Thermodynamic Characterization of the Folding Coupled DNA Binding by the Monomeric Transcription Activator GCN4 Peptide

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ABSTRACT Dimerization is a widely believed critical requirement for the yeast transcriptional activator GCN4 specifically recognizing its DNA target sites. Nonetheless, the binding of the monomeric GCN4 to DNA target sites AP-1 and ATF/CREB was recently detected by kinetic studies. Here, for the first time, we present a detailed description of the thermodynamics of a monomeric peptide GCN4-br, the basic region (226–252) of GCN4, binding to AP-1, and ATF/CREB. GCN4 specifically binds to AP-1 and ATF/CREB in the monomeric form as shown by our circular dichroism thermal unfolding measurements. Isothermal titration calorimetry experiments indicate that the binding process of GCN4-br with DNA is enthalpically driven, accompanied by an unfavorable entropy change. The temperature dependence of ΔH^0 reveals negative changes in heat capacity ΔC_p: $\Delta C_p = -0.92$ kJ · mol^{-1} K^{-1} and $\Delta C_p = -0.95$ kJ · mol^{-1} K^{-1} for GCN4-br binding to AP-1 and ATF/CREB, respectively, which is a striking manifestation of GCN4-br specifically recognizing DNA target sites. These thermodynamic characteristics may give new insight into the mechanism by which GCN4 protein binds to DNA target sites for its transcriptional regulation.

INTRODUCTION

Leucine zipper is a structural motif responsible for DNA binding in many transcriptional activator proteins (Landschulz et al., 1988). It consists of two functionally distinct regions: a C-terminal ''leucine zipper'', which is responsible for the dimerization by forming a coiled-coil structure and an N-terminal ''basic region'' adjacent to the leucine zipper region, directly involved in DNA target recognition and binding (O'Shea et al., 1989). Both regions form a functional unit and are highly conserved throughout the whole bZIP family (Vinson et al., 1989). The transcriptional activator GCN4 is an interesting representative of the large family of bZIP proteins (Hope and Struhl, 1986), which regulates many genes by binding to its specific DNA target sites as a dimer. The bZIP motif of GCN4 is located near its C-terminus, and ~60 residues are involved in dimer formation and site-specific DNA recognition (Hope and Struhl, 1987). The asymmetric palindrome AP-1 site 5′-ATGACTCAT-3′ and the symmetric palindrome ATF/CREB site 5′-ATGACGTCAT-3′ are the two optimal GCN4 targets (Hill et al., 1986). The crystal structures of the complexes of the GCN4 bZIP domain with AP-1 and ATF/CREB sites (Ellenberger et al., 1992; König and Richmond, 1993) have revealed that GCN4 dimerizes via the leucine zipper regions to form a Y-shaped dimer, and the basic regions are positioned almost parallel to the panel of the DNA bases, both of them forming identical contacts with bases in the major grooves of DNA's half sites. The DNA binding domain of GCN4 is flexible and partially disordered in the absence of DNA targets (Weiss, 1990; Weiss et al., 1990), however, the entire bZIP domain becomes fully helical when bound to DNA (Ellenberger et al., 1992; König and Richmond, 1993).

Many artificial sequence-specific DNA binding peptides have been designed and synthesized to elucidate the nature of GCN4-DNA interaction. Talanian and co-workers have reported a disulfide-bond linked dimer of the basic region, which specifically binds to the DNA targets as observed for the native bZIP domain (Talanian et al., 1990, 1992). Furthermore, the dimeric form of the basic region, which is linked by a C-terminal N^{α}-, N^{ε}-lysine linkage (Pellegrini and Ebright, 1996), metal complexes (Palmer et al., 1995), or C-terminal cyclodextrin-adamantyl complex (Morii et al., 1996), also specifically recognizes the DNA target sites of GCN4. These studies have demonstrated that when the leucine zipper is replaced by an artificial linker at the C-terminus of the basic region of GCN4, the basic region contains sufficient information for specific DNA binding and can be used as a DNA binding domain by itself. Moreover, CD experiments have indicated that the specific DNA binding region of GCN4 only consists of 15 residues, corresponding to residues 231–245 of the native protein GCN4 (Talanian et al., 1992). Understanding the energetics of this system can provide clues about molecular recognition and biological control mechanisms of the proteins binding to DNA system. Recent research in our laboratory has focused

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Abbreviations used: GCN4, gene control of amino acid synthesis nonderepressible mutant 4; GCN4-br, sequence of 226–252 of basic region of GCN4; AP-1, activation protein 1; ATF/CREB, activating transcription factor/cyclic AMP responsive element binding; CONT, control DNA; bZIP, basic leucine zipper; CD, circular dichroism; ITC, isothermal titration calorimetry; RP-HPLC, reversed phase high performance liquid chromatography; K_b, binding constant; ΔC_p, heat capacity change; ΔG^0, binding free energy change; ΔH^0, binding enthalpy determined by ITC.

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on the thermodynamic characterization of the binding of the disulfide-bond linked model peptide GCN4 (226–252) to its two DNA targets, AP-1 and ATF/CREB (Cao et al., 2000). Interestingly, we have found that the monomer of this model peptide, corresponding to residues 226–252 of GCN4, also can recognize the dimer DNA target sites specifically.

GCN4 forms a stable dimer in the absence of the specific DNA target sites, and binds to DNA as a dimer. Thus, it is widely believed that GCN4 has to form a dimer before it binds to its DNA targets and the monomer does not bind to DNA specifically (Hope and Struhl, 1987; Weiss et al., 1990). From the aspect of the molecular mechanism, it is also generally thought that the dimerization of bZIP precedes DNA binding during GCN4 recognizing its DNA targets. However, footprinting assays have recently shown that the leucine zipper basic region of the other bZIP proteins, such as v-Jun, can bind as monomers to the dimer-binding site (Park et al., 1996). Also the dimer pathway of DNA recognition for GCN4 protein has been challenged by recent kinetic evidence (Berger et al., 1998; Metallo and Schepartz, 1997). Moreover the kinetic studies of Fos · Jun · DNA complex formation have revealed that DNA binding is before dimerization (Kohler and Schepartz, 2001). Earlier studies mainly focused on the interaction of the GCN4 dimer and its DNA targets (Berger et al., 1996; Talanian et al., 1990, 1992). A detailed investigation of the molecular recognition between the monomer of the GCN4 and its DNA target sites has not been undertaken. To verify whether the monomer of the GCN4 recognizes the dimer DNA binding sites without dimerization and to better understand the recognition of this system, here we use ITC and CD spectroscopy to characterize the thermodynamics and specificities of the monomeric GCN4 peptide binding to DNA targets AP-1 and ATF/CREB. We have synthesized a 27-residue peptide corresponding to the basic region 226–252 of the native GCN4 protein, which is responsible for the DNA recognition. In this short peptide, residue Met-250 is replaced with Leu to inhibit oxidation without affecting DNA-binding affinity and specificity (Talanian et al., 1990), and the N-terminus is acetylated to avoid introduction of additional charge. Herein, we name this 27-residue model peptide GCN4-br (sequence shown in Fig. 1). Our studies have quantitatively addressed several issues relating to the interaction of the GCN4 monomer and its DNA targets, including: 1), manifestation of specific recognition of the GCN4-br and the DNA targets; 2), temperature dependence of the GCN4-br binding to DNA targets; and 3), comparison of the GCN4-br binding to the DNA targets AP-1 and ATF/CREB.

MATERIALS AND METHODS

Peptide synthesis

The model peptide GCN4-br (226–252) was synthesized on Rink resin (substitution of 0.6 mmol/g, Advanced Chem. Tech., Louisville, KY, USA) using Fmoc/Bu^t strategy and carboxyl group activation by HBTU/HOBT

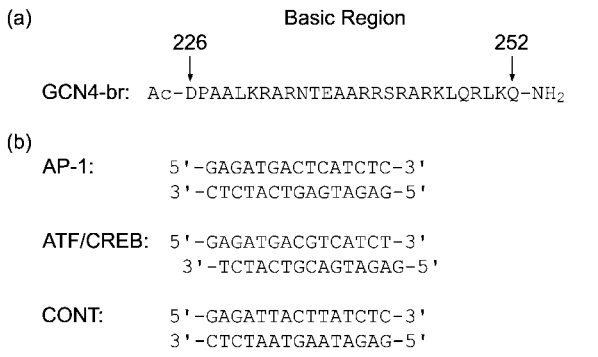

FIGURE 1 Sequences of the peptide and oligonucleotides studied. (*a*) Sequence of peptide GCN4-br. (*b*) Sequences of the oligonucleotides AP-1, ATF/CREB, and CONT.

(Knorr et al., 1989). The peptide was cleaved from the resin and the protecting groups of side chains were removed using trifluoroacetic acid method (Fields and Fields, 1993), and purified by RP-HPLC using semi-preparative Zorbax C18 column. The purity was confirmed by analytical RP-HPLC and MALDI-TOF mass spectra, giving molecular masses within ± 1 Da of calculated values.

Oligonucleotide synthesis

The oligonucleotides AP-1, ATF/CREB, and CONT (sequences shown in Fig. 1) were purchased from Sheng Gong Bioengineer, (Shanghai, China). The AP-1 has the DNA binding site (5′-ATGACTCAT-3′) and the ATF/CREB has the binding site (5′-ATGACGTCAT-3′), both are recognized by GCN4 specifically, and the CONT is a nonspecific control oligonucleotide. The dried individual component strands were synthesized on an automatic DNA synthesizer Model 391 (PerkinElmer, Wellesley, MA, USA) using standard phosphoramidite chemistry, and were purified by C18 RP-HPLC. The purity of the strands was checked by polyacrylamide gel electrophoresis and analytical RP-HPLC. The DNA duplexes were formed by mixing equal amounts of complementary strands and temperature annealing by heating at 80°C for 10 min, followed by slow cooling. The annealed samples were allowed to equilibrate at 4°C for 24 h before analysis. Oligonucleotide concentrations were determined spectrophotometrically by measuring the absorbance at 260 nm, the extinction coefficients (ε) were determined directly by using a nearest-neighbor analysis (Cantor et al., 1970).

CD spectroscopy experiments

CD spectra were obtained using a CD6 spectropolarimeter (Jobin Yvon, Longjumeau, France). This instrument is computerized and equipped with a programmable thermoelectrically controlled cell holder. Circular cells with 0.1-mm and 1.0-mm path length were used. All solutions contained 10 mM sodium phosphate buffer, 100 mM NaCl, and pH 7.4. Difference spectra were calculated by subtraction of the spectrum of the DNA from the spectra of the peptide-DNA complexes. The estimation of the secondary structure was made using VARSLC program according to the Johnson method (Johnson, 1990; Manavalan and Johnson, 1987), and the Yang method (Wu et al., 1981). Thermal stability was determined at peptide concentration of 36 μM for GCN4-br by monitoring the changes in $[\theta]_{222}$ as a function of temperature. The temperature was increased in steps of 0.2°C at scan rate 30°C/h from 5°C to 80°C. All thermal melts were reversible.

Isothermal titration calorimetry

The measurements of the heat of mixing model peptide GCN4-br with synthetic AP-1 target site (5′-GAGATGACTCATCTC-3′) and ATF/CREB

site (5′-GAGATGACGTCATCT-3′) were carried out with ITC from Calorimetry Sciences, American Fork, UT, USA. The instrument was electrically calibrated by means of a standard electric pulse as recommended by the manufacturer. For the peptide binding to DNA, solutions of peptide were used to titrate DNA. A 250-μL syringe was used for the titrant. Mixing was effected by stirring this syringe at 200 rpm during equilibration and experiment. Typically 25 injections of 10 μL each were performed with a 400-s interval between injections in a single titration at temperatures of 10, 15, 18, and 20°C, respectively. The reference cell of the calorimeter filled with buffer, acting as a thermal reference to the sample cell. To correct for GCN4 peptide heats of dilution, the control experiments were also performed using similar conditions with buffer solution only. All solutions were degassed by evacuation to reduce the noise. At each temperature, the instrument was electrically calibrated by means of a standard electric pulse as recommended by the manufacturer. The buffer contains 100 mM NaCl, 10 mM sodium phosphate, and pH 7.4.

ITC measurements were designed to obtain primarily the enthalpy of each complex formation and their stoichiometries. The heats of each reaction were determined by integration of the peaks observed. After the contribution from the heat of dilution of each injection was subtracted, the heat was plotted against the molar ratio of the peptide to DNA. The binding constant (K_b), enthalpy of binding (ΔH^0), and stoichiometry (N) of the formation of complex were determined by fitting the binding isotherm against the binding equation described by Freire et al. (1990) using an independent binding model. Data analysis was carried out with the software provided with the instrument.

RESULTS

Evidence of recognition of the GCN4-br to DNA target sites

We have studied the conformational changes of GCN4-br in the presence and absence of the two DNA target sites AP-1 and ATF/CREB. Fig. 2 shows the CD spectra of the peptide and the complexes with AP-1 and ATF/CREB at 20°C. CD spectra of the peptide exhibit characterization of α-helix with two minima at ~222 nm and 208 nm. The results suggest that the GCN4-br has partial helical conformation. A significant increase in the intensity of the CD signal at 222 nm and 208 nm is observed for the complexes of GCN4-br with the two DNA target sites (Fig. 2). The difference spectra indicate that the GCN4-br undergoes significantly conformational transitions from the extended conformation to helix when bound to the DNA targets AP-1 and ATF/CREB. The estimation of the secondary structures shows that the α-helix content of GCN4-br increases from ~10% to ~70% when bound to DNA. The results suggest that the recognition process of the monomeric basic region of GCN4 to its dimeric DNA target sites is possibly similar to that of the disulfide bond linked dimer basic region (Talanian et al., 1990; Cao et al., 2000).

Control CD spectra were obtained with the oligonucleotide CONT (Fig. 2). CONT has the base composition of the AP-1 site, but with two bases C and G replaced by A and T respectively (Fig. 1). The results show that the CONT also induces the conformational changes of the GCN4-br, which indicates that CONT has some interactions with the peptide GCN4-br, but the signal at 222 nm is much smaller than that AP-1 and ATF/CREB induced.

Thermal unfolding experiments

To further confirm whether the monomer of GCN4 can recognize its dimer DNA binding sites specifically, we have measured the thermal stability of the peptide GCN4-br and its two complexes with AP-1, ATF/CREB targets, and the complex with the control DNA CONT. The GCN4-br was incubated with DNA in 2:1 molar ratio (peptide: DNA) to make sure the peptide completely binds to DNA. The CD signals at 222 nm were monitored as a function of temperature, shown in Fig. 3. The thermal unfolding curve of the free peptide GCN4-br (curve *a* in Fig. 3) further suggests that the partial α-helix conformation existing at low temperature fully disrupts at ~30°C. However, the complexes of GCN4-br with AP-1 and ATF/CREB undergo cooperative and reversible thermal unfolding transitions. The increased thermal stability of GCN4-br:AP-1, GCN4-br:ATF/CREB, and the sigmoidal shape of the thermal unfolding curves in our studies demonstrate that GCN4-br binds to its dimer DNA target sites in a specific manner.

The thermal stability of the nonspecific DNA complex of GCN4-br with CONT was examined under the same conditions, shown in Fig. 3 (curve *d*). CONT induces the CD signal increase of GCN4-br at 222 nm at 20°C (Fig. 2), but unlike the results obtained for the AP-1 and ATF/CREB targets, the shape of the thermal transition suggests noncooperative binding. Comparison of the thermal unfolding curves of the complexes of GCN4-br with AP-1, ATF/CREB, and the control oligonucleotide CONT further confirms that the monomeric basic region of GCN4 can recognize its dimer targets specifically.

Isothermal titration calorimetry experiments

We have carried out ITC experiments on the formation of complexes between the peptide GCN4-br and DNA target sites AP-1 and ATF/CREB at a temperature range 10–20°C. Analysis above (see Figs. 2 and 3) indicates that the GCN4-br-DNA complexes begin to disrupt above 20°C and the peptide GCN4-br shows less changes in conformation at low temperature than at high temperature; thus, high limit temperature for ITC experiments was set at 20°C. And it is necessary to ensure that the DNA sequences are present in double-stranded form. This is particularly important for the ATF/CREB site, which is completely palindromic and has the potential to form hairpins (Fig. 1). The duplexes of AP-1 and ATF/CREB were achieved by very slow annealing after heating of the complementary oligonucleotides to 80°C (see Materials and Methods). CD thermal disruptions were carried out on the AP-1 and ATF/CREB used in ITC experiments to ensure both DNA target sites are in duplex form (data not shown). Additionally, we have studied the formation of the AP-1 duplex from its two complementary single strands using ITC and differential scanning calorimetry (Cao and Lai, 1999). In conclusion, both AP-1 and ATF/CREB were in duplex form under the conditions of all the

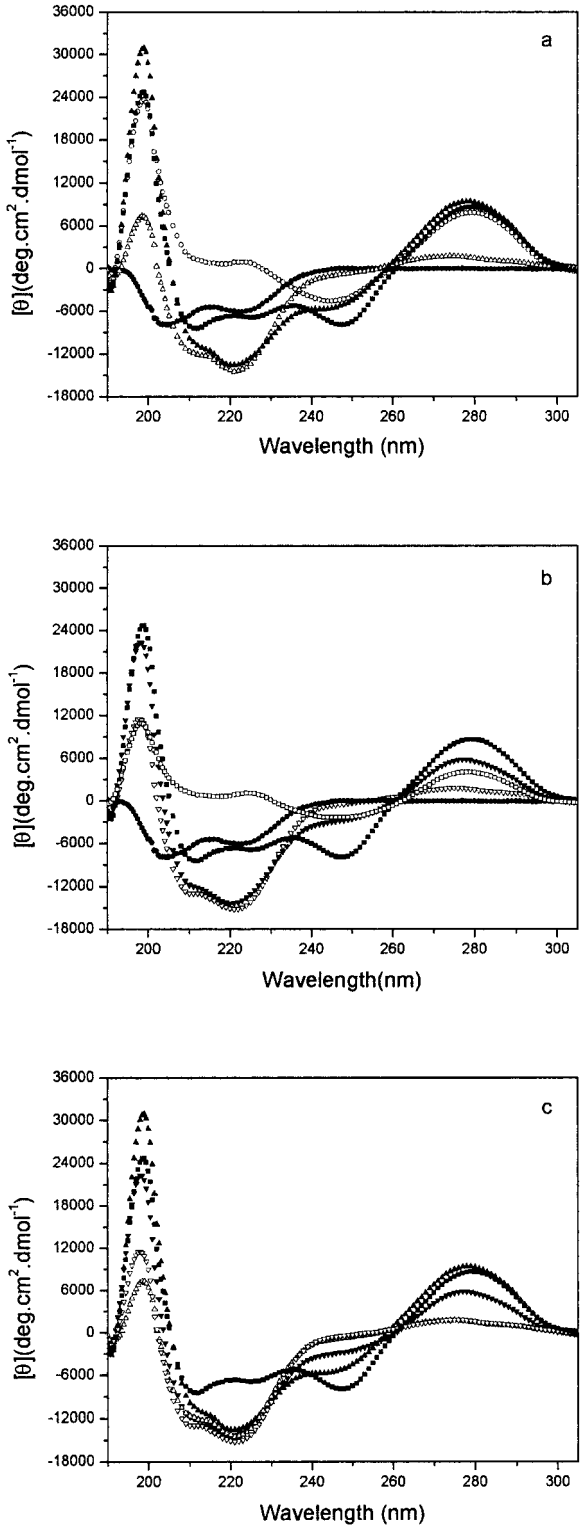

FIGURE 2 CD difference spectra indicate that GCN4-br changes into α-helical conformation upon binding to AP-1 and ATF/CREB target sites at 20°C. (*a*) GCN4-br binding to the AP-1 site. (*b*) GCN4-br binding to the ATF/CREB site. (*c*) GCN4-br binding to the AP-1 and ATF/CREB sites, respectively, along with the difference spectra. (●) GCN4 alone; (○) AP-1 alone; (□) ATF/CREB alone; (■) spectra of GCN4 bound to control DNA CONT; (▲) spectra of GCN4 bound to AP-1 site; (△) difference spectra of GCN4 bound to AP-1; (▼) spectra of GCN4 bound to ATF/CREB; (▽) difference spectra of GCN4 bound to ATF/CREB. The concentration of GCN4-br is 36 μM. The buffer contains 10 mM sodium phosphate, 100 mM NaCl, and 0.1 mM EDTA, pH7.4.

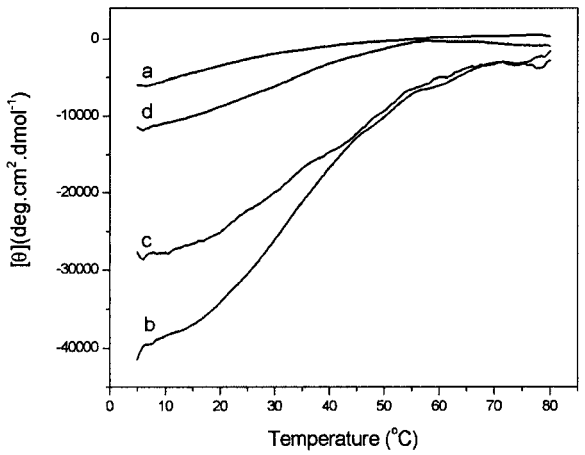

FIGURE 3 Thermal unfolding curves of GCN4-br and the complexes with AP-1 and ATF/CREB at 222 nm. (*a*) GCN4-br alone. (*b*) GCN4-br-AP-1. (*c*) GCN4-br-ATF/CREB. (*d*) GCN4-br-CONT. The concentration of GCN4-br is 36 μM and the concentration of DNA is 18 μM. The molar ratio of peptide/DNA is 2:1. The buffer is same as in Fig. 2.

ITC experiments carried out herein. Fig. 4 illustrates a typical ITC titration for GCN4-br binding to the AP-1 site at 20°C. Panel *a* shows the trace recorded for each of the 25 10-μL injections made at 400-s intervals. After each titration, an exothermic heat effect is observed. The area of each peak was integrated and corrected for the peptide heat of dilution, which was estimated by a separate experiment by injecting the peptide into the buffer. By fitting the titration curve with a nonlinear least-squares method, the enthalpy change ΔH^0 and the binding constant K_b of peptide binding to DNA can be estimated with the assumption of an independent binding site model. Panel *b* shows the fit of each integrated heat to a titration curve. The results obtained by this curve fitting using the calorimetric software supplied with the calorimeter were $N = 2.03\ (\pm 0.04)$ binding sites per DNA molecule, $K_b = 5.18\ (\pm 0.25) \times 10^4\ M^{-1}$, and $\Delta H^0 = -35.7 \pm 0.6\ kJ \cdot mol^{-1}$.

The thermodynamics of the GCN4-br binding to DNA targets AP-1 and ATF/CREB at 10, 15, 18, and 20°C in 100 mM NaCl and 10 mM phosphate buffer at pH 7.4 are summarized in Table 1. The values provided are the average of duplicate experiments. For the peptide binding to both DNA targets, we obtained exothermic enthalpy. The standard free energies (ΔG^0) were obtained from the equation $\Delta G^0 = -RT\ln K_b$, in which the K_b is the binding constants at each corresponding temperature. The ΔS^0 function was calculated from the standard thermodynamic relation $\Delta G^0 = \Delta H^0 - T\Delta S^0$. We obtained favorable free energies that result from partial compensation of favorable enthalpies with unfavorable entropy. The CD results reported above (Fig. 2) indicate

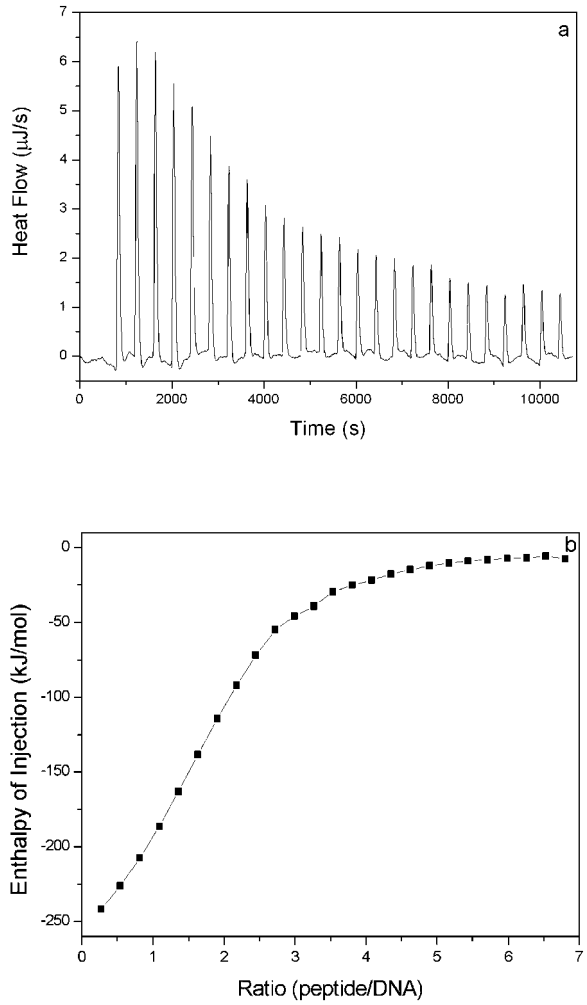

FIGURE 4 (*a*) Typical ITC profile of GCN4-br binding to AP-1 at 20°C. Each peak corresponds to a 10-μL injection containing 800 μM GCN4-br into the cell containing 40-μM DNA. (*b*) Results of curve fitting to an independent site model. The buffer is same as in Fig. 2.

that the peptide GCN4-br binds to the AP-1 and ATF/CREB target sites coupled with basic region folding. Thus, the thermodynamics of this protein-DNA recognition system studied here are the overall properties of binding and folding reactions.

The data obtained from ITC experiments were used to examine the temperature dependence of the binding enthalpy. Table 1 indicates that ΔH^0 are temperature dependent. The relationship between enthalpy and temperature is shown in Fig. 6. The slope of a linear least-squares fit of these data yields the heat capacity changes $\Delta C_p = -0.92$ kJ $\cdot$ mol^{-1} K^{-1}, and $\Delta C_p = -0.95$ kJ $\cdot$ mol^{-1} K^{-1} for GCN4-br binding to AP-1 and ATF/CREB, respectively. Here, the ΔC_p was obtained assuming that it was temperature independent in the range of 10–20°C. Fig. 6 also shows the effects of temperature on the entropy $T\Delta S^0$ and the free energy change ΔG^0 of the binding reaction, indicating that the binding of GCN4-br to the AP-1 and ATF/CREB target sites is enthalpically driven.

DISCUSSION

CD spectroscopy and ITC have been used to characterize the DNA binding of the monomeric GCN4. The objective of this work is to confirm whether the monomeric GCN4 recognizes its DNA targets as the dimer does in sequence-specific fashion. Most assays for this DNA binding system mainly use the filter-binding experiments, gel mobility retardation, and footprinting techniques other than the calorimetry. However, the accurate thermodynamic information is necessary to understand the sequence-specific recognition and affinity of the protein-DNA binding. To our knowledge, the thermodynamics of the monomer of GCN4 binding to its DNA targets have not been characterized. Our work reported here also is important to understand the mechanism of the binding of transcription factors to their target sites for transcriptional regulation.

Binding of GCN4-br to AP-1 and ATF/CREB is enthalpically driven

Enthalpies ΔH^0 measured for GCN4-br binding to AP-1 and ATF/CREB here were obtained by fitting the binding isotherms to an independent site model, on the basis of the assumption that two half DNA binding sites are independent and noninteracting. The best-fit curves for GCN4-br binding to AP-1 site at a temperature range 10–20°C are illustrated in Fig. 5. The sigmoidal binding isotherms indicate that

TABLE 1 Thermodynamic parameters of binding of GCN4-br to AP-1 and ATF/CREB sites

Temperature (°C)	N	$K_b \times 10^{-4}$ (M^{-1})	ΔH^0 (kJ/mol)	ΔG^0 (kJ/mol)	$T\Delta S^0$ (kJ/mol)
AP-1					
10	2.03 ± 0.02	15.7 ± 0.28	−26.2 ± 0.4	−28.2 ± 0.5	2.0 ± 0.9
15	1.99 ± 0.03	14.5 ± 0.37	−31.0 ± 0.5	−28.5 ± 0.3	−2.5 ± 0.8
18	2.00 ± 0.02	6.79 ± 0.16	−33.1 ± 0.5	−26.9 ± 0.1	−6.2 ± 0.6
20	2.03 ± 0.04	5.18 ± 0.25	−35.7 ± 0.6	−26.5 ± 0.2	−9.2 ± 0.8
ATF/CREB					
10	2.00 ± 0.05	15.3 ± 0.23	−25.1 ± 0.4	−28.1 ± 0.5	3.0 ± 0.9
15	2.04 ± 0.04	13.8 ± 0.45	−29.8 ± 0.6	−28.4 ± 0.4	−1.4 ± 1.0
18	2.02 ± 0.05	7.03 ± 0.37	−32.4 ± 0.8	−27.0 ± 0.2	−5.4 ± 1.0
20	2.01 ± 0.03	7.01 ± 0.20	−34.7 ± 0.6	−27.3 ± 0.1	−7.4 ± 1.7

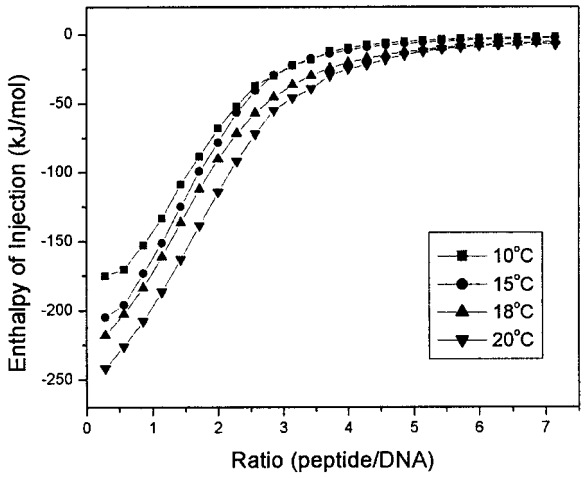

FIGURE 5 Calorimetric binding isothermals of GCN4-br binding to AP-1 site in temperature range 10–20°C. (■) 10°C; (●) 15°C; (▲) 18°C; (▼) 20°C.

the association of the GCN4-br with its DNA targets is exothermic in the temperature range 10–20°C under the conditions studied here. With this binding model, the best fit to the stoichiometry N is ~2.00 (±0.09) for the two targets at all temperatures studied, showing that two GCN4-br molecules bind to one DNA molecule. The concentration of GCN4-br was calculated as monomer quantities, and oligonucleotides were calculated as duplex molecules; thus, the determined ΔH^0 values are corresponding to the heat of binding of one molar GCN4-br molecule to its DNA target sites. By fitting the data using the independent binding model, we obtained the same thermodynamic parameters for the two half-site of DNA interacting with the peptide; whether the binding of the first GCN4-br molecule to one half-site of DNA influences the second peptide molecule binding needs further investigation. However, the quality of the fitting procedure illustrated by the curves in Fig. 5 may suggest that the model with independent site type used for this DNA-binding system is appropriate. The suitability of this model also can be confirmed by the findings from the crystal structures of the complexes GCN4 with AP-1 and ATF/CREB: that the leucine zipper orientated the basic region parallel to the panel of the DNA site and both basic regions identically contact with the major grooves of half-sites of DNA (Ellenberger et al., 1992; König and Richmond, 1993).

We have performed similar titration of the GCN4-br binding to the control oligonucleotide CONT under the same conditions, but the binding heat is too small to measure the thermodynamic parameters for its binding to GCN4-br (data not shown here), which further suggests that the binding of GCN4-br to CONT is nonspecific. CD difference spectrum has shown that the control DNA CONT induces the conformational changes of the GCN4-br (Fig. 2); however, our ITC results and the thermal unfolding experiments (Fig. 3) reported above indicate that the interactions between GCN4-br and CONT are presumably due to electrostatic attraction, rather than the specific interactions. Therefore, here we confirm the sequence-specificity for GCN4-br recognizing the DNA targets AP-1 and ATF/CREB.

Fig. 6 and Table 1 indicate that the entropy ΔS° of GCN4-br binding to AP-1 and ATF/CREB are negative and temperature dependent at all temperatures studied here. The overall thermodynamic profile for this interaction elucidates that GCN4-br binds to the DNA target sites accompanied by a favorable enthalpy change and an unfavorable entropy change. As discussed above, the GCN4-br binds to its DNA targets coupled with conformational transition of peptide from extended conformation to α-helix, thus the determined entropies are the overall values including not only the DNA binding but also the peptide folding. The peptide folding coupled with DNA binding entails the burial of hydrophobic surface and the formation of complementary peptide-DNA binding. The unfavorable contribution from a loss of conformational entropy must overcompensate a positive entropy contribution from the solvent effect, i.e., from the loss of structured water upon burial of the hydrophobic surface, therefore resulting in an unfavorable overall entropy for GCN4-br binding to DNA. The overall entropy may be mainly due to: 1), a favorable entropy caused by the hydrophobic effect; 2), an unfavorable entropy resulting from the reduction in the available rotational and translatory degrees of freedom of the protein and DNA on forming complex; and 3), an unfavorable entropy resulting from folding or other conformational changes in the protein and DNA. For the GCN4-br binding to the DNA targets, the unfavorable binding entropy originates primarily from the folding transitions of the basic region of the peptide upon binding to its DNA target sites.

The temperature dependence of both the observed enthalpies ΔH^0 and the derived $T\Delta S^0$ for GCN4-br binding

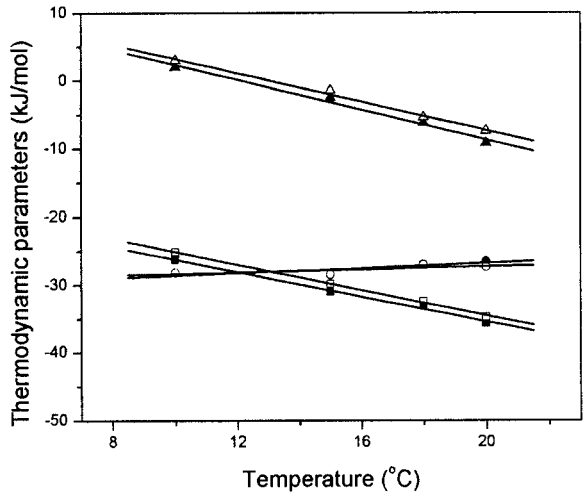

FIGURE 6 Temperature dependence of ΔH (□), $T\Delta S$ (△), and ΔG (○) of GCN4-br binding to its DNA target sites AP-1 (*solid*) and ATF/CREB (*open*).

to its two DNA target sites compensates to make ΔG^0 almost insensitive to temperature (Table 1 and Fig. 6). This behavior is typical of specific macromolecular interactions and has been consistently observed for a variety of specific protein-DNA interactions (Ladbury et al., 1994; Spolar and Record, 1994). This characteristic temperature dependence is the consequence of large negative heat capacity changes, ΔC_p (see discussion below). The determined free energy changes ΔG^0 reported here also include the components from the interaction of the peptide with DNA, and from the conformational changes in the peptide GCN4-br. The peptide folding coupled to DNA binding is necessary to form the uniquely and precisely stereocomplementary interface. In the absence of DNA, the conformation of the basic region of GCN4 in solution is flexible and mobile; this flexibility and mobility of peptide are required to facilitate the processes of association and disassociation. The DNA binding process for the monomeric peptide is driven by electrostatic attractions, hydrogen bonds, van der Waals bonds, hydrophobic effects, and so on.

Negative ΔC_p is characteristic of GCN4-br specifically binding to DNA

The temperature dependence of ΔH° indicates negative change in heat capacity ΔC_p upon GCN4-br binding to its two DNA targets. Assuming ΔC_p is temperature independent, we have lineally fit the temperature dependence of the binding enthalpies ΔH° to obtain the ΔC_p values: $\Delta C_p = -0.92$ kJ $\cdot$ mol^{-1} K^{-1} and $\Delta C_p = -0.95$ kJ $\cdot$ mol^{-1} K^{-1} for GCN4-br binding to AP-1 and ATF/CREB, respectively, in 100 mM NaCl, 10 mM phosphate buffer at pH 7.4. The negative ΔC_p is thought to be a manifestation of specific recognition based on stereospecific macromolecular interaction between large and complementary surfaces (Murphy and Freire, 1992; Spolar and Record, 1994). The major contribution is believed to come from changes in water-accessible surface, which are closely related to protein folding where a great part of the extended protein chain is buried from hydrating waters upon folding. As discussed above, the peptide GCN4-br undergoes a profoundly conformational transition from the extended conformation to α-helix upon binding to DNA, which is required to create a significant complementary peptide-DNA interface. The negative sign of ΔC_p also suggests that the reduction of solvent-accessible nonpolar and polar surfaces in the peptide and DNA drive the DNA binding process. However, the ΔC_p values of GCN4-br binding to DNA determined here are less negative half of the values reported by Berger et al. (1996) for the bZIP domain of GCN4 binding to the AP-1 ($\Delta C_p = -2.17 \pm 0.19$ kJ $\cdot$ mol^{-1} K^{-1}) and ATF/CREB ($\Delta C_p = -3.11 \pm 0.22$ kJ $\cdot$ mol^{-1}K^{-1}). Because we have known from the high-resolution X-ray structures of the complexes of the bZIP domain of GCN4 with DNA targets that, the basic region forms an α-helix and makes extensive contacts to base pairs and the sugar-phosphate backbone of the major groove upon binding to its DNA targets (Ellenberger et al., 1992; König and Richmond, 1993), the less negative ΔC_p values determined for GCN4-br are likely due to the less tight complementarity of the peptide-DNA interface, which might result from the weak binding affinities.

The GCN4-br binding to its DNA targets shows weak binding affinity (in level 10^4 M^{-1}), which is typical of nonspecific binding ($K_b \leq 10^6$ M^{-1}, Ladbury et al., 1994). As discussed above, the high-resolution x-ray studies have shown that the basic region of GCN4 forms an α-helix upon binding to DNA targets; however, our CD studies have shown that the helicity of the peptide GCN4-br only increases to ~70% upon DNA binding. This suggests that the dimerization formed by the leucine zipper orientates the basic region and therefore stabilizes the complex of GCN4-DNA. Without the orientation and restriction by the leucine zipper, the monomer basic region has higher degrees of freedom of flexibility and mobility, which may result in less negative ΔC_p values. However, it should be noted that specific interaction could be weak, as certainly as strong interaction could be nonspecific (Ladbury et al., 1994). The results presented here reinforce a suggestion that, irrespective of the overall stability of the complex, a negative ΔC_p is the hallmark of a biological reaction that forms a large highly complementary or specific interface.

Comparison of thermodynamics of GCN4-br binding to AP-1 and ATF/CREB

Pseudopalindromic AP-1 and palindromic ATF/CREB sites are two optimal DNA targets for GCN4. The two sites differ by a central G·C base pair between the two half-site ATGA sequences (Fig. 1). The crystal structures of the complexes of the bZIP domain of GCN4 with them have shown (Ellenberger et al., 1992; König and Richmond, 1993) that the additional G · C base pair is accommodated by a 20° bend of the DNA toward the leucine zipper and a larger base pair inclination in the GCN4-bZIP–ATF/CREB complex. The overall conformation of ATF/CREB in complex is B-form, but the central region shows features of A-DNA. In contrast, the DNA in the AP-1 complex is not bent and has little A-like characteristics. However, the structure of the GCN4 in the two complexes is highly similar. The thermodynamics of the binding of the GCN4-bZIP to the AP-1 and ATF/CREB sites have been previously studied to understand the molecular recognition process between GCN4 and its DNA target sites (Berger et al., 1996). The difference seen in the crystal structures of the GCN4:AP-1 and GCN4:ATF/CREB complexes are paralleled by differences in the thermodynamics of the association reaction (Berger et al., 1996). However, an analysis of the thermodynamic parameters obtained from our studies of the monomeric basic region, the GCN4-br, binding to its two DNA targets AP-1 and ATF/CREB does not

reveal obvious differences in the thermodynamics of the GCN4-br binding to the two targets within the uncertainties of our measurements. Moreover, the conformational changes of GCN4-br induced by DNA binding and the stability of the GCN4-br:AP-1 and GCN4-br:ATF/CREB are essentially same under the conditions studied here. This is to say that the additional G·C basepair in ATF/CREB does not induce much different binding properties from AP-1 binding for the monomeric basic region of GCN4. Compared with the binding affinities of GCN4-bZIP (10^7 M^{-1}, Berger et al., 1996), much weaker affinities have been observed for GCN4-br (10^4 M^{-1}). This may explain the similarity of GCN4-br binding process with AP-1 and ATF/CREB. For the bZIP domain containing leucine zipper, the DNA of ATF/CREB site is bent by 20° upon protein binding; this induced bending may have contributed to the different thermodynamics of the association reaction (Berger et al., 1996). However, the peptide GCN4-br may have much higher degrees of freedom of flexibility and mobility at the specific peptide-DNA interface without the restriction of a leucine zipper, which implies that, unlike the GCN4-bZIP, the more flexible GCN4-br does not distort the conformation of the ATF/CREB target, at least not so far. The binding of peptide GCN4-br to its DNA targets based on structural events deserves further study.

The weaker binding affinity of GCN4-br probably is also the reason that gel retardation assay failed to detect the monomer of GCN4 recognizing DNA target sites (Talanian et al., 1990), i.e., the much weaker DNA binding affinity of monomer might prevent the detection of the monomer binding during gel retardation assay at the concentrations used. Because GCN4 forms a stable dimer in the absence of DNA and binds to DNA as a dimer, the molecular mechanism by which GCN4 recognizes its DNA targets is widely believed to follow a dimer pathway, i.e., the dimerization of protein precedes DNA binding. Our findings presented here would give insight into the molecular recognition mechanism of GCN4 binding to its DNA targets.

CONCLUSION

We have demonstrated that the monomer of the basic region of GCN4, GCN4-br, can recognize the DNA target sites AP-1 and ATF/CREB in a specific form, and comprehensively analyzed the thermodynamics of the specific recognition of GCN4-br. The DNA binding process of GCN4-br is enthalpically driven, accompanied by an unfavorable entropy change. The temperature dependence of ΔH^0 has indicated negative changes of heat capacity ΔC_p upon GCN4-br binding to DNA, which is a strong manifestation of specific recognition of the monomeric GCN4 to its DNA targets. Compared with the bZIP domain of GCN4, the GCN4-br binding to its DNA targets shows much weaker affinity, which may result in no obvious differences existing in the thermodynamics of the interaction between GCN4-br and AP-1, ATF/CREB. Our results would provide important thermodynamic proofs for the monomer of GCN4 specifically binding to its DNA target sites. These findings that the monomeric GCN4 can recognize its DNA targets in specific fashion would give new insight into the mechanism by which GCN4 protein finds its target site for transcriptional regulation.

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