and characterization of folding intermediates. Antibodies are becoming powerful tools in the study of protein folding. Not only can antibodies be made against peptides, but antipeptide antibodies cross-react with the proteins from which the peptides were derived. These and other developments in technique will surely have a major impact in the next decade on work aimed at solving the folding problem.

[2] Scanning Microcalorimetry in Studying Temperature-Induced Changes in Proteins

By PETER L. PRIVALOV and SERGEY A. POTEKHIN

Introduction

The importance of calorimetric studies of changes in the state of proteins induced by various factors stems from the fact that calorimetry is the only method for the direct determination of the enthalpy associated with the process of interest. Calorimetry acquires special significance in studies of temperature-induced changes in the state of the protein, since temperature and enthalpy are coupled extensive and intensive variables. All temperature-induced changes in macroscopic systems always proceed with a corresponding change of enthalpy, i.e., they are accompanied by heat absorption if the process is induced by a temperature increase, or by the evolution of heat if it is caused by a temperature decrease. The functional relation between enthalpy and temperature actually includes all the thermodynamic information on the macroscopic states, accessible within the considered temperature range and this information can be extracted from the enthalpic function by its thermodynamic analysis.

The temperature dependence of the enthalpy can be determined experimentally by calorimetric measurements of the heat capacity of the studied objects over the temperature range of interest. Since the heat capacity determined at constant pressure is a temperature derivative of the enthalpy function

$$C_p = (\partial H/\partial T)_p \tag{1}$$

one can easily estimate the enthalpy function by integration of the heat capacity

$$H(T) = \int_{T_0}^{T} C_p(T)dT + H(T_0) \tag{2}$$

The so-called heat capacity calorimeters are used for heat capacity measurements over a particular temperature range. There are many modifications of this instrument designed for studies of various materials in different aggregate states, over different temperature ranges, and with different accuracies. As a material for calorimetric study protein has some characteristics which distinguish it from other objects.

Among the protein characteristics the size of these molecules should be mentioned first. The molecular weight of a protein is usually greater than 10,000. Thus, these molecules, consisting of many thousands of atoms, can be regarded in themselves as macroscopic systems. In this respect proteins are similar to nucleic acids, the other important representatives of biological macromolecules, the calorimetric studies of which attract no less attention.

The important feature in calorimetric studies of these biological macromolecules is that we are interested in their physical properties not in the isolated state—in vacuum, but in the dispersed state—in solution (particularly in aqueous solution) in which they can be regarded as individual macroscopic systems surrounded by the solvent medium. The solution concentration at which the interaction between macromolecules is sufficiently small to be neglected is of the order of 10^{-4} M for average proteins, i.e., of the order of one tenth of the weight percent. In such dilute solutions, however, the macromolecular contribution to the thermal properties of the entire samples should also be small: the protein heat capacity does not exceed 0.03%, while the excess heat capacity at the peak of denaturation is less than 1% of the solution heat capacity. At the same time, even dilute solutions of biological molecules are quite viscous so that they cannot be stirred to achieve rapid thermal equilibration during heating, as is usually done in studies of liquids.

The other important peculiarity of biological molecules is their poor availability and exceptionally high cost due to the difficulty of their isolation and purification. The amount of material which can be used practically in experiments for the most available materials does not exceed a few milligrams.

Thus, heat capacity studies of proteins actually boil down to calorimetric studies of very small heat effects which occur in a few milliliters of a viscous solution heated over a broad temperature range. These experiments cannot be done in any of the known heat capacity calorimeters used for physicochemical studies of nonbiological materials. The realization of these experiments has required the creation of a qualitatively new technique, which is known as heat capacity microcalorimetry.

We will consider in this chapter the experimental technique used in heat capacity studies of individual macromolecules in solution, experi-

ments with these materials, methods of treatment of experimental results, and their analysis.

Experimental Technique

Microcalorimeters for Heat Capacity Studies of Liquids

During the last few years a number of review papers have been devoted to supersensitive and superprecise instruments for heat capacity studies of small volumes of liquids.[1-6] Unfortunately, the technical problems inherent in heat capacity microcalorimetry have been treated rather briefly. Here we also cannot go into technical detail and readers interested in this aspect are referred to the original papers.[7-11] Nevertheless, some general principles of construction of such instruments will be considered here, to make clear what can be expected from this technique and how to work with it.

All contemporary heat capacity microcalorimeters have a number of features in common. First, they do not have a mechanical stirrer as do all the macrocalorimeters for the rapid redistribution of the introduced thermal energy over the sample volume. The stirring of a liquid with a high and variable viscosity produces uncontrollable Joule heat in an amount much greater than the measured heat effect. The elimination of the mechanical stirrer was made possible by the great decrease of the operational volume of the calorimeter. Therefore, a small volume of the calorimetric cell is a key requirement in the construction of heat capacity microcalorimeters.

All microcalorimeters measure heat capacity not in a discrete way at stepwise sample heating by discrete energy increments as do all the classical calorimeters, but continuously with continuous heating or cooling of the sample at a constant rate. In other words, they scan along the temperature scale by measuring continuously small changes in the heat capacity

[1] I. Wadsö, *Q. Rev. Biophys.* **3,** 383 (1970).
[2] J. M. Sturtevant, this series, Vol. 26, p. 227.
[3] J. M. Sturtevant, *Annu. Rev. Biophys. Bioeng.* **3,** 35 (1974).
[4] S. Mabrey and J. M. Sturtevant, *Methods Membr. Biol.* **9,** 237 (1978).
[5] K. C. Krishnan and J. F. Brandts, this series, Vol. 49, p. 3.
[6] P. L. Privalov, *Pure Appl. Chem.* **52,** 479 (1980).
[7] P. L. Privalov, J. R. Monaselidze, G. M. Mrevlishvili, and V. A. Magaldadze, *Zh. Eksp. Teor. Fiz. (USSR)* **47,** 2073 (1964); see also *Sov. Phys. JETP* **20,** 1399 (1965).
[8] P. L. Privalov and J. R. Monaselidze, *Prib. Tek. Eksp. (USSR)* **6,** 174 (1965).
[9] P. L. Privalov, V. V. Plotnikov, and V. V. Filimonov, *J. Chem. Thermodyn.* **7,** 41 (1975).
[10] S. J. Gill and K. Beck, *Rev. Sci. Instrum.* **36,** 274 (1965).
[11] R. Danford, H. Krakauer, and J. M. Sturtevant, *Rev. Sci. Instrum.* **38,** 484 (1967).

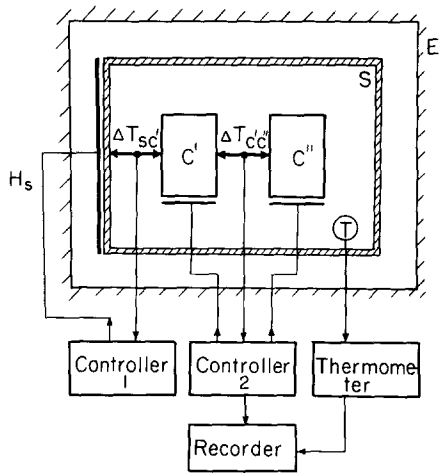


FIG. 1. Differential adiabatic scanning calorimeter with two identical cells (C' and C") heated at a constant rate and a controlled temperature of the surrounding shell (S).

of the samples. As a result, these instruments are usually called scanning microcalorimeters.

Continuous heating and measurement have great advantages over the discrete procedure: it gives more complete information on the heat capacity function and permits the complete automatization of all the measurement processes. Its disadvantage is that the studied sample is never in complete thermal equilibrium. This sets certain requirements for the samples and for instrument construction: the temperature-induced changes in the samples should not be too sharp and the relaxation process at these changes should be sufficiently fast, while the construction of the calorimetric cell should provide minimal thermal gradients which do not change with heating.

All scanning microcalorimeters measure not the absolute but the difference heat capacity, i.e., they are all differential instruments with two identical calorimetric cells, and measurements consist in a comparison of their heat capacities (Fig. 1). One of the cells is loaded with the solution to be studied and the other one with some standard liquid. Thus, the heat capacity of the studied liquid is determined relative to the chosen standard. In studying dilute solutions, it is convenient to take the solvent as a standard, since, in this case, the measured difference heat capacity will correspond directly to the heat capacity contribution of the molecules dissolved in the solution.

The heat capacity difference of the cells is usually measured by the compensation method: the controller automatically monitors the power in the electric heaters of both cells to maintain identity of their temperatures

at heating, and the difference of these powers is recorded as a temperature function. Such a feedback by the heat balance in the cells improves significantly the dynamic characteristics of the instrument which is especially important for a precise registration of the complicated heat capacity functions.

The constant rate of heating over the entire temperature range in scanning microcalorimetry is provided either by the controllable power which compensates for an increasing loss of energy by the cells on heating[12,13] or by the thermoisolation of the cells which prevents the loss of their energy. The first method is simpler to construct. However, the large amplitude of heating power regulation results in excessive noise and an unstable baseline. That is why this method is not used in precision scanning microcalorimetry where the normal practice is to use complete or partial adiabatization of cells, i.e., their controllable thermal isolation from the surrounding thermostat.[6]

The heat exchange of the cells with the thermostat is controlled by enclosing them in thermal shells with a controllable temperature (Fig. 1). The shells completely prevent the heat exchange between the cells and the thermostat if its temperature is maintained equal to that of the cells, while a constant difference between the cell and shell temperatures provides constant positive or negative heat flow into the cells. Such a heat flow is necessary for scanning in both directions along the temperature scale.

The thermal shell is made of metal with high thermal conductivity (silver, copper). Electric heaters insulated by a thin film are uniformly distributed on the outward surface of the shell. Precise instruments usually have several shells to provide a high symmetry and constancy of the thermal field around the twin cells over the entire operational temperature range.

One of the most difficult problems in scanning microcalorimetry is the loading of the cells with equal and definite amounts of the studied and standard materials and of the free volume. It is clear that when measuring heat capacity with an error of less than 10^{-5} J K^{-1}, the error in loading the cells with the sample should not exceed 10^{-6} g. It is practically impossible to load the calorimetric cells with such accuracy by weighing the sample. A calorimeter with extractable cells never gives reproducible results; each replacement of the cells results in a different slope and position of the baseline of the instrument. Therefore, such instruments, being quite sensitive to sharp changes of heat capacity with temperature, cannot be

[12] E. S. Watson, M. J. O'Neil, J. Justin, and N. Brenner, *Anal. Chem.* **36,** 1233 (1964).
[13] E. S. Watson and M. J. O'Neil, U.S. Patent, 3, 263, 484 (1966).

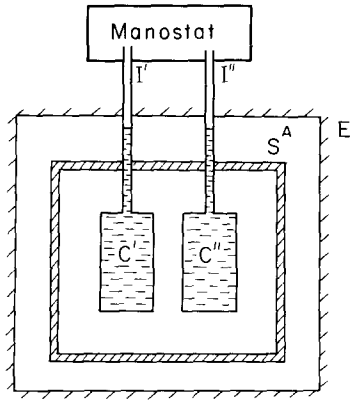


FIG. 2. Undismountable calorimetric block with the cells completely filled with liquid samples through the capillary inlets I′ and I″. The measuring volume of the cells is fixed by the point at which the adiabatic shell (S) contacts with the capillary inlet and shunts the difference heat effect. The manostat is used to apply extra pressure on the liquid for squeezing the bubbles which might be left in the cells and for preventing their appearance upon heating.

used for determining the absolute heat capacity difference and its dependence on temperature, i.e., they cannot be considered as precise instruments for the determination of the difference heat capacity of liquids.

The free volume in the loaded cells raises similar difficulties. It is evident that hermetic cells cannot be filled completely with a liquid sample, since the thermal expansions of the liquid and of the cell are usually different. On the other hand, the free volume in the cell leads also to numerous complications during heating caused by vaporization of the liquid. Although the vapor pressures of the solvent and the dilute solution do not differ greatly, the difference in the heat effect of their vaporization might be quite significant because of the large specific heat of vaporization (about 2 kJ g^{-1} for water).

The problem of loading a scanning microcalorimeter with a sample and that of the free volume was resolved by replacing heat capacity measurements of a sample of definite mass by heat capacity measurements of a sample of definite volume.[6,9] The volume of the studied sample can be fixed by the fixed operational volume of the calorimetric cell. The main requirement which must be fulfilled is that the cell should be filled completely and no microscopic bubbles should be left in it. It is clear that this can be done only by replacing the isolated hermetic cell, which has always been used in heat capacity calorimetry, by an open cell connected with the external vessel (Fig. 2). A thin capillary tube connects the cell with the external vessel so that the cell can be filled with the sample without

removing it from the adiabatization system of thermal shells. This permits the liquid expanded on heating to flow from the cell. The operational volume of the cell is determined by the thermal shell which is in thermal contact with the capillary tubes and which plays the role of a thermal shunt, cutting off the influence of the external part of the capillary tube on the cells.

To exclude bubbles from the cells, an excess pressure is applied to the external ends of the capillary tubes by a manostat and all measurements are performed under this constant pressure. A few atmospheres are sufficient to compress all the bubbles in the cells and to prevent their appearance on heating. Since the excess pressure raises the boiling temperature, this extends the operational range of the instrument. For example, 5 atmospheres of excess pressure are enough to heat an aqueous solution to 150°, which is very important for studies of thermostable macromolecules.

When the described cells are heated, the expanding liquid filling the cells is removed through the capillary tube, i.e., the mass of the liquid which is in the cell decreases with an increase of temperature. However, the flow of liquid from both cells is almost identical when studying dilute solutions, since the thermal expansion coefficients of the dilute solution and the solvent are almost the same. Therefore, expansion of the liquid does not affect the measured difference of heat capacity, although a decrease of the mass in the cells should be taken into account when calculating the specific heat capacities.

The principle of fixed volume of liquid heat capacity measurements was used in designing the precision scanning microcalorimeters DASM-1M and DASM-4, manufactured by the Bureau of Biological Instrumentation of the USSR Academy of Sciences. Later this principle was also used in the scanning microcalorimeter Biocal, manufactured by Setarum (France), and in the model MK-2 of the scanning microcalorimeter manufactured by Microcal (United States).

The undismountable calorimetric block of the DASM-1M instrument with the cells filled through the capillary tube and a double adiabatic shell is shown in Fig. 3. The golden 1 ml volume cells are made as flat disks. The capillary tubes with a 1.2 mm inner diameter are made of platinum. The thermal shells are made of silver.

The main difference between calorimeters DASM-4 and DASM-1M is in the construction of the cells. In DASM-4 the cells are made completely from capillary tubes wound into a helix (Fig. 4). Capillary cells have many advantages: they are easily washed and filled without bubbles and they provide a much more homogeneous thermal field with lower temperature gradients in the studied liquids. This permits use of higher heating rates

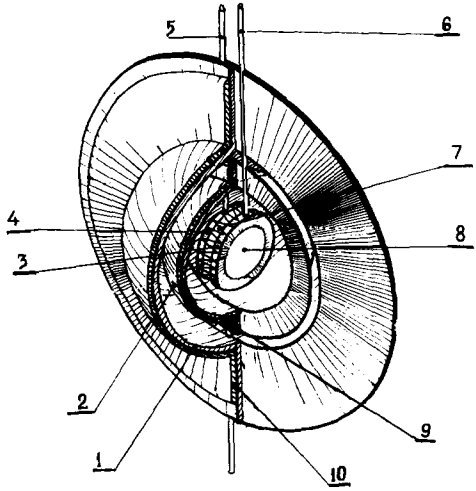


FIG. 3. Calorimetric block of the scanning microcalorimeter DASM-1M. (1,2) Internal and external adiabatic shells with heaters; (3) calorimetric cells; (4) shell thermosensor; (5, 6) capillary inlets; (7) thermopile; (8) cell heater; (9) internal shield rim; (10) external shield rim.

during measurements, and therefore increases the sensitivity of the instrument in heat capacity studies. In the capillary cell there is no thermal convection of the liquid during heating which is one of the sources of artifacts in scanning microcalorimetry. Moreover, thin capillary tubes can withstand much higher pressures than cells of any other shape and this is important for extending the operational temperature range of the instrument (Fig. 5).

Main Characteristics of Scanning Microcalorimeters

Usually the sensitivity of an instrument means the minimal signal which can be detected against the background noise. Therefore, it depends not only on the noise of the instrument, but also on the shape of the signal.

In the case of the scanning microcalorimetry of biological molecules, the spectrum of possible signals varies over a wide range, from fractions of a degree (melting of homopolymers and phospholipid bilayers) to dozens of degress (gradual changes of heat capacity, cooperative transitions with small enthalpies) and the problem of their isolation against the background noise requires detailed studies of noise characteristics, i.e., of the noise spectrum of the instrument. However, this is usually somewhat arbitrarily replaced by the evolution of a mean square deviation of the recording from an ideal line (noise level) and by the evolution of the

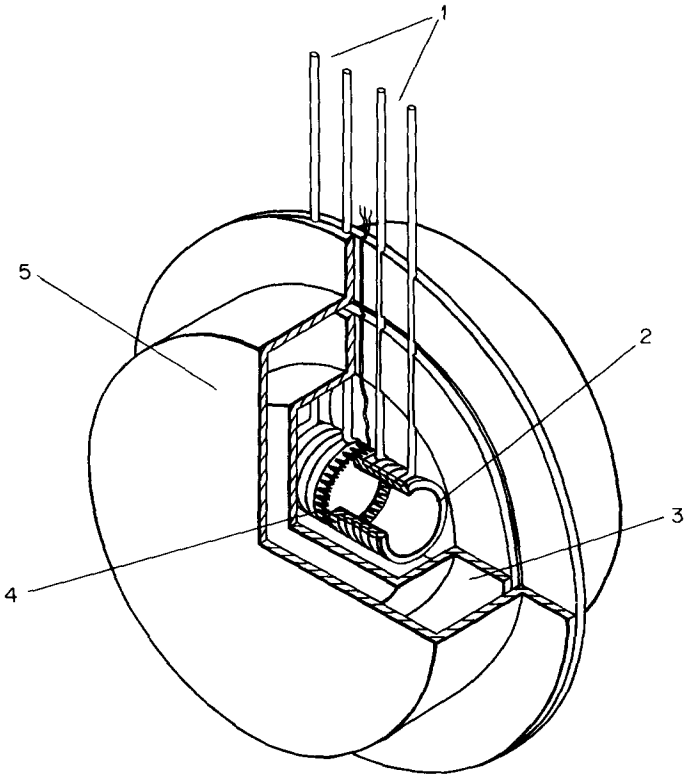


FIG. 4. Calorimetric block of the capillary scanning microcalorimeter DASM-4. (1) Capillary inlets; (2) helical capillary cell with heater; (3) internal shell; (4) thermopile measuring the temperature difference between the capillary cells; (5) external shell.

reproducibility of recordings at consecutive runs of the instrument without refilling the cells with the sample (convergency). But this is far from sufficient for characterizing the ability of the instrument to measure the difference heat capacity. The main parameter showing this ability of a scanning microcalorimeter is the reproducibility of the results after refilling the cells with the same sample. In contrast to the mean noise level and convergency which are usually estimated in power units (watts), the reproducibility of the results of difference heat capacity measurements should be estimated in heat capacity units (J K^{-1}) at the optimal heating rate.[6]

It is evident that the sensitivity of a scanning microcalorimeter depends directly on the amount of the sample studied. The smaller the amount of the sample and, consequently, its heat capacity, the smaller are the heat effects registered on heating. Therefore, one of the widespread

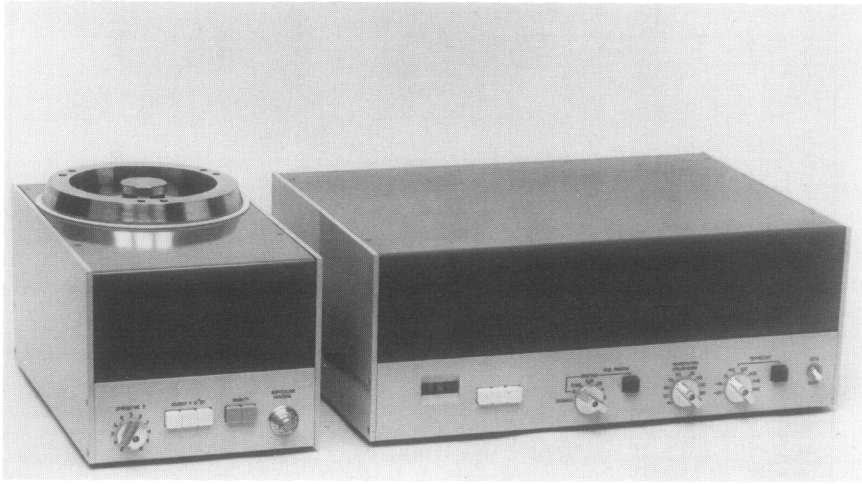


FIG. 5. Capillary scanning microcalorimeter DASM-4, manufactured by the Bureau of Biological Instrumentation of the Academy of Sciences of the USSR.

methods of increasing the apparent sensitivity of a scanning microcalorimeter is reduction of the volume of the calorimetric cells. However, in solution studies it is the sensitivity to the heat effect occurring within the sample of definite volume that is significant, and not the absolute sensitivity to the heat effect, since in solution studies it is the minimal concentrations and not the minimal volumes that are of importance. The characteristics of a scanning microcalorimeter reduced to a unit of volume are denoted as reduced characteristics.[6] It is clear that the reduced characteristics are basic in the assessment of the quality of an instrument designed for solution studies.

Although at present many companies manufacture scanning calorimeters, it is difficult to establish a clear idea of the potentials of these instruments from their descriptive brochures, since usually the descriptions give only one or two most favorable parameters, for example, only the noise level. The table summarizes the basic characteritics determined from the descriptions and illustrations of some manufactured instruments which at present are used most frequently in studies of macromolecules. It is evident that although there is only a 100-fold difference in the noise levels of these instruments, their reduced noise-levels, which per se determine their fitness for studying macromolecules in solution, differ more than 1000-fold, while the relative error in estimating heat capacity varies by two orders of magnitude. This qualitative difference compels us to divide scanning calorimeters into two classes. The dividing line can be the relative error in the estimation of the heat capacity. It is expedient to

MAIN CHARACTERISTICS OF COMMERCIAL SCANNING MICROCALORIMETERS

Quality	Characteristic	Unit	Perkin-Elmer DSC-2	Dupont 910 DSC	Daini Seikoshi SSC-50	Acad. Sci. USSR DASM-1M	Acad. Sci. USSR DASM-4	Microcal USA MC-2	Seteram France Biocal
Operational range	Volume of the cell	ml	0.03	0.03	0.07	1.0	0.5	1.3	1.0
	Temperature range	K	100–1000	100–1000	120–400	273–373	250–400	250–390	273–373
	Heating rates	K/min	0.3–320	0.5–100	0.01–5.0	0.1–2.0	0.1–2.0	0.16–1.5	0–1.0
Sensitivity	Noise level	μW	17	4	1.3	0.5	0.2	0.26	1.0
	Reduced noise level	μW/ml	600	150	20	0.5	0.4	0.2	1.0
Precision	Reproducibility without refilling of the cell	μW	—	20	2.0	2.0	0.5	1.3	—
	Reduced reproducibility without refilling of the cell	μW/ml	—	700	30	2.0	1.0	1.0	—
Accuracy	Reproducibility on refilling of the cell	mJ K^{-1}	—	—	—	0.3	0.05	0.13	0.3
	Reduced reproducibility on refilling of the cell	mJ K^{-1}/ml	—	—	—	0.3	0.1	0.1	0.3
	Relative error in the heat capacity determination	%	1.0	1.0	0.5	0.01	0.005	0.005	0.01

consider the instruments for which this error does not exceed 0.1% as precision instruments, as these instruments can be used in quantitative studies and, in particular, studies of macromolecules in solution. The other instruments, which we cannot call precision ones, have a number of advantages: a smaller cell volume and larger ranges of heating rates and working temperatures. In other words, they are more universal. It is evident that precision is the cost for the universality which has ensured their wide usage mainly in applied studies. Such instruments are also used in studies of macromolecules in solution: low sensitivity and accuracy is compensated by using high concentrations and heating rates, but the possibility of their increase is rather limited. The maximally admissible heating rate in studies of macromolecules should be 5 K/min, but in many cases it is much lower.

Calorimetric Experiment

Preparation of Samples

The calorimetric study of a protein usually begins with the choice of appropriate conditions and the preparation of protein solutions. We assume these conditions to be the required purity of the protein, the most suitable solvent (pH, salt content), the concentration, the temperature range, and the scanning rate.

The requirement for protein purity in calorimetric experiments is usually very rigid and not easily realizable, since even the most sensitive instruments require large amounts of material for the experiment. Homogeneity of the studied protein preparation is crucial for a thermodynamic analysis of calorimetric data, especially in studies of complex temperature-induced processes, since an admixture of small amounts of even the same modified protein can alter the result seriously. The homogeneity of the studied preparation must be checked by all possible tests: by electrophoresis of the native protein and of the unfolded one in SDS in the presence of reducing agents, by isoionic focusing, chromatography, ultracentrifugation, and terminal group analysis. None of these tests separately can guarantee the purity and homogeneity of the preparation, while a combination of several tests can give a more or less certain answer. In quantitative studies, the amount of contamination should not exceed 3%.

Arguments for the choice of solvent are as follows: (1) the protein should be soluble in the solvent over the entire studied temperature range, i.e., it should not aggregate on heating; (2) the states of protein to be studied should be realizable in the chosen solvent; and (3) protein transitions between the states should be reversible (at least partly).

Irreversibility of the protein intramolecular transitions is usually caused by various concomitant intermolecular processes, such as aggregation, chemical modification, and intermolecular cross-linking. Therefore, a search for conditions providing maximal reversibility of the considered changes in the state of the protein leads us, first of all, to conditions which prevent direct contact between protein molecules, i.e., a low concentration and maximal electrostatic repulsion between protein molecules, which is achieved by a proper choice of pH and salt content of solution.

In scanning calorimetric experiments pH stabilization is achieved by the use of various buffers which are added in minimally required concentrations to avoid any direct influence of the buffer on the protein. However, one should bear in mind that the pH of buffer solutions is a function of temperature. Since this dependence is proportional to the enthalpy of ionization of the buffering compound, it is preferable to choose buffers with a minimal enthalpy of ionization for scanning calorimetric experiments, so that they should contribute minimally to the calorimetrically measured heat effect. The buffers most preferable for studying conformational transitions of proteins are those which have the same ionization enthalpies as the protein group, since in this case both effects, the heat of protein ionization and the heat of buffer ionization, compensate each other, and we measure directly the net heat effect of the conformational transition. One such buffer is glycine, the carboxylic and amino groups of which are ionized in the pH regions of 2–4 and 8–10, respectively.

In working with proteins which bind specifically ligands other than protons, it is necessary to stabilize not only the pH but also the concentration of these ligands in solution during heating. For example, in studying temperature-induced changes of calcium-binding proteins (calmodulin, torponin C) it is necessary to stabilize the activity of calcium ions in solution with an EDTA/Ca^{2+} buffer. The concentration of free Ca^{2+} in solution $[Ca^{2+}]$ is determined in this case by the equation

$$[Ca^{2+}] \simeq \frac{[Ca]}{[EDTA] - [Ca]} K^{-1} \tag{3}$$

where K is the Ca^{2+} binding constant by EDTA, [Ca] is the concentration of calcium in solution, and [EDTA] is the concentration of EDTA in solution.[14]

The required concentration of protein depends on the sensitivity of the instrument and the experimental goals. The estimation of partial heat capacity requires a quite high concentration of protein (about 2–5 mg/ml

[14] T. N. Tsalkova and P. L. Privalov, *J. Mol. Biol.* **181,** 533 (1984).

for the DASM-4 instrument). The required concentration for studying conformational transitions of proteins depends on the sharpness of the transition and its specific enthalpy: for studying the relatively sharp transitions of globular proteins with a half-width of about 5–10 K and a specific enthalpy of about 20 J g^{-1} the concentration can be about 5 times lower than that indicated above (about 0.4–1 mg/ml). For studying sharp transitions, such as the melting of collagen and phospholipid bilayers, the concentration can be an order lower (about 0.1 mg/ml), while for studying multidomain proteins which melt over a close to 100 K temperature range, the concentration should be high (about 3 mg/ml).

The preparation of a protein solution for calorimetric experiments starts by dissolving the protein in the chosen solvent in amounts required to obtain the desired concentration and volume. Then, the protein solution is carefully dialyzed against the solvent for 12 hr with several changes of buffer to achieve complete equilibrium between the low molecular compounds in the protein solution and the pure solvent. This is an absolutely necessary procedure since the protein solution and solvent which are loaded into the two cells of the differential calorimeter should differ only in the protein content. (The small difference in the solvent composition caused by the Donnan effect is negligible for a dilute protein solution.)

After dialysis, the protein solution should be centrifuged (30 min, 15,000 *g*) to remove aggregates and dust, and its concentration should be determined by some standard method. For a quantitative thermodynamic analysis of calorimetric results, the error in determining the concentration should not exceed 3%.

In some cases the studied liquids are degassed under vacuum before loading into the calorimetric cells. However, one should bear in mind that this procedure could change significantly the concentrations of the studied solutions. If experiments are done on calorimeters which operate under excess pressure (DASM-1M, DASM-4), no degassing is needed, except for some special purpose, e.g., oxygen release from the solution.

The excess pressure which is used in scanning microcalorimetric experiments to prevent degassing and boiling of the liquids depends on the upper temperature to which the protein solution is to be heated. It is 1 atm for heating to 100°, 1.5 atm for 110°, 3 atm for heating to 130°, and 5 atm for heating to 150°.

When choosing the optimal scanning rate the following considerations should be taken into account: (1) the sensitivity of the scanning calorimeter increases with an increase of the heating rate; (2) an increase of the heating rate leads to a decrease in the uniformity of the temperature field of the sample; this results in smoothing of the studied effects; and (3) the

rate of the temperature-induced transition in protein is limited, it is about 10^3 sec^{-1} for the denaturation of globular proteins and only 10^{-2} sec^{-1} for the denaturation of fibrillar proteins.

The main criterion in choosing the optimal heating rate is the lack of dependence of the calorimetric recordings on the heating rate. If the heating rate is too large, the observed transition will appear broader, flatter, and will be shifted to higher temperatures. The optimal rates for calorimeters with capillary cells (DASM-4) are 1–2 K/min for globular proteins, 0.25–1.0 K/min for fibrillar proteins, and 0.1–0.25 K/min for phospholipid bilayers.

Difference Heat Capacity Determination

Although a differential scanning microcalorimeter is designed for the difference heat capacity determination of liquids, this cannot be done by single measurements of the heat capacity of its two cells loaded with the two liquids (solvent and solution). The first thing that should be done for a precise determination of their heat capacity difference is to load both cells with one of the liquids which is considered as a standard (solvent) and to determine the zero or the baseline of the instrument over the entire required temperature region (Fig. 6). This "line" in most cases is neither linear nor horizontal for supersensitive instruments, because it is practically impossible to make the two cells absolutely identical. However, in precise instruments, it is stable and reproducible in repeated runs of the instrument after refilling the cells with new aliquots of the same liquid. The slope of this line can be easily corrected by applying a temperature-controlled power into one of the cells. A more radical correction of the baseline, including its linearization, can be achieved by memorizing the result of the first run of the instrument and subtracting it from the results of all other runs. This can be done by a special electronic corrector with a memory unit or by a computer on line with the scanning microcalorimeter used for further processing of the data.

Once the baseline has been determined, one of the cells is filled with the studied solution. The deviation of the recording for the solution from the baseline should correspond to the heat capacity difference of the same volume of solvent and solution. Therefore, in difference heat capacity determinations we are comparing the heat capacity of the solution with that of the solvent which had been in the same cell previously, while the other cell with the solvent is used only as a reference one. This permits complete exclusion of the influence of nonidentity on the results of measurements.

To estimate the difference heat capacity in heat capacity units, the instrument must be calibrated. This cannot be done with standard liquids

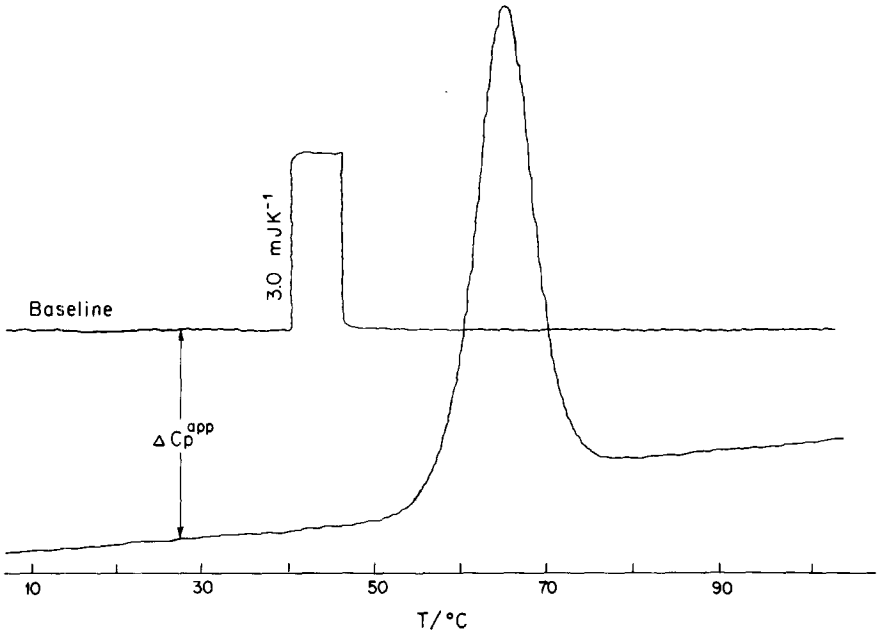


FIG. 6. Microcalorimetric recording of the difference heat capacity of 0.182% lysozyme solution against the solvent—40 m*M* glycine buffer, pH 2.5. The baseline was obtained on filling both cells with the solvent.

with known heat capacity, since absolute heat capacity has never been determined with the accuracy required for the calibration of scanning microcalorimeters. Therefore, it can be calibrated only electrically by applying some definite power, δW, to one of the cells, which imitates the heat capacity change of this cell. The apparent heat capacity change will be equal to $\delta C_p = \delta W/(dT/dt) = \delta W(dt/dT)$, where dT/dt is the heating rate and dt/dT is the time required for heating the cell by one degree. Dividing δC_p by the observed deviation of the recording from the baseline δl, we obtain the heat capacity value of a unit displacement on heat capacity versus temperature record, $(\delta W/\delta l)\ (dt/dT)$.

If the calibration power was applied for a duration, δt, the energy released in the cell is $\delta E = \delta W \delta t$ and it corresponds to the δS area of the calibration mark. Therefore $\delta W \delta T/\delta S$ will be the energy value of the square unit on the record.

Determination of Protein Partial Heat Capacity

The difference in heat capacity between a solution of biological macromolecules and a solvent measured by scanning microcalorimetry is al-

ways negative, i.e., the heat capacity of the solution is smaller than the heat capacity of the same volume of solvent (Fig. 6). It follows that the heat capacity of macromolecules in solution is smaller than that of the same volume of the solvent. For the observed difference in heat capacity between the solution and the solvent we have

$$\Delta C_p^{app}(T)_{pr,sol/solv} = C_p(T)_{pr} m(T)_{pr} - C_p(T)_{solv} \Delta m(T)_{solv} \quad (4)$$

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 $m(T)_{solv}$ is the mass of the solvent displaced by proteins in solution. The latter equals

$$\Delta m(T)_{solv} = m(T)_{pr} \frac{V(T)_{pr}}{V(T)_{solv}} \quad (5)$$

Here $V(T)_{pr}$ is the partial specific volume of protein at temperature T and $V(T)_{solv}$ is that of the solvent. From Eqs. (4) and (5) we obtain the partial specific heat capacity of protein:

$$C_p(T)_{pr} = C_p(T)_{solv} \frac{V(T)_{pr}}{V(T)_{solv}} - \frac{\Delta C_p^{app}(T)_{pr,sol/solv}}{m(T)_{pr}} \quad (6)$$

It should be noted that the heat capacity of protein determined by the above equation is not strictly the partial heat capacity since the latter is the value which 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 less than 10^{-4} M and this solution does not show any concentration dependence of the heat capacity it becomes evident that we can consider the value determined by Eq. (6) as the partial specific heat capacity of the protein in solution.

Since partial specific volumes of proteins and solvents do not change significantly with temperature, we can consider their ratio $V(T)_{pr}/V(T)_{sol}$ as temperature independent in the first approximation. As for the second term in Eq. (6), it also does not depend significantly on temperature because thermal expansion leads not only to a decrease of the mass of protein in the calorimetric cell (m_{pr}) but also to an increase of the sensitivity of the instrument as a result of the cell heat capacity decrease and these two effects greatly compensate each other. Therefore, if high precision is not required in the partial specific heat capacity determination one can use the values for the specific volumes of protein and solvent and for the mass of protein in the cell which had been estimated for 25°. For many proteins and solvents, the values of the specific volumes at 25° are tabu-

lated and can be found in handbooks. Specific volumes for proteins can be also calculated with quite reasonable accuracy from the known specific volumes of the amino acid residues.

If it is necessary to determine the partial specific heat capacity of a protein with a greater accuracy and over a larger temperature range, the specific volumes of the protein and the solvent should be determined experimentally in the same temperature range using precise densimeters, e.g., the digital vibrational densimeter DMA 02 (Anton Paar, Graz). Specific volumes must be determined in heat capacity studies of phospholipids, since their partial specific volumes change significantly with temperature variation.

In determining the partial specific heat capacity of macromolecules in solution with high accuracy, it is necessary to use precise data on the heat capacity of the solvent, as well as to take into account the change of the mass of macromolecules in the calorimetric cell due to thermal expansion of the solution and the cell.

The specific heat capacity of the solvent can be determined over the desired temperature range with the required accuracy using the same scanning microcalorimeter. To do this, we have to measure the difference heat capacity of the considered solvent in relation to distilled water:

$$\Delta C_p^{app}(T)_{solv/H_2O} = C_p(T)_{H_2O} m(T)_{H_2O} - C_p(T)_{solv} m(T)_{solv}$$

$$= C_p(T)_{H_2O} \frac{v(T)}{V(T)_{H_2O}} - C_p(T)_{solv} \frac{v(T)}{V(T)_{solv}} \qquad (7)$$

where $v(T)$ is the operational volume of a calorimetric cell at temperature T and $V(T)_{H_2O}$ and $V(T)_{sol}$ are the specific volumes of water and solvent at this temperature. From Eq. (7) we have

$$\frac{C_p(T)_{sol}}{V(T)_{sol}} = \frac{C_p(T)_{H_2O}}{V(T)_{H_2O}} - \frac{\Delta C_p^{app}(T)_{sol/H_2O}}{v(T)} \qquad (8)$$

and substituting it in Eq. (6) we get

$$C_p(T)_{pr} = \left[\frac{C_p(T)_{H_2O}}{V(T)_{H_2O}} - \frac{\Delta C_p^{app}(T)_{solv/H_2O}}{v(T)} \right] V(T)_{pr} - \frac{\Delta C_p^{app}(T)_{pr,sol/solv}}{m(T)_{pr}} \qquad (9)$$

The specific volume and the heat capacity of pure water (V_{H_2O} and C_{p,H_2O}) are known with high accuracy over the entire temperature range of its existence in the liquid phase and can be found in handbooks. As for the operational volume of a calorimetric cell $v(T)$ and the mass of protein in the cell $m(T)_{pr}$ at temperature T, they can be calculated from their values at room temperature T_0 and known coefficients of thermal expansion of the solution and the cell material. Bearing in mind that

$$m(T)_{pr} = v(T)\rho(T)_{pr} \qquad (10)$$

where $\rho(T)_{pr}$ is the protein concentration in solution at temperature T, we have

$$\rho(T)_{pr} = \rho(T_0)_{pr} \Big/ \left[1 + \frac{1}{V(T_0)_{solv}} \frac{dV_{sol}}{dT} (T - T_0)\right] \tag{11}$$

$$v(T) \simeq v(T_0)[1 + \beta(T - T_0)] \simeq v(T_0)[1 + 3\alpha(T - T_0)] \tag{12}$$

In Eq. (11) the specific volume of the solution is approximated by the specific volume of the solvent since they do not differ significantly for dilute solutions. In Eq. (12) the volumetric thermal expansion coefficient β is replaced by the linear thermal expansion coefficient α which is equal to 14.3×10^{-6} for gold.

Finally, we obtain the following equation for the partial specific heat capacity of protein in solution

$$C_p(T)_{pr} = \left[\frac{C_p(T)_{H_2O}}{V(T)_{H_2O}} - \frac{\Delta C_p^{app}(T)_{solv/H_2O}}{v(T)}\right] V(T)_{pr} - \frac{\Delta C_p^{app}(T)_{pr/sol}}{\rho(T_0)_{pr} v(T_0) \left[1 + \left(3\alpha - \frac{1}{V(T_0)_{sol}} \frac{dV_{sol}}{dT}\right)(T - T_0)\right]} \tag{13}$$

It should be noted that the difference heat capacity of solvent/water could be much larger than the difference heat capacity of protein solution/solvent, since a protein solution is dilute while the solvent can be a quite concentrated solution of a low-molecular-weight compound (salts, alcohol, urea, etc.). Therefore the difference heat capacity of the solvent is usually measured at low sensitivity of the scanning microcalorimeter.

The operational volume of a calorimetric cell v(T) is determined by measuring the difference heat capacity of two liquids with different known specific heat capacities, or just by measuring the heat capacity difference of an empty cell and the cell filled with water.

Changes of Partial Heat Capacity and Enthalpy in Temperature-Induced Changes of a Protein

The partial specific heat capacity of native proteins at 25° varies over the range of 1.2 to 2.3 J K^{-1} g^{-1} for various proteins and increases linearly with a temperature increase with a slope of the order of 10^{-3} J K^{-1} g^{-1}. The heat capacity of a protein in the denatured state is significantly higher than that in the native state (Fig. 7). For various proteins this difference is from 0.30 to 0.70 J K^{-1} g^{-1} and seems to be independent of temperature (for details see Ref. 15). In nucleic acids, the heat capacities of the native

[15] P. L. Privalov, *Adv. Protein Chem.* **33,** 179 (1979).
[16] V. V. Filimonov and P. L. Privalov, *J. Mol. Biol.* **122,** 465 (1978).

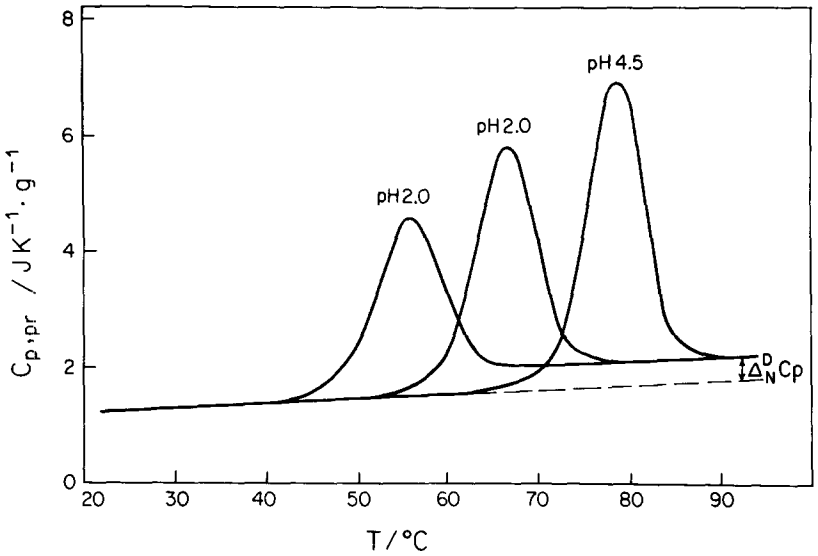


FIG. 7. Partial specific heat capacity of lysozyme in solutions with different pH values.

and denatured states do not differ noticeably, in any case this difference is not greater than 0.06 J K^{-1} g^{-1}.[16]

Since the pure native and denatured states of a protein in any given conditions are realized at different temperatures, it is evident that the heat capacity difference of these states can be estimated either by extrapolation of their heat capacity values to the considered temperature, or by varying solvent conditions if they do not affect the heat capacity of the protein as is true in the case of pH variation (Fig. 7).

If the heat capacity of the protein in the native state is known over a quite extended temperature range, we can calculate the so-called "excess" heat capacity of the protein by extrapolating it to the range in which it cannot be determined by direct measurements. The "excess" heat capacity of the protein relative to some state, considered as its "zero" state, is the difference between the heat capacity of the protein and the heat capcity of this "zero" state at the considered temperature T

$$\langle \Delta C_p(T) \rangle = C_p(T)_{pr} - C_{p,0}(T)_{pr} \tag{14}$$

Correspondingly for the excess enthalpy of protein we have

$$\langle \Delta H(T) \rangle = \int_0^T \langle \Delta C_p(T) \rangle dT \tag{15}$$

Although in the above equation the integration is from absolute zero temperature, in practice it is integrated from some temperature T_0 from

which the heat capacity of the protein is distinguished from the heat capacity of the protein in the zero state.

The importance of the excess heat capacity and the excess enthalpy functions follows from the fact that only a part of the observed heat effect, which is accounted by these functions, is related to changes of the macroscopic states of a system. Therefore, these functions actually include all the thermodynamic information on the states realized in the considered temperature range. This is expressed by a very general relation of statistical thermodynamics

$$\langle \Delta H(T) \rangle = RT^2 \frac{d \ln Q}{dT} \tag{16}$$

where $Q = \Sigma K_i$ is the partition function of a system and K_i is the probability of all accessible states.[17,18] Thus, for the partition function of a protein in solution we have

$$Q = \exp\left(\int_0^T \frac{\langle \Delta H(T) \rangle}{RT^2}\, dT\right) = \exp\left\{\int_{T_0}^T \frac{1}{RT^2}\left[\int_{T_0}^T \langle \Delta C_p(T) \rangle dT\right] dT\right\} \tag{17}$$

Thermodynamic Analysis of Calorimetric Data

Transition between Two States

The simplest temperature-induced process for macromolecules is the transition between two thermodynamically stable states—the native (N) and denatured (D) ones. A general scheme of this process for a biological macromolecule, not necessarily consisting of a single polymer chain, can be presented as

$$\mathrm{N} \rightleftharpoons m_1\mathrm{D}_1 + m_2\mathrm{D}_2 + \cdots + m_k\mathrm{D}_k$$

assuming that the transition results in the disintegration of the macromolecule into k different subunits (chains) each present in m_i copies. Designating the initial and final states by indices 0 and 1, we have for the equilibrium constant

$$K = \exp\left(-\frac{\Delta_0^1 G}{RT}\right) = \exp\left(\frac{\Delta_0^1 S}{R} - \frac{\Delta_0^1 H}{RT}\right) \tag{18}$$

Let F be the fraction of molecules that have undergone the transition at a given temperature T, and $[\mathrm{N}]_0$ be the initial concentration of the preparation in the native state. Then, the concentration of molecules in

[17] E. Freire and R. L. Biltonen, *Biopolymers* **17**, 463 (1978).
[18] R. L. Biltonen and E. Freire, *Crit. Rev. Biochem.* **5**, 85 (1978).

the native state can be presented as

$$[\mathrm{N}] = [\mathrm{N}]_0(1 - F) \tag{19}$$

The concentration of each subunit in the denatured state will be

$$[\mathrm{D}_i] = m_i[N]_0F \tag{20}$$

and for the equilibrium constant we will have

$$K = \frac{\prod_{i=1}^{k} [\mathrm{D}_i]^{m_i}}{[\mathrm{N}]} = \frac{\prod_{i-1}^{k} (m_i)^{m_i}[\mathrm{N}]_0^{m_i}F^{m_i}}{[\mathrm{N}]_0(1 - F)} \tag{21}$$

or

$$K = \frac{[\mathrm{N}]_0^{n-1}F^n}{1 - F} \prod_{i=1}^{k} (m_i)^{m_i} \tag{22}$$

where $n = \sum_{i=1}^{k} m_i$ is the order of the reaction. Thus, the equilibrium constants of reactions of identical order and various stoichiometries differ only by a constant multiplier. Consequently, the temperature derivatives of the equilibrium constant logarithm will be indistinguishable for such reactions

$$\frac{\partial \ln K}{\partial T} = \frac{\Delta_0^1 H}{RT^2} = (n/F)\frac{dF}{dT} + \frac{1}{1 - F}\frac{dF}{dT} = \frac{n - F(n - 1)}{F(1 - F)}\frac{dF}{dT} \tag{23}$$

Hence we get for the temperature derivative of parameter F

$$\frac{dF}{dT} = \frac{\Delta_0^1 H}{RT^2}\frac{F(1 - F)}{n - F(n - 1)} \tag{24}$$

At the same time for the excess enthalpy of the considered system we have

$$\langle \Delta H \rangle = \Delta_0^1 HF \tag{25}$$

and for its excess heat capacity

$$\langle \Delta C_\mathrm{p} \rangle = \frac{d\langle \Delta H \rangle}{dT} = \frac{d(\Delta_0^1 HF)}{dT} = \Delta_0^1 H\frac{dF}{dT} + \Delta_0^1 C_\mathrm{p}F \tag{26}$$

Substituting Eq. (24) into Eq. (26) we obtain

$$\langle \Delta C_\mathrm{p} \rangle = \frac{(\Delta_0^1 H)^2}{RT^2}\frac{F(1 - F)}{n - F(n - 1)} + \Delta_0^1 C_\mathrm{p}F \tag{27}$$

By determining the temperature dependence of F from Eq. (22), one can calculate the excess heat capacity function for a reaction of any order.

For example, for a monomolecular reaction N $\rightleftharpoons$ D for which $n = 1$ and

$$K = \frac{F}{1 - F} \tag{28}$$

we have

$$\langle \Delta C_p \rangle = \frac{(\Delta_0^1 H)^2}{4RT^2} Ch^{-2}(\Delta_0^1 G/2RT) + \frac{\Delta_0^1 C_p}{2} \exp(-\Delta_0^1 G/2RT) Ch^{-1}(\Delta_0^1 G/2RT) \tag{29}$$

This is an analytical expression for the dependence of the excess heat capacity on temperature and the thermodynamic parameters of the transition for a monomolecular reaction.

By comparing the observed excess heat capacity of a macromolecule with the calculated one from this equation, it is possible to find out, using the best fit procedure, whether the studied process indeed represents a monomolecular two-state transition and to determine the enthalpy and entropy of transition which should also satisfy the two basic requirements

$$\frac{\partial \Delta_0^1 H}{\partial T} = \Delta_0^1 C_p \tag{30}$$

and

$$\frac{\partial \Delta_0^1 S}{\partial T} = \frac{\Delta_0^1 C_p}{T} \tag{31}$$

In practice, however, this can be determined by a much simpler analysis of the main characteristics of the heat capacity peak—the area, height, and temperature of the maximum.

Let us estimate the position of the heat capacity peak maximum and its value for an arbitrary process. Differentiating Eq. (27) with respect to temperature and bearing in mind that $(\partial \Delta_0^1 C_p / \partial T)$ is likely to be very small for biopolymers and, therefore, can be neglected,[15] we get

$$\frac{\partial \langle \Delta C_p \rangle}{\partial T} = \frac{F(1 - F)}{1 - kF} \frac{2\Delta_0^1 H(T\Delta_0^1 C_p - \Delta_0^1 H)}{nRT^3} + \frac{(\Delta_0^1 H)^3}{n^2 R^2 T^4} + \frac{F(1 - F)(1 - 2F + kF^2)}{(1 - kF)^3} + \Delta_0^1 C_p \frac{\Delta_0^1 H}{nRT^2} \frac{F(1 - F)}{1 - kF} \tag{32}$$

where $k = (n - 1)/n$. At the extremum this equation is equal to zero. Dividing the above equation by $(\Delta_0^1 H)/(nRT^2)[F(1 - F)]/(1 - kF) \neq 0$,

we get at the maximum

$$\frac{2(T\Delta_0^1C_p - \Delta_0^1H)}{T} + \frac{(\Delta_0^1H)^2}{nRT^2}\frac{1 - 2F + kF^2}{(1 - kF)^2} + \Delta_0^1C_p = 0$$

or

$$\frac{1 - 2F + kF^2}{(1 - kF)^2} = -\frac{nRT(3T\Delta_0^1C_p - 2\Delta_0^1H)}{(\Delta_0^1H)^2} = -nX \tag{33}$$

where

$$X = \frac{RT(3T\Delta_0^1C_p - 2\Delta_0^1H)}{(\Delta_0^1H)^2} \tag{34}$$

Thus, F_{max}, which corresponds to the maximum of the heat absorption peak, must satisfy the equation

$$F_{max}^2k - 2F_{max} + \frac{1 + nX}{1 + knX} = 0 \tag{35}$$

Since for a first-order reaction $k = 0$, we have two quite different cases for Eq. (35): it is linear for first-order reactions and quadratic for higher order reactions.

Let us consider the first case when

$$F_{max} = \frac{1 + X}{2}$$

and the maximal heat capacity is

$$\langle\Delta C_p\rangle_{max} = \frac{(\Delta_0^1H)^2}{4RT_{max}^2}(1 - X^2) + \frac{\Delta_0^1C_p}{2}(1 + X) \tag{36}$$

Equation (36) is exact and can be used for an exact estimation of the enthalpy of the process if the latter represents a "two-state" transition. However, since we can never be certain about the correctness of this assumption, the enthalpy value estimated by this equation based on the van't Hoff relations, is regarded as an effective one and is usually called the van't Hoff enthalpy to distinguish it from the real one measured calorimetrically. Equation (36) can be simplified if the parameter X is sufficiently small relative to unity. In this case, it can be written as

$$\frac{(\Delta_0^1H)^2}{4RT_{max}^2} = \langle\Delta C_p\rangle_{max} - \frac{\Delta_0^1C_p}{2} \tag{37}$$

and the value of the van't Hoff enthalpy of the process can be expressed either as

$$\Delta_0^1 H^{vh} = \frac{4RT_{max}^2(\langle \Delta C_p \rangle_{max} - \Delta_0^1 C_p/2)}{\Delta_0^1 H^{cal}} \tag{38}$$

or as

$$\Delta_0^1 H^{vh} = 2T_{max}\sqrt{R\left(\langle \Delta C_p \rangle_{max} - \frac{\Delta_0^1 C_p}{2}\right)} \tag{39}$$

The advantage of the first expression is that the value of the van't Hoff enthalpy $\Delta_0^1 H^{vh}$ in this case does not depend on the absolute values $\langle \Delta C_p \rangle_{max}$ and $\Delta_0^1 H^{cal}$, and, consequently, neither on possible errors in determining the concentration of the studied preparation nor on the calibration of the scanning calorimeter.

The applicability of the simplified Eqs. (37)–(39) can be verified by evaluating the parameter X. As a rule, $3T\Delta_0^1 C_p \gg 2\Delta_0^1 H$ for biological macromolecules. Hence for X we obtain an approximate value

$$X = \frac{3RT_{max}^2 \Delta_0^1 C_p}{(\Delta_0^1 H)^2} = \frac{4RT_{max}^2}{(\Delta_0^1 H)^2}\frac{3\Delta_0^1 C_p}{4} \approx \frac{3}{4}\frac{\Delta_0^1 C_p}{\langle \Delta C_p \rangle_{max} - \dfrac{\Delta_0^1 C_p}{2}} \tag{40}$$

Let us estimate the error in evaluating the van't Hoff enthalpy using Eq. (37) instead of (36) induced by the nonzero value of the parameter X. From Eq. (36) we have

$$\begin{aligned}
\frac{(\Delta_0^1 H)^2}{4RT_{max}^2} &= \frac{\langle \Delta C_p \rangle_{max} - (\Delta_0^1 C_p/2)(1 + X)}{1 - X^2} \\
&= \left(\langle \Delta C_p \rangle_{max} - \frac{\Delta_0^1 C_p}{2}\right)\left[1 - \frac{\Delta_0^1 C_p}{2(\langle \Delta C_p \rangle_{max} - \Delta_0^1 C_p/2)} X\right] \Big/ (1 - X^2) \\
&\approx \left[\left(1 - \frac{2}{3}X^2\right)\left(\langle \Delta C_p \rangle_{max} - \frac{\Delta_0^1 C_p}{2}\right)\right] \Big/ (1 - X^2) \\
&\approx (\langle \Delta C_p \rangle_{max} - \Delta_0^1 C_p/2)(1 + X^2/3)
\end{aligned} \tag{41}$$

Thus

$$\Delta_0^1 H^{vh} = \frac{4RT_{max}^2}{\Delta_0^1 H^{cal}}\left(\langle \Delta C_p \rangle_{max} - \frac{\Delta_0^1 C_p}{2}\right)(1 + X^2/3) \tag{42}$$

In order for the error in the estimate of the van't Hoff enthalpy not to exceed 1%, it is necessary that $X^2 \leq 3 \times 10^{-2}$, and that the heat capacity difference $\Delta_0^1 C_p$ should not exceed approximately 20% of the maximal heat capacity value.

In the case when the transition between two states is a reaction of an order higher than the first one, we have from Eq. (35) for the value of F_{max} at a heat capacity maximum

$$F_{max} = \left(1 - \sqrt{1 - \frac{1 + nX}{1 + knX}k}\right)\Big/k = \left(1 - \sqrt{\frac{1 - k}{1 + knX}}\right)\Big/k$$

$$= \frac{1 - \sqrt{1 - k}}{k} + \left(\sqrt{1 - k} - \sqrt{\frac{1 - k}{1 + knX}}\right)\Big/k$$

$$= \frac{1 - \sqrt{1 - k}}{k} + \frac{\sqrt{1 - k}}{k}\left[1 - \frac{1}{\sqrt{1 + knX}}\right]$$

$$= \frac{n - \sqrt{n}}{n - 1} + \frac{\sqrt{n}}{n - 1}\left[1 - \frac{1}{\sqrt{1 + (n - 1)X}}\right] \tag{43}$$

Taking into consideration the small value of the parameter X, we get from Eq. (43) by expanding it into a series with an accuracy of a quadratic term

$$F_{max} = \frac{n - \sqrt{n}}{n - 1} + \frac{1}{2\sqrt{n}}X - \frac{3(n - 1)}{8n\sqrt{n}}X^2 \tag{44}$$

It is easy to see that even at a very low value of the parameter X the F_{max} value corresponding to the maximal heat capacity differs significantly from 0.5. Indeed, for a bimolecular reaction this value will correspond to approximately 0.59, and in the case of a third-order reaction it will be approximately 0.63.

Substituting the value F_{max} [Eq. (44)] in Eq. (27), we obtain

$$\langle \Delta C_p \rangle_{max} = \frac{(\Delta_0^1 H)^2}{RT_{max}^2(\sqrt{n} + 1)^2} + \frac{\Delta_0^1 C_p \sqrt{n}}{\sqrt{n} + 1} + \frac{\Delta_0^1 C_p X}{2\sqrt{n}}$$

$$+ \left[\frac{7(\Delta_0^1 H)^2}{4RT_{max}^2} - \frac{3\Delta_0^1 C_p(n - 1)}{2}\right]\frac{X^2}{4n\sqrt{n}} \tag{45}$$

If X is sufficiently small, the van't Hoff enthalpy values are expressed as

$$\Delta_0^1 H^{vh} = \frac{(\sqrt{n} + 1)^2 RT_{max}^2\left(\langle \Delta C_p \rangle_{max} - \dfrac{\Delta_0^1 C_p \sqrt{n}}{\sqrt{n} + 1}\right)}{\Delta_0^1 H_{cal}} \tag{46}$$

or

$$\Delta_0^1 H^{vh} = (\sqrt{n} + 1)T_{max}\sqrt{R\left(\langle \Delta C_p \rangle_{max} - \frac{\Delta_0^1 C_p \sqrt{n}}{\sqrt{n} + 1}\right)} \tag{47}$$

The errors related to the deviation of X from zero using Eqs. (46) and (47) are of the same order of magnitude as those for the monomolecular reaction.

The value of the transition entropy can be calculated easily if the temperature corresponding to the heat absorption peak is known. In this case, for the first-order reaction when $F_{max} = (1 + X)/2$ we have

$$K_{max} = \frac{F_{max}}{1 - F_{max}} = \frac{1 + X}{1 - X} \approx 1 + 2X \tag{48}$$

$$\ln K_{max} = \ln(1 + 2X) \approx 2X \tag{49}$$

$$\Delta_0^1 G_{max} = - RT \ln K_{max} = - 2RT_{max}X \tag{50}$$

$$\Delta_0^1 S_{max} = \frac{\Delta_0^1 H_{max} - \Delta_0^1 G_{max}}{T_{max}} = \frac{\Delta_0^1 H_{max} + 2RT_{max}X}{T_{max}}$$

$$= \frac{\Delta_0^1 H_{max}}{T_{max}} + 2RX \tag{51}$$

If we take into account the real values of the parameters included in this expression it becomes evident that the transition entropy at peak temperature amounts to with good accuracy

$$\Delta_0^1 S_{max} = \Delta_0^1 H_{max}/T_{max} \tag{52}$$

For higher order reactions we have

$$K_{max} = \frac{[N]_0^{n-1} F_{max}^n}{1 - F_{max}} \prod_{i=1}^{k} (m_i)^{m_i} \tag{53}$$

$$\ln K_{max} = (n - 1) \ln[N]_0 + \sum_{i=1}^{k} m_i \ln m_i + \ln \frac{F_{max}^n}{1 - F_{max}}$$

$$\ln \frac{F_{max}^n}{1 - F_{max}} = \ln \left\{ \frac{\left(\frac{n - \sqrt{n}}{n - 1} + \frac{1}{2\sqrt{n}} X \right)^n}{1 - \frac{n - \sqrt{n}}{n - 1} - \frac{1}{2\sqrt{n}} X} \right\}$$

$$= \ln\left\{\frac{\left(\frac{n-\sqrt{n}}{n-1}\right)^n \left(1+\frac{n-1}{2\sqrt{n}\,(n-\sqrt{n})}X\right)^n}{\frac{1}{\sqrt{n}-1}-\frac{1}{2\sqrt{n}}X}\right\}$$

$$= \ln\left\{\frac{\left(1+\frac{\sqrt{n}-1}{2n}X\right)^n}{1-\frac{\sqrt{n}-1}{2\sqrt{n}}X}\right\} + \ln\left\{\left(\frac{\sqrt{n}}{\sqrt{n}-1}\right)^n (\sqrt{n}-1)\right\}$$

$$= \ln\left\{\left(1+\frac{\sqrt{n}-1}{2}X\right)\left(1+\frac{\sqrt{n}-1}{2\sqrt{n}}X\right)\right\} + \ln\left\{\frac{n^{n/2}}{(\sqrt{n}-1)^{n-1}}\right\}$$

$$\approx \ln\left(1+\frac{n-1}{2\sqrt{n}}X\right) + \ln\left\{\frac{n^{n/2}}{(\sqrt{n}-1)^{n-1}}\right\}$$

$$\approx \ln\frac{n^{n/2}}{(\sqrt{n}-1)^{n-1}} + \frac{n-1}{2\sqrt{n}}X \tag{54}$$

$$\ln K_{\max} = \frac{\Delta_0^1 G_{\max}}{RT_{\max}} = \ln\frac{[\mathrm{N}]_0^{n-1}\prod_{i=1}^{k}(m_i)^{m_i}n^{n/2}}{(\sqrt{n}-1)^{n-1}} + \frac{n-1}{2\sqrt{n}}X \tag{55}$$

$$\Delta_0^1 S_{\max} = \frac{\Delta_0^1 H_{\max} - \Delta_0^1 G_{\max}}{T_{\max}} = \frac{\Delta_0^1 H_{\max}}{T_{\max}}$$

$$+ R\ln\left\{\frac{[\mathrm{N}]_0^{n-1}\prod_{i=1}^{i}(m_i)^{m_i}n^{n/2}}{(n-1)^{n-1}}\right\} + RX\frac{n-1}{2\sqrt{n}} \tag{56}$$

The ratio $\Delta_0^1 H^{\mathrm{cal}}/\Delta_0^1 H^{\mathrm{vh}}$ can be considered as a measure of the validity of the assumption that the studied process is a two-state transition. For heat denaturation of small compact globular proteins, this ratio is usually very close to 1.0.[15] The deviation from 1.0 in most cases does not exceed 5%. This means that the population of all intermediate states between the native and denatured ones of these proteins is rather low, and denaturation can be considered in the first approximation as an "all-or-none" process.

However, for the denaturation of large proteins, and in some cases even of not very large ones, the ratio $\Delta_0^1 H^{\mathrm{cal}}/\Delta_0^1 H^{\mathrm{vh}}$ exceeds significantly 1.0, which indicates that the process of disruption of their native structure

is not of an "all-or-none" character even in the first approximation, and that in this process several intermediate states are realized.[19]

It should be noted that this ratio can also be smaller than 1.0, but this can occur only in two cases: when the molecular weight of a cooperative unit is determined incorrectly and when the studied process is irreversible.

The first case is found frequently for molecules which exist in solution as dimers and not as monomers, and which denature in the dimeric form. For a reversible process, a value of the ratio $\Delta_0^l H^{cal}/\Delta_0^l H^{vh}$ smaller than 1.0 can be considered as an indisputable argument in favor of the existence of a specific complex of several molecules.

Influence of Irreversibility on the Value of the $\Delta_0^l H^{cal}/\Delta_0^l H^{vh}$ Ratio

The problem of irreversibility of a process and its influence on the estimated thermodynamic characteristics is rather complicated. In principle, a quantitative thermodynamic analysis is based on equilibrium studies. Nevertheless, in some cases, one can use this analysis for studying processes which are practically irreversible. If the process of protein unfolding is irreversible due to the metastability of the initial state of a molecule, then the equations of equilibrium thermodynamics are inapplicable for its analysis.

However, the observed irreversibility of protein unfolding can be caused also by some ancillary concomitant processes occurring significantly slower and with a significantly smaller enthalpy than the gross conformational changes. For example, this may be the process of proline isomerization, which is rather slow and is practically not accompanied by any enthalpy change, but which hinders sterically the refolding of the polypeptide chain into a compact native structure. This may be also the aggregation of unfolded protein molecules which proceeds much more slowly than the unfolding of the native structure; it depends greatly on concentration and ionic conditions and blocks the refolding of the polypeptide chain into a compact native structure.

Thus, the process of irreversible denaturation can be presented schematically as

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k_3} mD$$

where N is the initial native state, U is the unfolded state, and D is the aggregated state. The reaction U → mD results in a decrease of the

[19] P. L. Privalov, *Adv. Protein Chem.* **35**, 1 (1982).

concentration of active molecules participating in the equilibrium $N \rightleftharpoons U$, and the rate of decrease of the active concentration increases with an increase in temperature. This will lead to a sharpening of the observed heat absorption peak and, hence, to an increase of the apparent van't Hoff enthalpy of the transition and to a decrease of the $\Delta_0^1 H^{cal}/\Delta_0^1 H^{vh}$ ratio. It is clear that this change in the van't Hoff enthalpy value is more significant the slower the heating rate; if the reaction $U \rightarrow D$ is not a monomolecular one, the change in the van't Hoff enthalpy will grow with an increase of the sample concentration. Therefore, the influence of irreversibility can be decreased by increasing the heating rate and decreasing the concentration of the studied protein solution.

Since the process of unfolding of the compact protein structure $N \rightarrow U$ is rather fast ($\sim 10^{-3}$ sec^{-1}), the value of $\Delta_0^1 H^{vh}$ obtained upon extrapolation to infinite heating rate and zero concentration should be very close to the real values that could be obtained for the first stage of the process in the absence of the second irreversible stage. This was demonstrated in our laboratory by a comparison of the data obtained on the same protein at different pH values, namely at pH values at which denaturation is completely reversible and, in turn, irreversible.

Analysis of Complex Processes

Under a complex process we mean these cases in which a temperature-induced transformation of a macromolecule proceeds through a number of intermediate macroscopic states. The complex nature of a process can be noticed by the appearance of additional extrema and bends on the melting curve and by the deviation of the calorimetrically measured total melting enthalpy from the effective one estimated by the van't Hoff equation. The main goal of complex process analysis is the determination of its reaction scheme and the thermodynamic description of all the macroscopic states which are realized in the process. A general case, in which the stages of a complex multistage process are not necessarily first-order reactions, is too complicated to be considered here, especially since real cases are usually much simpler: most of their stages are, as a rule, first-order monomolecular reactions. Therefore, here we shall limit our consideration to multistage monomolecular reactions.

It should be noted that the reactions representing a release of ligands can be considered also as monomolecular ones if the concentration of free ligands does not change considerably during the experiments, i.e., if there are free ligands in large excess or if the solution is correspondingly buffered.

Thermodynamic Description of Multistage Processes

Let us assume that a temperature increase induces the macromolecule to pass through distinct macroscopic states. Arranging all these states according to increasing enthalpy values, we can formally describe the equilibrium existing between these states by the following sequential scheme

$$I_0 \underset{}{\overset{K_{0,1}}{\rightleftharpoons}} I_1 \overset{K_{1,2}}{\rightleftharpoons} I_2 \cdots I_{n-1} \overset{K_{n-1,n}}{\rightleftharpoons} I_n$$

Denoting the equilibrium constant between the states i and j by $K_{i,j}$, we can write the following thermodynamic relations for this system

$$K_{0,i} = \prod_{j=1}^{i} K_{j-1,j} \tag{57}$$

$$\Delta_0^i G(T) = G_i(T) - G_0(T) = \sum_{j=1}^{i} \Delta_{j-1}^j G(T) \tag{58}$$

$$\Delta_0^i H(T) = H_i(T) - H_0(T) = \sum_{j=1}^{i} \Delta_{j-1}^j H(T) \tag{59}$$

$$\Delta_0^i S(T) = S_i(T) - H_0(T) = \sum_{j=1}^{i} \Delta_{j-1}^j S(T) \tag{60}$$

$$\Delta_0^i C_p(T) = C_{p,i}(T) - C_{p,0}(T) = \sum_{j=1}^{i} \Delta_{j-1}^j C_p(T) \tag{61}$$

The partition function of this system is

$$Q = \sum_{i=0}^{n} K_{0,i} = \sum_{i-0}^{n} \exp(-\Delta_0^i G/RT) \tag{62}$$

and the population of states is

$$F_i = K_{0,i}/Q = \frac{\exp(-\Delta_0^i G/RT)}{Q} \tag{63}$$

The mean excess enthalpy of the system in relation to the initial state can be presented as

$$\langle \Delta H(T) \rangle = \sum_{i=1}^{n} \Delta_0^i H F_i$$

$$= \left[\sum_{i=1}^{n} \Delta_0^i H_{\exp} \left(-\frac{\Delta_0^i H}{RT} + \frac{\Delta_0^i S}{R}\right)\right] \Big/ \left[\sum_{i=1}^{n} \exp\left(-\frac{\Delta_0^i H}{RT} + \frac{\Delta_0^i S}{R}\right)\right] \tag{64}$$

and the excess heat capacity as

$$\langle \Delta C_p(T) \rangle = \frac{d\langle \Delta H \rangle}{dT} = \sum_{i=1}^{n} \Delta_0^i H \frac{dF_i}{dT} + \sum_{i=1}^{n} \Delta_0^i C_p F_i$$

$$= \frac{\langle \Delta H^2 \rangle - \langle \Delta H \rangle^2}{RT^2} + \sum_{i=1}^{n} \Delta_0^i C_p F_i = \langle \delta C_p^{exc}(T) \rangle + \langle \delta C_p^{int}(T) \rangle \quad (65)$$

where

$$\langle \Delta H^2 \rangle = \sum_{i=1}^{n} (\Delta_0^i H)^2 F_i \quad (66)$$

is the mean value of the enthalpy squared.

Our task is to find the number of realizable states and to determine their thermodynamic parameters in such a way that the experimental curve would coincide with the calculated one within the accuracy of the experiment. Since each of the states is specified by three thermodynamic parameters, $(\Delta_0^i H, \Delta_0^i S, \Delta_0^i C_p)$, this task actually comes down to the minimization of the following function

$$\phi(\Delta_0^i H, \Delta_0^i S, \Delta_0^i C_p, n) = \int_0^\infty [\langle \Delta C_p(T) \rangle_{theor} - \langle \Delta C_p(T) \rangle_{exp}]^2 dT \quad (67)$$

which depends on $(3n + 1)$ parameters. In the analysis of calorimetric data, the dependence of the transition enthalpy on temperature is usually neglected, since it is weak and does not lead to significant distortion of the $\langle \delta C_p^{exc}(T) \rangle$ term in Eq. (65). However, the second term in this equation, $\langle \delta C_p^{int}(T) \rangle$, should be taken into account and subtracted from $\langle \Delta C_p(T) \rangle$.

The simplest is to approximate $\langle \delta C_p^{int}(T) \rangle$ by a straight line connecting the beginning and the end of the melting curve (Fig. 8). Such an approximation works well in the case of processes which are extended over a broad temperature and which consist of many overlapping broad transitions. A more accurate approximation can be achieved by assuming that the changes of specific enthalpy and specific heat capacity are proportional for all transitions:

$$\frac{\Delta_0^i C_p}{\Delta_0^i H} = \kappa = \frac{\Delta C_p^{tot}}{\Delta H^{tot}}$$

where ΔC_p^{tot} is the heat capacity difference of the final and initial states and ΔH^{tot} is the total denaturation enthalpy. In this case we get the

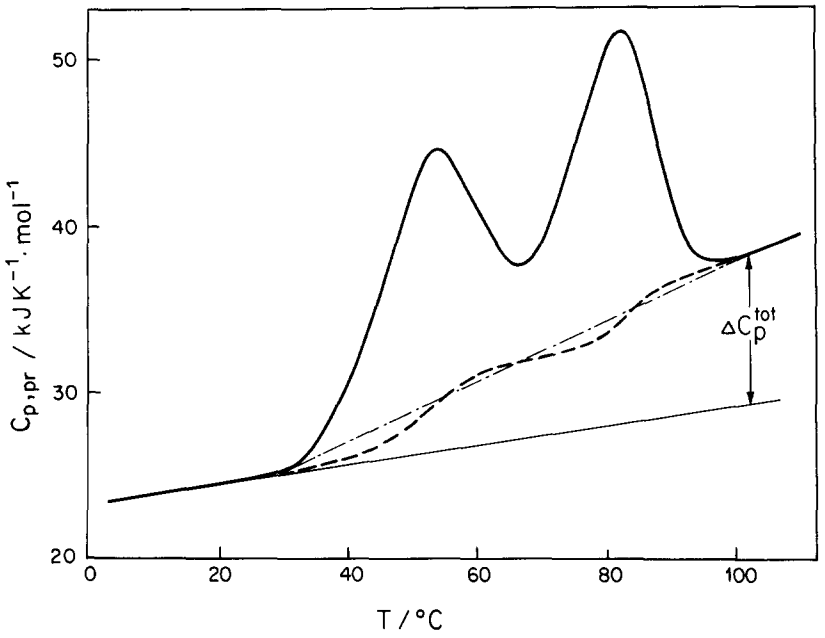


FIG. 8. Evaluation of δC_p^{int} in the transition temperature range, using as example troponin C (10 m*M* cacodylate buffer, pH 7.25, 10 m*M* EDTA, 9.5 m*M* $CaCl_2$) according to Tsalkova and Privalov.[14] Dot-dash line, first approximation of $\delta C_p^{int}(T)$; dashed line, final approximation of $\delta C_p^{int}(T)$ by Eq. (68).

expression

$$\langle \delta C_p^{int}(T) \rangle = \sum_{i=1}^{n} \Delta_0^i C_p F_i = \kappa \sum_{i=1}^{n} \Delta_0^i H F_i = \kappa \langle \Delta H(T) \rangle$$

$$= (\Delta C_p^{tot} / \Delta H^{tot}) \int_0^T \langle \delta C_p^{exc}(\tau) \rangle d\tau \qquad (68)$$

which relates $\langle \delta C_p^{int}(T) \rangle$ to $\langle \delta C_p^{exc}(T) \rangle$. Although this relation is only an approximate one, the error in the $\langle \delta C_p^{exc} \rangle$ value introduced by such an approximation of $\langle \delta C_p^{int} \rangle$ is not great, since $\langle \delta C_p^{exc}(T) \rangle$ is much larger than $\langle \delta C_p^{int}(T) \rangle$.

The determination of $\langle \delta C_p^{int} \rangle$ and its subtraction from $\langle \Delta C_p \rangle$ can be performed in the following way. The straight line connecting the beginning and the end of the excess heat capacity is taken as a zero aproximation of $\langle \delta C_p^{int} \rangle'$. Then $\langle \delta C_p^{exc} \rangle = \langle \Delta C_p \rangle - \langle \delta C_p^{int} \rangle'$ is integrated, and the complete integral is accepted to be ΔH^{tot}. The value of ΔC_p^{tot} is known; it

is equal to the difference between the heat capacities of the initial and final states; hence the coefficient κ is determined and the more accurate function $\langle \delta C_p^{int} \rangle''$ can be calculated by Eq. (68). The procedure should be repeated until the values of $\langle \delta C_p^{int} \rangle$ and $\langle \delta C_p^{exc} \rangle$ do not change. The obtained value of $\langle \delta C_p^{exc} \rangle$ is used for further analysis. Thus, we eliminate the parameters $\Delta_0^i C_p$ in Eq. (67) and decrease to $(2n + 1)$ the number of parameters which have to be found.

In using the best fit procedure for this analysis of calorimetric data one should bear in mind that the approximation of the experimental heat capacity curve by the calculated curve improves with an increase in the number of considered states, but this improvement significantly slows down after some definite number of states. This takes place due to a too low population of states added (for details see Ref. 20). Therefore, the states above this critical number can be neglected.

The best fit procedure for approximation of calorimetric data can be simplified significantly in the case when the considered process can be represented as the sum of independent transitions and the system can be considered as consisting of independent subsystems. In this case, the partition function of the system can be represented as the product of the subsystem partition functions

$$Q = \prod_{i=1}^{n} Q_i = \prod_{i=1}^{n} (1 + K_i) \tag{69}$$

The enthalpy and heat capacity of such a system is equal to the sum of the enthalpies and heat capacities of each of the subsystems

$$\langle \Delta H(T) \rangle = RT^2 \frac{\partial \ln Q}{\partial T} = RT^2 \frac{\partial \ln \left(\prod_{i=1}^{n} Q_i \right)}{\partial T}$$

$$= RT^2 \sum_{i=1}^{n} \frac{\partial \ln Q_i}{\partial T} = \sum_{i=1}^{n} \langle \Delta H_i(T) \rangle \tag{70}$$

$$\langle \Delta C_p(T) \rangle = \frac{\partial \langle \Delta H(T) \rangle}{\partial T} = \sum_{i=1}^{n} \langle \Delta C_{pi}(T) \rangle \tag{71}$$

while the heat capacity of each subsystem is described by Eq. (27) for a "two-state" transition. The model of independent subsystems was efficiently used, for example, in studying the melting of transfer RNA.[21]

[20] S. J. Gill, B. Richey, G. Bishop, and J. Wyman, *Biophys. Chem.* **21,** 1 (1985).
[21] P. L. Privalov and V. V. Filimonov, *J. Mol. Biol.* **122,** 447 (1978).

Recommendations for the Best Fit Procedure

What has been said can be summarized by the following recommendations. Determine the excess heat capacity function. Extract from it the effect of the intrinsic heat capacity change $\langle \delta C_p^{int} \rangle$. Minimize the function [Eq. (67)], assuming the most probable number of states. If the experimental curve cannot be approximated by the calculated one, increase the number of states. If the shape of the calculated curve is close to the experimental one, eliminate the states with a low maximal population and repeat the minimization. Obtain the minimal number of well-populated states required for a description of the melting curve. One should bear in mind that the lower the level of the maximal population in the given state, the higher is the error in determining its thermodynamic parameters.

For minimization of function [Eq. (67)] one can use practically any algorithm. However, it should be noted that, since the dependence of this function on variables is of a higher order than quadratic, a change of the second derivatives of this function is significant. Therefore, methods suggesting constant values of second derivatives will give satisfactory results only near the minimum. In regions remote from the minimum the method of steepest descent can be used.

Sequential Analysis of the Excess Heat Capacity Function

An alternative approach to the analysis of the excess heat capacity function was suggested by Freire and Biltonen[17,18] in 1978. The starting point in this approach is that, at sufficiently low temperature, only the first transition contributes noticeably to the observed excess heat capacity. The thermodynamic parameters of the first transition can be determined by using the following function

$$\varphi_1 = \frac{\langle \Delta H \rangle}{1 - F_0} = \left(\sum_{i=1}^{n} \Delta_0^i H F_i \right) \Big/ \left(\sum_{i-1}^{n} F_i \right) \tag{72}$$

For a system which has only two states, this function comes down to $\Delta_0^1 H$, while, for a more complicated system, it is equal to

$$\varphi_1 = \Delta_0^1 H + \frac{\sum_{i=1}^{n} (\Delta_0^i H - \Delta_0^1 H) F_i}{\sum_{i-1}^{n} F_i} = \Delta_0^1 H + \frac{\sum_{i=2}^{n} \Delta_1^i H F_i / F_1}{1 + \sum_{i=2}^{n} F_i / F_1}$$

$$= \Delta_0^1 H + \frac{\sum_{i=2}^{n} \Delta_1^i H \exp(-\Delta_1^i G / RT)}{1 + \sum_{i=2}^{n} \exp(-\Delta_1^i G / RT)} = \Delta_0^1 H + \langle \Delta H \rangle_1 \tag{73}$$

The second term of this function is nothing else than an averaged excess enthalpy for the system consisting of $(n - 1)$ states, and the initial one is considered here as state 1, but not zero. At rather low temperatures at which only the zero state and state 1 are noticeably populated, $\langle \Delta H \rangle_1 = 0$ and φ_1 approaches $\Delta_0^1 H$. Bearing also in mind that $F_0 = 1/Q$ and Q is determined by the excess enthalpy function according to Eq. (17), we get

$$\lim_{T \to 0} \frac{\langle \Delta H \rangle}{1 - F_0} = \lim_{T \to 0} \frac{\langle \Delta H \rangle}{1 - \exp\left(- \int_0^T \frac{\langle \Delta H \rangle}{RT^2} \, dT \right)} = \Delta_0^1 H \tag{74}$$

The partition function of the new subsystem consisting of $(n - 1)$ states can be estimated from the enthalpy of this subsystem

$$\langle \Delta H \rangle_1 = \varphi_1 - \Delta_0^1 H \tag{75}$$

$$Q_1 = \exp\left(\int_0^T \frac{\langle \Delta H \rangle_1}{RT^2} \, dT \right) \tag{76}$$

The enthalpy of the second transition can be obtained in the same way as that of the first transition using the function

$$\varphi_2 = \frac{\langle \Delta H \rangle_1}{1 - Q_1^{-1}} = \Delta_1^2 H + \frac{\sum_{i=3}^{n} \Delta_2^i H \exp(-\Delta_2^i G/RT)}{1 + \sum_{i=3}^{n} \exp(-\Delta_2^i G/RT)} = \Delta_1^2 H + \langle \Delta H \rangle_2 \tag{77}$$

for which

$$\lim_{T \to 0} \varphi_2 = \Delta_1^2 H$$

and

$$\langle \Delta H \rangle_2 = \varphi_2 - \Delta_1^2 H$$

Repeating the above procedure and using recurrent relations

$$\varphi_{i+1} = \frac{\langle \Delta H \rangle_i}{1 - Q_i^{-1}} \tag{78}$$

$$Q_i(T) = \sum_{j=i}^{n} \exp(-\Delta_i^j G/RT) = \exp\left(\int_0^T \frac{\langle \Delta H \rangle_i}{RT^2} \, dT \right) \tag{79}$$

$$\langle \Delta H \rangle_i = \varphi_i - \Delta_{i-1}^i H \tag{80}$$

$$\lim_{T \to 0} \varphi_i = \Delta_{i-1}^i H \tag{81}$$

one can obtain the thermodynamic parameters of all the realizable states. The equilibrium constant for these states, as is clear from Eq. (79), is

$$K_{i-1,i} = \frac{Q_{i-1} - 1}{Q_i} \tag{82}$$

and the population of the ith state, consequently, will be

$$F_i = F_{i-1}K_{i-1,i} = F_{i-1}\frac{Q_{i-1} - 1}{Q_i} \tag{83}$$

The thermodynamic parameters of transitions between the $(i - 1)$th and ith states can be obtained from the condition of equality of their population. Assuming under $T_{t,i}$ a temperature at which $F_{i-1} = F_i$ and the equilibrium constant $K_{i-1,i}$ becomes unity, we can determine $T_{t,i}$ from the condition

$$Q_{i-1}(T_t) - 1 = Q_i(T_t) \tag{84}$$

On the other hand, since

$$K_{i-1,i} = \exp(-\Delta^i_{i-1}H/RT_{t,i} + \Delta^i_{i-1}S/R) = 1$$

we have for the entropy of this transition

$$\Delta^i_{i-1}S = \frac{\Delta^i_{i-1}H}{T_{t,i}} \tag{85}$$

This algorithm is mathematically strict and, as has been shown by Freire and Biltonen, gives the single solution if the function $\langle \Delta C_p \rangle$ and, consequently, $\langle \Delta H \rangle$ are known over the entire temperature range from absolute zero up to temperatures at which the last transition is completed. However in reality the $\langle \Delta C_p \rangle$ value is not known from absolute zero temperature and is estimated with some error.

Actually the $\langle \Delta C_p \rangle$ function is determined only from some temperature T_0 at which the difference $(C_p - C_{p,0})$ begins to exceed the experimental error. Therefore the value of the experimentally determined $\langle \Delta H \rangle_0$ function equals zero at T_0, whereas Q_0 and F_0 are equal to unity. Correspondingly, φ_1 at this point cannot be determined since it tends to $+\infty$ at $T \rightarrow T_0$ (Fig. 9). Therefore the function φ_1 approaches the first transition enthalpy value with a reasonable accuracy (of about 7% error) only at

$$T = T_0 + \frac{4RT^2_{t,1}}{\Delta^1_0 H} \approx T_0 + \Delta T_{t,1} \tag{86}$$

i.e., at a distance of half-width of the first transition from T_0. Thus, if we

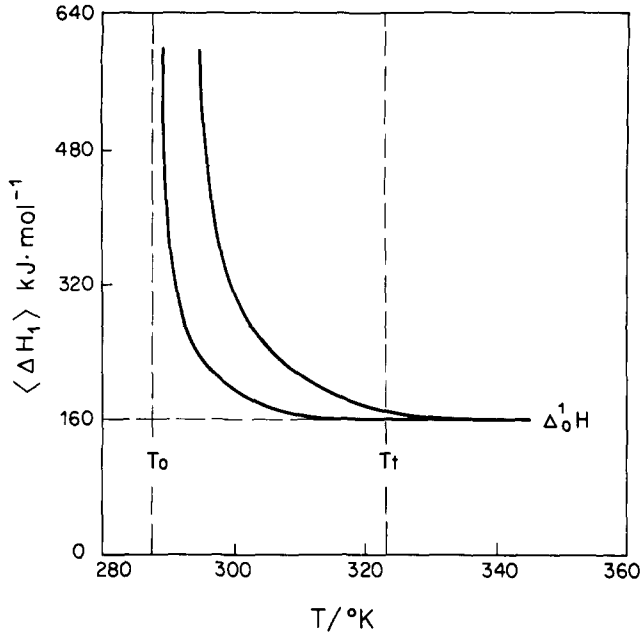


FIG. 9. Temperature dependence of functions $\varphi = \langle \Delta H \rangle / (1 - F_0)$ and $\varphi' = \langle \Delta H^2 \rangle / \langle \Delta H \rangle$ for a two-state transition with $\Delta_0^1 H = 160$ kJ mol^{-1}, $T_t = 323$ K, and $T_0 = 288$ K.

have several transitions, and the first transition is followed by a second one, the determination of its enthalpy value becomes a problem, since the contribution of the subsequent transitions to φ_1 can become noticeable before the value φ_1 approaches $\Delta_0^1 H$. That is why in reality one has to search not for the limit but for the minimum of function φ_1 and to accept this value as the enthalpy of the first transition. Hence, it becomes clear that the extent of transition overlap imposes certain limitations on the applicability of this method to excess heat capacity analysis. A serious obstacle to this method arises also from the accumulation of errors at consecutive steps of the analysis.

In order to decrease the influence of the enumerated factors, the possibility was studied of using for the deconvolution analysis, instead of function φ, some other one which could give also a transition enthalpy at the limit $T \to 0$.[22] In particular, it has been shown that the function $\varphi' = \langle \Delta H^2 \rangle / \langle \Delta H \rangle$ approaches the transition enthalpy value faster than function

[22] V. V. Filimonov, S. A. Potekhin, S. V. Matveyev, and P. L. Privalov, *Mol. Biol. (USSR)* **16,** 551 (1982).

φ (see Fig. 9), and, consequently, is much more useful for sequential analysis of complicated processes. Nevertheless, even with the use of this function, the error accumulation is significant in sequential analysis.

The results of the analysis can be improved by an optimization procedure carried out at each stage of sequential deconvolution.[22] After determining $\Delta_0^1 H$ and $T_{t,1}$, the heat absorption peak corresponding to this parameter is synthesized, and the initial and synthesized curves are compared in the temperature region from T_0 to T_1, where T_1 corresponds to the temperature at which the minimum of the function φ', corresponding to $\Delta_0^1 H$, is achieved. The optimization procedure consists in a search for the minimum of the function

$$\phi(\Delta_0^1 H, T_{t,1}) = \int_{T_0}^{T_1} (\langle \Delta C_p \rangle_{theor} - \langle \Delta C_p \rangle_{exp})^2 dT \tag{87}$$

The refined values of $\Delta_0^1 H$ and $T_{t,1}$ are used to determine $\langle \Delta H \rangle_1$. Then the parameters of the second transition are optimized in the same manner and the parameters of both transitions are optimized in the temperature range from T_0 to T_2 which includes the first two transitions. The same procedure is then repeated. The efficiency of optimization at each stage of the sequential deconvolution analysis of the complex function was demonstrated on synthesized model functions.

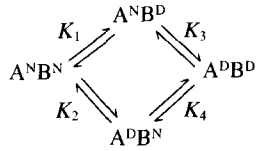
Interpretation of the Results of Heat Capacity Function Analysis

The problem which arises after the determination of the number of states of a macromolecule realized in the temperature-induced process and the thermodynamic parameters of the transition which constitutes this process is the interpretation of the found values, their physical and structural meaning.

In many cases, discrete states of macromolecules result in their structural discreteness, i.e., in the subdivision of their structure into cooperative structural blocks, which disrupt on heating in an "all-or-none" way. Thus, each of the realized states represents some definite combinations of blocks in the ordered and disordered states. If there are N such blocks in the molecule, the number of possible states will be 2^N. However, far from all possible states are realized upon heating. The number of such states is determined both by the thermodynamic parameters of stabilization of the blocks and by their interaction.

Let us consider as an example a macromolecule consisting of two

cooperative blocks A and B which can be either in state N or D. The scheme of possible transitions in such a molecule is



Not all the equilibrium constants in this scheme are independent, since $K_1K_3 = K_2K_4$. It is clear that, if there is no interaction between the transitions, then $K_1 = K_4$ and $K_2 = K_3$.

However, all these transitions can be represented formally by a linear scheme of sequential reactions, as well

$$A^NB^N \overset{K_1^*}{\rightleftharpoons} A^NB^D \overset{K_2^*}{\rightleftharpoons} A^DB^N \overset{K_3^*}{\rightleftharpoons} A^DB^D$$

The equilibrium constants K_i^* are related to the equilibrium constants K_i in the previous scheme in the following way

$$\begin{array}{lll} K_1^* = K_1 & & K_1 = K_1^* \\ K_2^* = K_2/K_1 & \text{or} & K_2 = K_1^*K_2^* \\ K_3^* = K_4 & & K_4 = K_3^* \\ & & K_3 = K_2^*K_3^* \end{array} \tag{88}$$

Using these relations, we can obtain the enthalpy and entropy of transition of blocks A and B with the "neighbor" in the ordered or disordered state from the thermodynamic parameters for the linear scheme, which can be determined by the sequential deconvolution analysis of the calorimetric melting curve

$$\begin{array}{ll} \Delta^{A^DB^N}_{A^NB^N}H = \Delta H_1^* + \Delta H_2^* & \Delta^{A^DB^N}_{A^NB^N}S = \Delta S_1^* + \Delta S_2^* \\ \Delta^{A^DB^D}_{A^NB^D}H = \Delta H_2^* + \Delta H_3^* & \Delta^{A^DB^D}_{A^NB^D}S = \Delta S_2^* + \Delta S_3^* \\ \Delta^{A^NB^D}_{A^NB^N}H = \Delta H_1^* & \Delta^{A^NB^D}_{A^NB^N}S = \Delta S_1^* \\ \Delta^{A^DB^D}_{A^DB^N}H = \Delta H_3^* & \Delta^{A^DB^D}_{A^DB^N}S = \Delta S_3^* \end{array} \tag{89}$$

Then, for the enthalpy and entropy of interaction between blocks A and B, we have

$$\begin{array}{l} \delta H_{\text{inter}} = \Delta^{A^DB^N}_{A^NB^N}H - \Delta^{A^DB^D}_{A^NB^D}H = \Delta H_1^* - \Delta H_3^* \\ \delta S_{\text{inter}} = \Delta^{A^DB^N}_{A^NB^N}S - \Delta^{A^DB^N}_{A^NB^N}S = \Delta S_1^* - \Delta S_3^* \end{array} \tag{90}$$

In real cases, the enthalpy of interaction between the blocks is much lower than the enthalpy of structure stabilization of individual blocks.

Nevertheless, it can be easily determined by measuring the heat effect of melting at conditions with different transition sequences of the blocks (see for example Ref. 23).

Increase of Analysis Reliability

The incorrectness in the determined transition enthalpy values, caused by the inaccuracy of the experimental curve, increases with a decrease of the maximal population of the corresponding state. At the limit when the population of intermediate states tends to zero, the shape of the melting curve becomes indistinguishable from that of the "two-state" transition, and, in this case, the thermodynamic parameters of only the final state can be determined reliably. The population of the intermediate states decreases with an increase of the transition overlap. Therefore, one of the possibilities to increase the reliability of the analysis consists in carrying out experiments at solvent conditions which assure a maximal spread of the studied process over the temperature scale, and, as a result, a minimal overlap of its constituent transitions.

The criterion of reliability of the results of the deconvolution analysis can be the continuity in the changes of the found parameters with variation of environmental conditions. Indeed, variation in the stability of individual states results usually in significant changes in the observed overall melting profile (Fig. 10). However, if the analysis is carried out correctly, the temperature and enthalpy of individual transitions should vary continuously, and their functional dependence on environmental conditions should be interpreted physically (see for example Ref. 24).

When the studied macromolecular system is too complicated, consisting of many subsystems undergoing transitions in the overlapping temperature ranges, it is expedient to simplify it by fragmentation. Studies of melting of a set of fragments permits identification of the found transitions with the melting of a definite part of the molecule, i.e., to carry out actually a structural analysis of the molecule at the domain level (Fig. 11). However, fragmentation of a large molecule into quite definite parts and isolation of highly homogeneous fragments in amounts required for calorimetric experiments by biochemical procedures is much more time consuming than calorimetric measurements and analysis of results.

[23] S. V. Matveyev, V. V. Filimonov, and P. L. Privalov, *Mol. Biol. (USSR)* **6,** 1234 (1982).
[24] V. V. Novokhatny, S. A. Kudinov, and P. L. Privalov, *J. Mol. Biol.* **179,** 215 (1984).

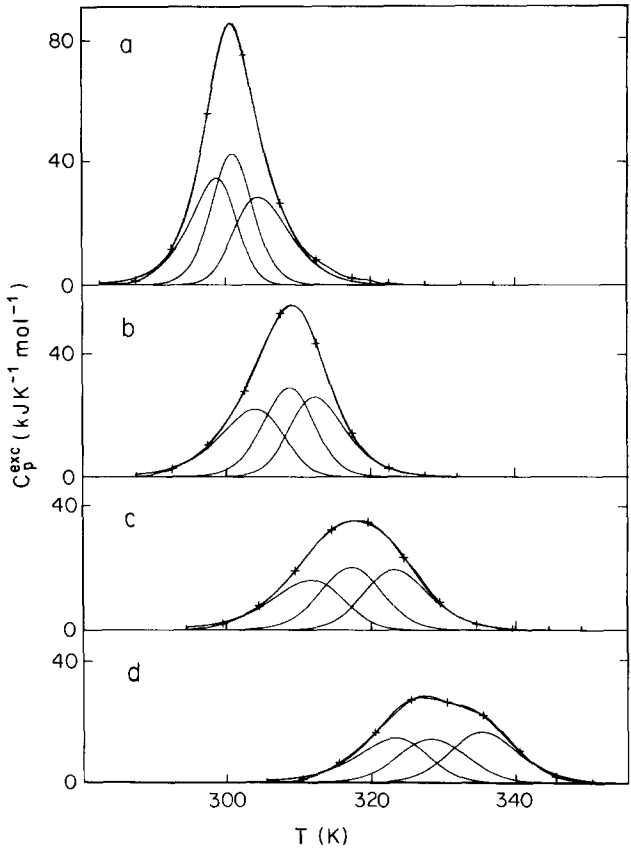


FIG. 10. Deconvolution of the excess heat capacity functions of plasminogen fragment K1-3 in solutions with different pH values: (a) pH 7.4, (b) pH 5.4, (c) pH 4.0, and (d) pH 3.4. Crosses indicate the experimentally determined function to distinguish it from the calculated one using the parameters of the transition estimated by the convolution analysis (see for details Novokhatny *et al.*[24]).

Additional Information Obtained from Melting Curves

Information Obtained from Calorimetric Melting Curves

Carrying out calorimetric experiments at various solvent conditions permits us not only to increase the reliability of the deconvolution analysis, but also to obtain some additional information on the revealed transitions, particularly on the amount of ligands released during the transition.

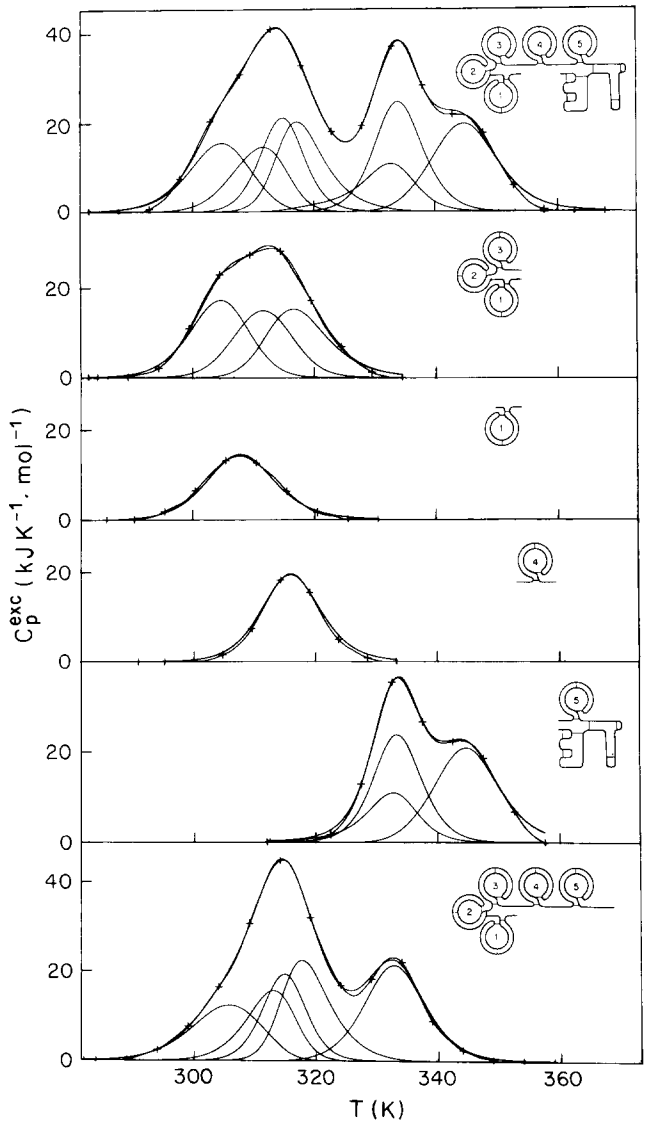


FIG. 11. Deconvolution of the excess heat capacity functions of Lys-plasminogen and its various proteolytic fragments which are given schematically in the upper right-hand corner of each panel (see for details Novokhatny *et al.*[24]).

The equilibrium constant of a reaction which proceeds with ligand binding, such as

$$N \rightleftharpoons D + jL$$

where N and D are the native and denatured states of the macromolecule and L is the ligand released at denaturation, can be expressed as

$$K = \exp\left(-\frac{\Delta G}{RT} + j \ln a_L\right) \tag{91}$$

where a_L is the activity of the ligand in solution. The conditions corresponding to some fixed value of the equilibrium constant, for example, conditions at which the equilibrium constant is equal to unity, i.e., transition conditions, can be found from the requirement

$$\frac{d \ln K}{d \ln a_L} = \left[d\left(-\frac{\Delta_N^D G}{RT} + j \ln a_L\right)\right] \Big/ (d \ln a_L)$$
$$= -\frac{\Delta_N^D H}{RT_t^2} \frac{dT_t}{d \ln a_L} + j = 0 \tag{92}$$

From this equation, we get immediately

$$\frac{d(1/T_t)}{d \ln a_L} = -\frac{jR}{\Delta_N^D H} \tag{93}$$

Therefore, knowledge of the functional dependence of the reciprocal of the transition temperature on the logarithm of the ligand activity in solution permits us to determine the amount of ligands released during the transition (see for example Refs. 19 and 25).

Information Obtained from Noncalorimetric Melting Curves

In order to carry out a structural interpretation of the stable states that are realized on heating the macromolecule, it is useful to have as many various physicochemical characteristics of these states. However, although obtaining the temperature dependence of some physicochemical parameter characterizing the molecule usually does not present any experimental problems, their deconvolution analysis, intended for the determination of a characteristic specific value for each realizable state, is practically impossible without scanning microcalorimetry data.

Indeed, if a temperature increase induces the molecule to pass through n macrostates which are specified by definite values of the considered parameter $\Delta_0^i A$, the observed overall melting profile of the quantity A will

[25] P. L. Privalov, O. B. Ptitsyn, and T. M. Birshtein, *Biopolymers* **8**, 559 (1969).

be

$$\langle \Delta A(T) \rangle = \sum_{i=0}^{n} \Delta_0^i A F_i \tag{94}$$

$$= \left[\sum_{i=0}^{n} \Delta_0^i A \exp\left(- \frac{\Delta_0^i H}{RT} + \frac{\Delta_0^i S}{R} \right) \right] \Big/ \left[\sum_{i=0}^{n} \exp\left(- \frac{\Delta_0^i H}{RT} + \frac{\Delta_0^i S}{R} \right) \right]$$

The principal difference between any parameter A and enthalpy, i.e., between Eqs. (94) and (64), is that in the case of enthalpy it figures not only as a coefficient but also in the exponents. Therefore, this equation is solvable only if A is the enthalpy and not any other parameter. The enthalpy function is unique in this respect. For any arbitrary A, this equation can be solved only if we know the thermodynamic characteristics of all the realized states, i.e., their population over the entire considered temperature range. The latter can be determined only by deconvolution analysis of the calorimetric melting curve. If the population of intermediate states, F_i, is already determined from calorimetric experiments all other characteristics of these states can be found from the observed melting curve, $\langle A(T) \rangle$, just by minimization of the function

$$\phi(\Delta_0^i A) = \int_{T_0}^{T} \left[\langle \Delta A(T) \rangle - \sum_{i=1}^{n} \Delta_0^i A F_i(T) \right]^2 dT \tag{95}$$

For this purpose, ϕ is differentiated with respect to parameter $\Delta_0^k A$ and equated to zero, assuming $\Delta_0^k A$ to be independent of temperature.

$$\frac{\partial \phi}{\partial \Delta_0^k A} = 2 \int_{T_0}^{T} \left\{ \langle \Delta A(T) \rangle - \sum_{i=1}^{n} \Delta_0^i A F_i(T) \right\} F_k(T)\, dT$$

$$= 2 \left\{ \int_{T_0}^{T} \langle \Delta A(T) \rangle F_k(T)\, dT \right.$$

$$\left. - \sum_{i=1}^{n} \Delta_0^i A \int_{T_0}^{T} F_i(T)\, F_K(T)\, dT \right\} = 0 \tag{96}$$

Thus, the problem is reduced practically to the solution of the linear system of equations

$$\begin{aligned} L_{11}\Delta_0^1 A + L_{12}\Delta_0^2 A + \cdots + L_{1n}\Delta_0^n A &= M_1 \\ L_{n1}\Delta_0^1 A + L_{n2}\Delta_0^2 A + \cdots + L_{nn}\Delta_0^n A &= M_n \end{aligned} \tag{97}$$

where

$$L_{jk} = L_{kj} = \int_{T_0}^{T} F_j(T) F_k(T) dT, \qquad M_k = \int_{T_0}^{T} \langle A(T) \rangle F_k(T) dT$$

(for details see Ref. 26).

[26] S. V. Matveyev, V. V. Filimonov, and P. L. Privalov, *Mol. Biol. (USSR)* **17**, 172 (1983).

Thermodynamic Description of a Cooperative Unit

Although the enthalpy and entropy of the transition of a cooperative unit between its two realizable states (native and denatured) can be measured calorimetrically only within the transition temperature range, the enthalpy, entropy, and Gibbs energy difference of these states can be estimated over a much broader temperature range if the heat capacities of these states or their differences are known. Indeed, bearing in mind that $(\partial S/\partial T) = C_p/T$ and, at transition temperature, T_t

$$\Delta_N^D G(T_t) = \Delta_N^D H(T_t) - T_t \Delta_N^D S(T_t) = 0 \tag{98}$$

we have for these difference functions at temperature T

$$\Delta_N^D H(T) = \int_T^{T_t} C_p^N(T)dT + \Delta_N^D H(T) + \int_{T_t}^{T} C_p^D(T)dT$$

$$= \Delta_N^D H(T_t) - \int_T^{T_t} \Delta_N^D C_p(T)dT \tag{99}$$

$$\Delta_N^D S(T) = \int_T^{T_t} \frac{C_p^N(T)}{T}\,dT + \frac{\Delta_N^D H(T_t)}{T_t} + \int_{T_t}^{T} \frac{C_p^D(T)}{T}\,dT$$

$$= \frac{\Delta_N^D H(T_t)}{T_t} - \int_T^{T_t} \Delta_N^D C_p(T)d \ln T \tag{100}$$

$$\Delta_N^D G(T) = \Delta_N^D H(T) - T\Delta_N^D S(T) = \Delta_N^D H(T_t)\frac{T_t - T}{T_t}$$

$$- \int_T^{T_t} \Delta_N^D C_p(T)dT + T\int_T^{T_t} \frac{\Delta_N^D C_p(T)}{T}\,dT \tag{101}$$

Usually the heat capacity difference between the native and denatured states, $\Delta_N^D C_p(T)$, does not depend significantly on temperature[15] and can be considered in the first approximation as a constant specific for the considered cooperative unit. This permits us to simplify the above equations and to rewrite them in the following way:

$$\Delta_N^D H(T) = \Delta_t H - \Delta_t C_p(T_t - T) \tag{102}$$

$$\Delta_N^D S(T) = \frac{\Delta_t H}{T_t} - \Delta_t C_p \ln \frac{T_t}{T} \tag{103}$$

$$\Delta_N^D G(T) = \Delta_t H \frac{T_t - T}{T_t} - \Delta_t C_p(T_t - T) + \Delta_t C_p T \ln \frac{T_t}{T} \tag{104}$$

where $\Delta_t H \equiv \Delta_N^D H(T_t)$ is the calorimetrically measured transition enthalpy at transition temperature T_t and $\Delta_t C_p \equiv \Delta_N^D C_p(T_t)$ is the heat capac-

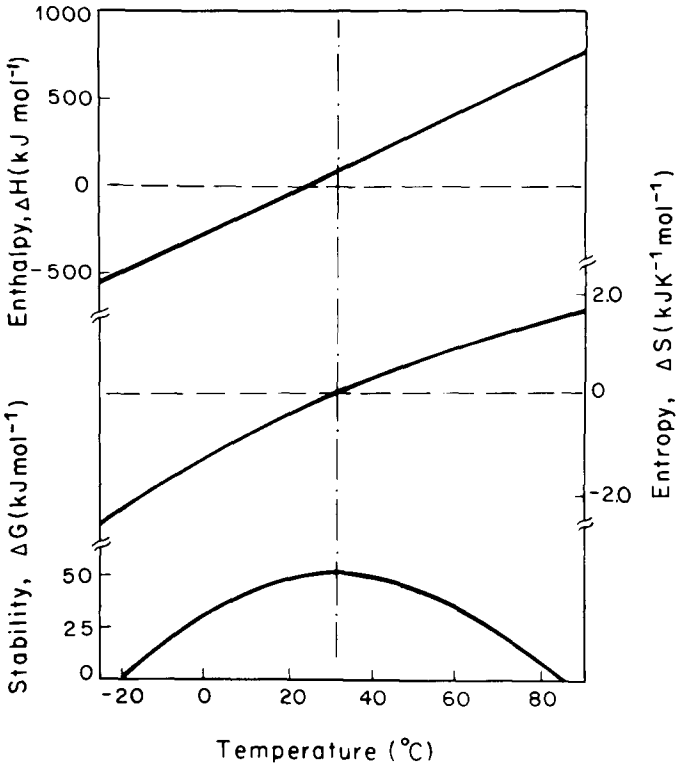


FIG. 12. The enthalpy, entropy, and Gibbs energy differences between native and denatured states of CN-metmyoglobin in 40 mM glycine solution, pH 10.7.

ity difference extrapolated to the transition midpoint between the native and denatured states. As already shown, the transition temperature T_t is usually close to the temperature of the maximal heat absorption T_{max} [see Eq. (52)].

Figure 12 shows the enthalpy, entropy, and Gibbs energy differences of the native and denatured states of a typical small compact globular protein, myoglobin, whose denaturation is very close to the two-state transition. As can be seen, the enthalpy and entropy differences are both increasing functions of temperature, while the Gibbs energy difference is a function with an extremum. The maximal value of the Gibbs energy difference is achieved at the temperature at which

$$\frac{\partial \Delta_N^D G(T)}{\partial T} = -\Delta_N^D S(T) = 0 \tag{105}$$

Since the Gibbs energy difference between the native and denatured states determines actually the stability of the cooperative structure, since

it is the work required for its disruption, it follows that the maximal stability of the cooperative unit structure is achieved at conditions at which the native and denatured states do not differ in their entropy values (for details see Refs. 27 and 28).

[27] P. L. Privalov and N. N. Khechinashvili, *J. Mol. Biol.* **86,** 665 (1974).
[28] W. Pfeil and P. L. Privalov, *Biophys. Chem.* **4,** 23 (1976).

[3] Kinetic Mechanisms of Protein Folding

By HIROYASU UTIYAMA and ROBERT L. BALDWIN

Introduction

The main purpose in working out the kinetic mechanism of folding of a protein is to take the first step in determining its pathway of folding. The second step is to find conditions in which structural folding intermediates are well populated. The third step is to characterize the structures of the intermediates and to place the intermediates in order on the folding pathway. This chapter considers how to determine the kinetic mechanism of folding; later chapters consider how to characterize the folding intermediates (see, for example, the chapters on circular dichroism[1] and on amide proton exchange[2]). Another chapter[3] considers the use of subzero temperatures as a means of populating folding intermediates for long times. If a folding reaction shows no populated intermediates, the only direct information that can be learned about the folding pathway is that the process of folding is highly cooperative. The nature of the rate-limiting step can, however, be studied by investigating the dependence of the folding rate on temperature, pH, and denaturant concentration.

The goal of determining the pathway of folding by characterizing the structures of intermediates has now been pursued by protein chemists for more than two decades. Attention has centered on small, single-domain proteins in an effort to find out how the simplest proteins fold up. The conclusion has been widely accepted for a decade that folding intermediates can be detected only with difficulty, if at all, in equilibrium studies of single-domain proteins in aqueous solvents. (It remains to be seen whether the use of high-resolution two-dimensional (2-D) NMR will change this conclusion.) Consequently, there has been wide interest in the kinetic intermediates found in the folding reactions of single-domain pro-

[1] A. M. Labhardt, this volume [7].
[2] P. S. Kim, this volume [8].
[3] A. L. Fink, this volume [10].