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Thermodynamic analysis of the heterodimerization of leucine zippers of Jun and Fos transcription factors

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ABSTRACT

Jun and Fos are components of the AP1 family of transcription factors and bind to the promoters of a diverse multitude of genes involved in critical cellular responses such as cell growth and proliferation, cell cycle regulation, embryonic development and cancer. Here, using the powerful technique of isothermal titration calorimetry, we characterize the thermodynamics of heterodimerization of leucine zippers of Jun and Fos. Our data suggest that the heterodimerization of leucine zippers is driven by enthalpic forces with unfavorable entropy change at physiological temperatures. Furthermore, the basic regions appear to modulate the heterodimerization of leucine zippers and may undergo at least partial folding upon heterodimerization. Large negative heat capacity changes accompanying the heterodimerization of leucine zippers are consistent with the view that leucine zippers do not retain α-helical conformations in isolation and that the formation of the native coiled-coil α-helical dimer is attained through a coupled folding-dimerization mechanism.

Jun and Fos are components of the AP1 transcription factor and are expressed in a wide variety of tissues [1,2]. Upon activation by a diverse multitude of mitogenic signals, including up-regulation by MAP kinases, Jun and Fos heterodimerize and bind to specific DNA sequences found in the promoters of genes such as metallothionein IIa, collagenase, interleukin 2 and cyclin D1. In this manner, the Jun–Fos heterodimeric transcription factor plays a central role in coupling mitogenic stimuli to DNA transcription and, in so doing, regulates a wide array of cellular processes such as cell growth and proliferation, cell cycle regulation and embryonic development [1].

The ability of Jun and Fos to recognize specific DNA sequences at the promoter regions resides in a region that has come to be known as the basic zipper (bZIP) domain (Fig. 1). The bZIP domain can be further dissected into two well-defined functional subdomains termed the basic region (BR) at the N-terminus followed by the leucine zipper (LZ) at the C-terminus. The LZ subdomain is a highly conserved protein module found in a wide variety of transcription factors and structural proteins and contains a signature leucine at every seventh position within the five successive heptads of amino acid residues. The LZ subdomains adapt continuous α-helical conformations and induce heterodimerization of Jun and Fos by virtue of their ability to wrap around each other in a coiled-coil dimer [2,3]. Such intermolecular arrangement brings the BR subdomains at the N-termini of bZIP domains into close proximity and thereby enables them to insert into the major grooves of DNA at the promoter regions in an optimal fashion in a manner akin to a pair of forceps [4].

Although the mechanism of the binding of bZIP domains to DNA is well-established, little is known about how protein–protein interactions dictate the heterodimerization of leucine zippers. To address this important issue, we have employed here the powerful technique of isothermal titration calorimetry (ITC) to measure and characterize thermodynamics of heterodimerization of leucine zippers of Jun and Fos from their respective homodimers in the context of the LZ subdomains and bZIP domains. Our data provide novel insights into the thermodynamics of a key protein-protein interaction pertinent to cellular transcriptional machinery.

Materials and methods

Protein preparation. LZ subdomain (residues 277–331) and bZIP domain (residues 251–331) of human Jun as well as LZ subdomain (residues 162–216) and bZIP domain (residues 136–216) of human Fos were cloned into pET102 bacterial expression vector, with an N-terminal thioredoxin (Trx)-tag and a C-terminal polyhistidine (His)-tag, using Invitrogen TOPO technology. Recombinant proteins were expressed, purified, and characterized as described previously [5].

ITC measurements

Isothermal titration calorimetry (ITC) measurements were performed on Microcal VP-ITC instrument and data were acquired and processed using fully automized features in Microcal ORIGIN soft-

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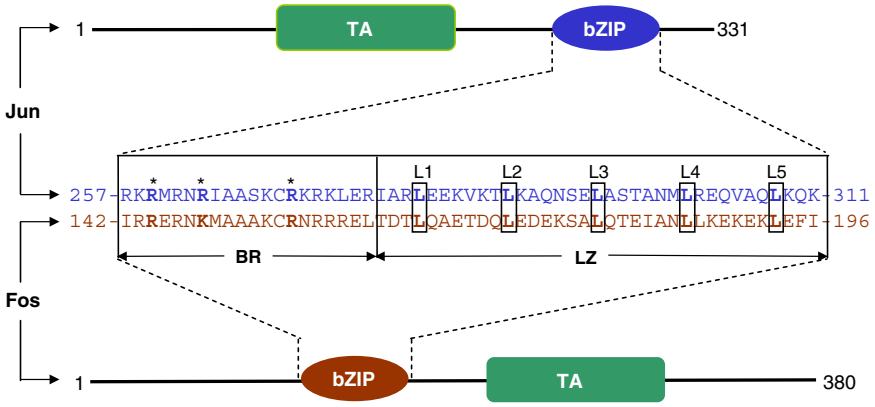

Fig. 1. A schematic showing domain organization of Jun and Fos transcription factors containing the basic zipper (bZIP) domain and the transactivation (TA) domain. The positions of the N-terminal basic region (BR) and the C-terminal leucine zipper (LZ) subdomains relative to each other are indicated. The five signature leucines (L1–L5) characteristic of LZ subdomains are boxed and bold faced.

ware. All measurements were repeated 3–4 times. Briefly, the protein samples were prepared in 50 mM Tris, 200 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol at pH 8.0 and de-gassed using the ThermoVac accessory for 10 min. The experiments were initiated by injecting 25 × 10 μl injections of 50–100 μM of LZ subdomain or bZIP domain of Fos from the syringe into the calorimetric cell containing 1.8 ml of 5–10 μM of LZ subdomain or bZIP domain of Jun at a fixed temperature in the narrow range 20–30 °C. The change in thermal power as a function of each injection was automatically recorded using Microcal ORIGIN software and the raw data were further processed to yield ITC isotherms of heat release per injection as a function of Fos to Jun molar ratio. The negligible heats of mixing and unfolding were subtracted from the heat of binding per injection by carrying out a control experiment in which the same buffer in the calorimetric cell was titrated against the LZ subdomain or bZIP domain of Fos in an identical manner. All other control experiments were performed as necessary. To extract the apparent equilibrium constant (K_d) and the enthalpy change (ΔH) associated with heterodimerization, the ITC isotherms were iteratively fit to the following built-in function by non-linear least squares regression analysis using the integrated Microcal ORIGIN software:

$$q(i) = (n\Delta HVP/2)[1 + (L/nP) + (K_d/nP)] - [[1 + (L/nP) + (K_d/nP)]^2 - (4L/nP)]^{1/2} \quad (1)$$

where $q(i)$ is the heat release (kcal/mol) for the ith injection, n is the stoichiometry of heterodimerization, V is the effective volume of Jun in the calorimetric cell (1.46 ml), P is the total Jun concentration in the calorimetric cell (μM) and L is the total Fos concentration in the calorimetric cell at the end of each injection (μM). The above equation is derived from the binding of two molecules using the law of mass action assuming a 1:1 stoichiometry [6].

Results and discussion

Heterodimerization of leucine zippers is under enthalpic control

In an attempt to elucidate the thermodynamic forces governing the heterodimerization of leucine zippers of Jun and Fos, we employed the powerful technique of ITC. To shed light on the role of basic regions in the heterodimerization of leucine zippers, we performed ITC analysis on both the LZ subdomains (containing leucine zippers only) and bZIP domains (containing basic regions located N-terminal to leucine zippers). The basic regions and leucine zippers are also alternatively referred to as BR subdomains and LZ subdomains, respectively, throughout this study. Our ITC data indicate that the heterodimerization of leucine zippers in the context of both the LZ subdomains and bZIP domains is under strong enthalpic control and accompanied by an unfavorable loss of entropy at physiological temperatures (Fig. 2 and Table 1). However, the heterodimerization of LZ subdomains proceeds with an affinity (0.06 μM) that is over 2-fold greater than that observed for the heterodimerization of bZIP domains (0.13 μM)—implying that the BR subdomains play an inhibitory role in the heterodimerization of leucine zippers. These results are in good agreement with previous studies based on non-calorimetric methods [7,8]. Our study, however, provides complete thermodynamic signatures of the heterodimerization of the leucine zippers of Jun and Fos that hitherto have not been reported.

What is the molecular basis of a largely enthalpy-driven nature of the heterodimerization of leucine zippers with unfavorable entropic contributions observed here? The leucine zippers of Jun and Fos adopt a coiled-coil conformation comprised of parallel α-helices wound around each other like a pair of forceps [4]. The α-helices are held together by molecular glue comprised of largely hydrophobic contacts with minor but important contributions from electrostatic interactions due to the salt bridging of oppositely charged residues. In light of such structural observations, it is thus highly likely that the establishment of an extensive network of hydrophobic contacts and electrostatic interactions between a pair of α-helices could account for the favorable enthalpic contributions to the free energy of heterodimerization. The molecular basis of heat release upon the heterodimerization of leucine zippers may seem intuitive but that of entropic penalty begs a little more thought. The entropy change observed here is the net change resulting from a combination of favorable and unfavorable entropic forces upon molecular associations. The major favorable entropic force upon molecular associations is the enhancement in the degrees of freedom of water molecules as a result of their restructuring and displacement from molecular surfaces coming into contact with each other, particularly from around the apolar groups—this favorable contribution is usually denoted ΔS_{solv} or the change in solvent entropy. However, the favorable ΔS_{solv} is largely offset by the loss of conformational degrees of freedom of the backbone and sidechain atoms upon molecular associations—this unfavorable contribution is usually denoted ΔS_{conf} or the change in conformational entropy. There is also a slight negative contribution to the overall entropic change due to the restriction of translation, rotational, and vibrational degrees of freedom of molecules upon association. In light of the foregoing argument, we attribute the large unfavorable entropy change observed here upon the heterodimer-

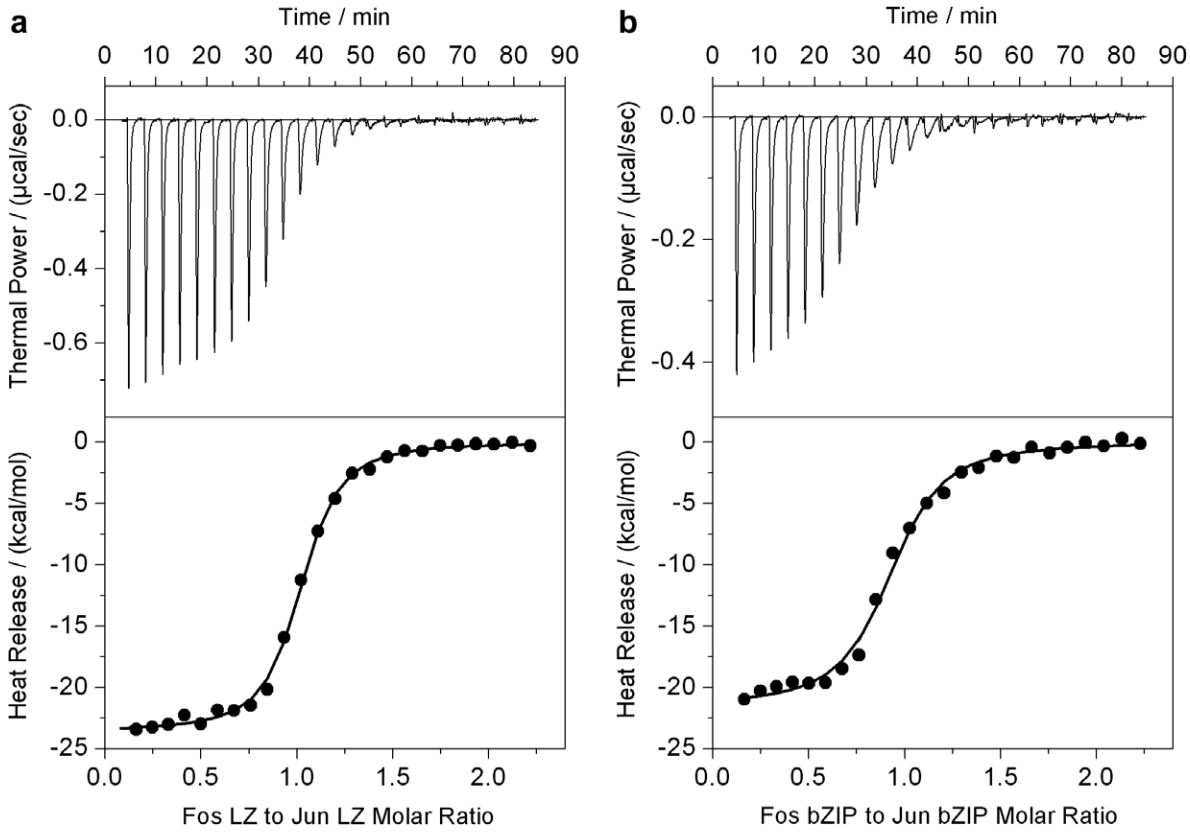

Fig. 2. ITC analysis of the heterodimerization of LZ subdomains (a) and bZIP domains (b) of Jun and Fos. LZ subdomains or bZIP domains of Jun in the calorimetric cell were titrated with 25 × 10 µl injections of corresponding LZ subdomains or bZIP domains of Fos from the injection syringe at 25 °C. The solid lines to the data in the lower panel represent the fit to expression [1].

Table 1
Experimentally determined thermodynamic parameters for the heterodimerization of LZ subdomains and bZIP domains of Jun and Fos using ITC at 25 °C and pH 8.0

	K_d/µM	ΔH/kcalmol^{-1}	$T\Delta S$/kcalmol^{-1}	ΔG/kcalmol^{-1}
LZ	0.06 ± 0.01	−23.21 ± 0.23	−13.52 ± 0.42	−9.69 ± 0.26
bZIP	0.13 ± 0.03	−21.75 ± 0.28	−12.35 ± 0.42	−9.40 ± 0.14

The values for the apparent equilibrium constant (K_d) and the enthalpy change (ΔH) associated with heterodimerization were obtained from the fit of expression [1] to the ITC isotherms shown in Fig. 2. Free energy of heterodimerization (ΔG) was calculated from the relationship $\Delta G = RT\ln K_d$, where R is the universal molar gas constant (1.99 cal/mol/K) and T is the absolute temperature (K). Entropic contribution ($T\Delta S$) to heterodimerization was calculated from the relationship $T\Delta S = \Delta H - \Delta G$. The stoichiometries to the fits agreed to within ±10%. Errors were calculated from 2 to 3 independent measurements. All errors are given to one standard deviation.

ization of LZ subdomains and bZIP domains to the loss of conformational degrees of freedom of the backbone and sidechain atoms as embodied in the term ΔS_{conf}.

Enthalpic and entropic factors compensate the effect of temperature on the heterodimerization of leucine zippers

Thermodynamics of protein–protein interactions can be highly dependent on the ambient temperature and knowledge of how thermodynamics vary as a function of temperature can provide invaluable insights into the mechanism of protein oligomerization. In an effort to determine the effect of temperature on the various thermodynamic parameters, we analyzed heterodimerization of LZ subdomains and bZIP domains of Jun and Fos in the narrow temperature range 20–30 °C using ITC (Fig. 3). Our data indicate that both the enthalpic (ΔH) and entropic ($T\Delta S$) contributions to the overall free energy of heterodimerization (ΔG) show strong temperature-dependence and that both ΔH and $T\Delta S$ largely compensate each other to generate ΔG that is virtually independent of temperature—while ΔH and $T\Delta S$ experience nearly 20 kcal/mol change in their contributions to heterodimer formation in going from 20 °C to 30 °C, ΔG gains no more than about 1 kcal/mol over the same temperature range. Consistent with this observation is the relatively constant nature of the apparent equilibrium constant of heterodimerization (0.05–0.3 µM) over the same temperature range.

The linear and opposing dependence of ΔH and $T\Delta S$ as a function of temperature, while maintaining a more or less constant ΔG, is a common feature observed in protein folding and binding reactions. This phenomenon gives rise to two key temperature points T_H and T_S—the temperatures where enthalpic (ΔH) and entropic ($T\Delta S$) contributions to the free energy change sign, respectively. In the case of the heterodimerization of leucine zippers of Jun and Fos, ΔH will become negative and hence thermodynamically favorable above T_H, while $T\Delta S$ will become negative and hence thermodynamically unfavorable above T_S. Table 2 provides the values for T_H and T_S accompanying the heterodimerization of leucine zippers of Jun and Fos in the context of LZ subdomains and bZIP domains. As evidenced in Table 2, both T_H and T_S fall well below the physiological temperature of 37 °C, implying that the heterodimerization of leucine zippers of Jun and Fos will be largely under enthalpic control accompanied by entropic penalty at physiological temperatures.

The temperature-dependence of ΔH is related to heat capacity of binding (ΔC_p) by Kirchhoff's relationship $\Delta C_p = d(\Delta H)/dT$—the slope of a plot of ΔH versus temperature equates to ΔC_p. Heat capacity is an important thermodynamic parameter in that it is related to the extent of the burial and dehydration of molecular surfaces from surrounding solvent molecules upon intermolecular

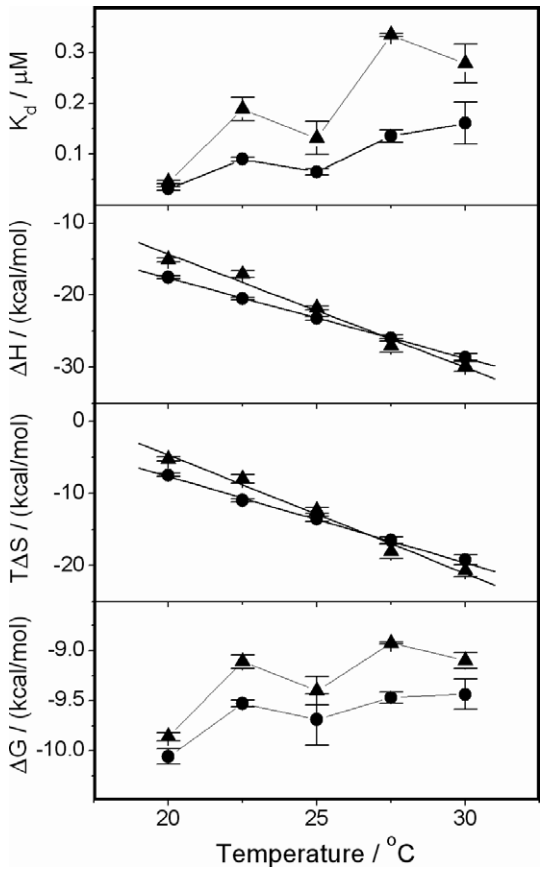

Fig. 3. Dependence of thermodynamic parameters K_d, ΔH, $T\Delta S$ and ΔG on temperature for the heterodimerization of LZ subdomains (●) and bZIP domains (▲) of Jun and Fos. Each data point is the arithmetic mean of 3–4 experiments. All error bars are given to one standard deviation. The solid lines for the ΔH and $T\Delta S$ plots show linear fits to the data, while the solid lines for the K_d and ΔG plots show straight lines connecting the data points for clarity.

Table 2
Experimentally determined thermodynamic parameters for the heterodimerization of LZ subdomains and bZIP domains of Jun and Fos obtained from ITC measurements at various temperatures in the narrow range 20–30 °C and pH 8.0

	T_H/°C	T_S/°C	ΔC_p/kcalmol^{-1} K^{-1}
LZ	+4.15 ± 0.42	+13.36 ± 0.39	−1.11 ± 0.03
bZIP	+10.78 ± 0.88	+17.10 ± 0.46	−1.57 ± 0.10

The values for the various parameters shown were obtained as follows. The values for T_H, the temperature at which ΔH is zero, were obtained from the extrapolation of linear fits to the ΔH versus temperature plots (Fig. 3). The values for T_S, the temperature at which $T\Delta S$ is zero, were obtained from the extrapolation of linear fits to the $T\Delta S$ versus temperature plots (Fig. 3). The values for ΔH_{60}, the enthalpy at 60 °C, were obtained from the extrapolation of linear fits to the ΔH versus temperature plots (Fig. 3). The values for ΔC_p, the heat capacity change, were obtained from the slopes of linear fits to the ΔH versus temperature plots (Fig. 3). Errors were calculated from 3 to 4 independent measurements. All errors are given to one standard deviation.

association [9–12]. As indicated in Table 2, the association of leucine zippers of Jun and Fos into a heterodimer results in large negative changes in heat capacity. What might be the significance of such large negative values of ΔC_p observed here? A positive value of ΔC_p implies that the residues being occluded from the solvent and hence residing at the interface of two molecular surfaces coming together are largely of polar nature with little or negligible contributions from apolar groups. The fact that the heat capacity changes are largely negative suggests strongly that the heterodimerization of LZ subdomains and bZIP domains involves substantial burial of hydrophobic residues with little contributions from polar residues. It should be noted here that protein–ligand interactions typically result in the magnitude of ΔC_p of less than—1000 cal/mol/K, while values of ΔC_p in the range −1000 to −2000 cal/mol/K are characteristic of proteins undergoing folding due to burial of a large number of apolar groups as a result of hydrophobic effect. Several lines of evidence suggest that the LZ subdomains are unstable as α-helices when in isolation and only fold into α -helices in the context of a coiled-coil dimer [13,14]. This is believed to be due to the fact that the dimer interface of a coiled-coil is comprised of hydrophobic residues, created largely by the interdigitation of signature leucines from each α-helix, and thus each LZ α-helix is thermodynamically unstable in isolation. In light of these arguments, we believe that the large negative changes in heat capacity observed here most likely arise due to the association of unfolded leucine zippers of Jun and Fos into α -helical heterodimers. In other words, heterodimerization of Jun and Fos appears to be coupled to folding of α-helices of their respective leucine zippers.

Basic regions modulate the heterodimerization of leucine zippers

Although it is widely believed that electrostatic repulsions between basic residues in the BR subdomains prevent them from becoming structured in the absence of DNA [15,16], our demonstration that the affinity of heterodimerization of leucine zippers is 2-fold greater in the context of LZ subdomains relative to bZIP domains suggests that the basic regions inhibit the heterodimerization of leucine zippers (Table 1). In an attempt to understand the thermodynamic basis of such differences, we generated differential thermodynamic signatures for the heterodimerization of LZ subdomains relative to bZIP domains (Fig. 4a). In this analysis, a negative value of $\Delta\Delta H$ implies that the enthalpy change is more favorable for the heterodimerization of LZ subdomains relative to bZIP domains, a negative value of $T\Delta\Delta S$ implies that the entropy change is less favorable for the heterodimerization of LZ subdomains relative to bZIP domains, and a negative value of $\Delta\Delta G$ implies that the free energy change is more favorable for the heterodimerization of LZ subdomains relative to bZIP domains.

As evidenced in Fig. 4a, the enthalpy of heterodimerization is more favorable by about −1.46 kcal/mol for the LZ subdomains relative to the bZIP domains. This small but clearly more favorable release of heat upon the heterodimerization of LZ subdomains could contribute to the magnitude of affinity of heterodimerization by as much as 10-fold instead of 2-fold relative to bZIP domains observed here. That this is not the case is best understood in terms of the more unfavorable entropic contribution of about −1.17 kcal/mol for the heterodimerization of LZ subdomains relative to the bZIP domains. In other words, the favorable gain of enthalpy for the heterodimerization of LZ subdomains relative to bZIP domains is largely offset by nearly an equal but opposing loss of entropy. In contrast, the unfavorable loss of enthalpy for the heterodimerization of bZIP domains relative to LZ subdomains is largely counterbalanced by nearly an equal but opposing gain of entropy. While the slightly less favorable enthalpy change observed for the heterodimerization of bZIP domains relative to LZ subdomains could be rationalized in terms of the unfavorable electrostatic repulsions between the basic residues in the BR subdomains, the rationale for the slightly less unfavorable loss of entropy observed for the heterodimerization of bZIP domains relative to LZ subdomains begs further tuition.

In Fig. 4b, we decompose the additional loss of entropy ($\Delta\Delta S$) upon the heterodimerization of LZ subdomains relative to bZIP domains observed here into its two major constituent components $\Delta\Delta S_{solv}$ and $\Delta\Delta S_{conf}$. In this analysis, a negative value of $\Delta\Delta S$ indicates that the heterodimerization of LZ subdomains is entropically less favorable relative to heterodimerization of bZIP domains, a

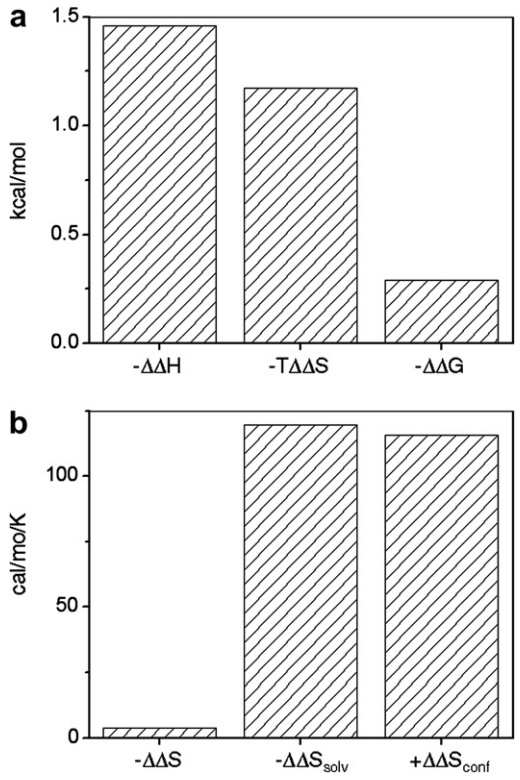

Fig. 4. Differential energetics for the heterodimerization of LZ subdomains versus bZIP domains. (a) Differential thermodynamic signature for the heterodimerization of LZ subdomains relative to bZIP domains. $\Delta\Delta H$, $T\Delta\Delta S$, and $\Delta\Delta G$ were calculated from the relationships $\Delta\Delta H = \Delta H_{LZ} - \Delta H_{bZIP}$, $T\Delta\Delta S = T\Delta S_{LZ} - T\Delta S_{bZIP}$, and $\Delta\Delta G = \Delta G_{LZ} - \Delta G_{bZIP}$, where the subscripts LZ and bZIP denote the corresponding thermodynamic parameters for the heterodimerization of LZ subdomains and bZIP domains, respectively (Table 1). (b) Differential entropic signature for the heterodimerization of LZ subdomains relative to bZIP domains. $\Delta\Delta S$, $\Delta\Delta S_{solv}$, and $\Delta\Delta S_{conf}$ were calculated from the relationships $\Delta\Delta S = \Delta S_{LZ} - \Delta S_{bZIP}$, $\Delta\Delta S_{solv} = \Delta S_{solv(LZ)} - \Delta S_{solv(bZIP)}$, and $\Delta\Delta S_{conf} = \Delta S_{conf(LZ)} - \Delta S_{conf(bZIP)}$, where the subscripts LZ and bZIP denote the corresponding thermodynamic parameters for the heterodimerization of LZ subdomains and bZIP domains, respectively. ΔS_{solv} was calculated from the relationship $\Delta S_{solv} = \Delta C_p \ln[298/385]$ and ΔS_{conf} from the relationship $\Delta S_{conf} = \Delta S\text{-}\Delta S_{solv}$ for the heterodimerization of LZ subdomains or bZIP domains with the ΔS and ΔC_p being the corresponding thermodynamic parameters (Tables 1 and 2).

negative value of $\Delta\Delta S_{solv}$ indicates that the change in solvent entropy upon the heterodimerization of LZ subdomains is less favorable relative to the heterodimerization of bZIP domains, and a positive value of $\Delta\Delta S_{conf}$ indicates that the change in conformational entropy upon the heterodimerization of LZ subdomains is more favorable relative to the heterodimerization of bZIP domains. That $\Delta\Delta S_{solv}$ is less favorable and $\Delta\Delta S_{conf}$ is more favorable for the heterodimerization of LZ subdomains relative to the heterodimerization of bZIP domains suggests strongly that the basic regions undergo at least partial folding upon heterodimerization of bZIP domains and, in so doing, are likely to modulate the heterodimerization of leucine zippers.

Conclusions

Thermodynamics is a powerful tool to gain insights into the energetic components that define protein-protein interactions relevant to biological function. Despite their discovery over two decades ago, the thermodynamics of heterodimerzation of leucine zippers of Jun and Fos hitherto have not been characterized. Our thermodynamic analysis here shows that the heterodimerization of leucine zippers of Jun and Fos is under enthalpic control and accompanied by entropic penalty at physiological temperatures. We have reasoned herein that the nature of the entropic penalty is likely to be largely due to the restriction in the conformational degrees of freedom of the backbone and sidechain atoms upon heterodimerization. We attribute large negative changes in heat capacity observed upon the heterodimerization of Jun and Fos to the formation of α-helical heterodimers from the corresponding unfolded leucine zippers. One additional key finding of our study is that the basic regions in the bZIP domains modulate the heterodimerization of leucine zippers and may undergo some degree of folding though their complete folding may necessitate the binding of DNA in agreement with previous studies [15,16]. Taken together, our study provides novel insights into the thermodynamics of a key protein–protein interaction pertinent to cellular transcriptional machinery.

Acknowledgments

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