ANALYTICAL BIOCHEMISTRY **232,** 79–85 (1995)

Precise Scanning Calorimeter for Studying Thermal Properties of Biological Macromolecules in Dilute Solution[1]

George Privalov, Vincent Kavina, Ernesto Freire,[2] and Peter L. Privalov[2]
Department of Biology and Biocalorimetry Center, The Johns Hopkins University, Baltimore, Maryland 21218

Received June 30, 1995

A precise scanning calorimeter for studying the heat capacity of liquids in a broad temperature range has been developed. By its design and capabilities this calorimeter is the first of a new generation for this type of instrument. This new scanning calorimeter operates differentially, is equipped with a pair of gold capillary cells and semiconductor sensors, and is able to scan up and down in temperature at user-selected rates. This instrument is completely operated by an integrated computer which also provides a full thermodynamic analysis of the results. Its construction does not involve the use of organic compounds, thus eliminating a source of baseline noise that has affected previous calorimeters. The operational temperature range of the instrument can be varied between 0 and 120°C. The gold capillary cells (operational volume 0.8 ml) minimize temperature gradients in the heated/cooled liquid sample and permit easy washing and reloading without air bubbles. These features are crucial for the accuracy of difference heat capacity measurements and determination of the absolute value of the partial heat capacity of solute molecules. The measurements can be performed under an excess constant pressure (up to 3 atm) to prevent formation of gas bubbles and boiling of aqueous solutions above 100°C. The noise level of the recorded heating/cooling power difference is below 50×10^{-9} W (i.e., below 10 ncal/s) with a response half-time of 5 s. The reproducibility of the baseline without refilling the capillary cells is on the order of 0.5×10^{-6} W. Reproducibility of the baseline upon refilling the cell is of the same order of magnitude. This provides an accuracy in difference heat capacity determination on the order of 10 μcal/°K · ml at a heating rate of 1°K/min. **© 1995 Academic Press, Inc.**

The importance of the heat capacity function of temperature, or its temperature integral, the enthalpy function, for understanding the energetics of any macroscopic system is obvious. The functional dependence between the enthalpy and temperature, two fundamental conjugate extensive and intensive variables, includes all thermodynamic information on the macroscopic states of a system which are populated in the temperature range under consideration. This is particularly true in the case of biological macromolecules. By analyzing the heat capacity of these molecules, e.g., the temperature dependence of their enthalpy function, one can obtain a full thermodynamic description of their native state and the intermediate states that appear upon temperature-induced unfolding (1). For this reason, the study of the heat capacity function of biological macromolecules, proteins, and nucleic acids and their complexes has generated a great deal of interest. The experimental determination of this function has been extremely difficult, because it requires measurements of the heat capacity contribution of solute molecules in very dilute solutions, i.e., under conditions in which the background heat capacity (solvent) largely exceeds that of the molecule under consideration. This requires extremely precise instrumentation. To achieve this goal superprecise heat capacity calorimeters have been developed. These instruments are now known as differential scanning microcalorimeters.

The first differential scanning microcalorimeters appeared in the mid sixties (2–4). Their sensitivity was three orders of magnitude higher than that of existing instruments at that time (see for review 5–10). This qualitative increase of sensitivity was reached by a combination of various methods: differential measurement, power compensation, continuous heating (scanning in temperature), and complete thermal isolation of the calorimetric cells (adiabatization). The most important step for realization of high sensitivity consisted of the miniaturization of the measuring unit, the calorimetric cells. This circumvented the problem of temperature gradients in the continuously heated liquid and avoided the necessity of mechanical stirring.

[1] This work was supported by NIH Grant RR04328.
[2] To whom correspondence may be addressed. Fax: (410) 516 5213.

0003-2697/95 $12.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

In the first scanning microcalorimeters, the calorimetric cells were heated electrically by a constant power. The twin cells were completely isolated from the surroundings. In the instrument described by Privalov *et al.* (2) adiabatization was achieved by using high vacuum and a system of thermal screens (thermal jacket) which closely followed the temperature of the cells. Therefore, those calorimeters could scan only up in temperature (heating mode). After each measurement the cells had to be removed from the adiabatization system, washed, reloaded with the new sample, and placed back inside the system. Since it is impossible to reload the cells with exactly the same amount of sample and completely restore the surroundings within the adiabatization system, the baseline of the instrument was not reproducible. Therefore, this instrument was able to measure only sharp temperature heat effects (e.g., the heat of melting of nucleic acids, proteins, and lipids) but not the absolute values of the partial heat capacity of solute macromolecules.

The important step in the development of heat capacity microcalorimetry involved the use of samples with a consistent volume defined by the reaction cell itself and not by the user measurement (11). This permitted the construction of a calorimeter with a nonremovable measuring block in which the calorimetric cells are placed permanently inside the instrument. The cells were washed and reloaded through capillary tubes. The baseline of this instrument was highly reproducible upon refilling of the cells. This made possible for the first time the determination of the absolute values of the partial heat capacity of proteins and nucleic acids in dilute aqueous solutions in a broad temperature range (8,12). The instrument with a nonremovable calorimetric block appeared rather compact and technologically advanced. In the early seventies, the Bureau of Biological Instrumentation of the Russian Academy of Sciences started its serial production under the trademark DASM-1M.

The next development in scanning microcalorimetry involved the invention of the capillary calorimetric cell (6). The main advantage of the capillary cell in comparison with the cylindrical one is in the smaller temperature gradient inside the cell. It also permits raising considerably the excess pressure and thus expanding the upper temperature range. Also important was that capillary cells are much easier to wash and reload without bubbles. The importance of the latter requirement is clear because even microscopic gas bubbles can lead to significant errors in the determination of the absolute value of the partial heat capacity of solute molecules in dilute solutions. In the late seventies, the Bureau of Biological Instrumentation of the Russian Academy of Sciences started producing scanning microcalorimeters with platinum capillary cells under the trademark DASM-4. This instrument represented the third generation of scanning microcalorimeters. It gained great popularity because of its ease of operation, high sensitivity, and precision.

However, the DASM-4 instrument had some drawbacks. With some solutions, the record of the first heating scan sometimes differed from the second one. This irreproducibility seemed to be connected with some surface effects caused by platinum which is known to be a strong catalyst and active absorbent of hydrogen. However, all attempts to replace platinum by gold failed because of the technological difficulties in constructing the cell from a pure gold capillary tube.

In the DASM-4, as in all previous precise scanning microcalorimeters, the cell was heated with constant power and was followed in temperature by the thermal jacket (adiabatization system) in order to minimize heat exchange with the environment. Therefore, the heating rate of these instruments was not perfectly constant and decreased upon increasing the heat capacity of the cells with temperature. The measurement of the heat capacity in cooling scans was also a problem. Cooling scans requires removal of energy from the cells. In the DASM-4 this was achieved by shifting the temperature of thermal jacket several degrees below the cell temperature. The same method was used later in other instruments, particularly Microcal-2.

The other defect of the DASM-4 is that the baseline is not linear and that it changes with time. This was caused by the organic glues used to fix the thermosensors and electric heaters and to insulate them electrically from the calorimetric block. These organic materials were used also in all previous microcalorimetric instruments but did not present a problem due to their lower sensitivity. Organic glues and organic electric insulation need to be avoided in the design of instruments with higher sensitivities. This task is, however, not easy.

Here we describe a differential scanning calorimeter in which all defects mentioned above are solved. In addition, this instrument introduces a new principle of operation that permits both heating and cooling scans with no difference in sensitivity and with outstanding baseline reproducibility. By design and new capabilities to be described below, it actually represents the next, fourth generation of scanning microcalorimeters.

THE PRINCIPAL FEATURES OF THE NEW DESIGN

The main difference of the new instrument with all previous ones resides in the implementation of the heating/cooling process. In previous scanning microcalorimeters the cell is heated with constant power and is followed in temperature by the thermal jacket (adiabatization system) in order to exclude heat exchange. In the new instrument, the thermal jacket plays a leading role in the heating and cooling of the cells (Fig. 1).

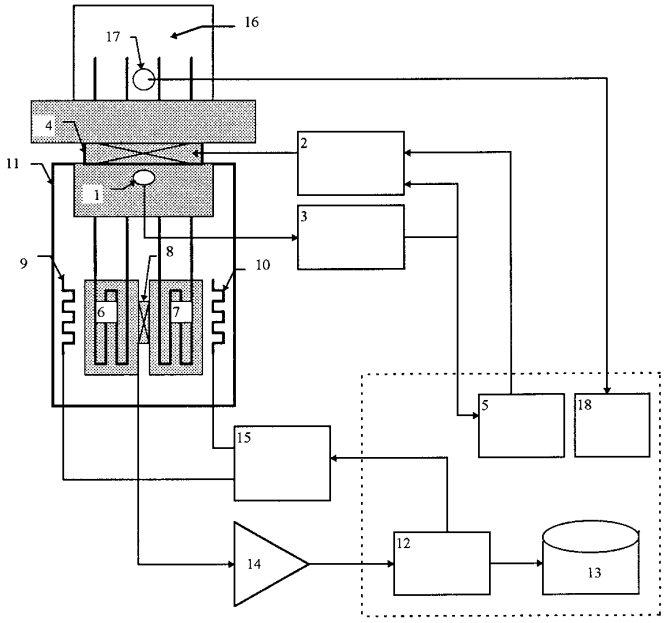

FIG. 1. Block diagram of the new scanning microcalorimeter. Dashed rectangle represents the components implemented in software. 1, platinum thermometer; 2, temperature control block; 3, temperature measuring circuit; 4, heating and cooling Peltier elements; 5, temperature control algorithm; 6 and 7, calorimetric cells; 8, thermosensor; 9 and 10, power compensation heaters; 11, jacket; 12, feedback control algorithm; 13, data file; 14, signal amplifier; 15, power compensation bridge; 16, manostat; 17, pressure sensor; 18, pressure gauge.

The idea of using the thermal jacket as the driving heating force is not new in scanning calorimetry. It was implemented before in various instruments (13,14). In those instruments a massive copper jacket was heated electrically with constant power and the cells heated passively by the heat flow from the jacket. If the heat capacity of the jacket is large and the heating power significantly exceeds the heat exchange with the environment, the temperature of the jacket will increase at a rather constant rate; however, the rate of cooling will be temperature dependent. Accordingly, this instrument allowed measurements of difference heat effects upon heating but not cooling.

In the instrument that we describe here (Fig. 1), the thermal jacket (11) is heated and cooled at a constant rate. The temperature of the jacket is controlled by the computer using the reference voltage that is translated directly to the temperature. The temperature control circuit compares the reference voltage from the computer with the voltage provided by the platinum thermometer (1) and determines the power response for the heating and cooling Peltier elements (4) necessary to minimize the difference between the two voltages. As a result, the computer has a direct linear control of the temperature of the jacket via one of the digital to analog converters (5). Therefore, the cells (6,7) are heated and cooled at any desired constant rate. However, for practical reasons, the maximal heating rate of the new instrument is set at 2°K/min. The minimal heating rate actually does not have a limit.

The other important feature of the new instrument is that semiconductor thermobatteries are used as sensors (8). Although semiconductors are much more efficient that their bimetal counterparts, the trade-off is that some properties of semiconductor thermobatteries (heat conductivity and efficiency) are temperature dependent. To avoid these problems, the power compensation approach was used. The temperature difference between the two cells measured by semiconductor thermobattery (8) and amplified by signal amplifier (14) is maintained near zero by applying appropriate power difference using electric heaters (9,10) attached directly to the cell body and controlled by a feedback control algorithm (12) and power compensation bridge (15). This power difference is also recorded as the main signal by the computer (13).

The use of semiconductor batteries as sensors required a complete redesign in the construction of the calorimetric cells (Fig. 2). In the new instrument the

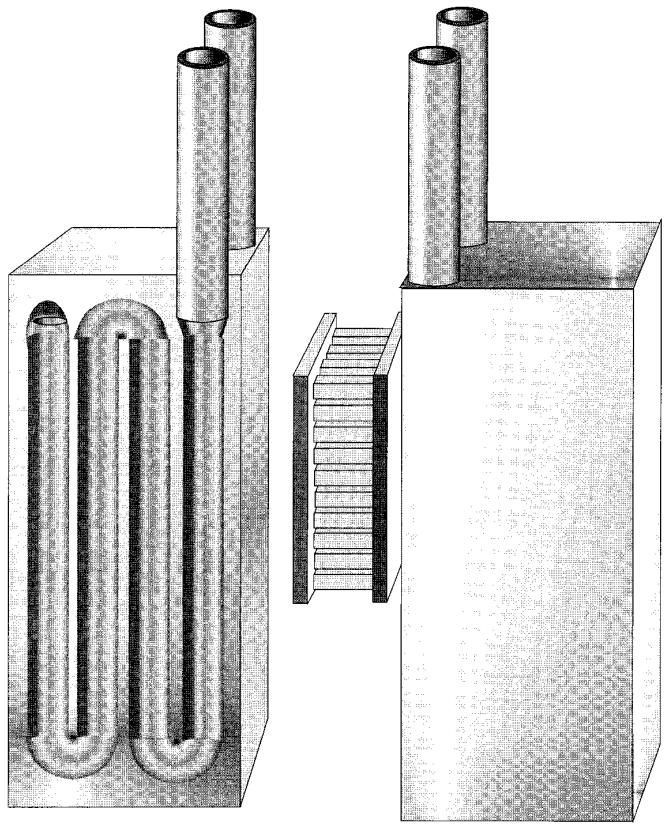

FIG. 2. The capillary calorimetric cells from solid blocks of gold with platinum inlet and outlet tubes and a semiconductor thermosensor.

cells are made from solid blocks of pure 24-karat gold. The capillary channels are drilled inside each block and connected sequentially to form a thorough tightly packed capillary channel (Fig. 1). The inlet and outlet of each capillary channel are both terminated in platinum tubes which end in the manostate to maintain constant excess pressure. The sensors and balancing heaters (resistors) are soldered on the surface of the cells.

The heating of the cells is provided by the heat flow through the platinum capillary tubes that are in thermal contact with the jacket. The temperature of the jacket is measured by the platinum thermometer and is monitored by the computer. The excess pressure in the manostat (16) is set by a piston and its magnitude, measured by a piezoelectric sensor (17), is displayed by the computer monitor (18). The excess pressure of up to 3 atm is sufficient to heat the aqueous solution up to 130°C without appearance of gas bubbles and boiling.

In the ideal case the properties of the sample cell and reference cell are absolutely identical. The ideal instrument, filled with water in both cells, will yield zero power baseline. Due to technological reasons, the ideal match between the two cells is impossible to achieve. Differences between the cells result in differences between the heating and cooling baselines. To eliminate this problem an additional power difference must be provided to compensate for the differences in cell properties. This additional power is automatically calculated by the computer at the first calibration run of the instrument and is applied during the heating and cooling cycles.

The instrument is incorporated in a single box with the computer. It does not require water supply and is indifferent to normal variations in room temperature.

SPECIFICATIONS OF THE INSTRUMENT

The instrument is characterized by highly stable and reproducible baselines as demonstrated by performing heating/cooling scans of buffer solutions that are commonly used in the study of biological macromolecules. Figure 3 displays original recordings of consecutive baselines obtained after refilling both cells with 50 mM glycine buffer, pH 2.5. As indicated in the figure, the noise level is less than 50×10^{-9} W, i.e., ~5 ncal/s at the response half-time of 5 s. Most important, the difference between repeated scans obtained after evacuating, cleaning, and refilling the cells does not exceed 0.5×10^{-6} W, i.e., 100 ncal/s.

For measurements of the absolute value of the partial heat capacity of a solute, the most important instrument specification is the reproducibility of recordings upon refilling the cells with the same sample (6). This characteristic permits accurate measurements of the difference in heat capacity between solution and

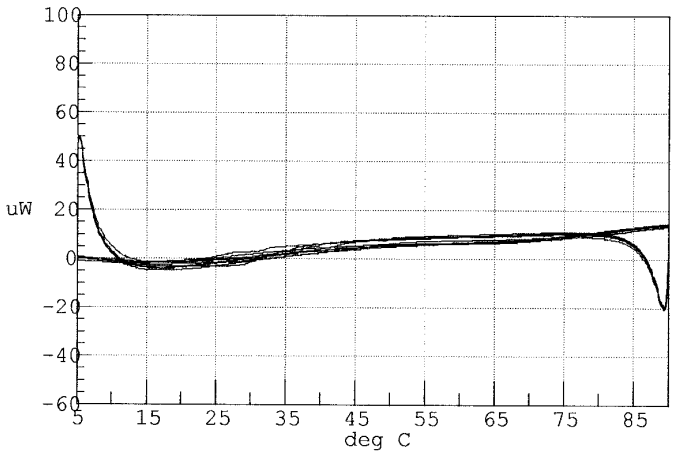

FIG. 3. The recordings of the baselines at consecutive heating and cooling of the calorimetric cells refilled for each scan by the solvent (50 mM glycine buffer, pH 2.5). The deviation of recording at start indicates its correspondence to heating or cooling experiment.

solvent and, consequently, the partial heat capacity of solute. Our test using degassed water showed that the baseline reproducibility upon refilling the cells is actually of the same order of magnitude as the reproducibility obtained without refilling the cells. This provides an accuracy in the difference heat capacity determination of ~10 μcal/°K upon heating at a rate of 1°K/min. The same reproducibility was found using protein solutions (Figs. 4 and 5).

The extensive heat absorption peak upon heating of lysozyme corresponds to the heat of its thermal denaturation (unfolding). The peak of heat release upon cooling the same solution corresponds to the effect of its renaturation (refolding). For lysozyme at pH 2.5 this process of unfolding/refolding is not completely reversible. Therefore, the observed heat effects upon heating and cooling lysozyme solution are similar (although reverse) but not quite identical in magnitude. The shift in the temperature of the maximum and the apparent broadening of the peak observed upon cooling are caused by the slower kinetics of refolding.

INSTRUMENT PERFORMANCE

The baseline. Although a differential scanning calorimeter is designed for measuring the difference in heat capacities of two liquids (solvent and solution), this cannot be done by a single measurement in which one of the cells is loaded with one liquid (solvent) and the other cell with the other liquid (solution) under study. For difference heat capacity determinations it is necessary first to load both cells with solvent and to determine the baseline of the instrument over the entire required temperature range (see Ref. 6). This baseline is usually neither linear nor close to zero since it is

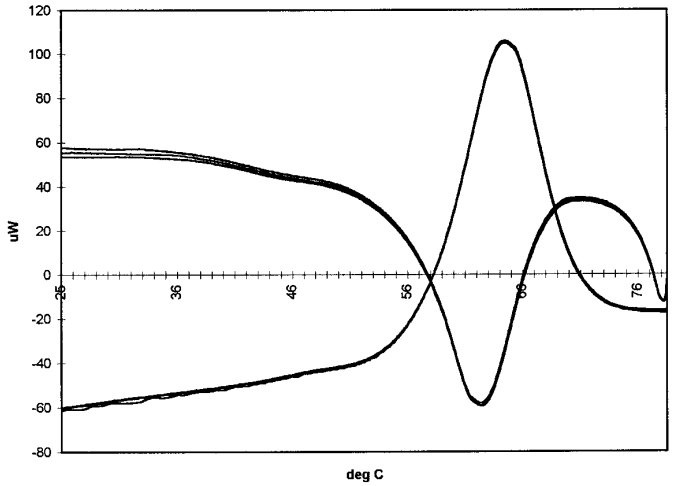

FIG. 4. Three different scans of hen egg white lysozyme (20 mM glycine buffer, pH 2.5, concentration of protein 2.17 mg/ml) superimposed on top of each other. These scans were performed after cleaning and refilling the calorimetric cell with different aliquots of the same protein solution. The cell was cleaned by 1 M formic acid washed by distilled water. These experiments demonstrate the reproducibility between scans after refilling. For the heating scans the average standard deviation was 0.56 μW. For the cooling scan the average standard deviation was 1.07 μW. Also important to note is the noise level on the order of 50 nW standard deviation. The variation in the temperature of the peak is within 0.1K. The baseline of this experiment is given in Fig. 3.

practically impossible to make two cells absolutely identical. In the instrument described here the computer analyzes this function during the calibration run, approximates it by a polynomial, and determines the power necessary to compensate the deviation of baseline from zero. Later, upon subsequent scanning this correction power is automatically applied to the cells. The resulting baseline is almost linear and close to zero. The baseline obtained for the given solvent can be automatically subtracted from the results of scanning the solutions of studied solutes with the same solvent. In that case the recorded deviation from zero will correspond to the difference heat capacity between the studied solution and solvent throughout the entire temperature range.

Determination of the partial heat capacity of solute molecule. The difference in heat capacity between a solution containing a biological macromolecule and the pure solvent is negative because the heat capacity of the solution (Fig. 4) is smaller than the heat capacity of the same volume of solvent (Fig. 3). For the observed difference in heat capacity between the solution and solvent we have

$$\Delta C_p^{app}(T)_{pr.sol/solv} = C_p(T)_{pr}\, m(T)_{pr} - C_p(T)_{solv}\Delta m(T)_{solv}, \quad [1]$$

where $C_p(T)_{pr}$ is the partial specific heat capacity of protein at temperature T, $m(T)_{pr}$ is the mass of protein which is in the calorimetric cell at temperature T, and $\Delta m(T)_{solv}$ is the mass of the solvent displaced by proteins in solution. The latter equals

$$\Delta m(T)_{solv} = m(T)_{pr}[V(T)_{pr}/V(T)_{solv}]. \quad [2]$$

Here $V(T)_{pr}$ is the partial specific volume of proteins at temperature T and $V(T)_{solv}$ is that of the solvent. From Eqs. [1] and [2] we obtain the partial specific heat capacity of the protein:

$$C_p(T)_{pr} = C_p(T)_{solv}[V(T)_{pr}/V(T)_{solv}] - \Delta C_p^{app}(T)_{pr.sol/solv}/m(T)_{pr}. \quad [3]$$

The specific heat capacity of the solvent can be determined over the desired temperature range by the same method using as a reference pure water, the specific volume and heat capacity of which are well known. As for the operational volume of the calorimetric cell and the mass of protein in the cell at temperature T, they can be calculated from their values at room temperature and known coefficients of thermal expansion of the solution and cell material (see for details Ref. 6).

It should be noted that the protein heat capacity determined by the above equation is not strictly the partial heat capacity since the latter is the value that is obtained by extrapolation to an infinitely dilute solution. However, if we take into account that the protein concentration in the solution used for the scanning calorimetric experiment is usually less than 10^{-4} M and this solution does not show any concentration dependence of the heat capacity, it becomes evident that the value determined by Eq. [3] can be considered as a

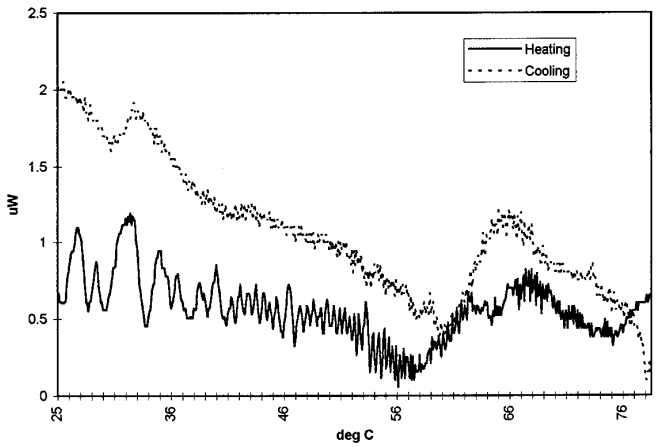

FIG. 5. Standard deviation of the three different scans of hen egg lysozymes (Fig. 3). Standard deviation was calculated for each point on the temperature scale.

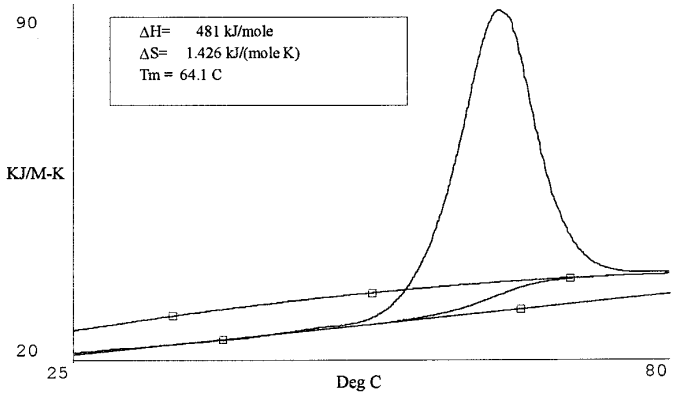

FIG. 6. Temperature dependence of the partial heat capacity of lysozyme. Also shown are the approximations of the heat capacity functions of the native and denatured state and the sigmoidal baseline that corresponds to the transition region. Inset shows the calorimetric enthalpy ΔH, entropy ΔS, and transition temperature Tt.

partial heat capacity of protein in solution (see for details Ref. 6). The instrument mentioned here permits accurate measurements of the absolute value of the partial molar heat capacities of protein and other macromolecules in a broad temperature range. The partial heat capacity of lysozyme calculated from the calorimetric recording given in Fig. 4 is presented in Fig. 6.

Deconvolution of the partial molar heat capacity. Having a differential scanning calorimeter with the capability to measure accurately the partial molar heat capacity of a macromolecule provides the opportunity of applying statistical thermodynamic methods without the need for ad hoc assumptions. From a rigorous statistical thermodynamic point of view, the partial molar heat capacity of a macromolecule is given by the equation

$$C_P = C_{P,0} + \frac{\partial}{\partial T}\left(\sum_{i=1}^{N} P_i \Delta H_i\right), \qquad [4]$$

where $C_{P,0}$ is the partial molar heat capacity of the reference state, usually the native state; ΔH_i is the relative enthalpy of state i, $\Delta H_i = H_i - H_0$; and P_i is the population of state i. In Eq. [4], the summation runs over all accessible states of the macromolecule. Expanding Eq. [4], one obtains

$$C_P = C_{P,0} + \sum_{i=1}^{N} \Delta H_i \frac{\partial P_i}{\partial T} + \sum_{i=1}^{N} P_i \Delta C_{P,i}, \qquad [5]$$

where $\Delta C_{P,i}$ is the relative heat capacity of state i. Equation [5] provides a complete representation of the entire heat capacity function that can be analyzed to provide information regarding the number of states populated during the transition and the thermodynamics of those states (1,15–17). The experimental partial heat capacity function can be analyzed in terms of Eq. [5] without arbitrary baseline subtractions. An example of this type of analysis is presented in Fig. 7. It shows the calorimetrically determined heat capacity function of lysozyme in the temperature range of its temperature-induced transition to the denatured state and the best approximation to this function assuming that the denaturation process is a two-state transition. Since the protein used in this experiment was highly purified (the content of impurities less than 1% according to electrophoretic test), the difference between these two functions is an indication of the deviation of a real process, lysozyme denaturation, from a two-state transition. A precise fit between the measured and calculated heat capacity functions is obtained if the process is considered as a three-state transition. This deviation from two-state behavior is reflected in the ratio of the calorimetric and van't Hoff enthalpies. The enthalpy of the real process (which is usually called calorimetric enthalpy) exceeds the enthalpy of its theoreti-

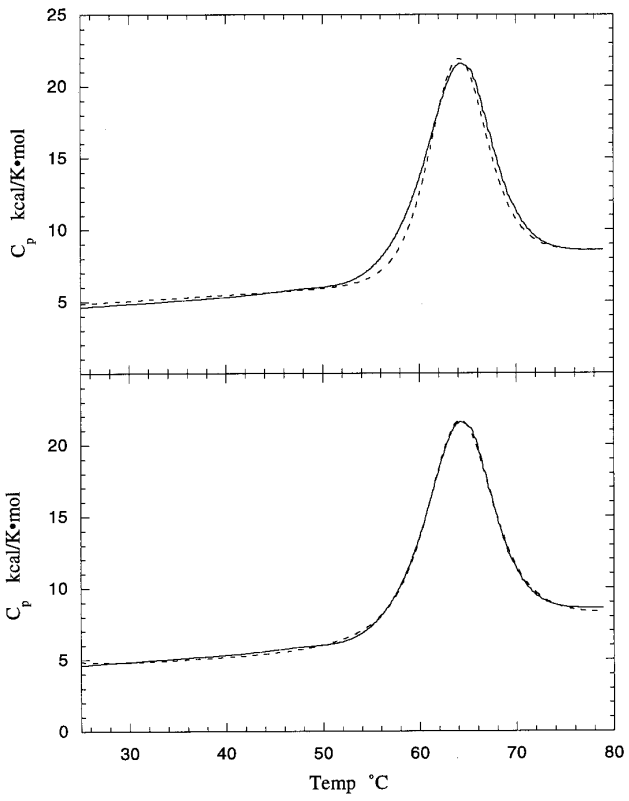

FIG. 7. Comparison of calorimetrically measured and calculated transition heat capacity functions of lysozyme. The best approximation using a two-state model is depicted (top) by a dashed line. The experimental data and the fit to a three-state function are indistinguishable (dashed line, bottom). The ratio between the calorimetric and van't Hoff enthalpies is 1.05. This deviation from unity is indicative of the presence of intermediates during the thermal transition.

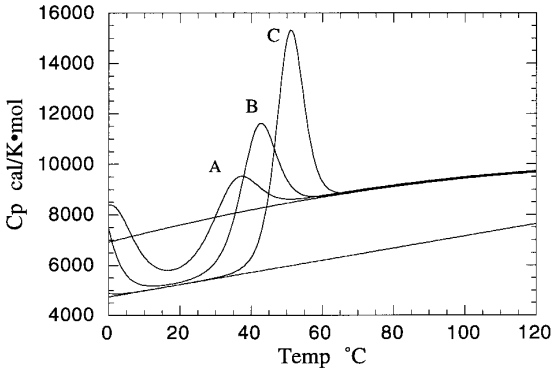

FIG. 8. The heat capacity functions of hypothetical marginally stable protein which unfolds with significant heat capacity increment (for the real examples see Ref. 19). When stability of protein is low the native state might not be fully populated because of cold denaturation which starts at low temperatures. In this case an arbitrary baseline subtraction will cause significant error in the data analysis of the heat denaturation peak.

cal two-state approximation (i.e., van't Hoff enthalpy) by 5%. Deviations of this magnitude have been previously reported in the literature for lysozyme and other small globular proteins (18) and are indicative of the presence of intermediates. Unfortunately, in the past these small deviations could not be accurately analyzed due to instrument limitations. It is expected that this new generation of instruments will permit a rigorous analysis of the intermediates present in the folding/unfolding process of small globular proteins.

Analysis of marginally stable proteins. Another major advantage of having access to the partial molar heat capacity is on the analysis of protein denaturation under conditions in which the protein displays low stability. An example of this type of behavior is shown in Fig. 8. In this figure, curve A exhibits very low stability and consequently a significant degree of cold denaturation such that the native state is never fully populated at any temperature. Under those conditions a conventional baseline subtraction cannot be performed and the calculated enthalpies, entropies, heat capacity changes, and transition temperatures will be in error. More importantly, a DSC instrument that is not accurate enough for partial molar heat capacity determination will not detect the existence of such a situation, resulting in serious interpretation errors.

REFERENCES

1. Freire, E., and Biltonen, R. L. (1978) *Biopolymers* **17,** 463–479.
2. Privalov, P. L., Monaselidze, D. R., Mrevlishvili, G. M., and Magaldadze, V. A. (1964) *Zh. Eksp. Teor. Fiz. (USSR)* **47,** 2073–2079; see also *Sov. Phys. JTP* (1965) **20,** 1393–1396.
3. Gill, S. J., and Beck, K. (1965) *Rev. Sci. Instrum.* **36,** 274–276.
4. Danford, R., Krakauer, H., and Sturtevant, J. M. (1967) *Rev. Sci. Instrum.* **38,** 484–491.
5. Privalov, P. L., and Plotnikov, V. V. (1989) *Thermochim. Acta* **139,** 257–277.
6. Privalov, P. L., and Potekhin, S. A. (1986) *Methods Enzymol.* **131,** 2–51.
7. Privalov, P. L. (1980) *Pure Appl. Chem.* **52,** 479–490.
8. Privalov, P. L. (1976) *Pure Appl. Chem.* **47,** 293–304.
9. Sturtevant, J. M. (1974) *Annu. Rev. Biophys. Bioeng.* **3,** 35–41.
10. Wadso, I. (1970) *Q. Rev. Biophys.* **3,** 380–450.
11. Privalov, P. L., Plotnikov, V. V., and Filimonov, V. V. (1975) *J. Chem. Thermodyn.* **7,** 41–47.
12. Privalov, P. L., and Khechinashvili, N. N. (1974) *J. Mol. Biol.* **86,** 665–684.
13. Ross, P. D., and Goldebrg, R. N. (1974) *Therm. Chem. Acta* **10,** 143–151.
14. Suurkuusk, R. J., Lentz, B. R., Barenholz, Y., Biltonen, R. L., and Thompson, T. E. (1976) *Biochemistry* **15,** 1393–1399.
15. Griko, Y., Freire, E., and Privalov, P. L. (1994) *Biochemistry* **33,** 1889–1899.
16. Xie, D., Fox, R., and Freire, E. (1994) *Protein Sci.* **3,** 2175–2184.
17. Gomez, J., Hilser, J. V., Xie, D., and Freire, E. (1995) *Proteins* **22,** 404–412.
18. Privalov, P. L. (1979) *Adv. Protein Chem.* **33,** 167–239.
19. Privalov, P. L. (1990) *CRC Crit. Rev. Biochem. Mol. Biol.* **25,** 281–305.
20. Freire, E. (1995) *Methods Enzymol.* **259,** 144–168.