ELSEVIER

Biophysical Chemistry 126 (2007) 16–24

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Microcalorimetry of biological macromolecules

Peter L. Privalov *, Anatoly I. Dragan

Johns Hopkins University, Baltimore, MD 21218, USA

Received 22 February 2006; accepted 8 May 2006
Available online 13 May 2006

Dedicated to the memory of Professor Julian Sturtevant.

Abstract

The capabilities of contemporary differential scanning and isothermal titration microcalorimetry for studying the thermodynamics of protein unfolding/refolding and their association with partners, particularly target DNA duplexes, are considered. It is shown that the predenaturational changes of proteins must not be ignored in studying the thermodynamics of formation of their native structure and their complexes with partners, particularly their cognate DNA duplexes.

Keywords: Microcalorimetry; Thermodynamics; Proteins; DNA; Unfolding; Association; Hydration

1. Introduction

Since it was realized that the formation of the unique spatial structures of biological macromolecules, i.e. proteins, nucleic acids and their specific complexes are in principal reversible, thermodynamically driven reactions, investigation of the thermodynamics of these processes has gained high priority. This has required direct measurements of the heat effects associated with these intra- and inter-macromolecular processes using highly dilute solutions of these molecular objects. Realization of this program required development of special super-sensitive calorimetric techniques, differential scanning (DSC) and isothermal titration (ITC) microcalorimetry, for measuring the heats associated with change in temperature at fixed solvent conditions or with change in solvent conditions at fixed temperature, particularly on forming specific complexes of proteins with other molecules.

Contemporary DSC instruments are characterized not only by high sensitivity but by the high stability of their baseline and the ability to scan aqueous solutions up to and above 100 °C under excess pressure and by supercooling down below 0 °C. The wide operational range is important because changes of many macromolecules take place over a very broad temperature range. As for the stability of the baseline, this is important since it permits determination of the partial heat capacity of the solute (macromolecule) in dilute solution. As shown below, knowledge of the absolute partial heat capacity of macromolecule over a wide temperature range opens new prospects for studying their thermodynamic properties.

The main specificities of the contemporary ITC instruments are their sensitivity (i.e. the nanomolar consumption of material for experiment), the stable work at various fixed temperatures and fast equilibration at reloading the sample and changing the temperature at which measurements are carried out.

Below, we consider examples of using these contemporary microcalorimetric techniques in solving problems of structural molecular biology.

2. Heat capacity studies of proteins

The very first calorimetric studies of the temperature induced unfolding of compact globular proteins showed that this process is associated with extensive heat absorption over a temperature range that depends on the solvent conditions and results in a significant heat capacity increment (Fig. 1a,b). Increase of pH increases the stability of proteins, the heat effect of unfolding and its sharpness.

* Corresponding author.
E-mail address: privalov@jhu.edu (P.L. Privalov).

0301-4622/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.bpc.2006.05.004

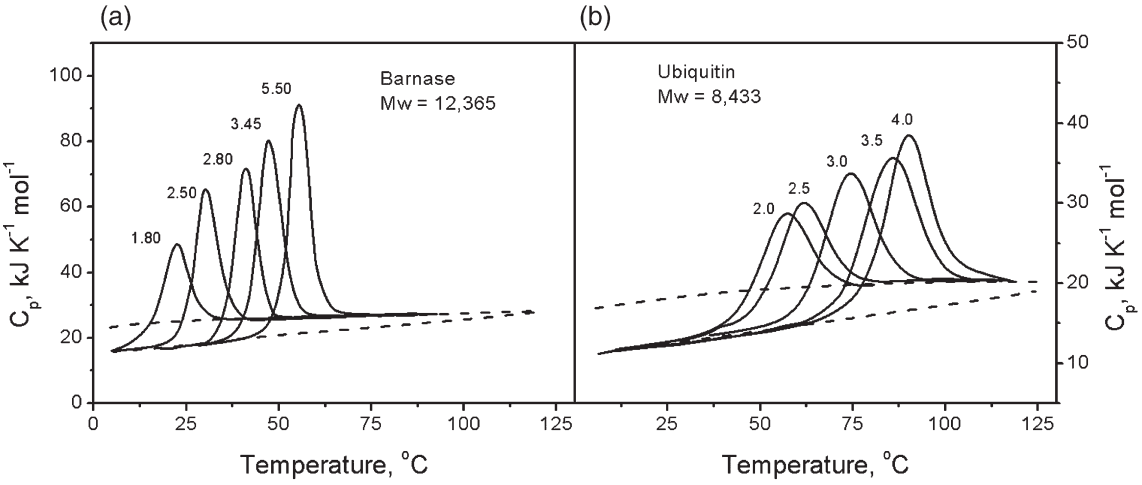

Fig. 1. The partial molar heat capacity functions of (a) barnase (Mw=12.4 kDa) and (b) ubiquitin (Mw=8.4 kDa), in solutions with different pH. The dashed lines represent the partial molar heat capacity of native and unfolded proteins. For the native state, it is obtained by the linear extrapolation of the initial slope of the heat capacity function; for the unfolded state, it is a curved function calculated by summing up the heat capacities of the amino acid residues constituting its polypeptide chain. For details, see [1,2].

The area of the heat absorption peak represents the calorimetric enthalpy of the unfolding process, $\Delta^{\mathrm{U}}_{\mathrm{N}}H^{\mathrm{cal}}=H^{\mathrm{U}}-H^{\mathrm{N}}$. From the sharpness of unfolding (the shape of the heat absorption peak), one can derive the van't Hoff enthalpy, $\Delta^{\mathrm{U}}_{\mathrm{N}}H^{\mathrm{vH}}$, assuming that this process represents a two-state transition [3–5]. For small, single domain globular proteins, the calorimetric enthalpy was found to be in a good correspondence with the van't Hoff enthalpy of unfolding. The correspondence of these two enthalpies, the calorimetric and van't Hoff, is the strongest indication that the unfolding of single domain globular proteins is a highly cooperative transition, proceeding without noticeable intermediates [4]. In that case, the Gibbs energy difference between the native and unfolded states, $\Delta^{\mathrm{U}}_{\mathrm{N}}G=G^{\mathrm{U}}-G^{\mathrm{N}}$, is zero at the transition midpoint T_{t},

$$\Delta^{\mathrm{U}}_{\mathrm{N}}G(T_{\mathrm{t}})=\Delta^{\mathrm{U}}_{\mathrm{N}}H(T_{\mathrm{t}})-T_{\mathrm{t}}\Delta^{\mathrm{U}}_{\mathrm{N}}S(T_{\mathrm{t}})=0 \tag{1}$$

and for the transition entropy we have:

$$\Delta^{\mathrm{U}}_{\mathrm{N}}S(T_{\mathrm{t}})=\Delta^{\mathrm{U}}_{\mathrm{N}}H(T_{\mathrm{t}})/T_{\mathrm{t}} \tag{2}$$

Since according to Kirchhoff's relation, $\partial\Delta H/\partial T=\Delta C_{\mathrm{p}}$, and similarly $\partial\Delta S/\partial T=\Delta C_{\mathrm{p}}/T$, using the difference between the heat capacities of the folded and unfolded forms of the protein, $\Delta^{\mathrm{U}}_{\mathrm{N}}C_{\mathrm{p}}(T)=C_{\mathrm{p}}(T)^{\mathrm{U}}-C_{\mathrm{p}}(T)^{\mathrm{N}}$, one can determine the temperature dependencies of the enthalpy and entropy of protein unfolding:

$$\Delta^{\mathrm{U}}_{\mathrm{N}}H(T)=\Delta^{\mathrm{U}}_{\mathrm{N}}H(T_{\mathrm{t}})+\int_{T_{\mathrm{t}}}^{T}\Delta^{\mathrm{U}}_{\mathrm{N}}C_{\mathrm{p}}(T)\mathrm{d}T \tag{3}$$

$$\begin{aligned}\Delta^{\mathrm{U}}_{\mathrm{N}}S(T)&=\Delta^{\mathrm{U}}_{\mathrm{N}}S(T_{\mathrm{t}})+\int_{T_{\mathrm{t}}}^{T}\Delta^{\mathrm{U}}_{\mathrm{N}}C_{\mathrm{p}}(T)\mathrm{d}\ln T\\&=\Delta^{\mathrm{U}}_{\mathrm{N}}H(T_{\mathrm{t}})/T_{\mathrm{t}}+\int_{T_{\mathrm{t}}}^{T}\Delta^{\mathrm{U}}_{\mathrm{N}}C_{\mathrm{p}}(T)\mathrm{d}\ln T\end{aligned} \tag{4}$$

$$\Delta^{\mathrm{U}}_{\mathrm{N}}G(T)=\Delta^{\mathrm{U}}_{\mathrm{N}}H(T)-T\Delta^{\mathrm{U}}_{\mathrm{N}}S(T) \tag{5}$$

Thus, the main problem in determining these thermodynamic functions specifying protein unfolding/refolding is in specifying the heat capacity of the native and unfolded states of the protein over the whole considered temperature range. In the first calorimetric experiments, when the precision of the calorimetric measurements was not high, it was assumed that the difference between the heat capacity of the native and unfolded state does not depend on temperature and the enthalpy of protein unfolding is a linear function of temperature [3]. With the appearance of precise scanning calorimeters having a stable base line over a broad temperature range [6,7], it was found that the heat capacities of the native and unfolded states of proteins do not change in parallel [8]. Detailed studies of the heat capacity of unfolded proteins showed that this is represented by a curved function of temperature (Fig. 1a,b). As regards the partial heat capacities of native compact globular proteins (such as BPTI, barnase, myoglobin, lysozyme and ubiquitin), they appeared to be a rather similar linear function of temperature. Thus, the difference between the heat capacities of the native and unfolded states appeared to be a decreasing function of temperature, vanishing at about 120 °C. Correspondingly, enthalpies of protein unfolding calculated using Eq. (3) were described by asymptotic functions approaching some level at this temperature (Fig. 2, curves 1 and 2).

However, with an increase in the number of calorimetrically studied proteins, it became evident that there are many proteins with a much steeper slope of the heat capacity function in the predenaturational temperature range than previously found when studies were restricted to compact globular proteins with rigid native structures (Fig. 3a,b). In these cases, extrapolating the heat capacity function of the initial state to higher temperatures resulted in its crossing the heat capacity function of the final state at temperatures very much lower than 120 °C. Thus, the difference between the heat capacities of the unfolded state and the (apparent) heat capacity of the folded state, $\Delta^{\mathrm{U}}_{\mathrm{N}}C_{\mathrm{p}}(T)$, appeared

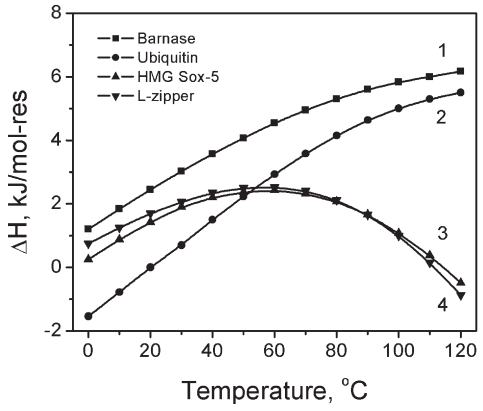

Fig. 2. Temperature dependencies of the specific enthalpy of unfolding of barnase (1), ubiquitin (2), HMG Sox-5 DBD (3), leucine zipper GCN4 (4), calculated by the linear extrapolation of the initial heat capacity function.

to change sign at low temperature significantly lower than 120 °C. This led to the confusing conclusion that, according to Eqs. (3) and (4), above this "crossing over" temperature, the enthalpy and entropy of cooperative protein unfolding become decreasing functions of temperature (Fig. 2, curves 3 and 4). This shows that the straightforward extrapolation of the apparent heat capacity function of the native protein cannot be correct. It became clear that the increasing heat capacity of the native protein with temperature rise is not simply due to intensifying vibrations of the protein structure, but reflects an accumulation of energy upon heating that results from other changes in the protein's structure. Larger slopes of the partial specific heat capacity function are particularly seen for proteins that form complexes with other molecules and especially for DNA binding-domains (Table 1). Fig. 4 illustrates the range of initial slopes of partial specific heat capacity functions of various proteins. It is remarkable that all these heat capacities converge as the temperature decreases, implying that at low temperatures (about −20 °C) they must be rather similar.

Fig. 4 and Table 1 include also the specific heat capacity of an anhydrous protein (chymotrypsinogen) measured by an absolute heat capacity calorimeter [9]. In the absence of water, the specific heat capacity functions of all studied proteins and polypeptides (i.e. calculated per gram or mole of residue) are

Table 1
Thermodynamic characteristics of proteins calculated per mole of amino acid residue assuming the molecular mass of residue 115 Da

N	Protein (reference)	C_p (0 °C)	dC_p/dT	The gross cooperative unfolding				ΔH^{tot} (120 °C)
				T_t	$\Delta C_{p,t}$	ΔH_t	ΔH_{120}	
1	Anhydrous protein [9]	133	0.47					
2	BPTI [19]	141	0.65	100.5	22	5.3	5.7	5.7
3	Barnase [19]	140	0.70	54.3	53	4.8	6.2	6.7
4	Myoglobin [19]	138	0.80	70.8	63	3.4	5.8	7.0
5	Lysozyme [19]	140	0.82	75.3	57	4.3	5.7	7.3
6	Cytochrome C [19]	143	0.90	72.1	42	2.8	5.6	6.3
7	Ubiquitin [19]	143	0.90	90.0	33	4.0	5.0	6.6
8	T_4 lysozyme [19]	148	0.90	64.4	50	3.6	5.2	7.0
9	RNase T1 [19]	140	0.92	61.2	45	4.88	6.3	7.3
10	RNase A [19]	144	1.10	57.9	43	3.9	5.2	6.7
11	Engrailed [50]	154	1.30	48.7	25	2.0	1.1	7.5
12	Mat α2 [50]	163	1.43	52.2	19	1.7	0.6	8.0
13	Antennapedia [50]	145	1.48	55.0	23	3.3	2.1	6.5
14	HMGD-74 [38]	145	1.54	41.6	0	2.3	−0.4	7.7
15	LZ-GCN4 [15]	144	1.75	62.5	−16	0.8	−0.5	9.3
16	HMG SOX5 [33]	148	1.88	46.1	14	2.23	0	7.2
17	Zn-finger TFIIIA [52]	153	1.73					
18	NHP6A [39]	155	2.07	38.6	−0.6	1.5	−2.5	7.2
19	SRY [39]	172	2.65	38.0	−37	0.23	−10.0	8.3
20	Lef-79 [39]	176	2.73	44.5	−40	−0.3	−8	7.3

T_t, the transition temperature; ΔH_t, the enthalpy of cooperative unfolding at the transition temperature calculated by linear extrapolation of the initial heat capacity function; ΔH_{120}, the enthalpy of gross cooperative unfolding extrapolated to 120 °C; ΔH_{120}**, the total enthalpy of unfolding at 120 °C calculated by the excess heat capacity integration from −20 °C using as a baseline the heat capacity of the folded BPTI.
T_t in °C; C_p and ΔC_p in J/K(mol-res), dC_p/dT in J/K^2(mol-res); ΔH in kJ/(mol-res).

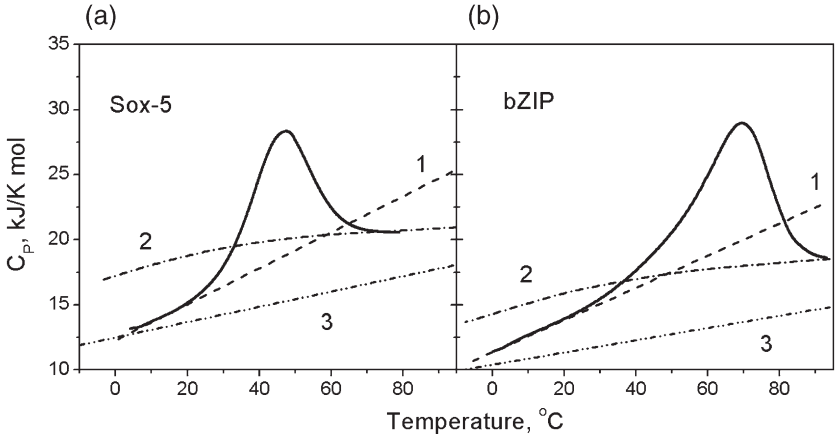

Fig. 3. Temperature dependencies of the molar partial heat capacity of (a) the DNA binding domain (DBD) of Sox-5 [14] and (b) the dimerization domain (leucine zipper) of the GCN4 bZIP [15]. Line 1 shows the linear extrapolation of the initial heat capacity slope; line 2 shows the heat capacity function calculated for the fully unfolded protein; line 3 shows the heat capacity function expected for the fully folded protein (i.e. the partial specific heat capacity of BPTI recalculated to the molecular weights of Sox-5 GCN4-bZIP).

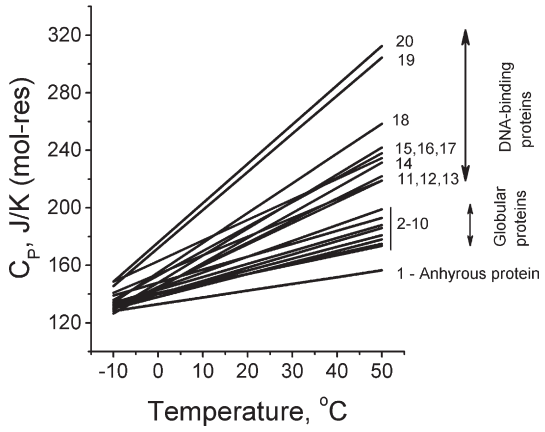

Fig. 4. Temperature dependencies of the initial partial specific heat capacities of various native proteins calculated per mole of amino acid residue, assuming that the averaged molecular mass of a residue is 115 Da. For the proteins and references, see Table 1: the numbers on the curves correspond to those given in the first column of the table.

very similar and in the considered temperature range appear to be perfectly linear [9–11]. It is known that the structure of anhydrous protein is very rigid and does not unfold at any specific temperature. The slight increase of its heat capacity with heating results from the increasing thermal vibrations of its structure [12]. In the presence of water, protein structure becomes more labile. The hydrated protein is more vulnerable to rapid and reversible local disruptions of its structure, which increase in intensity as the temperature rises [13]. It appears therefore that the slope of the heat capacity function depends on the lability of the native protein structure. It is clear that in considering the energetic characteristics of protein unfolding one has to take into account the all energy which is accumulated upon heating and not only the very substantial heat effect associated with gross conformational transitions, i.e. all the excess heat effects must be integrated.

The main problem which is faced here is how to determine this excess heat effect? Previously, it was determined simply by extrapolation of the initial slope of the heat capacity function, which was supposed to correspond to the heat capacity of the completely folded native protein, as shown by the dashed line in Fig. 1. We now know that for most proteins such a line does not represent the heat capacity of the completely folded state. Among the studied proteins, the heat capacity function of bovine pancreatic trypsin inhibitor, BPTI, the structure of which is highly stable being heavily S–S crosslinked, has the minimal slope (Table 1 and Fig. 4). It is sensible therefore to use the partial specific heat capacity of BPTI for determining the excess heat capacity of all other proteins. In Fig. 3, this standard heat capacity is shown by the dashed-and-dotted line 3.

To determine the enthalpy of protein unfolding is also needed the heat capacity of the unfolded state, i.e. the heat capacity function of the completely unfolded polypeptide chain of protein over a broad temperature range (line 2 in Fig. 3). It can be calculated by summing up the partial specific heat capacities of the individual amino acid residues the values of which are well known in the temperature range from 0 to 120 °C [16–18]. If we now assume that at temperature T_o the protein is completely folded, the total enthalpy and entropy of its unfolding can be determined by the integrations:

$$\Delta^{U}_{N}H(T) = \int_{T_o}^{T_{max}} [C_p - C_p^F]dT - \int_{T}^{T_{max}} [C_p^U - C_p^F]dT \quad (6)$$

$$\Delta^{U}_{N}S(T) = \int_{T_o}^{T_{max}} [(C_p - C_p^F]d\ln T - \int_{T}^{T_{max}} [C_p^U - C_p^F]d\ln T \quad (7)$$

Here C_p is the heat capacity of the protein, C_p^U is the heat capacity of unfolded protein and C_p^F is the heat capacity of the completely folded state of the protein calculated using as a standard the specific heat capacity of BPTI, and T_{max} is the temperature to which the protein was heated in the calorimetric experiment and at which it is assumed to be completely unfolded. It is reasonable to start integration from $T_o = -20$ °C where the heat capacities of all the considered proteins are likely to converge and one can assume that all these proteins are in thermodynamically similar states (Fig. 4). Although the partial heat capacities of proteins in aqueous solutions are usually determined down to 0 °C and in some cases by supercooling aqueous solutions down to −10 °C, their linear extrapolation to −20 °C will not result in significant error in the overall enthalpy and entropy of unfolding.

As follows from Table 1, the total enthalpies of unfolding calculated per mole of amino acid residue for 120 °C, ΔH^{tot} (120 °C), differ considerably from the enthalpies of the gross conformational transition at that temperature, ΔH_{120}, determined by the linear extrapolation of the initial heat capacity: for

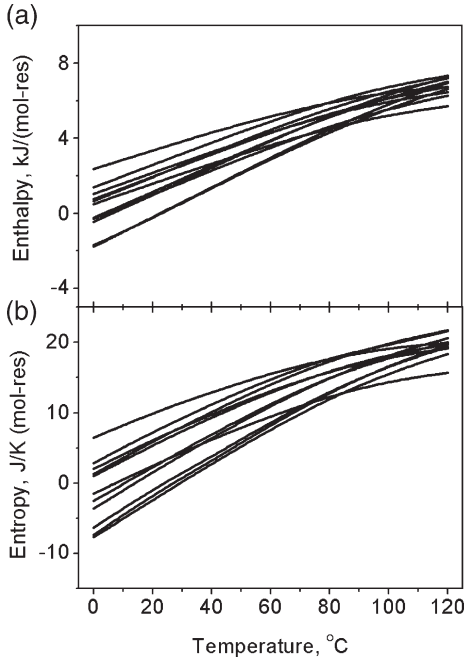

Fig. 5. Temperature dependencies of (a) the total enthalpy and (b) entropy of unfolding of various proteins calculated per mole of amino acid residue using as standard the fully folded state of the protein with the partial specific heat capacity of BPTI. The averaged molecular mass of an amino acid residue is assumed to be 115 Da.

the proteins with loose structure the linear extrapolation of the initial heat capacity function gives totally unrealistic values, e.g. for SRY HMG box one finds that the enthalpy of unfolding is −10 kJ/(mol-res). The functions of total enthalpy and entropy of unfolding are presented in Fig. 5a,b. It is remarkable that these functions for very different globular proteins converge as the temperature increases. Some exception presents BPTI which, being heavily S–S crosslinked does not unfolds completely even at 120 °C. For all other proteins, the averaged asymptotic limit at 120 °C for the enthalpy of unfolding is about 7±1 kJ/(mol-res) and for the entropy about 20±2 J/K(mol-res). These values of the total enthalpies and entropies of unfolding are 30% larger than the enthalpies and entropies of the gross cooperative transitions of globular proteins that were previously determined for compact globular proteins by linear extrapolation of the initial heat capacity function, i.e. ignoring predenaturational changes [19].

The values of the enthalpy and entropy of protein unfolding at 120 °C are of special interest because the enthalpy and entropy of hydration of apolar groups do not contribute at that temperature [19–21]. This is because the heat capacity effect of hydration of apolar groups is positive and, therefore, the negative enthalpy and entropy of hydration of these groups decrease in magnitude with heating and vanish at about 120 °C. In contrast, the heat capacity effect of hydration of polar groups is negative and the negative enthalpy and entropy of hydration of these groups increases in magnitude upon heating [19,22–24]. The enthalpy of unfolding at 120 °C therefore comes from disruptions of van der Waals contacts and hydrogen bonds between polar groups of protein and their hydration. Similarly, the entropy of unfolding at that temperature includes the positive entropy resulting from increase of conformational freedom of the polypeptide chain and the negative entropy of hydration of exposed polar groups. The later explains why the entropy of unfolding reached at 120 °C is significantly lower than the entropy expected for the increase of conformational freedom on protein unfolding that, according to theoretical estimates [25–32], should be about 40 J/K(mol-res).

It is curious that the hydration entropy of polar groups was almost completely ignored in the literature. This is surprising because in explaining the observed small value of the enthalpy of hydrogen bonding in proteins at room temperature, nobody doubted that this is due to the large negative enthalpy of hydrating polar groups compensating almost completely the enthalpy of breaking the hydrogen bonds between protein groups. However, when water molecules interact strongly with polar groups, this should result in a significant decrease in their freedom, i.e. in a decrease of the entropy of water surrounding these groups.

Another temperature of interest is around 20 °C. This is because at that temperature the negative enthalpy of forming van der Waals contacts between nonpolar groups is compensated by the positive enthalpy of their dehydration, i.e. the net enthalpy of hydrophobic interactions is close to zero [19]. At that temperature, the electrostatic interactions also do not contribute much to the enthalpy of protein unfolding since in aqueous solution these interactions are largely entropic. The enthalpy of protein unfolding at 20 °C should therefore be attributed mostly to the hydrogen bonds that stabilize the protein's structure.

3. Analysis of the predenaturational changes in proteins

In the case of some small globular proteins the initial increase of heat capacity proceeds so steeply that one might indeed doubt this results from a gradual process of intensification of local fluctuations, rather than a cooperative transitions of some sub-parts of these proteins [33]. The great advantage of calorimetry in analysis of the melting profiles of biological macromolecules is that enthalpy and temperature are conjugate extensive and intensive thermodynamic parameters specifying the system and their functional dependence includes all the information on the states of the system populated over the considered temperature range [34–37]. Therefore, this information can be revealed by the deconvolution analysis of the heat capacity function. Such analysis showed that small globular proteins with compact rigid structure, such as barnase, lysozyme, ribonuclease, ubiquitin, unfold in a single cooperative stage, i.e. their structure represents a single highly cooperative domain [4,19]. However, in the cases of many other small globular proteins, the temperature induced change of their structure proceeds not in one but in several cooperative stages. For example, the DNA binding domains (DBDs) of the HMG box family, the molecular mass of which is only about 10 kDa, unfold in several cooperative stages (Fig. 6a,b): the non-sequence-specific DBDs unfold in two stages, while the sequence-specific DBDs unfold in three stages [33,38,39].

The low temperature transitions in these HMG DBDs proceed with small enthalpy and are correspondingly spread out over a wide temperature region, strongly overlapping. Thus, without deconvolution analysis of the heat capacity profiles it would be impossible to show that unfolding of these proteins proceeds in several cooperative stages. Since a two-state transition in proteins usually corresponds to unfolding of a cooperative domain organized around a hydrophobic core [40], one can conclude that the L-shaped structure of the HMG box DBDs is subdivided into several cooperative subdomains, three for SRY and two for NHP6A. Correspondingly, it follows that the sequence-specific SRY-DBD has a more flexible structure

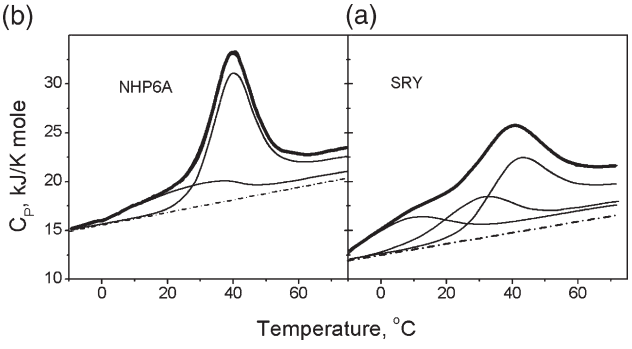

Fig. 6. Deconvolution analysis of the heat capacity functions of the HMG box DNA binding domains of (a) Sry and (b) NHP6A showing that the structure of these two small proteins (Mw about 10 kDa) is subdivided into three (in the case of SRY) and two (in the case of NHP6A) subdomains. For details, see: [39].

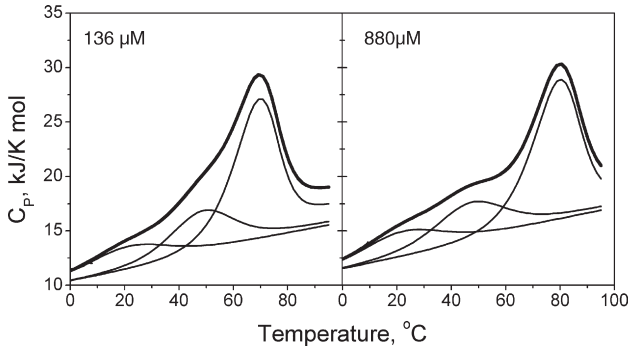

Fig. 7. Heat capacity functions of the GCN4 leucine zipper (Leu-ZIP GCN4) determined by DSC for protein concentrations of 136 μM and 880 μM and the deconvolution into their component transitions according to [15].

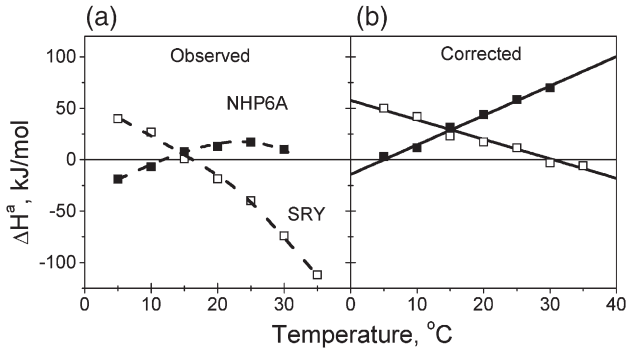

Fig. 8. (a) The ITC-measured association enthalpies of the DNA binding domains of SRY and NHP6A with their optimal DNAs plotted as functions of temperature. (b) The association enthalpies corrected for the refolding of proteins upon binding. For details, see [39].

than the non-sequence-specific NHP6A-DBD. For details of calorimetric studies of the stability of HMG DBDs, see [33,38,39].

Another example of a small protein that unfolds upon heating in several discreet stages is the leucine zipper, the dimerization domain of the yeast GCN4 transcription factor. This dimeric coiled-coil is of only ~7 kDa mass and many authors considered its rather sharp temperature induced unfolding as a perfect two-state transition [41–47]. However, deconvolution analysis of the excess heat capacity function showed that GCN4 leucine zipper unfolds in three stages, among which only the last one depends on concentration, i.e. represents the cooperative dissociation of strands (Fig. 7b,c). Optical studies of this process showed that the first transition is associated with unfolding of the N-terminus of the leucine zipper and the second with repacking of the interface of this coiled-coil. For details, the reader is referred to the original paper [15,48].

4. Formation of protein–DNA complexes

The energetics of formation of specific protein complexes with other proteins or nucleic acids is now attracting increasing attention since it actually represents a most basic biological function. It is widely accepted practice to determine the enthalpy of complex formation by an isothermal titration microcalorimeter (ITC) at different fixed temperatures (Fig. 8a). Assuming that Kirchhoff's relation ($\partial\Delta H/\partial T = \Delta C_p$) is applicable to the studied case, the dependence of the enthalpy of the association reaction on temperature is usually regarded as the heat capacity effect of complex formation, ΔC_p^a.

The heat capacity effect of binding is a very important parameter because it is known that the change of heat capacity in the reaction between macromolecules in aqueous solution is mainly caused by the change in hydration, and since hydration effects are proportional to the exposed surface areas of polar and apolar groups, the heat capacity change yields information on the extent and polarity of the interface formed between the components of the complex [49]. In using the temperature dependence of the binding enthalpy for estimating the intrinsic heat capacity effect of binding and the associated dehydration effect, it is not always appreciated that this assumes that the components of this process do not change their state over the considered temperature range, i.e. the heat capacities of all the components of the reaction are constant. However, association into a complex results in significant stabilization of the all components of binding reaction. This is especially true for DNA binding proteins since the structure of most of them is rather unstable in the absence of DNA and at ambient temperatures they are partly unfolded. Fig. 9a,b presents some typical examples: the heat capacity profiles show that on heating, the protein/DNA complexes unfold cooperatively at somewhat higher temperature than the free proteins and free DNA duplexes. Thus, protein refolds upon association with DNA. Therefore, the heat of association measured by ITC at some temperature includes the heat of protein refolding. If we wish to determine the enthalpy of DNA association with the as folded protein as it is in the complex, we have to exclude the heat of protein refolding from the ITC-measured heat effect of association. Correction for these heat effects of temperature-induced changes in the components of the association reaction was considered in detail in [14].

The enthalpies of association for the SRY and NHP6A HMG DBDs with their cognate DNA duplexes, corrected on refolding, are shown in Fig. 8b. One can see that correction for the refolding of protein significantly alters not only the absolute values of the association enthalpy but also its

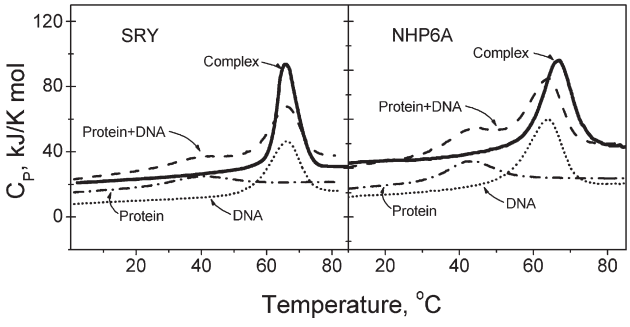

Fig. 9. The partial molar heat capacity functions of the free DBDs of SRY and NHP6A, their free cognate DNA duplexes, their complexes and the sum of the heat capacities of free protein and DNA. For details, see [39].

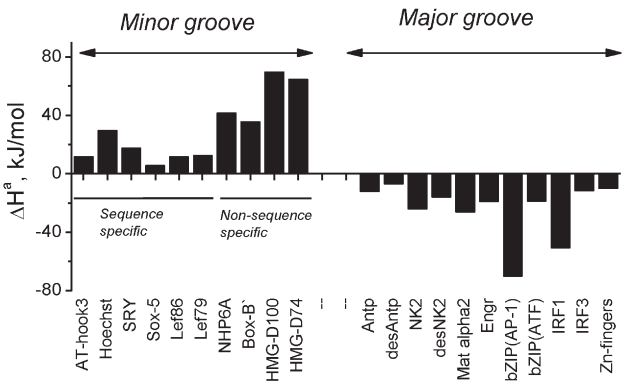

Fig. 10. The enthalpy of association of various DNA-binding proteins to their target DNA duplexes at 20 °C in 100 mM KCl, 10 mM potassium phosphate, pH 6.0. The data were taken from: HMG boxes SRY, Sox5, Lef86, Lef79, NHP6A and Box-B′ [39]; HMGD-100 and HMGD-74 [38]; homeodomains Antennapedia, desAntp, MATα2, engrailed [50]; bZIP [39]; IRFs [51]; zinc fingers [52].

dependence on temperature, i.e. the heat capacity effect of association. In the case of the sequence-specific association of the SRY HMG box with its target DNA duplex, while the *apparent* heat capacity effect is $\Delta C_p = -4.0$ kJ/K mol, the corrected value is only half of this, −2.0 kJ/K mol [14,39].

Correction of the calorimetrically measured thermodynamic characteristics for refolding is especially important when making a structural analysis of the energetic basis of forming the complex. This is because in the structure of the complex determined by crystallography or NMR the protein is folded and the interface is formed by the folded protein. Thus the structural parameters of forming the complex (e.g. the change in water accessible surface area (ΔASA) of the apolar and polar groups at the interface) would be expected to correlate with the thermodynamic characteristics of complex formation only after correcting for refolding.

5. Thermodynamics of protein binding to the minor and major groves of DNA

Fig. 10 presents the corrected on refolding enthalpies of binding of various proteins to their target DNAs. It is most remarkable that binding to the major and minor grooves of DNA differs qualitatively: the enthalpies of binding to the minor groove are always positive, while the enthalpies of binding to the major groove are invariably negative.

The key question is therefore why is there a difference in the sign of the enthalpy of association for binding to the minor and major grooves? One of the possible explanation is that binding to narrow minor groove results in significant deformation of DNA, which requires some work [14,53,54]. The other, perhaps more substantiated explanation is that these two grooves differ in hydration: water in the minor groove is much more ordered than water in the major groove and its removal upon protein binding requires more enthalpy. The presence of a spine of well-ordered water molecules in the minor groove of AT-rich DNA sequences has been shown indeed by high-resolution crystallography and neutron diffraction [55–57].

6. Conclusion

In studying the energetics of protein, unfolding/refolding attention has usually been concentrated on the most pronounced stage of this process, the gross cooperative transition. This is because this stage takes place over a relatively short range of external variables (e.g. temperature); it can be observed by a variety of experimental techniques and can easily be analyzed and specified quantitatively in thermodynamic parameters. The physics of this cooperative process of protein unfolding/refolding does indeed represent an interesting and important problem. This interest, however, has almost completely obliterated other, more subtle changes of protein structure under the influence of changing environmental conditions, particularly temperature. These changes are less impressive than those of the gross cooperative conformational transition because they are not so pronounced but are gradual, or proceed with low cooperativity and with many overlapping stages; correspondingly, their experimental investigation is more difficult and the theoretical analysis is more complicated. However, they take place at physiological temperatures and may therefore have functional significance. These changes show that the structures of small proteins, which were usually considered as a single domain, are not uniform in stability and flexibility and might have unstable parts that fluctuate intensively. The stability of a protein, or its subdomains, can change upon association with its functional partners: another protein, nucleic acid or specific ligand. These dynamic properties of protein structure which are displayed at physiological temperatures are now of particular interest because of their possible functional significance. As shown here, without studying the thermodynamics of these subtle predenaturational changes of proteins one cannot get a proper understanding of the energetic basis of their structure nor of their specific complexes with partners. For such studies, the combined use of the isothermal titration and differential scanning microcalorimeters is critical.

The other important result of the calorimetric studies is the revealed qualitative difference in the energetic signatures of protein binding to the minor and major grooves. Its nature certainly requires the most thorough investigation to understand the mechanism of DNA recognition.

Acknowledgement

The financial support of NIH GM48036 and NSF 04-23 is acknowledged.

References

[1] Y.V. Griko, G.I. Makhatadze, P.L. Privalov, R.W. Hartley, Thermodynamics of barnase unfolding, Protein Sci. 3 (1994) 669–676.
[2] P.L. Wintrode, G.I. Makhatadze, P.L. Privalov, Thermodynamics of ubiquitin unfolding, Proteins 18 (1994) 246–253.
[3] P.L. Privalov, N.N. Khechinashvili, A thermodynamic approach to the problem of stabilization of globular protein structure: a calorimetric study, J. Mol. Biol. 86 (1974) 665–684.
[4] P.L. Privalov, Stability of proteins: small globular proteins, Adv. Protein Chem. 33 (1979) 167–241.

[5] P.L. Privalov, Protein stability, in: T. Creighton (Ed.), Encyclopedia of Molecular Biology, Wiley, New York, 1999, pp. 2016–2020.
[6] G. Privalov, V. Kavina, E. Freire, P.L. Privalov, Precise scanning calorimeter for studying thermal properties of biological macromolecules in dilute solution, Anal. Biochem. 232 (1995) 79–85.
[7] P.L. Privalov, V.V. Plotnikov, Three generations of scanning microcalorimeters for liquids, Thermochim. Acta 139 (1989) 257–277.
[8] P.L. Privalov, S.J. Gill, Stability of protein structure and hydrophobic interaction, Adv. Protein Chem. 39 (1988) 191–234.
[9] J.O. Hutchens, A.G. Cole, J.W. Stout, Heat capacities from 11 to 305 degrees K and entropies of hydrated and anhydrous bovine zinc insulin and bovine chymotrypsinogen A. Entropy change for formation of peptide bonds, J. Biol. Chem. 244 (1969) 26–32.
[10] G.M. Mrevlishvili, Heat capacities of biological macromolecules, in: H.-J. Hinz (Ed.), Thermodynamic Data for Biochemistry and Biotechnology, Springer-Verlag, 1986, pp. 148–176.
[11] J. Wen, Heat capacity of polymers, in: J.E. Mark (Ed.), Physical Properties of Polymers Handbook, American Institute of Physics, Woodbury, NY, 1996.
[12] J. Gomez, V.J. Hilser, D. Xie, E. Freire, The heat capacity of proteins, Proteins 22 (1995) 404–412.
[13] J.S. Milne, Y. Xu, L.C. Mayne, S.W. Englander, Experimental study of the protein folding landscape: unfolding reactions in cytochrome *c*, J. Mol. Biol. 290 (1999) 811–822.
[14] P.L. Privalov, I. Jelesarov, C.M. Read, A.I. Dragan, C. Crane-Robinson, The energetics of HMG box interactions with DNA: thermodynamics of the DNA binding of the HMG box from mouse sox-5, J. Mol. Biol. 294 (1999) 997–1013.
[15] A.I. Dragan, P.L. Privalov, Unfolding of a leucine zipper is not a simple two-state transition, J. Mol. Biol. 321 (2002) 891–908.
[16] M. Hackel, H.J. Hinz, G.R. Hedwig, A new set of peptide-based group heat capacities for use in protein stability calculations, J. Mol. Biol. 291 (1999) 197–213.
[17] G.I. Makhatadze, P.L. Privalov, Heat capacity of proteins: I. Partial molar heat capacity of individual amino acid residues in aqueous solution: hydration effect, J. Mol. Biol. 213 (1990) 375–384.
[18] P.L. Privalov, G.I. Makhatadze, Heat capacity of proteins: II. Partial molar heat capacity of the unfolded polypeptide chain of proteins: protein unfolding effects, J. Mol. Biol. 213 (1990) 385–391.
[19] G.I. Makhatadze, P.L. Privalov, Energetics of protein structure, Adv. Protein Chem. 47 (1995) 307–425.
[20] G.I. Makhatadze, P.L. Privalov, Contribution of hydration to protein folding thermodynamics: I. The enthalpy of hydration, J. Mol. Biol. 232 (1993) 639–659.
[21] P.L. Privalov, G.I. Makhatadze, Contribution of hydration to protein folding thermodynamics: II. The entropy and Gibbs energy of hydration, J. Mol. Biol. 232 (1993) 660–679.
[22] K.P. Murphy, S.J. Gill, Solid model compounds and the thermodynamics of protein unfolding, J. Mol. Biol. 222 (1991) 699–709.
[23] P.L. Privalov, G.I. Makhatadze, Contribution of hydration and non-covalent interactions to the heat capacity effect on protein unfolding, J. Mol. Biol. 224 (1992) 715–723.
[24] R.S. Spolar, J.R. Livingstone, M.T. Record Jr., Use of liquid hydrocarbon and amide transfer data to estimate contributions to thermodynamic functions of protein folding from the removal of nonpolar and polar surface from water, Biochemistry 31 (1992) 3947–3955.
[25] A.J. Doig, M.J. Sternberg, Side-chain conformational entropy in protein folding, Protein Sci. 4 (1995) 2247–2251.
[26] K.H. Lee, D. Xie, E. Freire, L.M. Amzel, Estimation of changes in side chain configurational entropy in binding and folding: general methods and application to helix formation, Proteins 20 (1994) 68–84.
[27] G. Nemethy, S.J. Leach, H.A. Scheraga, The influence of amino acid side-chains on the free energy of helix-coil transitions, J. Phys. Chem. 70 (1966) 998–1004.
[28] S.D. Pickett, M.J. Sternberg, Empirical scale of side-chain conformational entropy in protein folding, J. Mol. Biol. 231 (1993) 825–839.
[29] J.A. Schellman, The stability of hydrogen bonded peptide structures in aqueous solution, Trav. Lab. Carlsberg. Ser. Chim. 29 (1955) 230–259.
[30] M.J. Sternberg, J.S. Chickos, Protein side-chain conformational entropy derived from fusion data–comparison with other empirical scales, Protein Eng. 7 (1994) 149–155.
[31] J. Wang, Z. Szewczuk, S.Y. Yue, Y. Tsuda, Y. Konishi, E.O. Purisima, Calculation of relative binding free energies and configurational entropies: a structural and thermodynamic analysis of the nature of non-polar binding of thrombin inhibitors based on hirudin55–65, J. Mol. Biol. 253 (1995) 473–492.
[32] A.S. Yang, B. Honig, Free energy determinants of secondary structure formation: I. Alpha-helices, J. Mol. Biol. 252 (1995) 351–365.
[33] C. Crane-Robinson, C.M. Read, P.D. Cary, P.C. Driscoll, A.I. Dragan, P.L. Privalov, The energetics of HMG box interactions with DNA. Thermodynamic description of the box from mouse Sox-5, J. Mol. Biol. 281 (1998) 705–717.
[34] E. Freire, R.L. Biltonen, Statistical mechanical deconvolution of thermal transitions in macromolecules: I. Theory and application to homogeneous systems, Biopolymers 17 (1978) 463–479.
[35] E. Freire, Statistical thermodynamic analysis of differential scanning calorimetry data: structural deconvolution of heat capacity function of proteins, Methods Enzymol. 240 (1994) 502–530.
[36] P.L. Privalov, V.V. Filimonov, Thermodynamic analysis of transfer RNA unfolding, J. Mol. Biol. 122 (1978) 447–464.
[37] P.L. Privalov, S.A. Potekhin, Scanning microcalorimetry in studying temperature-induced changes in proteins, Methods Enzymol. 131 (1986) 4–51.
[38] A.I. Dragan, J. Klass, C. Read, M.E. Churchill, C. Crane-Robinson, P.L. Privalov, DNA binding of a non-sequence-specific HMG-D protein is entropy driven with a substantial non-electrostatic contribution, J. Mol. Biol. 331 (2003) 795–813.
[39] A.I. Dragan, C.M. Read, E.N. Makeyeva, E.I. Milgotina, M.E. Churchill, C. Crane-Robinson, P.L. Privalov, DNA binding and bending by HMG boxes: energetic determinants of specificity, J. Mol. Biol. 343 (2004) 371–393.
[40] P.L. Privalov, Stability of proteins. Proteins which do not present a single cooperative system, Adv. Protein Chem. 35 (1982) 1–104.
[41] H.R. Bosshard, E. Durr, T. Hitz, I. Jelesarov, Energetics of coiled coil folding: the nature of the transition states, Biochemistry 40 (2001) 3544–3552.
[42] J.A. D'Aquino, J. Gomez, V.J. Hilser, K.H. Lee, L.M. Amzel, E. Freire, The magnitude of the backbone conformational entropy change in protein folding, Proteins 25 (1996) 143–156.
[43] B. Ibarra-Molero, G.I. Makhatadze, C.R. Matthews, Mapping the energy surface for the folding reaction of the coiled-coil peptide GCN4-p1, Biochemistry 40 (2001) 719–731.
[44] I. Jelesarov, E. Durr, R.M. Thomas, H.R. Bosshard, Salt effects on hydrophobic interaction and charge screening in the folding of a negatively charged peptide to a coiled coil (leucine zipper), Biochemistry 37 (1998) 7539–7550.
[45] D. Krylov, I. Mikhailenko, C. Vinson, A thermodynamic scale for leucine zipper stability and dimerization specificity: e and g interhelical interactions, EMBO J. 13 (1994) 2849–2861.
[46] D.N. Marti, I. Jelesarov, H.R. Bosshard, Interhelical ion pairing in coiled coils: solution structure of a heterodimeric leucine zipper and determination of pKa values of Glu side chains, Biochemistry 39 (2000) 12804–12818.
[47] K.S. Thompson, C.R. Vinson, E. Freire, Thermodynamic characterization of the structural stability of the coiled-coil region of the bZIP transcription factor GCN4, Biochemistry 32 (1993) 5491–5496.
[48] A.I. Dragan, L. Frank, Y. Liu, E.N. Makeyeva, C. Crane-Robinson, P.L. Privalov, Thermodynamic signature of GCN4-bZIP binding to DNA indicates the role of water in discriminating between the AP-1 and ATF/CREB sites, J. Mol. Biol. 343 (2004) 865–878.
[49] R.S. Spolar, M.T. Record Jr., Coupling of local folding to site-specific binding of proteins to DNA, Science 263 (1994) 777–784.
[50] A.I. Dragan, Z. Li, E.N. Makeyeva, E.I. Milgotina, Y. Liu, C. Crane-Robinson, P.L. Privalov, Forces driving the binding of homeodomains to DNA, Biochemistry 45 (2006) 141–151.

[51] V.V. Hargreaves, E.N. Makeyeva, A.I. Dragan, P.L. Privalov, Stability and DNA binding ability of the DNA binding domains of interferon regulatory factors 1 and 3, Biochemistry 44 (2005) 14202–14209.
[52] J.R. Liggins, P.L. Privalov, Energetics of the specific binding interaction of the first three zinc fingers of the transcription factor TFIIIA with its cognate DNA sequence, Proteins, Suppl. 4 (2000) 50–62.
[53] L. Jen-Jacobson, L.E. Engler, L.A. Jacobson, Structural and thermodynamic strategies for site-specific DNA binding proteins, Structure 8 (2000) 1015–1023.
[54] W.B. Peters, S.P. Edmondson, J.W. Shriver, Thermodynamics of DNA binding and distortion by the hyperthermophile chromatin protein Sac7d, J. Mol. Biol. 343 (2004) 339–360.
[55] S. Arai, T. Chatake, T. Ohhara, K. Kurihara, I. Tanaka, N. Suzuki, Z. Fujimoto, H. Mizuno, N. Niimura, Complicated water orientations in the minor groove of the B-DNA decamer d(CCATTAATGG)2 observed by neutron diffraction measurements, Nucleic Acids Res. 33 (2005) 3017–3024.
[56] T.K. Chiu, M. Kaczor-Grzeskowiak, R.E. Dickerson, Absence of minor groove monovalent cations in the crosslinked dodecamer C-G-C-G-A-A-T-T-C-G-C-G, J. Mol. Biol. 292 (1999) 589–608.
[57] X. Shui, C.C. Sines, L. McFail-Isom, D. VanDerveer, L.D. Williams, Structure of the potassium form of CGCGAATTCGCG: DNA deformation by electrostatic collapse around inorganic cations, Biochemistry 37 (1998) 16877–16887.