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Heat capacities of protein functional groups

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Abstract

Using a precise technique of scanning calorimetry the heat capacities of a series of carboxylic acids and their sodium salts, alcohols, and *N*-substituted amides have been measured from 5 to 100 °C. From these data, the partial molar heat capacities of $-CH_2-$, –CONH–, –COOH, and –COONa groups have been determined. It is shown that the heat capacity of the $-CH_2-$ group in aqueous solution is independent of the type of compound used for its determination, is positive at low temperature, and is linearly decreasing in magnitude with an increase in temperature. In contrast, the heat capacities of –COOH and –COONa groups in aqueous solution are negative at room temperature and their magnitude non-linearly decreases with an increase in temperature. It appears that the partial heat capacity of –CONH– group in aqueous solution depends on the type of model compound used for its determination. These differences correlate with the difference in the water accessible surface area of atoms in the –CONH– group in different model compounds. © 1997 Elsevier Science B.V.

Keywords: Scanning calorimetry; Partial heat capacity; Model compounds; Unfolded proteins

1. Introduction

The partial heat capacity of proteins in an aqueous solution is one of the basic thermodynamic parameters specifying the state of a protein. The difference in the heat capacities of native and unfolded states determines the temperature dependencies of all thermodynamic functions describing protein folding/unfolding, i.e. stability of its native structure [1]. With the realization that the difference in the heat capacities of native and unfolded protein is caused mainly by hydration of exposed groups it became clear that the heat capacity of the unfolded state can be used as an indicator of the extent of protein unfolding. This raised interest in the determination of the partial heat capacity of the fully unfolded polypeptide chain in an aqueous solution.

Several years ago an empirical method for calculating the partial molar heat capacities of proteins in the unfolded state over a broad temperature range was developed [2–5]. This was done by careful measurements of the partial molar heat capacities of over two dozen different small organic compounds which contain functional groups identical to those in proteins [3]. Based on these data and using the additivity of heat capacity we have calculated the expected heat capacities of proteins in the unfolded state for a broad range of temperatures from 5 to 125 °C. Initial comparison with the experimentally mea-

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sured heat capacities of four proteins (lysozyme, cytochrome c, ribonuclease A and myoglobin) in the unfolded states, have shown that there is excellent agreement between experimental and calculated values of the heat capacity of unfolded proteins for the entire experimentally accessible temperature range [4,5]. Later the ability of our method to predict the heat capacities of proteins in the unfolded state was demonstrated for more than twenty different proteins by our group [6–10] and many others [11–16]. Still there were several questions that needed more detailed examination. First, is the additivity principle for calculation of the partial molar heat capacities of organic compounds, which contain polar and charged groups, valid? Second, what is the effect of ionization on the heat capacity of charged groups? In this paper we present the results of the measurements of the partial molar heat capacities of the several organic compounds with the aim of answering these questions.

2. Materials and methods

Methanol, ethanol, n-propanol, n-butanol, n-pentanol, propionic acid, butanoic acid, pentanoic acid, sodium propanoate, sodium butanoate, propionamide, *N*-ethylacetamide, *N*-methylpropionamide, and *N*-methylacetamide were purchased from Aldrich Chemical Co. (Milwaukee, WI) and were used without further purification. Aqueous solutions of these compounds in water were prepared by mixing the weighed components, the compound of interest and Milli-Q Plus (Millipore) water. Five solutions for each compound were prepared at the concentrations ranging from 0.3 to 1.5 wt.%.

The apparent molar heat capacity, $C_{p,\phi}$, of the solute at temperature T was determined by measuring the heat capacity difference, $\Delta C_{p,app}$, between the solvent (water) and the solution at this temperature using the differential scanning microcalorimeter developed in The Johns Hopkins University (operating principles are described elsewhere [17], using the equation:

$$C_{p,\phi} = \frac{C_{p,H_2O} \cdot V_\phi^o}{V_{H_2O}} - \frac{M \cdot \Delta C_{p,app}}{m} \quad (1)$$

where C_{p,H_2O} and V_{H_2O} are the molar heat capacity and molar volume of water, respectively. V_ϕ^o is the partial molar volume of the solute, m is the mass of the solute in the calorimetric cell, and M is molar mass. All experiments were performed at a heating rate of 1 °C min^{-1}. In the concentration range studied, no concentration dependence for the apparent molar heat capacity was found. Thus the measured values of $C_{p,\phi}$ were considered as the partial molar heat capacity values, i.e. $C_{p,\phi o}$. Reported values of $C^o_{p,\phi}$ are thus averaged values obtained at five different concentrations.

The apparent molar volume of the studied compounds in water, V_ϕ, was determined from the density of solution, ρ, and the density of the solvent (water), ρ_o, using the equation:

$$V_\phi = \frac{M}{\rho} - \frac{1000 \cdot (\rho - \rho_o)}{c \cdot \rho \cdot \rho_o} \quad (2)$$

where c is the molality of the solution. The density was measured by vibrational densitometer DMA-60/602 (Anton Paar, Austria) at a fixed temperature using water and air as a standard for calibration. The

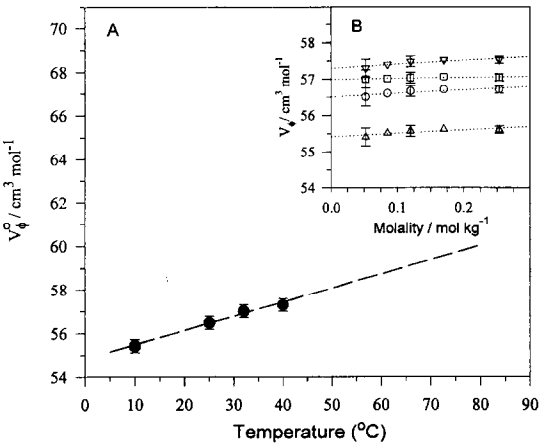

Fig. 1. (a) Temperature dependence of the partial molar volume of *N*-methylformamide in aqueous solution. The symbols represent experimental results, the dotted lines are the linear fit. Coefficients for the linear fit are listed in Table 1. (b) Dependence of the apparent partial molar volume of *N*-methylformamide in aqueous solution on the molality of solution at different temperatures: (△) 10 °C; (○) 25 °C; (□) 32 °C; (▽) 40 °C. Dashed lines represent linear extrapolation to the infinite dilution, i.e. partial molar volume, V_ϕ^o.

Table 1
Partial molar volume, V_ϕ°, and its temperature dependence $V_\phi^\circ = b(0) + b(1) \cdot t(°C) + b(2) \cdot t(°C)^2$ for the studied compounds, in aqueous solution

Compound	V_ϕ° ($cm^3\ mol^{-1}$)	$V_\phi^\circ = b(0) + b(1) \cdot t(°C) + b(2) \cdot t(°C)^2$		
	25 °C	$b(0)$	$b(1)$	$b(2)$
Methanol	38.10 (38.1 [a], 38.25 [c])	38.00	1.01×10^{-4}	1.59×10^{-4}
Ethanol	55.09 (55.0 [b], 55.12 [c])	55.52	−0.037	7.94×10^{-4}
n-Propanol	70.77 (70.8 [b], 70.63 [c])	70.41	-5.47×10^{-3}	7.97×10^{-4}
n-Butanol	86.52 (86.5 [b], 86.48 [c])	85.39	0.031	5.74×10^{-4}
n-Pentanol	102.47 (102.3 [b], 102.88 [c])	100.69	0.0545	6.65×10^{-4}
n-Propionic acid	67.35 (67.8 [a], 67.9 [d])	65.24	0.0847	–
n-Butanoic acid	83.61 (84.6 [d])	80.70	0.1163	–
n-Pentanoic acid	99.34 (100.5 [d])	95.71	0.1453	–
Sodium propanoate	53.22 (53.84 [d])	51.00	0.0887	–
Sodium butanoate	68.71 (69.19 [d])	64.97	0.1496	–
n-Propionamide	71.10 (71.3 [a], 71.54 [d])	69.18	0.0766	–
N-methylformamide	56.46 (56.78 [c])	54.84	0.0648	–
N-methylacetamide	73.89 (74.04 [c])	72.93	0.0383	–
N-methylpropionamide	89.65 (89.75 [c])	88.41	0.0497	–
N-ethylacetamide	90.21 (90.72 [c])	88.56	0.0658	–

[a] Ref. [18].
[b] Ref. [19].
[c] Ref. [41].
[d] Ref. [42].
Estimated error in the V_ϕ° values is in the order of 0.5–1%.

partial molar volume at infinite dilution, V_ϕ°, was obtained by linear extrapolation of the apparent molar volumes, V_ϕ, to zero concentration (Fig. 1(b)). Resultant partial molar volumes, V_ϕ°, obtained at different temperatures have been extrapolated for the entire temperature range from 5 to 100 °C (Fig. 1(a)) using a first- or second-order polynomial (Table 1). The details of the partial molar volume determinations are considered elsewhere [18].

3. Results

The partial molar volumes of methanol, ethanol, n-propanol, n-butanol, n-pentanol, n-propionic acid, n-butanoic acid, n-pentanoic acid, sodium propanoate, sodium butanoate, propionamide, *N*-ethylacetamide, *N*-methylpropionamide, and *N*-methylacetamide at 25 °C are listed in Table 1. In the same table the data on these compounds obtained by various groups are also listed for comparison.

The partial molar heat capacities of methanol, ethanol, n-propanol, n-butanol, n-pentanol, n-propionic acid, n-butanoic acid, n-pentanoic acid, sodium propanoate, sodium butanoate, propionamide, *N*-ethylacetamide, *N*-methylpropionamide, and *N*-methylacetamide are listed in Table 2 and are presented in Figs. 2–4. Fig. 2 also presents the partial molar heat capacities of alcohols obtained by us earlier [19]. There is an excellent agreement between partial heat capacities obtained on different preparations of alcohols using different generations of scanning calorimeters. The same is valid for propionic acid and propionamide (Figs. 3 and 4), for which the partial molar heat capacities in the broad temperature range were reported by us earlier [3].

Fig. 5 presents the dependence of the partial molar heat capacity of the $-CH_2-$ group obtained from the homologous series of the carboxylic acids, sodium salts of carboxylic acids, and *N*-substituted amides. This was done by considering differences between partial molar heat capacities of homologous compounds with different numbers of methyl groups. There is good correlation between the values for

Table 2
Partial molar heat capacity, $C^{o}_{p,\phi}$, and its temperature dependence $C^{o}_{p,\phi} = b(0) + b(1) \cdot t(°C) + b(2) \cdot t(°C)^2 + b(3) \cdot t(°C)^3$ for the studied compounds in aqueous solution

Compound	$C_{p,\phi}$ (J K^{-1} mol^{-1})	$C^{o}_{p,\phi} = b(0) + b(1) \cdot t(°C) + b(2) \cdot t(°C)^2 + b(3) \cdot t(°C)^3$			
	25 °C	$b(0)$	$b(1)$	$b(2)$	$b(3)$
Methanol	156 (158.3 [a],158.2 [c])	155.1	0.0365	–	–
Ethanol	268 (262.5 [b],260.3 [c])	285.2	−0.8400	6.27×10^{-3}	–
n-Propanol	361 (355.2 [b], 352.9 [c])	376.2	−0.7085	3.73×10^{-3}	–
n-Butanol	447 (445.9 [b], 437 [c])	467.3	−0.8743	3.05×10^{-3}	–
n-Pentanol	544 (539.5 [b], 523.8 [c])	574.9	−1.3202	2.59×10^{-3}	–
n-Propionic acid	252 (257 [b], 253 [c])	244.6	0.3427	-1.10×10^{-3}	–
n-Butanoic acid	335 (337 [c])	328.1	0.2875	-1.15×10^{-3}	–
n-Pentanoic acid	419 (432 [c])	416.6	0.1240	-1.27×10^{-3}	–
Sodium propanoate	158	83.3	4.1410	−0.050	1.74×10^{-4}
Sodium butanoate	241	170.5	3.8870	−0.046	1.44×10^{-4}
n-Propionamide	257 (258.2 [a], 253.6 [c])	243.5	0.6000	-2.50×10^{-3}	–
N-methylformamide	163 (164 [c])	142.6	0.8904	-2.86×10^{-3}	–
N-methylacetamide	253 (258 [c])	247.0	0.4785	-8.86×10^{-3}	–
N-methylpropionamide	344 (334 [c])	338.8	0.2033	4.40×10^{-5}	–
N-ethylacetamide	347 (343 [c])	337.2	0.4026	-8.41×10^{-4}	–

[a] Ref. [3].
[b] Ref. [19].
[c] Ref. [41].
Estimated error in the $C^{o}_{p,\phi}$ values is in the order of 3%.

$C^{o}_{p,\phi}(-CH_2-)$ obtained from these compounds and the ones calculated from a different set of a homologous series of model compounds [3]. This once more indicates additivity of the group contribution to the partial molar heat capacity of organic molecules in aqueous solution. Using the partial molar heat capacities of $-CH_3$ and $-CH_2-$ groups, reported earlier [3], we can calculate the partial molar heat capacity of the peptide unit, i.e. $C^{o}_{p,\phi}(-CONH-)$ as:

$$C^{o}_{p,\phi}(-CONH-) = C^{o}_{p,\phi}(\text{amide}) - N_{CH_3} \cdot C^{o}_{p,\phi}(-CH_3) - N_{CH_2} \cdot C^{o}_{p,\phi}(-CH_2-) - N_H \cdot C^{o}_{p,\phi}(-H) \quad (3)$$

where N_{CH_3}, N_{CH_2}, and N_H are the number of $-CH_3$, $-CH_2-$, and $-H$ groups in the molecule of amide, respectively, and $C^{o}_{p,\phi}(-CH_3-)$, $C^{o}_{p,\phi}(-CH_2-)$, and $C^{o}_{p,\phi}(-H)$ are corresponding partial molar heat capacities for these groups. The temperature dependence of $C^{o}_{p,\phi}(-CONH-)$ is presented in Fig. 6, which also presents the heat capacities of the –CONH– group calculated from two different model compounds, glycine peptides and cyclodiglycine, reported by us earlier [20]. There is a significant difference in the heat capacity of the –CONH– group depending on the type of model compound used for its evaluation. A possible explanation for this is discussed below.

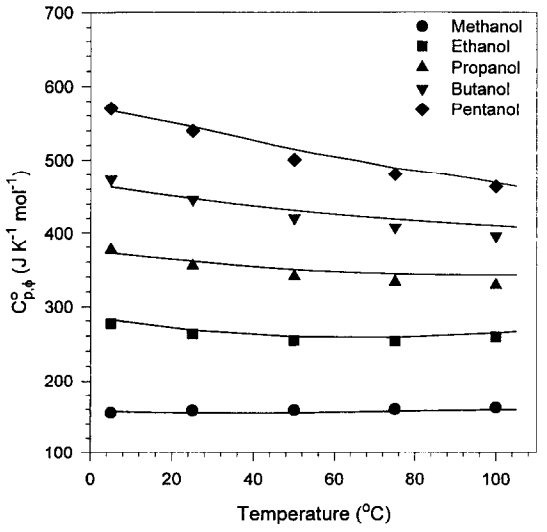

Fig. 2. Temperature dependence of the partial molar heat capacity of linear alcohols. The solid lines represent results of this work, the symbols represent results of previous measurements (Ref. [3] for methanol; Ref. [18] for the remaining alcohols).

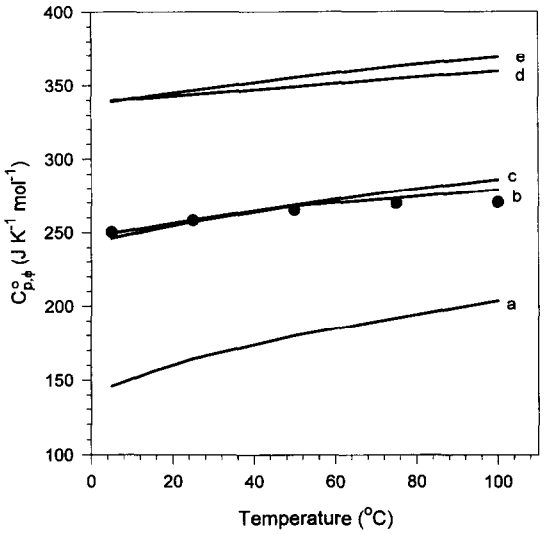

Fig. 3. Temperature dependence of the partial molar heat capacity of *N*-substituted amides. The solid lines represent results of this work, The symbols represent results of previous measurements for n-propionamide [3]. a, *N*-methylformamide; b, *n*-propionamide; c, *N*-methylacetamide; d, *N*-methylpropionamide; e, *N*-ethylacetamide.

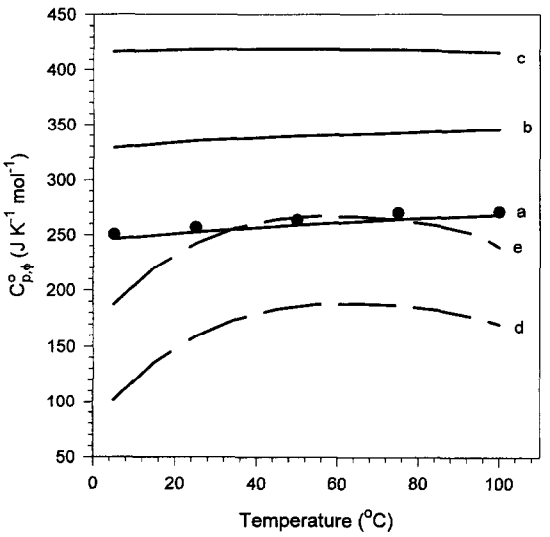

Fig. 4. Temperature dependence of the partial molar heat capacity of linear carboxylic acids (————) and their sodium salts (---). The lines represent results of this work, the symbols show the results of previous measurements for *n*-propionic acid [3]. a, propionic acid; b, butanoic acid; c, pentanoic acid; d, sodium propionate; e, sodium butanoate.

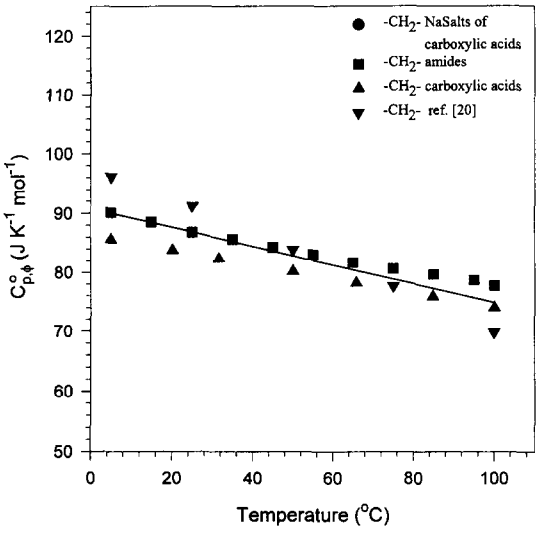

Fig. 5. Temperature dependence of the partial molar heat capacity of the $-CH_2-$ group, obtained from different model compounds. The line represents the best fit over all points: $C^{o}_{p,\phi}(-CH_2-) = 90.85-0.16 \cdot t(°C)$.

Using an equation similar to Eq. (3) we can calculate the partial molar heat capacities of –COOH and –COONa groups which are presented in Fig. 7.

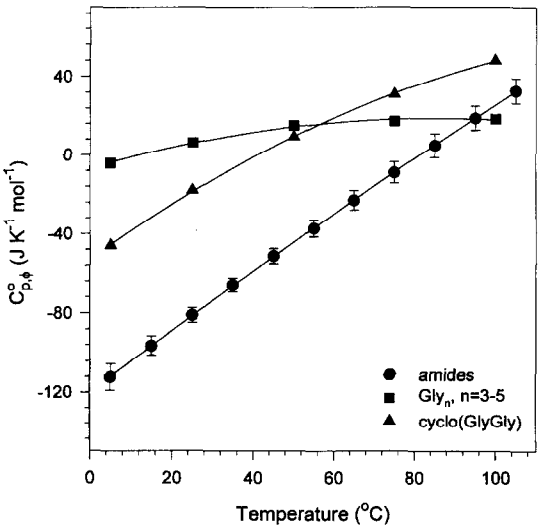

Fig. 6. Temperature dependence of the partial molar heat capacity of the –CONH– group obtained from different model compounds.

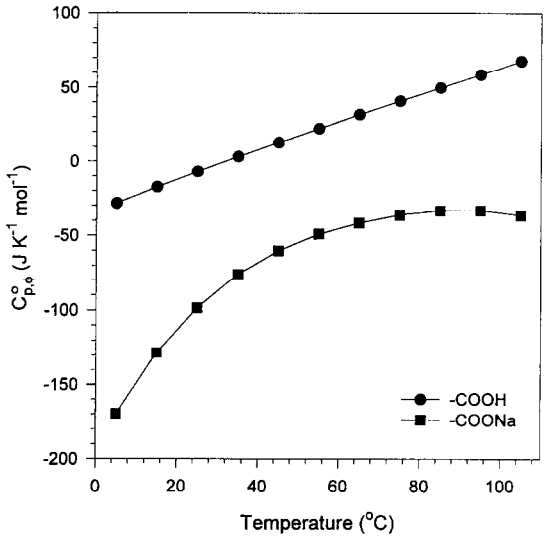

Fig. 7. Temperature dependence of the partial molar heat capacity of the –COOH and –COONa groups.

As one can see, the partial molar heat capacity of the sodium salt is always lower than the corresponding acid. It should be noted that the $C^{o}_{p,\phi}$(–COOH) are in good agreement with those obtained previously [3].

4. Discussion

4.1. Temperature dependence of the heat capacity of the peptide unit

The peptide group, –CONH–, is the most omnipresent group in proteins. However, its contribution to the partial molar heat capacity of protein in the unfolded state is only in the order of 7% [4,5]. Nevertheless, it is important to know the partial molar heat capacity of this group with reasonable precision. This raises the question of which values of the heat capacity of the –CONH– group one should use for the calculations of the partial molar heat capacities of proteins in the unfolded state (see Fig. 6). For this, one has to first understand the possible reasons for the observed differences in the heat capacities of –CONH– groups obtained from different model compounds, i.e. glycine peptides, cyclodiglycine, and *N*-substituted amides.

The heat capacity of any compound in aqueous solution, $C^{o}_{p,\phi}$, can be represented by a sum of two terms [3]:

$$C^{o}_{p,\phi} = C^{anh}_{p} + \Delta C^{hyd}_{p} \quad (4)$$

where C^{anh}_{p} corresponds to the "intrinsic" heat capacity and depends only on the nature of constituting atoms or covalent bonds, i.e. is additive [21]. The second term, ΔC^{hyd}_{p}, reflects the heat capacity effects of interactions with the solvent (water) [3–5]. The hydration heat capacity, ΔC^{hyd}_{p}, is proportional to the water accessible surface area (ASA) of the molecule [3–5,22–27]. Thus one can suggest that the difference in the partial molar heat capacities of the –CONH– group obtained from different model compounds arises from the difference in the water accessible surface areas of this group. Indeed calculations show (Table 3) that there is a significant difference in the ASA of atoms of the –CONH– group in glycine peptides, cyclodiglycine and *N*-substituted amides. This difference is most pronounced for N and O atoms. The absolute ASA values for these two atoms vary from 12 Å^2 to 27 Å^2, and 29 Å^2 to 40 Å^2, respectively (Table 3). For comparison, the surface areas of N and O atoms in the extended polypeptide chain are 5 and 24 Å^2, respectively. These calculations provide a qualitative description for the observed differences in the values of $C^{o}_{p,\phi}$(–CONH–) obtained from different model compounds. A quantitative description will be possible with the accumulation of a larger set of model compounds and with the improvements of the calculations of ASA. Currently in most cases ASA is calculated for a given static

Table 3
Water accessible surface areas for the atoms in the –CONH– group, calculated for different model compounds

Compound	ASA of –CONH– (Å^2)		
	C	O	N
N-ethylacetamide	5	34	25
N-methylacetamide	6	34	26
N-methylpropionamide	7	28	27
Cyclo(GlyGly)	5	40	23
Glycine peptide (Gly$_n$, n = 3–5)	4	29	12
Unfolded protein [a]	2	24	5

[a] Unfolded polypeptide chain is modeled in a fully extended conformation as described in Ref. [43].

structure which was generated using a particular molecular modeling software package. Of course this is a crude approximation of the dynamic structure of the molecule in solution. More reliable surface area calculations promise to be obtained on the Monte Carlo ensemble of structures [28,29].

The question, however, remains as to which model compound provides more reliable estimates for the partial molar heat capacity of the peptide unit, $C^{\circ}_{p,\phi}$(–CONH–). We believe that so far the best studied model compounds are glycine peptides. This is based on two major considerations: (i) glycine peptides are closest in chemical nature to the protein backbone; (ii) the surface area of the peptide unit in glycine peptides is the closest to the exposure of the peptide backbone in the extended state of polypeptides.

Based on the analysis of our experimental data on model compounds [3], it has been argued by Khechinashvili et al. [30] that the heat capacity of the unfolded proteins cannot have a non-linear dependence. This conclusion seems to be unjustified based on our previous results [3,20], the experimental results presented here, as well as numerous direct experimental measurements of the temperature dependence of the partial molar heat capacities of proteins in the unfolded state [6–16]. It seems quite clear from the data on the model compounds that the partial molar heat capacities of polar groups including peptide unit have a non-linear dependence on temperature.

4.2. Temperature dependence of the heat capacity of non-polar groups

The partial molar heat capacities of the non-polar groups (i.e. aliphatic and aromatic) appear to be significantly dependent on temperature. It has been shown on a number of model compounds for the temperature range from 5 to 150 °C, that the partial molar heat capacity of non-polar compounds monotonically decreases with an increase of temperature [19,20,22–24,31,32]. However, the heat capacity functions for aliphatic and aromatic compounds differ both in the specific absolute values and in the shape. For aliphatic compounds it appears that the partial molar heat capacity can be described by the linearly decreasing function of temperature (see, for example, Refs. [19,20,22–24] and Fig. 5). In contrast, the heat capacity of aromatic groups seems to be a non-linearly decreasing function of temperature [31,33]. The heat capacity of aromatic group decreases faster at lower temperatures than at higher. Nevertheless, for both aromatic and aliphatic compounds the temperature dependence of the partial molar heat capacity is a monotonic function. These experimental observations are in contradiction with the recent calculations by Gomez et al. [32]. They fit experimental data on the partial molar heat capacities of nine proteins and their water accessible surface areas to 10 independent parameters. From these Gomez et al. concluded that the heat capacity contribution of non-polar groups should have a maximum at about room temperature and should decrease significantly only above 40 °C. Unfortunately such behavior of the heat capacity of non-polar groups have not been yet observed experimentally. It appears that even statistically sound solutions of global fitting of experimental results should always be considered cautiously in terms of the physical meaning that they contain.

4.3. Temperature dependence of the heat capacity of ionizable residues

Comparison of the heat capacities of carboxylic acids in the free and sodium salt forms show that ionization effects can significantly affect the absolute values of the partial molar heat capacities as well as their temperature dependencies. It appears from Figs. 4 and 7 that the heat capacity of the –COOH group is always higher than the corresponding sodium salt, i.e. –COONa. Similar effects have been observed for the analogs of the positively charged amino acid residues Lys and Arg [3]. Thus protonation/deprotonation can affect the partial molar heat capacity very significantly.

Unfortunately such effects are not always taken into account which results in artifacts. This is particularly significant in the case of small compounds such as small peptides. Contemporary differential scanning microcalorimetry is a very sensitive tool and can easily register very small differences in solvent composition [1,17]. These small differences affect both the absolute value of the measured heat capacity and to a larger extent the temperature de-

pendence of the partial heat capacity [1]. The sample of interest prior to the calorimetric experiments should be equilibrated with a corresponding solvent to obtain equal chemical potential between solution of compound studied and the solvent. In the case of large solute molecules such as proteins, DNA, and RNA this can be achieved by extensive dialysis against a corresponding buffer. In the case of small compounds such as Gly–X–Gly, this can be done only by using gel filtration. Direct dissolution of a peptide or protein sample in the corresponding buffer gives an equimolar concentration of low molecular weight component (buffer). In the case of ionic compounds this will lead to the non-identical conditions in terms of buffer composition. Ionic solute will directly interact with the buffer components, thus decreasing its bulk concentration. The relative heat capacity difference of the solution and the solute measured in DSC experiment in the case of equimolar solution cannot be used for the determination of the partial molar heat capacity of the solute according to Eq. (1). It appears that direct dissolution of glycine-based tripeptides in buffered aqueous solution produced significant "unexplained" contradictions with the earlier reported values in the measurements of partial molar heat capacity by Vogl et al. [34].

Another important implication of the observed changes in the heat capacities of charged residues under different ionization states is its possible influence of the partial molar heat capacities of unfolded proteins.

4.4. Heat capacity of proteins in the unfolded state

It has been experimentally observed on several proteins (staph. nuclease [35], apomyoglobin [36], α-lactalbumin, [37], and ubiquitin [38]) that the absolute values of the partial molar heat capacity in the unfolded state appear to decrease with a decrease of pH from neutral to acidic. Two explanations for this can be suggested. First, the changes in the heat capacity upon decrease in pH are related to the protonation of carboxylic groups which are charged at neutral pH and neutral at acidic pH. Second, the unfolded state can become more compact with the decrease in the overall number of charges on the molecule, as it leads to an increase of the hydrophobicity of side chains of aspartic and glutamic acids [35]. Such compactization will lead to the decrease in ASA and correspondingly the decrease in the heat capacity. Data on the model compounds presented here rules out the first explanation because protonation of the carboxyl groups actually leads to an increase in the heat capacity. Based on this we think that the decrease of the overall dimensions of the unfolded polypeptide is more probable. This is also consistent with the theoretical calculations on the compactness of the unfolded state [39,40].

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