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An alternative interpretation of the heat capacity changes associated with protein unfolding

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Abstract

The present study devises a method to get quantitative information for proteins on the theoretically important heat capacity at constant volume. For this purpose expansion coefficients of both the native and unfolded state of a variety of proteins have been determined and used together with compressibility coefficients to calculate the difference between isobaric and isochoric heat capacity, $(c_p - c_v)$, for the unfolding transition. This difference $\Delta(c_P - c_V) = (c - c) - (c - c)$ turns out to be a positive number that is larger than the experimental isobaric heat capacity change $\Delta_N^D c_P = c_P^D - c_P^N$ for the proteins studied. The generally observed positive heat capacity change on unfolding can therefore alternatively be interpreted as resulting from the difference in work involved in changing the intra- and intermolecular interactions including the weak, highly distance-dependent, van der Waals interactions, for the unfolded and native state, respectively. The difference in expansion work against the atmospheric pressure is negligible. This macroscopic interpretation cannot rule out that part of the denaturational heat capacity increase is also due to the different interaction with water of the native and unfolded conformations of the protein.

Keywords: Protein unfolding; Heat capacity change; Expansion coefficients; Compressibility; Heat capacity at constant pressure and constant volume

1. Introduction

Ever since the pioneering studies of Sturtevant [1,2], Privalov [3] and Privalov and Gill [4] the heat capacity change associated with protein unfolding has been an intriguing physical parameter. Generally $\Delta_N^D c_P$ has been observed a positive quantity and its magnitude has been found proportional to the change in accessible surface area on unfolding [5,6]. The $\Delta_N^D c_P$ value has been interpreted in molecular terms as resulting from the positive and negative heat capacity increments caused by the interaction with hydration water of hydrophobic and hydrophylic residues, respectively [7,8]. Since a large body of experimental evidence appears to support the idea that the combined effect of hydrophobic and hydrophylic hydration fully explaines, $\Delta_N^D c_P$ fundamentally different concepts have been explored much less. In this study we propose an alternative explanation for the observed heat capacity increase on protein unfolding.

We conclude from expansion and compressibility data for the native and unfolded state of a variety of proteins that there is a positive difference $\Delta_N^D(c_P - c_V)$ between denatured and native protein which could easily account for the experimental $\Delta_N^D c_P$ value. According to this purely thermodynamic hypothesis, the positive

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[1] Dedicated to Professor Dr. E. Wicke on the occasion of his 80th birthday.

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heat capacity change would result from the difference in the expenditure of work against cohesive forces (electrostatic, van der Waals etc.) in the two states rather than from the difference in hydration. On the basis of the present measurements it cannot, however, be excluded that part of this difference stems from the different degree of hydration of the native and unfolded proteins, respectively.

2. Materials and methods

2.1. Density measurements

Differential scanning density measurements (DSD) have been performed using a Kratky–Paar densitometric system consisting of two matched DMA 602 HT external cells in combination with a DMA 60 measuring unit (A. Paar, Graz, Austria) [9,10]. Temperature scans between 2–95°C were controlled with a Haake PG20 temperature controller and a Haake F3 thermostated bath. The effective scan rate was 0.4°C/min. Temperature was measured with a platinum resistance thermometer placed in a capillary tube in the sample cell compartment. Temperature readings were made using a Keithley 195 A multimeter. Data pairs of density and temperature were collected every 0.2 deg employing a personal computer. The temperature scans were interrupted for the time of the density measurement to allow for temperature equilibration. Partial specific volumes were calculated according to Eq. (1), since no concentration dependence of the apparent specific volume was observed in the concentration range studied,

$$\bar{v}=\frac{1}{\rho_{bu}}\left(1-\frac{\rho_{sol}-\rho_{bu}}{c_{Prot}}\right), \tag{1}$$

ρ_{bu} and ρ_{sol} are the densities of the buffer and protein solution, respectively. Protein solutions were dialysed to equilibrium before use. Expansion coefficients were calculated according to the equation

$$\alpha=\frac{1}{\bar{v}}\frac{\Delta\bar{v}}{\Delta T} \tag{2}$$

where $\bar{v}$ is the partial specific volume at the specified temperature. $\Delta\bar{v}$ is the volume change occuring in the temperature interval ΔT. $\Delta\bar{v}/\Delta T$ is the slope of the linear pre- or postdenaturational $\bar{v}(T)$ curve. Since the partial specific volume curves consist essentially of two linear parts, the calculation of the expansion coefficients could be based on differences instead of differentials.

Protein concentrations between 5 and 32 mg/ml have been used. The overall accuracy of the measurements is better than 0.005 ml/g.

2.2. Heat capacity measurements

Differential scanning heat capacity measurements (DSC) have been performed with the use of DASM-1M or DASM-4 calorimeters [11]. Runs were made in the same temperature range as the DSD studies using protein concentrations between 5 and 32 mg/ml and heating rates of 0.5°C/min or 1°C/min. Apparent specific heat capacity of the protein, $c_{p,P}$, was calculated using the relationship

$$c_{p,P}=c_{p,L}\cdot\frac{\bar{v}_P}{\bar{v}_L}+\frac{\Delta c_p}{m_p} \tag{3}$$

where $c_{p,L}$ is the specific heat capacity of the solvent, $\bar{v}_P$ and $\bar{v}_L$ are the temperature dependent partial specific volumes of protein and solvent, respectively, m_p is the mass of the protein in the sample cell in g, and Δc_p is the excess heat capacity in J/K of the protein solution. Heating rates and protein concentrations had no influence on the thermodynamic parameters derived from the c_p curves. Therefore the apparent specific heat capacity, $c_{p,P}$, can be considered a partial specific quantity.

Heat capacity and temperature data were routinely registered every tenth of a degree by a computer. Each sample measurement was preceded by a calibration run with the equilibrium dialysis buffer. Thermodynamic parameters were evaluated numerically.

3. Results and discussion

Fig. 1a shows the variation with temperature of the partial specific heat capacity and volume of lysozyme at pH = 2.0 and Fig. 1b shows the corresponding temperature course of the expansion coefficient in addition to the $\bar{v}(T)$ curve.

The excess heat capacity curve (Fig. 1a) exhibits the well known profile of a highly cooperative 2-state

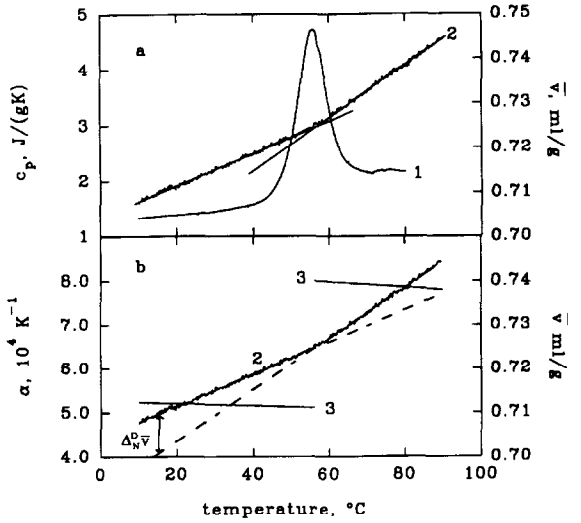

Fig. 1. DSC and DSD studies on lysozyme. The graphs show the unsmoothed experimental data. (a) 1: DSC measurement, $c = 8.3$ mg/ml, 0.1 M potassium phosphate buffer pH = 1.9, heating rate 0.5 K/min; 2: DSD measurement, $c = 32.2$ mg/ml, 0.1 M potassium phosphate buffer pH = 2.0, effective heating rate 0.4 K/min; (b) 2: same as under (a) 2: the dashed lines are extrapolations of the linear branches of the $\bar{v}$ curve, that correspond to native and denatured protein, respectively. Below the transition temperature the partial specific volume of the unfolded state is lower than that of the native state. The difference is indicated by $\Delta^D_N\bar{v}$. 3: temperature dependence of the expansion coefficient derived from curve 2 under the assumption of a linear increase of $\bar{v}(T)$ with temperature before and after the transition. A higher degree polynomial fit does not appear to be justified.

transition with a positive heat capacity change of 6.0 kJ/(mol K) involved in the unfolding reaction. The graph of the dependence on temperature of the partial specific volume of lysozyme consists of two linear parts. They intersect near the transition temperature of the heat capacity curve. It is most remarkable that the transition from the native to the denatured state occurs within a very small temperature range. It almost appears, as if the volume function were discontinuous. This behaviour is characteristic for the majority of proteins studied so far. Fig. 1b displays the same $\bar{v}(T)$ function (labeled (2)) and the expansion coefficient α (labeled (3)) calculated from these data using Eq. (2). The variation with temperature of the expansion coefficient shows actually a discontinuity, since we approximated the pre- and postdenaturational branch of the $\bar{v}(T)$ curve by two straight lines. Their intersection defines the transition temperature $T_{m,v}$, which in most of our measurements is identical with the transition temperature T_m defined by the heat capacity curves as the temperature of 50% conversion.

A variant is seen in Fig. 2, which reports DSC and DSD curves of ribonuclease A. The heat capacity curve is fully compatible with an all-or-none transition and exhibits a heat capacity increase on unfolding of 4.5 kJ/(mol K) in agreement with published data. The pattern of the $\bar{v}(T)$ curve is, however, different from that of lysozyme. The predenaturational part is not linear but the slope decreases slightly before the transition temperature. Above $T_{m,v}$ we find the higher slope characteristic of the unfolded protein. As with lysozyme the transition from native to unfolded is very abrupt, when followed by the partial specific volume, while the heat capacity curve displays the usual transition range dictated by the van't Hoff equation of a two-state process. A very similar temperature dependence of $\bar{v}$ of ribonuclease has been observed by Holcomb and van Holde [12], with the only exception that they find an even higher slope of the $\bar{v}(T)$ curve above the transition temperature than we observe. It is worth mentioning that the change in slope characteristic of the unfolded protein occurs again at a temperature equal to T_m.

The characteristic pattern of the variation with temperature of the partial specific volume is difficult to reconcile with some of the molecular mechanisms proposed to determine protein unfolding. Our results demonstrate clearly that at the transition temperature the partial specific volume of the unfolded protein is practically identical or – as for lactalbumin which unfolds partially irreversibly at pH = 7.0 – slightly larger than that of the native protein. Above the transition temper-

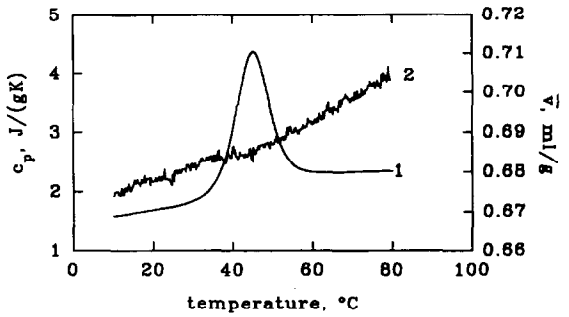

Fig. 2. DSC and DSD studies of ribonuclease A. The graphs show the unsmoothed experimental data. 1: DSC measurement, c = 14.4 mg/ml, 0.1 M potassium phosphate buffer, pH = 2.5, heating rate 0.5 K/min. 2: DSD measurement, $c = 14.4$ mg/ml, same buffer, effective heating rate 0.4 K/min.

ature, $\bar{v}$ increases linearly with increasing temperature.

As pointed out by Zipp and Kauzmann [13], if the thermodynamic effects associated with protein denaturation are dominated by hydrophobic interactions, one would expect the thermodynamic changes on denaturation to parallel those that occur when nonpolar groups are transferred from a nonpolar environment, the protein interior, to water. Now transfer of small nonpolar molecules from a liquid hydrocarbon environment to water involves an increase in heat capacity, but a volume decrease of 1 to 2 ml per mol of methylene group [14]. Similar volume changes of -2 to -20 ml/mol are expected to result from dissociation of a salt bridge [15]. Therefore, if the interior of the protein molecule resembles a liquid hydrocarbon and if a significant number of groups are exposed on unfolding, $\Delta_N^D c_P$ should be large and positive, which one observes, and ΔV should be negative or at least not positive. What we observe is a zero volume change but a positive change of the expansion coefficient. Since undoubtedly the degree of exposure of hydrophobic residues to water increases with unfolding, the volume changes expected on the basis of the model compound studies can either not apply quantitatively and/or compensating positive volume changes are associated with the unfolding process which render the experimentally observed zero or slightly positive change of the partial specific volume understandable. There are findings that could account for the positive volume changes. Suzuki et al. [16] have found from the effect of pressure on the water solubility of diketopiperazine, a model for the peptide bond, that for the dissolution of solid material to give aqueous diketopiperazine ΔV is about $+5$ ml/mol. This is in linc with the estimates given by Finney for the volume changes associated with breaking of hydrogen bonds ($+2$ to $+6$ ml/mol) [17]. Thus, provided the model is valid, a correspondingly large number of peptide groups could, when hydrated on unfolding, account for the necessary positive volume contribution.

A different mechanism is suggested by studies of Boje and Hvidt [18–20], which evaluated the effect of local concentration of hydrophobic groups on the volume change expected from the rupture of the hydrophobic bonds. They show that, because the local concentration of nonpolar groups is quite high even in a randomly coiled protein, ΔV for this process could even be positive.

Now the very fact that proteins can be unfolded by pressure implies by the general relationship

$$\left(\frac{\partial \ln K}{\partial P}\right)_T = -\frac{\Delta V}{RT} \tag{4}$$

that at constant temperature an overall negative volume change should be involved in the denaturation process. This is not at variance with our finding that the change in the partial specific volume of the protein at 1 bar is zero or even slightly positive at the transition temperature. As a matter of fact our results offer an explanation for the striking sequence of folding and unfolding processes observed by Zipp and Kauzmann in their pressure studies on metmyoglobin. Metmyoglobin at pH = 5.5 and 3000 kg/cm^2 is denatured at low temperatures, but on heating at this pressure undergoes a transition to the native state at 20°C. The temperature must then be raised to 80°C in order for the protein to revert to the denatured state.

It is known that above pH = 4.0 myoglobin shows only heat denaturation in the accessible temperature range from 0 to 100°C [21]. We have measured $\bar{v}(T)$ at pH = 4.1 and have observed a 'regular' temperature profile of the $\bar{v}(T)$ curve analogous to that of lysozyme. Therefore the results are not shown. This pattern of the $\bar{v}(T)$ curve provides, however, a rationale for the experimental observation of Zipp and Kauzmann that at 3000 kg/cm^2 pH = 5.5 with increasing temperature myoglobin undergoes the renaturation–denaturation sequence mentioned above. Below the transition temperature the unfolded state will have a smaller partial specific volume than the native state, provided one can linearly extrapolate the high-temperature part of the $\bar{v}(T)$ curve to low temperatures. That this is a valid procedure is suggested by the DSD measurement on α-lactalbumin at pH = 2.5 as shown later. We anticipate the result here to justify the linear extrapolation procedure. At pH = 2.5 α-lactalbumin occurs in the molten globular state at low temperature and fully unfolded at high temperature [22–25]. The curve is linear over the whole temperature range which indicates that the expansion coefficient of the molten globular form is apparently identical to that of the high temperature form of the protein. Obviously the denatured and molten globular forms of the protein have very similar expansion coefficients. The absence of any discontinuity in the curve demonstrates that extrapolation of the high

temperature branch of the $\bar{v}(T)$ curve to temperatures below the transition temperature is permissible. We assume that the linear extrapolations of both the high and low temperature branches of the $\bar{v}(T)$ curve are generally valid.

With these assumptions the folding–unfolding transitions of metmyoglobin under pressure observed by Zipp and Kauzmann [13] can be understood. Increase of pressure on the native protein at low temperature will shift equilibrium according to the le Châtelier principle to the system having the smaller volume, which is the unfolded state. Thus the protein will unfold. Increase of temperature results in a decrease of $\Delta_N^D\bar{v}$ (see Fig. 1b) and accordingly ΔV. On the basis of Eq. (4) the ability of pressure to shift the equilibrium towards the unfolded state will decrease with decreasing ΔV. Since the unfolding tendency of temperature in the temperature range below T_m is negligible, it is the magnitude of $\Delta_N^D\bar{v}$ that governs the unfolding equilibrium at a given overpressure.

Bringing the temperature up to above the transition temperature in the presence of the excess pressure reverses the conditions. Now the volume of the native state is smaller than that of the denatured state and the overpressure may renature the protein, provided the pressure is high enough to overcome the unfolding tendency of temperature. At high enough temperature the pressure is no longer sufficient to keep the protein in the native state as observed by Zipp and Kauzmann with metmyoglobin at pH = 5.5 above 80°C.

Fig. 3a shows DSC and DSD studies on myoglobin at pH = 3.70. At this pH both cold and heat denaturation occur in the experimentally accessible temperature range. The heat capacity measurements of myoglobin at pH = 3.7 show that increase of temperature from 4 to 20°C causes renaturation of the cold-denatured protein as indicated by the excess heat capacity peak at about 10°C and also by the strong decrease in slope of the partial specific volume curve. At intermediate temperatures between about 20 and 40°C the native protein starts unfolding again, however, the strong changes in excess heat capacity occur only above 50°C.

The partial specific volume curve exhibits in this temperature range an intermediate slope, smaller than both that of the cold and heat denatured protein. The transition to the heat unfolded species is apparent in the volume curve at approximately 55°C, which is in good

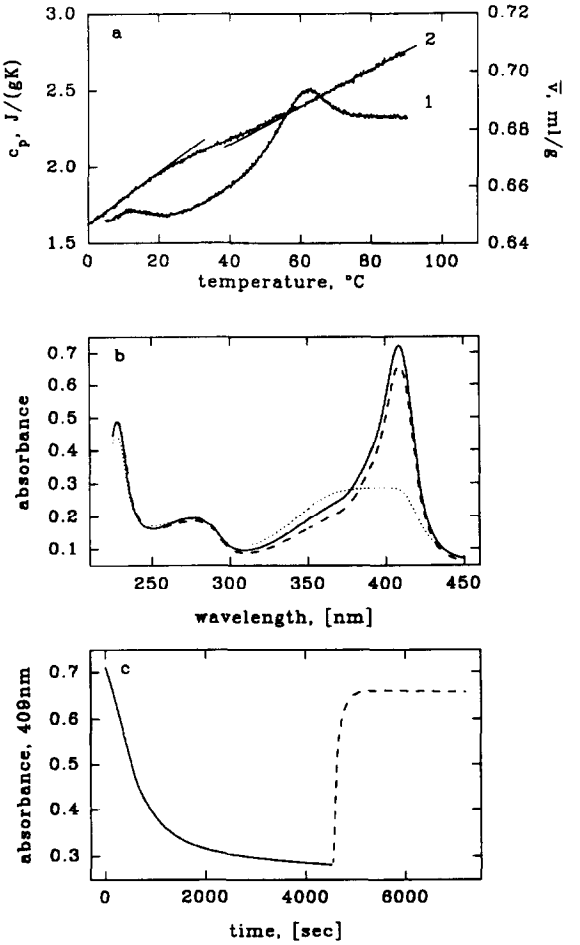

Fig. 3. DSC, DSD and UV studies on myoglobin. The curves show unsmoothed experimental data; (a) 1: DSC measurement, $c = 18.3$ mg/ml, 10 mM sodium acetate buffer pH = 3.7, heating rate 0.5 K/min. 2: DSD curve, same conditions as under 1, effective heating rate 0.4 K/min; (b) UV absorbance spectra of myoglobin in the native state (solid line), cold denatured state (dotted line) and renatured state (dashed line), $c = 0.1$ mg/ml, 10 mM sodium acetate buffer, pH 3.70; (c) UV absorbance measurements at 409 nm of the kinetics of cold denaturation (solid line) and cold renaturation (dashed line) of myoglobin, same conditions as under Fig. 3b; temperature-jump for denaturation kinetics was from 20 to 1°C and for renaturation from 1 to 20°C.

agreement with the half conversion temperature that can be estimated from the heat capacity curve.

It is noteworthy that the expansion coefficients of low and high temperature unfolded protein are clearly different, as demonstrated by the higher slope of the cold denatured species. We interprete this as an indication that these two unfolded states of the protein are not identical.

In principle there is the possibility that the low temperature at which cold denaturation occurs slows down the kinetics of unfolding to such an extent that attainment of the equilibrium state of unfolding is prevented. To exclude this possibility we performed UV absorption measurements of the unfolding and refolding kinetics of myoglobin. The results of these studies are shown in Figs. 3b and 3c.

Fig. 3b exhibits UV spectra at different temperatures comprising the protein and heme absorption range. The lowest temperature at which we could measure is 1°C. That spectrum (dotted curve in Fig. 3b) is very similar to the spectrum shown by Privalov et al. in Fig. 5 of their first study on cold denaturation of metmyoglobin in the presence of GdnHCl, i.e. in the unfolded state [21]. Actually the absorption we obtain at 1°C is even lower than that given in their study for −7°C, indicating that we reached practically complete unfolding under the experimental conditions of pH = 3.70.

To follow cold temperature unfolding kinetics we rapidly decreased temperature from 20 to 1°C and the resulting time course of the heme absorption at 409 nm is shown in Fig. 3c as a solid line. It is quite obvious that after approximately 1 h the unfolding equilibrium has been reached. Density and calorimetric studies were always started only after waiting for at least 1 to 2 hours of equilibration time at the low temperature.

Therefore one can conclude that the calorimetric and the density curves shown in Fig. 3a reflect the equilibrium state of the cold denaturated myoglobin. In view of the low level of UV absorption at 409nm at 1°C shown in Fig. 3b, we can also be confident that the degree of unfolding is very high. But even if one inferred that a small percentage of structure is still retained at this temperature, it would mean that the slope of the $\bar{v}(T)$ curve of the cold denatured protein would be even more different from that of the heat denatured form than we observed it. Thus the conclusion that cold and heat denatured protein behave differently with regard to expansion coefficients is valid.

Renaturation kinetics is shown by the dashed part of the absorbance versus time curve in Fig. 3c, and the spectrum of the renatured protein is shown as a dashed curve in Fig. 3b.

Obviously refolding occurs within 2 min when temperature is rapidly increased from 1 to 20°C. Therefore when employing a heating rate of 0.5 K/min in the calorimetric measurements refolding kinetics do not limit attainment of equilibrium conditions.

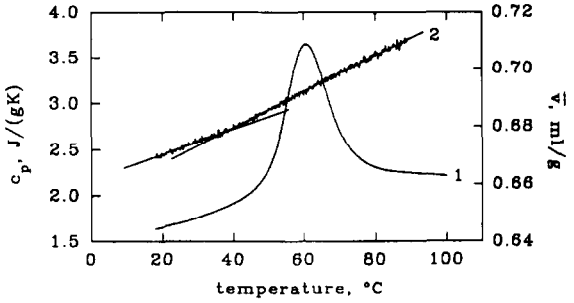

Fig. 4. DSC and DSD studies on cytochrome c. The curves show unsmoothed experimental data. 1: DSC curve, $c = 16.1$ mg/ml, 0.1 M potassium phosphate buffer pH = 3.2, heating rate 0.5 K/min. 2: DSD curve, identical solution as under 1, effective heating rate 0.4 K/min.

Fig. 4 displays DSC and DSD measurements of cytochrome c solutions.The DSC curve of the protein are identical with those published in literature [26,27]. The intriguing feature of the partial specific volume curve of cytochrome c is the occurrence of a very early transition from the native to the unfolded state. When compared with the heat capacity peak, the discontinuity in the volume curve occurs almost at the beginning of the transition seen by excess heat capacity. We have no explanation for this phenomenon and further extensive studies will be required to rationalize the unusual density function of cytochrome c.

Another interesting aspect of protein unfolding is addressed by the measurements presented in Fig. 5.

Fig. 5a exhibits volume and heat capacity measurements on α-lactalbumin at pH = 7.0, where the protein assumes the native tertiary structure. Accordingly one observes the regular heat capacity transition including the proper heat capacity increase on unfolding. A novel feature appears, however, in the partial specific volume curve. One realizes a small volume increase approximately up to the transition temperature observed calorimetrically, concomitant with an increased expansion coefficient. At higher temperatures the linear increase of volume with temperatures signals the unfolded state. This pattern of the $\bar{v}(T)$ curve is typical of partially reversible proteins, aggregating after unfolding. The partial reversibility under the given experimental conditions has been demonstrated by the fact, that a second microcalorimetric measurement with the same sample results in a significantly smaller excess heat capacity

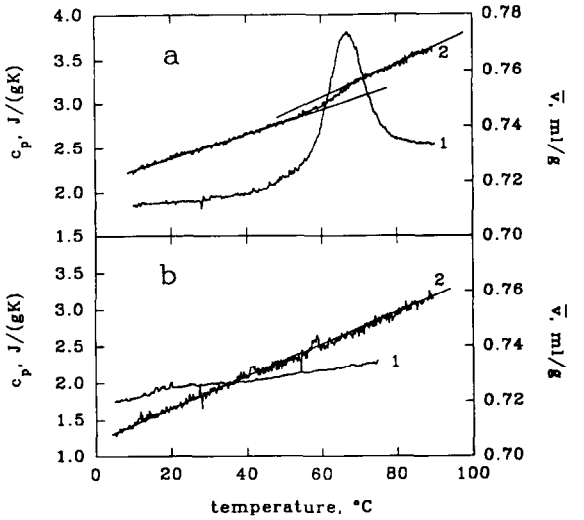

Fig. 5. DSC and DSD studies on α-lactalbumin. The curves show unsmoothed experimental data; (a) 1: DSC curve, $c = 7.17$ mg/ml, water adjusted to pH = 7.0, heating rate 0.5 K/min, Ca^{2+} to protein ratio about 1:1. 2: DSD curve, $c = 18.3$ mg/ml, water adjusted to pH = 7.0, effective heating rate 0.4 K/min, Ca^{2+} to protein ratio about 1:1; (b) 1: DSC curve, $c = 10.4$ mg/ml, water adjusted to pH = 2.5, heating rate 0.5 K/min. 2: DSD curve, $c = 12.2$ mg/ml, water adjusted to pH = 2.5, effective heating rate 0.4 K/min.

peak. The characteristic change to the linear $\bar{v}(T)$ curve of the unfolded protein occurs at a temperature almost identical to T_m observed by microcalorimetry.

Fig. 5b reports the results of DSC and DSD studies on a α-lactalbumin at pH = 2.5, where the protein is known to assume the molten globule state [28]. By definition this state is characterized by an absence of tertiary interactions while secondary structure is considered intact. This is verified by the CD spectra shown in Fig. 6.

They were recorded with diluted samples of the solutions used for the calorimetric and density studies and demonstrate that secondary structure at pH = 7.0 (Fig. 6a) and pH = 2.5 (Fig. 6b) is obviously similar to a high degree.

The calorimetric and volume measurements at pH = 2.5 (Fig. 5b) show no cooperative transition and clearly a linear increase of the partial specific volume over the temperature range from 5 to 90°C without any change in slope that would be indicative of a transition. Close inspection of the heat capacity curve reveals a shallow uncooperative increase in heat capacity between approximately 10 and 30°C that could arise from the dissociation of the residual secondary structure, since under these conditions no native structure is maintained. This minute excess heat capacity peak approximately coincides with the continuous decrease with temperature of the circular dichroism (CD) signal in the peptide region of the molten globule form of α-lactalbumin above 20°C [28]. If this decrease is considered to reflect a breakdown of the secondary structure with temperature increase, one would expect to see also some effect on the partial specific volume curve one way or the other.

Increase of exposure of peptide groups as a result of secondary structure break down ought to result in volume increases [16,17], an increased level of hydrophobic hydration should cause a volume decrease. Fig. 5b shows, however, indication of neither, the experimental volume curve is linear over the full temperature range. This may indicate one or all of three things: (i) molten globular structures are optimally hydrated so that further unfolding does not significantly improve hydration, (ii) the dissolution of diketopiperazine is no valid model for the volume changes involved in the hydration of peptide groups and (iii) exactly compensating volume changes occur in unfolding the secondary structure of molten globular α-lactalbumin. Although we have no unambiguous answer, we tend to believe that the molten globular state is characterized by practically the same hydration as the fully unfolded protein.

The slope of the volume curve at pH = 2.5 is rather similar to that of the unfolded protein at pH = 7.0. If the close similarity of the temperature dependence of the partial specific volume of heat denatured and molten globular forms of α-lactalbumin reflects a general feature of these two states of a protein, DSD studies can

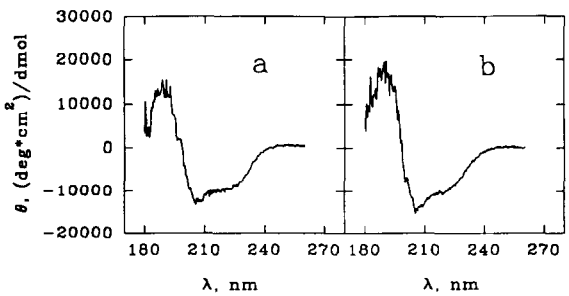

Fig. 6. Circular dichroism spectra of α-lactalbumin; (a) $c = 0.47$ mg/ml, pH = 7.0 in water; (b) $c = 0.47$ mg/ml, pH = 2.5 in water. A mean residue molecular weight of 115.2 has been used for the calculation of the ellipticity θ.

Table 1
Densitometric data of proteins; $\alpha_{25°C}$, $\alpha_{80°C}$: expansion coefficients at 25°C and 80°C; $\bar{v}_{25°C}$, $\bar{v}_{80°C}$: partial specific volumes at 25°C and 80°C; $\bar{v}^{lit.}_{25°C}$: literature values of the partial specific volume at 25°C;

protein	MW	$\alpha_{25°C}$ 10^4 K^{-1} ±0.5	$\alpha_{80°C}$ 10^4 K^{-1} ±0.5	$\bar{v}_{25°C}$ ml/g ±0.005	$\bar{v}_{80°C}$ ml/g ±0.005	$\bar{v}^{lit.}_{25°C}$ ml/g	pH
lysozyme	14300	5.1	8.2	0.715	0.740	0.719 [34]	2.0 [a]
ribonuclease A	13700	5.0	7.7	0.679	0.703	0.697 [12]	2.5 [a]
α-lactalbumin	14200	6.0	8.6	0.731	0.764	0.736 [14]	7.0 [b]
		7.9	7.6	0.721	0.755		2.5 [b]
cytochrome c	12400	5.4	11.7	0.708	0.741	0.710 [35]	7.0 [a]
		5.0	8.8	0.695	0.728	0.700 [36]	3.2 [a]
ovalbumin	45000	5.6	7.9	0.743	0.768	0.746 [14]	7.0 [a]

[a] 0.1M potassium phosphate.
[b] H_2O adjusted to the respective pH.

be considered a good additional diagnostic tool for the existence of molten globular states.

A summary of some characteristic partial specific volumes and expansion coefficients is given in Table 1. Since no complete temperature scans of the partial specific volume of proteins existed so far, we can compare our data only with literature values for low temperature [29,30]. Generally good agreement with published data is observed with the exception of ribonuclease A. We have no explanation for the difference in the results with ribonuclease A.

The expansion coefficients of all native proteins studied by us are between 5 to 6×10^{-4} K^{-1} in agreement with literature data. The expansion coefficients of the unfolded proteins show a larger variation and range from 7.5×10^{-4} K^{-1} to 12×10^{-4} K^{-1}. It is particularly interesting that the cold denatured myoglobin exhibits a considerably larger expansion coefficient (10.0×10^{-4} K^{-1}) than the heat denatured protein (7.9×10^{-4} K^{-1}). As mentioned before, we interprete this finding as being suggestive of structural differences between the two states.

The increased slope of the partial specific volume curve above the transition temperature which is associated with an increase in the expansion coefficient is in qualitative agreement with the results obtained for the expansivity, $\Delta V/\Delta T$ ($cm^3/(mol\ K)$), of 2-propanol, as a function of the mole fraction x [14]. In the range $0 < x \cong 0.1$ an increase in the mole fraction of 2-propanol causes a drastic increase of the apparent expansivity. In analogy we find that, on unfolding the protein, which involves an increase in the concentration of hydrophobic residues exposed to water, the expansion coefficient abruptly increases.

It is of course evident that the partial specific volume and the expansion coefficient are both composite quantities. They comprise at least changes of three contributions that may differ in sign (i) the contribution from the intrinsic expansion coefficient of the macromolecule (ii) that of bound water and (iii) that of free i.e. bulk water. In the course of the denaturation a redistribution of bound and free water occurs concomitant with the corresponding changes in the relative contributions of the various expansion coefficients. Of the three contributions only the temperature dependence of the expansion coefficient of free water is known precisely, it varies from 0.0001 K^{-1} to 0.0006 K^{-1} between 10 to 70°C. If one refers to water bound to hydrophobic residues as to ice-like clathrate structures, one might assign an ice-like expansion coefficient to such bound water. From −5 to 0°C the expansion coefficient of ice is 2.3×10^{-4} K^{-1}, i.e. about 10% larger than that of water at 20°C (2.07×10^{-4} K^{-1}). Electrostrictively bound water will have yet another expansion coefficient and also an unknown variation with temperature of this quantity. Therefore, in summary, any splitting of the experimental expansion coefficient into its constituing quantities appears to involve too many unknowns and has therefore not been attempted.

Apart from the inherent significance of the knowledge of expansion coefficients and partial specific volumes at different temperatures for the precise determination of molecular masses via ultracentrifugation, for proper evaluation of heat capacity curves,

and as a valuable diagnostic method for the characterization of volume changes associated with phase transitions, we consider the results obtained in this study to be of fundamental importance for the following reason. They permit together with compressibility data calculation of the difference between the isobaric and isochoric heat capacity for both the native and the denatured state of the protein in the following manner.

The difference between the heat capacity at constant pressure, c_p, and that at constant volume, c_V, for any system is given by the equation

$$c_P - c_V = \alpha\left[p + \left(\frac{\partial U}{\partial V}\right)_T\right]V \tag{5}$$

in which α is the expansion coefficient, p the external pressure, V the volume, and $(\partial U/\partial V)_T$ the isothermal change of the internal energy with volume. The product αpV describes the pressure–volume work produced in the surroundings, while $\alpha V(\partial U/\partial V)_T$ characterizes the work involved in separating the molecules against cohesive forces. This equation can be applied to both the native and unfolded protein. Since we measured $\alpha(T)$ and $\bar{v}(T)$, the only unknown in the equation is $(\partial U/\partial V)_T$. It can be shown that

$$(\partial U/\partial V)_T = T(\partial P/\partial T)_V - p \tag{6}$$

$$\text{and } (\partial P/\partial T)_V = \alpha/\kappa_T$$

Insertion of these results into Eq. (5) yields the relationship

$$c_P - c_V = \frac{\alpha^2}{\kappa_T}TV, \tag{7}$$

where α is the expansion coefficient, $\kappa_T = -(1/V)(\partial V/\partial P)_T$ the isothermal compressibility, T the absolute temperature, and V the volume. Therefore the difference $(c_P - c_V)$ can be calculated from our results and literature data for the compressibility. Since the relation between the heat capacities is entirely general, it can be applied to both the native and denatured state of the protein. Denoting the difference between the heat capacity at constant pressure and constant volume in the denatured state by $(c_P^D - c_V^D)$ and that in the native state by $(c_P^N - c_V^N)$, we obtain for the change in this quantity, $\Delta_N^D(c_P - c_V)$, that originates from the unfolding process:

$$\begin{aligned}\Delta_N^D(c_P - c_V) &= (c_P^D - c_V^D) - (c_P^N - c_V^N)\\ &= (c_P^D - c_P^N) - (c_V^D - c_V^N)\\ &= \Delta_N^D c_P - (c_V^D - c_V^N)\\ &= \Delta_N^D\left(\frac{\alpha^2}{\kappa_T}TV\right)\end{aligned} \tag{8}$$

If we know the expansion coefficients, the partial specific volumes, the isothermal compressibilities for both the native and the unfolded state, we can calculate $\Delta_N^D(c_P - c_V)$. The isothermal compressibility κ_T can be calculated from the isentropic compressibility κ_S, which is generally the experimentally accessible quantity, by the relation

$$\kappa_T = \kappa_S + \frac{\alpha^2 T}{dc_P} \tag{9}$$

here the density d may be regarded as $(1/\bar{v})$, c_P is the specific isobaric heat capacity at temperature T, κ_S the isentropic compressibility and α the expansion coefficient. Since we know the isobaric heat capacity change from the DSC studies we can also determine the difference between the heat capacities at constant volume $(c_V^D - c_V^N)$.

With the expansion coefficients determined in this study and the isothermal compressibility coefficients for native ($\kappa_{T,N} = 5.48 \times 10^{-11}$ Pa^{-1} (14)) and unfolded ribonuclease A ($\kappa_{T,D} = 6.96 \times 10^{-11}$ Pa^{-1}), from the studies of Brandts [31] and Gekko and Noguchi [14] we obtain a $\Delta_N^D(c_P - c_V)$ value of 1.004 J/(g K). The calorimetrically observed specific isobaric heat capacity change of RNAse A is only 0.33 J/(g K).

The calculation is based on the assumption, that no volume change occurs during the transition, which is obviously in agreement with our experimental results.

$\bar{v}$ and α values at the transition temperature $T_m = 44.7$°C were used in the calculation. For κ_T the value at 25°C was employed, since no significant variation with temperature was found for native proteins [32].

Table 2 summarizes the thermodynamic parameters relevant to the calculations and Table 3 shows the calorimetrically observed heat capacity changes and the calculated values for $\Delta_N^D(c_P - c_V)$. The most remarkable conclusion from these studies is the finding that the positive heat capacity change observed for protein

Table 2
Thermodynamic parameters used for the calculation of $\Delta_N^D(c_p - c_V)$

Protein	$\alpha_{25°C}$ $10^4\,K^{-1}$	$\alpha_{T_m}^N$ $10^4\,K^{-1}$	$\alpha_{T_m}^D$ $10^4\,K^{-1}$	κ_T^N $10^{11}\,Pa^{-1}$	κ_T^D $10^{11}\,Pa^{-1}$	$\bar{v}_{T_m}$ ml/g	T_m K	$T_{m,v}$ K
ribonuclease A	5.0	4.9	7.9	5.48 [14]	6.96	0.689	317.8	317.4
lysozyme	5.1	5.0	8.4	10.40 [29]	11.90	0.725	328.0	327.6
α-lactalbumin	6.0	5.9	8.8	12.46 [14]	13.96	0.748	338.3	336.9
cytochrome c	5.4	4.9	9.1	4.27 [30]	5.77	0.707	333.7	310.7

$\alpha_{T_m}^N$: expansion coefficient of the native protein; $\alpha_{T_m}^D$: expansion coefficient of the denaturated protein; κ_T^N: isothermal compressibility of the native protein (literature values); κ_T^D: isothermal compressibility of the denaturated protein; $\bar{v}_{T_m}$: partial specific volume of the protein at T_m; T_m calorimetric transition temperature; $T_{m,v}$: temperature assigned to the change in the expansion coefficient on the basis of linear extrapolations of the two branches of the $\bar{v}(T)$ curve.

unfolding can be easily rationalized by the difference in work that has to be invested to overcome the intramolecular forces in the denatured and native state.

Another surprising conclusion can be drawn from these calculations. Since $\Delta_N^D(c_P - c_V)$ is more positive than $\Delta_N^D c_P$ Eq. (8) requires $(c_V^D - c_V^N)$ to be a negative quantity. This implies the heat capacity at constant volume of the native protein, c_V^N, to be larger than the analogous value, c_V^D, for the denatured state. Unusual, as this conclusion appears at first sight, there is a possible rationale for it. If one assumes intact tertiary interactions with correlated vibrational modes before the transition and disappearance of these collective modes after the transition, it is imagineable that the native state has a higher heat capacity at constant volume than the denatured state. Reasoning along the same lines a decrease of c_V on denaturation can be envisaged, if a transformation of vibrational modes into rotational modes occurs due to the loss of tertiary coupling. Such a phenomenon would decrease the heat capacity due to the reduced contribution of $\frac{1}{2}R$ per rotational degree of freedom compared with R of the vibrational degree of freedom.

We are not aware of theoretical studies directly addressed to heat capacity of native and denatured proteins. There is one article, however, by Karplus et al. [33] that discusses the related problem of configurational entropy of native proteins. The results of calculations of the internal vibrational entropy suggest 'that the fluctuations, and therefore the configurational entropy in a folded protein, are similar to those of a random coil in a single-potential well'. However, the authors suggest themselves 'that the large magnitude of S_P^{vib} raises the possibility that it may have to be considered explicitly in some cases'. In other words ΔS, being a small difference between two large numbers, cannot quantitatively be predicted yet from such calculations. In view of the close relation between heat capacity and entropy similar arguments might be applied to a discussion of heat capacity differences. It may be reasoned that at least theory does not preclude the native state heat capacity at constant volume to be larger than the value of the unfolded state under the same conditions.

It is evident that our studies still leave a number of questions unanswered. There is no reasonable mecha-

Table 3
Calorimetric heat capacities ($\Delta c_{p,exp}$) and calculated $\Delta_N^D(c_p - c_V)$ values of protein denaturation

Protein	MW	$\Delta c_{p,exp}$ J/(g K)	$\Delta C_{p,exp}$ kJ/(mol K)	$\Delta_N^D(c_p - c_V)$ J/(g K)	$\Delta_N^D(C_p - C_V)$ kJ/(mol K)	pH
ribonuclease A	13700	0.33	4.52	1.004	13.8	2.5 [a]
lysozyme	14300	0.57	6.15	0.85	12.1	2.0 [a]
α -lactal bumin	14200	0.47	6.67	0.70	9.9	7.0 [b]
cytochrome c	12400	0.11	1.36	2.06	25.5	3.2 [a]

[a] 0.1 M potassium-phosphate.
[b] H_2O.

nism for the increase of the expansion and compressibility coefficent in the unfolded state, and we cannot provide yet an explanation for the molecular origin of the larger native-state heat capacity at constant volume. Nevertheless they offer an alternative rationalization of the denaturational heat capacity change of proteins that appears to be worth considering.

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