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Unfolding of bZIP dimers formed by the ATF-2 and c-Jun transcription factors is not a simple two-state transition

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ABSTRACT

The varied selectivity of bZIP transcription factors stems from the fact that they are dimers consisting of two not necessarily identical subunits held together by a leucine zipper dimerization domain. Determining their stability is therefore important for understanding the mechanism of formation of these transcription factors. The most widely used approach for this problem consists of observing temperature-induced dissociation of the bZIPs by any means sensitive to their structural changes, particularly optical methods. In calculating thermodynamic characteristics of this process from such data it is usually assumed that it represents a two-state transition. However, scanning micro-calorimetric study of the temperature-induced unfolding/dissociation of the three bZIPs formed by the ATF-2 and c-Jun proteins, i.e. the two homodimers (ATF-2/ATF-2) and (c-Jun/c-Jun) and the heterodimer (ATF-2/c-Jun), showed that this process does not represent a two-state transition, as found previously with the GCN4 homodimeric bZIP protein. This raises doubt about all indirect estimates of bZIP thermodynamic characteristics based on analysis of their optically-observed temperature-induced changes.

1. Introduction

Among transcription factor families the bZIPs attract attention for several reasons. Firstly, they function as dimers, not necessarily consisting of identical partners, so the number of their combinations is exceptionally large. Secondly, all members of this family have a well-defined structure: a common part responsible for their dimerization (the leucine zipper domain) and the part responsible for DNA recognition (the basic segments). The latter are highly charged and therefore partly unfolded in free solution but, being neutralized by the DNA phosphates, they fold and enter the major groove [1–5].

The energetic basis of leucine zipper dimerization is of particular interest and its understanding has required investigation of the unfolding/dissociation of the bZIPs under changing environmental conditions, e.g. temperature. The heat-induced unfolding/dissociation of synthetic regular α-helical coiled-coils proceeds in a highly cooperative manner [6], so it was tempting to assume that natural coiled-coil proteins also unfold in a highly cooperative process. In that case, the equilibrium analysis of any parameter sensitive to protein conformation, e.g. the circular dichroism, can provide all the thermodynamic characteristics of the folded state: the enthalpy, entropy and Gibbs energy of its formation. This approach has led to a number of studies on the stability and thermodynamics of bZIPs based on the observed temperature-induced change of their optical characteristics [7–12]. However, thermodynamic analysis of the melting profiles is possible only if the considered process indeed represents a reversible reaction closely approximated by a two-state transition. The visible sharpness of the temperature-induced changes in the optical characteristics of the state of protein cannot be regarded as an ultimate proof of the all-or-none character of a transition. For a temperature-induced process, the ultimate answer can be provided only by scanning calorimetric analysis of its heat effect since enthalpy and temperature are conjugate extensive and intensive thermodynamic parameters and their functional dependence bears all the statistical information on the macroscopic states which are realized over the considered temperature range [13–15]. Calorimetric studies of temperature-induced unfolding of the GCN4 leucine zipper have shown that its unfolding proceeds in several discreet steps, i.e. it does not represent a two-state transition [16,17]. Despite this, extensive investigations of the stabilities of a hundred or more homo- and hetero-leucine zippers were subsequently published, specifying the Gibbs energies determined from the observed sharpness of temperature-induced changes of their ellipticity [18].

Here we present the results of calorimetric studies of three other bZIPs, namely those formed by the ATF-2 and c-Jun transcription factors: two homodimers (ATF-2/ATF-2), (c-Jun/c-Jun) and one heterodimer (ATF-2/c-Jun). The data show that GCN4 bZIP is not an exception: the temperature-induced unfolding of all four natural leucine zippers does not represent a two-state transition.

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2. Results

The bZIP parts of the ATF-2 and c-Jun transcription factors used in this study were the same as those used in studying their interaction with target DNAs (Fig. 1) [19]. These are somewhat modified versions of the natural proteins (see Materials and methods). Correspondingly, we designate them as ATF2 and c-Jun. Shown in blue are the basic segments and in black are the dimerization parts that form the leucine zipper. The latter consists of five heptad repeats, each with a leucine residue located on the contacting face of these segments when in a coiled-coil α-helical conformation. SS crosslinking of the two components prevents their dissociation and reshuffling, which is particularly important in studying the heterodimer. As shown in our previous paper, disulfide crosslinking at the C-terminus of the leucine zipper does not affect the structure of the bZIPs or their ability to interact with DNA [19].

Upon heating the SS crosslinked bZIPs' ellipticity starts to change from the very beginning of heating and proceeds up to 100 °C (Fig. 2). One might assume that the initial change of ellipticity is associated with the gradual unfolding of the basic segments of the bZIPs, which are known to be partly unfolded at room temperature, while the leucine zipper domain starts to melt at higher temperatures, where the most significant change of ellipticity takes place and the temperature derivative of the ellipticity shows a peak (60–80 °C).

The partial molar heat capacity functions for the considered bZIPs are shown in Fig. 3. In all three cases the solid lines represent the heat capacity functions expected if the protein were completely folded and the dashed lines are the heat capacity functions expected for the completely unfolded proteins. The standard heat capacity of completely folded protein is taken as the specific heat capacity of the highly stable and compact protein pancreatic trypsin inhibitor (BPTI) [14,15]. The heat capacity functions of the completely unfolded bZIPs were calculated by summing up the molar heat capacities of all their amino acid residues [20]. Comparison of the observed heat capacity functions of the considered proteins (blue lines) with those expected for the fully folded and fully unfolded proteins shows that in all three cases excess heat absorption takes place from the very beginning of heating, which ends at 100 °C where the proteins are in a completely unfolded state. This excess heat effect is perfectly reproducible in subsequent cooling and heating experiments. Furthermore, it does not depend on the concentration of the SS-crosslinked bZIPs, i.e. it is not associated with aggregates. Thus, in all three cases we have a highly reversible intramolecular process extending over a one hundred K temperature range. The question then is whether it is a gradual process or a complex process consisting of several overlapping cooperative stages? This could be answered only by modeling the observed heat effect as a combination of discrete transitions.

Attempts to deconvolute the observed temperature-induced process into three cooperative stages were completely unsuccessful. Deconvolution into four stages also showed a large difference between the real and the best-fit heat capacity profiles (Fig. 4). An acceptable result was achieved only with deconvolution into five stages (Fig. 5) where the fitness of approximation is described by the parameter

$$\chi = \sqrt{\int_{T(0)}^{T(100)} \left(Cp^{cal}(T) - Cp^{Fit}(T)\right)^2 dT} \quad (1)$$

which is used in the CpCALC deconvolution program and shows the deviation between the measured and constructed heat capacity profiles. One can see that this parameter drops twenty fold from about 100 kJ/mol for four-stage unfolding to about 6 kJ/mol for a five-stage unfolding. A heat of 6 kJ/mol is only 1% of the total excessive heat absorption associated with unfolding of the bZIPs. Thus, 5 transitions are the minimal number of cooperative stages required for an appropriate description of the temperature-induced unfolding of the three bZIPs.

The difference in the partial molar heat capacity functions for crosslinked and non-crosslinked bZIP homodimers at identical concentrations of 3 mg/ml protein shows that removal of the disulfide bridge between protein subunits results in a decrease in T_t values, particularly for the higher temperature transitions, together with a corresponding drop in their enthalpy (Table 1). The (ATF2/c-Jun) heterodimer was not studied without cross linking because reshuffling of the subunits produces a mixture of the homo- and heterodimers, which cannot be quantitatively analyzed.

Among the five transitions revealed for the crosslinked bZIPs, the first two are presumably associated with unfolding of the basic segments, which are known to be partly unfolded at room temperature. This was shown in detail for the example of GCN-4 [17]. To confirm this conclusion, we studied the temperature induced changes of the anisotropy of tryptophan and tyrosine fluorescence of the (ATF2/c-Jun) heterodimer. There is one tryptophan in this protein in the N-terminal DNA-recognition basic segment and one tyrosine at the C-terminus of the dimerization domain (see Fig. 1). It was found that the N-terminal tryptophan fluorescence anisotropy is more temperature sensitive than the C-terminal fluorescence tyrosine signal (Fig. 6). This shows that the low temperature unfolding of the (ATF2/c-Jun) bZIP proceeds from the N-terminal DNA-recognition basic segments.

3. Conclusions

To date we have studied in detail the temperature induced unfolding of four bZIPs: the GCN4 bZIP homodimer [16,17], the (ATF2/ATF2) and (c-Jun/c-Jun) homodimers and the (ATF2/c-Jun) heterodimer. In all four cases we found that their temperature-induced unfolding does not represent a single cooperative transition. It was rather surprising to observe that, when crosslinked, all four cases unfold in five transitions. Bearing in mind that two transitions can be associated with the unfolding of the unstable DNA-recognition basic segments, it appears that in all four cases the leucine zipper domain

ATF2

MPDEKRRKFLERNRAAASRSRQKRKVWVQSL[1]EKKAEDL[2]SSLNGQL[3]QSEVTLL[4]RNEVAQL[5]KQLLLAHKD-GSGC

cJun

MERIKAERKRMRNRIAASKSRKRKLERIARL[1]EEKVKTL[2]KAQNSEL[3]ASTANML[4]REQVAQL[5]KQKVMNHVN-YSGC

Fig. 1. The modified sequences of the bZIP fragments from the ATF-2 and c-Jun transcription factors. The basic DNA-recognition segments are shown in blue; in black are the five heptads of the dimerization segments that form the leucine zipper domains. ATF2 includes one tryptophan residue (in violet). To distinguish c-Jun, a tyrosine was included in its C-terminal segment (in green). Additionally, the tetra-peptide *GSGC* was placed at the C-terminus of the ATF2 chain and the tri-peptide SGC on the c-Jun chain to provide cysteines for cross linking the dimers and maintaining an identical overall length for both chains. The flexible link *GSG* was used to avoid stresses in the dimerization domain that could result from the disulfide link.

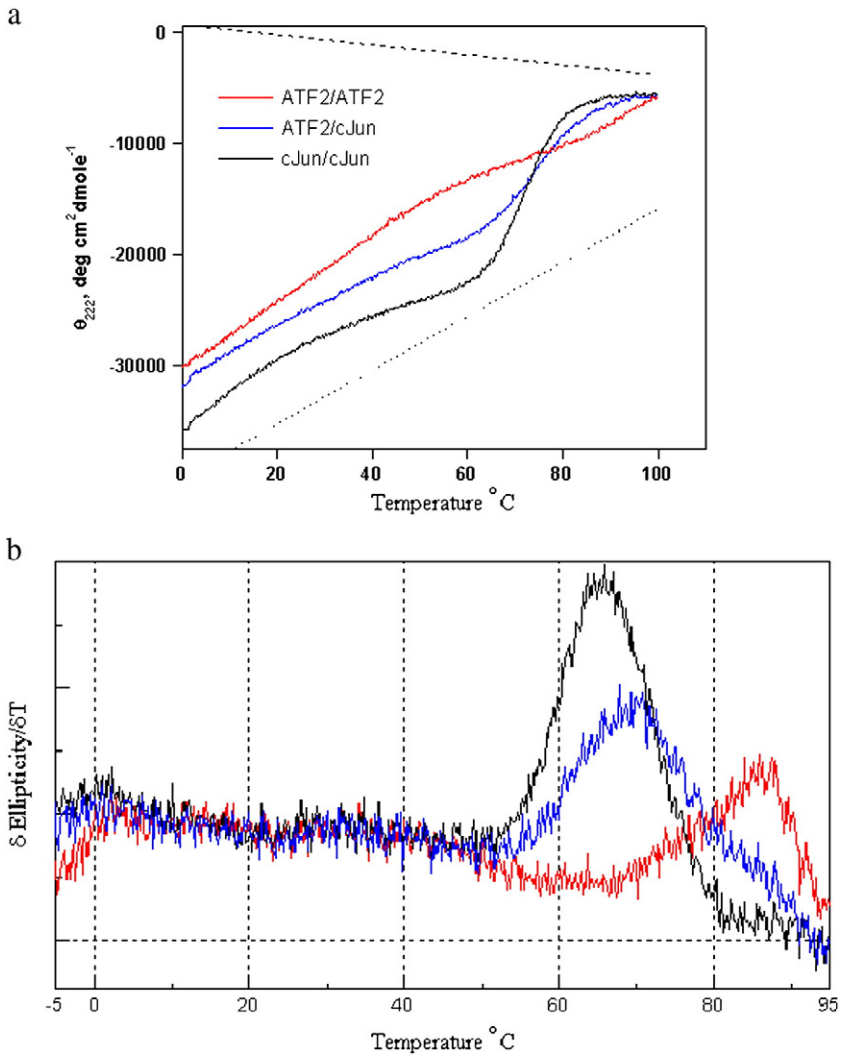

Fig. 2. (a). Temperature dependencies of the ellipticities of the free S-S cross linked bZIP proteins at 222 nm. Dashed and dotted lines are the calculated unfolded and folded dependencies, respectively. (b). The temperature derivative of the ellipticity profile indicating that the sharp changes in ellipticity take place in the temperature range from 60 to 80 °C.

undergoes three cooperative transitions upon temperature increase. Detailed study of the isolated GCN4 leucine zipper domain showed that the first of these three transitions is associated with local disruptions at the N-terminus; the second transition involves the whole dimer and is associated with its repacking, while the dissociation of strands takes place at the third transition [16]. It is very reasonable therefore to suppose that this is a general property of all leucine zippers.

4. Materials and methods

4.1. Proteins

Construction of the ATF-2 and c-Jun polypeptides utilized expression plasmids encoding the human bZIP domains for these proteins, were kindly provided by Dr. Vinson. The sequences of ATF-2 and c-Jun were modified to make them identical in length, distinguish them optically and enable them to be SS crosslinked. For that the tetra-peptide GSGC was added to the C-terminus of ATF-2 and the tetra-peptide YSGC added to c-Jun (Fig. 1). The corresponding plasmids, *pATF2 and pcJ2* were constructed using the QuikChange Multi Site-Directed Mutagenesis Kit from Stratagene, following the manufacturer's protocols and the plasmids generated, *pAj2 and pcJ2*, were sequenced and transformed into BL21(DE3)pLysS or Rosetta (DE3)pLysS for protein expression. Following expression, sonication and purification of the individual bZIP proteins ATF2 and c-Jun, equimolar amounts of these polypeptides were combined, mixed and placed in 5 M GdmCl in the presence of 3 mM DTT as reducing agent, 100 mM NaCl, 5 mM Na-Phosphate, 0.5 mM EDTA and 1 mM PMSF, (pH 6) at 4 °C for 1 h to completely unfold the dimers. Dialysis was performed over three days to remove denaturant and reductant and allowed for slow formation of dimers. The dimers were then characterized with MALDI Mass Spectroscopy and PAGE polyacrylamide gel electrophoresis. Their molecular mass closely corresponded to the expected from the chemical structures of monomers shown in Fig. 1: for the c-Jun/c-Jun and ATF2/ATF2 homo-dimers the values were 16,277 and 16,908 Da, respectively.

The dimers formed were isolated by fast protein liquid chromatography (*FPLC Pharmacia)* using a Hi-Trap SP XL cationic exchange column with a salt gradient (0.1M → 1 M NaCl), optically identifying the chains by their Tyr and Trp spectra. Additionally, the purity of all samples was checked by EMSA and mass spectroscopy.

4.1.1. Spectropolarimetry

CD measurements were carried out using a Jasco-710 spectropolarimeter equipped with a Peltier temperature controller PTC-3481.

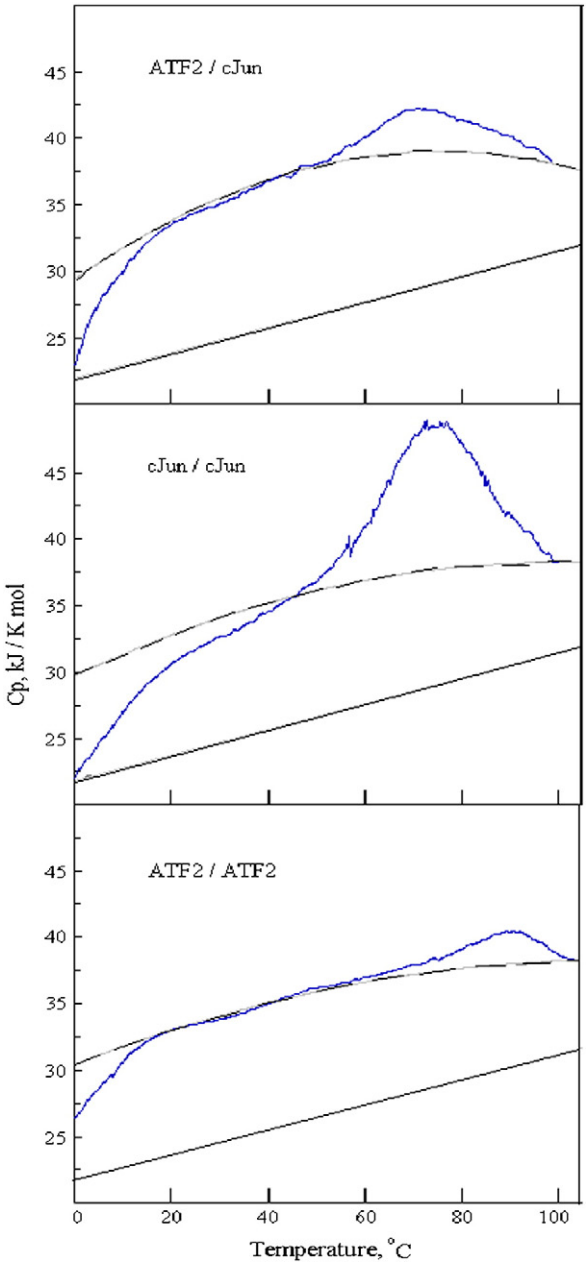

Fig. 3. The partial molar heat capacity functions of the ATF2/c-Jun hetero-dimer and the c-Jun/c-Jun and ATF2/ATF2 homodimers. The blue line shows the observed partial molar heat capacity functions for each bZIP protein. The dashed line shows the expected heat capacity function for the fully unfolded bZIP protein, calculated by summing up the partial molar heat capacities of all the amino acid residues. The solid line shows the heat capacity function expected for the fully folded protein (i.e. the partial specific heat capacity of bovine pancreatic trypsin inhibitor recalculated to the molecular weight for each bZIP protein).

The temperature dependence of the mean residue ellipticity of the unfolded peptide is expressed by the equation:

$$[\Theta]_{222} = 640 - 45 \times T \quad (2)$$

The mean residue ellipticity of the helix depends on the number of residues in the polypeptide (N_r) and the temperature, T, according to the equation [21]:

$$[\Theta]_{222} = (-40,000 + 250 \times T)(1 - 2.5 / N_r) \quad (3)$$

These functions are shown in Fig. 3(c) by the dashed lines.

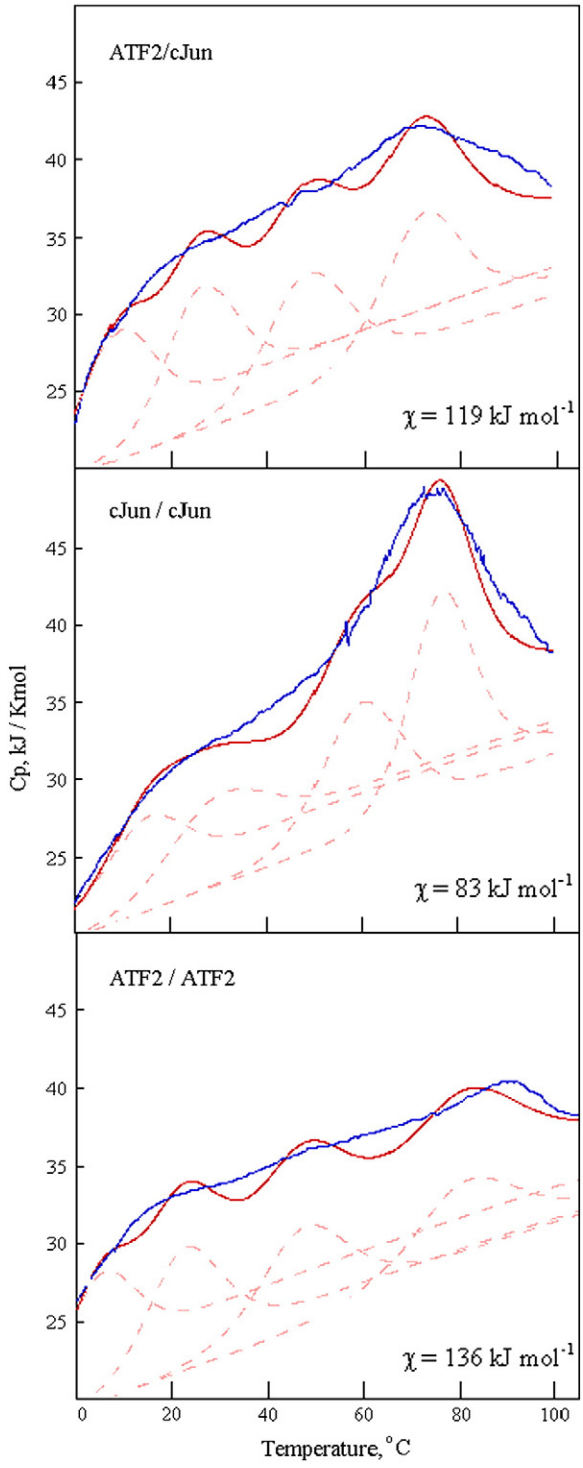

Fig. 4. The partial molar heat capacity functions of the three considered dimers (blue lines) and their best-fit approximations (red lines) to four component transitions (red dashed lines). The χ parameters show the deviation between the measured and simulated heat capacity functions.

4.1.2. Spectrofluorimetry

Fluorescence spectra of 40 μM of the ATF2/c-Jun heterodimer were measured on a SPEX FluoroMax-3 spectrofluorimeter under control of DataMax software (version 2.10). The instrument has a thermostated cell holder and software-controlled water bath. A 0.4 cm path-length Suprasil quartz cell was used. The fluorescence of tryptophan was excited at 288 nm and the emission followed at 352 nm with a slit

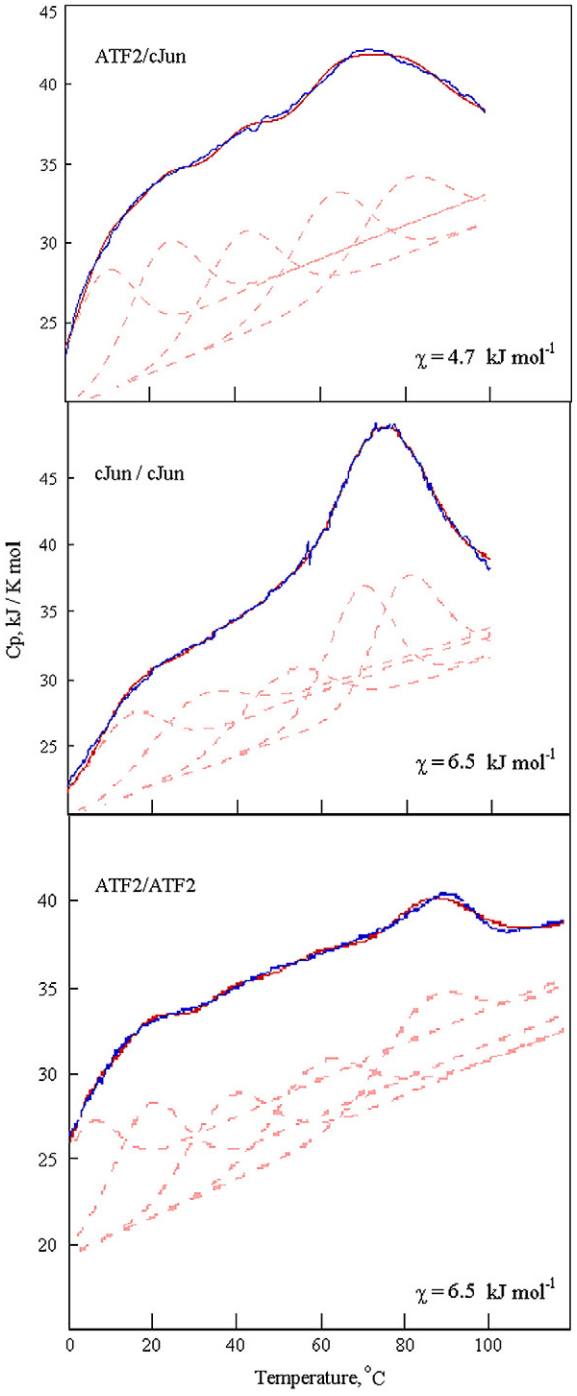

Fig. 5. The partial molar heat capacity functions of the three considered dimers (blue lines) and their best-fit approximations (red lines) fit to five component transitions (red dashed lines). The χ parameters show the deviation between the measured and simulated heat capacity functions.

width of 6 nm. The fluorescence of tyrosine was excited at 274 nm and the emission followed at 303 nm with a slit width of 6 nm.

4.2. Differential scanning calorimetry

DSC was performed on a Nano-DSC calorimeter from Calorimetry Sciences Corporation (Utah, USA) with a cell volume of 0.328 ml. The experiments used a scanning rate of 1 K/min and concentrations of proteins of about 3 mg/ml, determined optically with an error not exceeding 3%. Details of the performance of this instrument and the experimental procedures are given elsewhere [15]. Solutions for the calorimetric experiments were extensively dialyzed against solvent: 30 mM Na-Phosphate, 100 mM NaCl, pH 7.4, for 12 h at 5 °C with three replacements of dialyzate. The heat capacity was measured in both heating and cooling scans and in all cases the results were very similar, showing that unfolding and refolding of the bZIPs is highly reversible. The advantage of measuring in the cooling regime was that the heat capacity function could be determined down to 0 °C, or even lower by supercooling the aqueous solution. Results were analyzed using the C_pCALC program which is supplied with the Nano-DSC. For deconvolution analysis, the heat capacity of a fully folded protein was taken as that of bovine pancreatic trypsin inhibitor (BPTI) which, because of its high thermostability, is the most appropriate standard for a fully folded protein[14,15].

Table 1
Thermodynamic parameters of the component transitions.

bZIP		Parameter	Transitions				
			1	2	3	4	5
ATF2/ATF2	SS	T_t	0	18	37	54	83
		ΔH_t	99	120	107	91	121
		ΔC_p	3.4	1.5	1.0	1.8	1.8
		χ					6.5
	Non-SS	T_t	2	2	35	51	68
		ΔH_t	38	34	73	29	58
		ΔC_p	2.3	2.3	1.0	2	2
		χ					18
c-Jun/c-Jun	SS	T_t	12	25	50	69	80
		ΔH_t	102	86	119	168	174
		ΔC_p	2.5	3.0	0.9	1	1
		χ					6.5
	Non-SS	T_t	8	12	16	50	
		ΔH_t	93	39	51.3	115	
		ΔC_p	1.6	1.6	1.7	3.5	
		χ					5.0
ATF2/c-Jun	SS	T_t	7	21	41	62	78
		ΔH_t	119	125	132	133	126
		ΔC_p	2.6	2.7	1.0	1.3	1.3
		χ					4.7

T_t in °C; ΔH_t in kJ/mol; ΔC_p in kJ/Kmol, χ in kJ/mol.

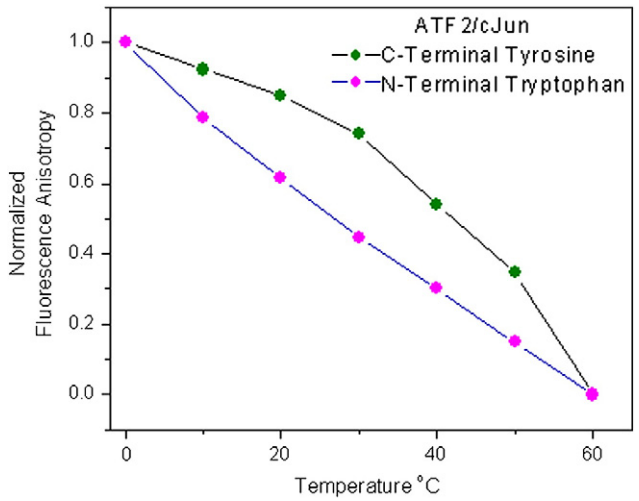

Fig. 6. Temperature induced changes of the (normalized) fluorescence anisotropy of the N-terminal tryptophan (violet) and the C-terminal tyrosine (green) of the ATF2/c-Jun heterodimer. This shows that the N-terminal tryptophan fluorescence signal changes at lower temperatures than does the C-terminal fluorescence tyrosine signal.

Acknowledgments

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