1 particle, binds to the soluble form of the cell receptor CD4 (sCD4) with an affinity of 5 nM and an enthalpy of -35 kcal/mol (Myszka et al., 2000) at 25°C. Before any meaningful information can be obtained from a titration of gp120 with sCD4, the active protein fraction must be determined for at least one of the proteins. DSC, as suggested in the section "DSC and the Determination of Active Protein Fraction" above, can obtain a measure of the degree of activity. Earlier studies have shown that the thermal denaturation of fully active gp120 is characterized by an enthalpy of 2.3 cal/(g \times K) (Leavitt, 2003), which serves as our reference enthalpy according to Equation 10. The ratio between the enthalpy from the unknown and standardized protein batch according to Equation 10 (see also Figures 5 and 6) is used to adjust the concentration of the gp120 prior to any ITC

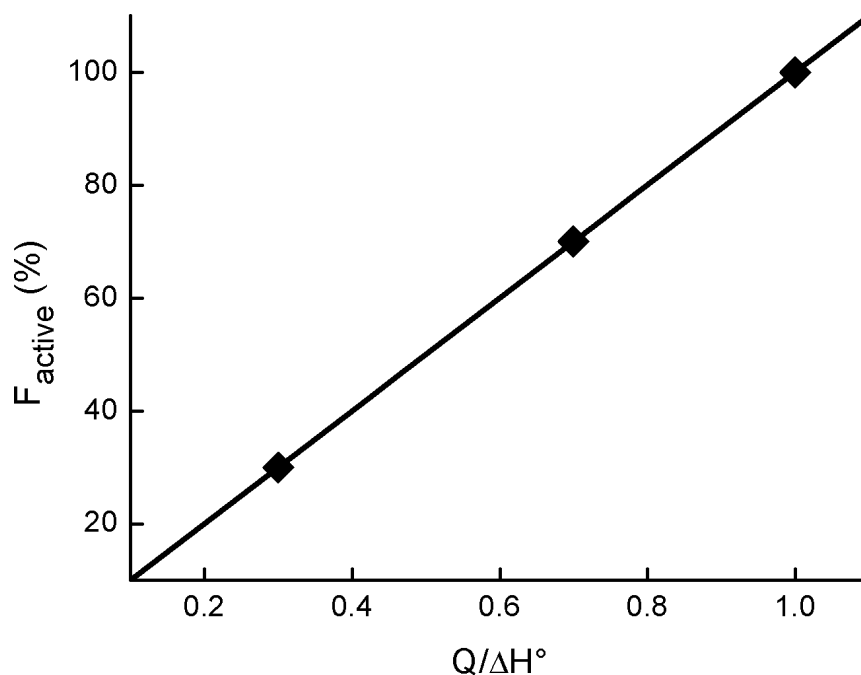


Figure 6 Fraction active protein as a function of the ratio between the experimental enthalpy Q and the standardized enthalpy ΔH° according to Equation 10. The plot was based on the areas derived from the simulated curves in Figure 5. The value for DH° was 6.5 cal/g.

experiment with sCD4. Any deviation in the apparent stoichiometry obtained in subsequent ITC experiment can thus be attributed to an uncertainty in the concentration of sCD4.

Example: Determination of Active Fraction HIV-1 Protease

HIV-1 protease is an aspartic protease crucial in the maturation steps in the HIV-1 virus life cycle. Biochemical studies on this enzyme require the assessment of the concentration of active protein. As mentioned above, in the case of proteins with measurable enzymatic activity, the activity (determined by spectrophotometric or fluorometric assays with appropriate substrates) can be used to monitor active fraction concentration. However, ITC provides an excellent methodology for performing active site titrations using both moderate and high-affinity ligands ($K_a \sim 10^6$ to 10^{10} M⁻¹).

Calorimetric studies with acetyl-pepstatin, a universal aspartic-protease inhibitor, have shown that it binds with moderate affinity and endothermically to HIV-1 protease ($K_a = 2.3 \times 10^6$ M⁻¹ and $\Delta H = 7.7$ kcal/mol, in acetate buffer 10 mM, pH 5, DMSO 2%, 25°C) (Todd and Freire, 1999; Velazquez-Campoy et al., 2001). Figure 7 shows the result from an ITC experiment in which the inhibitor is injected into a

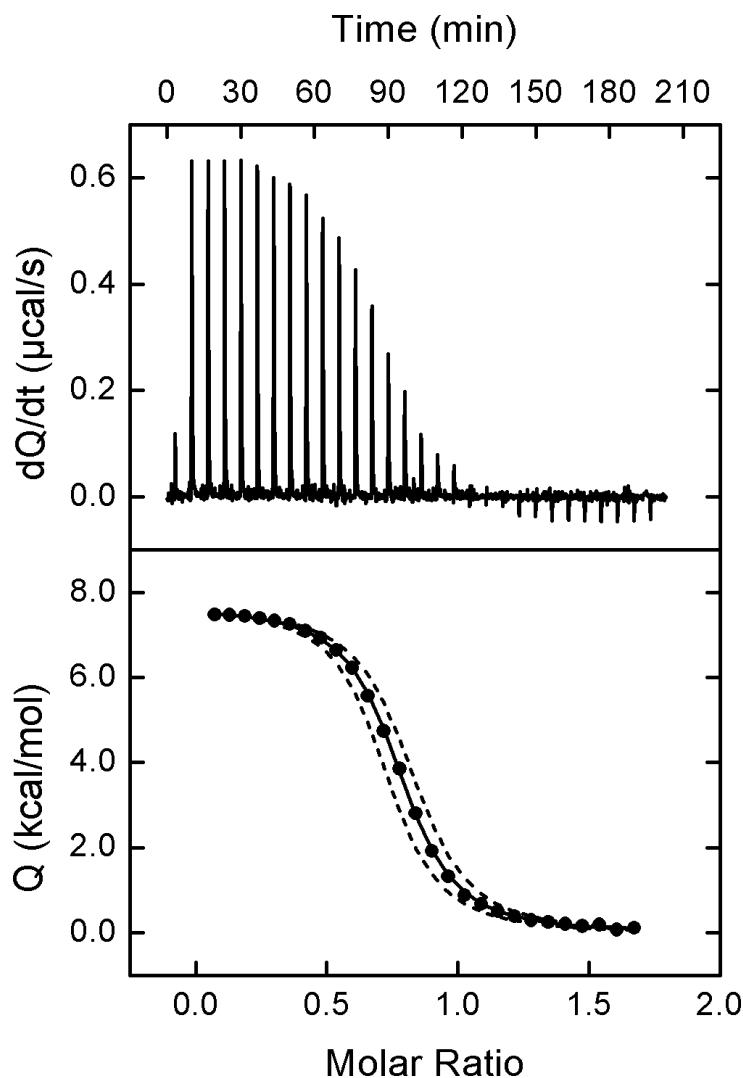


Figure 7 Results from a titration experiment in which 230 μM acetylpepstatin is injected into a solution of 29 μM HIV-1 protease. The regression analysis provides the following results: $K_a = 2.3 \pm 0.2 \times 10^6 \text{ M}^{-1}$, $\Delta H = 7.7 \pm 0.1 \text{ kcal/mol}$, and $n = 0.76 \pm 0.01$. The apparent stoichiometry of 0.76 indicates that only 76% of the total protein concentration, as determined spectrophotometrically, is active. If the analysis is repeated using 22 μM (corrected for the inactive fraction) as the concentration of protein, the analysis yields a value of 1 for the stoichiometry (100% active). The two dashed lines represent the titration plot with an n value of 0.76 ± 0.05 , in order to observe the precision obtained in this parameter. The value obtained for the binding enthalpy corresponds to the expected one, indicating that the concentration of acetylpepstatin was well determined.

solution of HIV-1 protease. An apparent stoichiometry of less than one obtained from the regression analyses using the known inhibitor concentration and the nominal protein concentration determined from its extinction coefficient at 280 nm reflects the presence of impurities or inactive protein.

It is interesting to point out that for an active site titration to be accurate, it is not necessary to use highly potent ligands. That is not the case in traditional enzymatic active site titrations where (sub)nanomolar inhibitors are employed in order to minimize the error in determining, usually by graphic extrapolation, the concentration of active fraction. Besides, as mentioned before, the enthalpy value obtained through the analysis of the experiments can be used to check the concentration of titrant. Any deviation from the expected value will reflect an error in the determination of such concentration.

Abbreviations

DSC	Differential scanning calorimetry
DMSO	Dimethyl sulfoxide
gp120	Glycoprotein 120
HIV-1	Human immunodeficiency virus type 1
ITC	Isothermal titration calorimetry
NMR	Nuclear magnetic resonance spectroscopy
sCD4	Soluble form of the cell surface receptor,
CD4	

Acknowledgments

Arne Schön and Adrian Velazquez-Campoy as well as the research work presented in this paper were supported by grants GM 57144 and GM 56550 from the National Institutes of Health and grant MCB-0131241 from the National Science Foundation to Ernesto Freire.

References

1. Boudker O, Todd MJ, and Freire E. The Structural Stability of the Co-chaperonin GroES. *J Mol Biol* 1997; 272:770-779
2. Freire E. Differential Scanning Calorimetry. *Methods Mol Biol* 1995a; 40:191-218
3. Freire E. Thermodynamics of Partly Folded Intermediates in Proteins. *Annu Rev Biophys Biomol Struct* 1995b; 24:141-165
4. Freire E, and Murphy KP. Molecular Basis Of Co-operativity In Protein Folding. *J Mol Biol* 1991; 222:687-698

5. Freire E, Mayorga OL, and Straume M. Isothermal Titration Calorimetry. *Anal Chem* 1990a; 62:950A-959A
6. Freire E, van Osdol WW, Mayorga OL, and Sanchez-Ruiz JM. Calorimetrically Determined Dynamics of Complex Unfolding Transitions in Proteins. *Annu Rev Biophys Biophys Chem* 1990b; 19:159-188
7. Johnson CR, Morin PE, Arrowsmith CH, and Freire E. Thermodynamic Analysis of the Structural Stability of the Tetrameric Oligomerization Domain of p53 Tumor Suppressor. *Biochemistry* 1995; 34:5309-5316
8. Kasimova MR, Milstein SJ, and Freire E. The Conformational Equilibrium of Human Growth Hormone. *J Mol Biol* 1998; 277:409-418
9. Leavitt S, and Freire E. Direct Measurement of Protein Binding Energetics by Isothermal Titration Calorimetry. *Curr Opin Struct Biol* 2001; 11:560-566
10. Leavitt SA, Schon A, Klein JC, Manjappara U, Chaiken IM, and Freire E. Interactions of HIV-1 Proteins gp120 and Nef with Cellular Partners Define a Novel Allosteric Paradigm. *Curr Protein Peptide Sci* 2004; 5:1-8
11. Manning MC, Patel K, and Borchardt RT. Stability of Protein Pharmaceuticals. *Pharm Res* 1989; 6:903-918
12. Myszka DG, Sweet RW, Hensley P, Brigham-Burke M, Kwong PD, Hendrickson WA, Wyatt R, Sodroski J, and Doyle ML. Energetics of the HIV gp120-CD4 Binding Reaction. *Proc Natl Acad Sci U S A* 2000; 97:9026-9031
13. Privalov PL, and Potekhin SA. Scanning Microcalorimetry in Studying Temperature-Induced Changes in Proteins. *Methods Enzymol* 1986; 131:4-51
14. Remmele RL Jr, and Gombotz WR. Differential Scanning Calorimetry: A Practical Tool for Elucidating stability of Liquid Pharmaceuticals. *Biopharm Eur* 2000; 56:58-60
15. Remmele RL Jr, Nightlinger NS, Srinivasan S, and Gombotz WR. Interleukin-1 Receptor (IL-1R) Liquid Formulation Development Using Differential Scanning Calorimetry. *Pharm Res* 1998; 15:200-208
16. Robertson AD, and Murphy KP. Protein Structure and the Energetics of Protein Stability. *Chem Rev* 1997; 97:1251-1268
17. Todd MJ, and Freire E. The Effect of Inhibitor Binding on the Structural Stability and Cooperativity of the HIV-1 Protease. *Proteins* 1999; 36:147-156
18. Todd MJ, Semo N, and Freire E. The Structural Stability of the HIV-1 Protease. *J Mol Biol* 1998; 283:475-488
19. Velazquez-Campoy A, Kiso Y, and Freire E. The Binding Energetics of First- and Second-Generation HIV-1 Protease Inhibitors: Implications for Drug Design. *Arch Biochem Biophys* 2001; 390:169-175

20. Velazquez-Campoy A, Leavitt SA, and Freire E. Characterization of Protein-Protein Interactions by Isothermal Titration Calorimetry. In: Fu H. *Protein-Protein Interactions. Methods and Applications*. Totowa, NJ: Humana Press; 2004:35-54.
21. Wiseman T, Williston S, Brandts JF, and Lin LN. Rapid Measurement of Binding Constants and Heats of Binding Using a New Titration Calorimeter. *Anal Biochem* 1989; 179:131-137

