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The Effect of *C*-Terminal Helix Stabilization on Specific DNA Binding by Monomeric GCN4 Peptides†

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Abstract: DNA binding by a 29-residue, monomeric, GCN4 basic region peptide, GCN4br, as well as by peptide br-C, a monomeric basic-region analogue that is helix stabilized at its *C*-terminal end by a Lys^{25}, Asp^{29} side-chain lactam-bridged alanine-rich sequence, was studied at 25 °C in an aqueous buffer containing 100 mM NaCl. Mixing of both peptides with duplex DNA containing the cAMP-responsive element (CRE) was accompanied by significant helix stabilization in the peptides, whereas mixing of the peptides with duplex DNA containing a scrambled CRE site was not. Peptide NBD-br-C was synthesized as a fluorescent probe to evaluate these peptide–DNA interactions further. Quantitative analysis of the fluorescence quenching of peptide NBD-br-C by CRE half-site DNA indicated the formation of a 1 : 1 complex with a dissociation constant of 1.41 ± 0.22 μM. Competitive displacement fluorescence assays of CRE half-site binding gave dissociation constants of 0.65 ± 0.09 μM for peptide br-C and 3.9 ± 0.5 μM for GCN4br, which corresponds to a free energy difference of 1.1 kcal/mol that is attributed to the helix stabilization achieved in peptide br-C. This result indicates that helix initiation by the α-helical leucine zipper dimerization motif in native bzip proteins, such as GCN4, contributes significantly to the affinity of basic region peptides for their recognition sites on DNA. Our fluorescence assay should also prove useful for determining dissociation constants for CRE binding by other GCN4 basic region analogues under equilibrium conditions and physiological salt concentrations.

Keywords: basic region; DNA binding; fluorescence assay; GCN4; helix stabilization; lactam bridge; bzip protein

INTRODUCTION

There is considerable interest in the design and synthesis of small peptides and peptidomimetics that bind to specific nucleotide sequences with high affinity and specificity [1,2]. Such compounds have potential utility as model systems for basic research into mechanisms of nucleotide recognition, as well as the design of molecular tools for genomics research and the development of drugs acting as artificial regulators of transcription and translation [3]. Specific DNA binding by protein transcription regulators incorporating a basic region-leucine zipper (bzip) motif [4] represent perhaps the simplest system to study as a model for specific DNA recognition. The yeast transcription activator, GCN4 [5,6],

Abbreviations: Boc, *tert*-butyloxycarbonyl; BOP, benzotriazolyloxy tris(dimethylamino)phosphonium hexafluorophosphate; bzip, basic region-leucine zipper; Bzl, benzyl; CD, circular dichroism; Cl-Z, 2-chlorobenzyloxycarbonyl; CRE, cAMP-responsive element; DIEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; Fmoc, fluorenylmethyloxycarbonyl; HBTU, O-benzotriazolyl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; HPLC, high-pressure liquid chromatography; MBHA, *p*-methylbenzhydrylamine resin; NBD-, 7-nitrobenz-2-oxa-1,3-diazol-4-yl-; NBD-F, 4-fluoro-7-nitrobenzofurazan; OFm, fluorenylmethyl ester; Tos, N^{ω}-*p*-toluenesulfonyl.

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is one of the most intensively studied proteins in this class. Bzip proteins bind to palindromic recognition sites on DNA as dimers. The leucine zipper motif is the only peptide determinant of dimerization, which is achieved through formation of a coiled coil of parallel α helices [7]. The leucine zipper motif is connected directly to the two basic region peptides of the dimer in the *N*-terminal direction, and these basic region peptides are believed to provide the only protein–DNA contacts. Crystal structures of the GCN4 bzip peptide in complexes with the AP-1 specific recognition site, ATGA[C/G]TCAT [8], and the CRE site, ATGACGTCAT [9], show that the dimerized bzip peptides form a continuous, forked α-helical structure with the basic-region peptides extending in opposite directions into the major groove of each half site in the cognate DNA.

In the absence of DNA, the GCN4 basic region appears to be largely unstructured, although the dimeric leucine zipper motif is fully α-helical [10–12]. DNA binding by GCN4 or bzip peptides is, therefore, accompanied by a significant entropic penalty resulting from the formation of an ordered α-helical conformation by the basic region upon binding. The magnitude of this entropic penalty is estimated from calorimetric measurements and NMR studies as $\Delta S_{conf} = -1.2$ kJ/mol/K (-0.29 kcal/mol/K) at 25 °C [13,14]. Approximately 40%–45% of this conformational change may be attributed to the loss of backbone conformational entropy [15,16]. This is equivalent to an unfavourable contribution to the free energy of formation of the dimeric GCN4–DNA complex at 25 °C of $\Delta G = 35$–40 kcal/mol, due to helix formation by the basic region peptide backbone alone. Specific DNA binding by GCN4 bzip peptides is, therefore, driven by large favourable enthalpies of complex formation, as demonstrated experimentally [17].

The leucine zipper dimerization motif, since it is fully α-helical in the dimerized GCN4 protein or in a bzip peptide, might also function as a helix initiating structure favouring α-helix formation in the basic region. In support of this potential effect, NMR studies of GCN4 bzip peptides identify short- and medium-range ^{1}H nuclear Overhauser effects [12] and temperature-dependent ^{13}C-carbonyl chemical shifts [14] that are characteristic of α-helical structure, extending from the zipper domain into the basic-region peptide from its *C*-terminal end. Helix initiation at the *C*-terminal end of the basic region is expected to stabilize DNA binding by the basic region, but the magnitude of this effect is unknown. GCN4 basic-region peptides alone, when dimerized through disulphide-bridge formation, metal-ion chelation, or other linkers, have been shown by gel-shift assay and CD spectropolarimetry to bind to their cognate DNA sequences with low-nanomolar, and even subnanomolar, dissociation constants comparable to those of the intact GCN4 protein itself [18–21]. More recently, gel-shift assays have demonstrated DNA binding by the monomeric basic-region peptide in buffers at low ionic strength, but not under physiological salt conditions [22]. However, by stabilizing the α-helical conformation throughout the basic region in order to overcome the backbone entropic penalty associated with binding, Zondlo and Schepartz [23] have shown that tight binding by monomeric basic-region peptides can be achieved, even in buffers at physiological ionic strength.

We have also reported that the monomeric basic region peptide from GCN4, GCN4br (Figure 1), shows specific binding in the micromolar concentration range to a DNA sequence containing the CRE site in buffers containing 100 M NaCl, and that this binding may be followed by circular dichroism spectropolarimetry [24]. Our initial results indicated that modest increases in the stability of the specific GCN4br–DNA complex could be achieved through modification of the peptide structure with lactam bridges linking the side chains of Lys^{i}, Asp^{i+4} residue pairs. This modification is known to favour the α-helical conformation [25,26], and may also, therefore, be favouring DNA binding by lowering the unfavourable ΔS_{conf} associated with the binding reaction. In the present study, we have chosen to use this lactam-based approach to investigate in detail the effect of *C*-terminal helix stabilization in GCN4br on the affinity of the monomeric basic-region peptide for the CRE-12 binding site. For this purpose, an Ala-rich lactam-bridged sequence has been introduced into the *C*-terminus of monomeric GCN4br, in order to mimic the *C*-terminal helix initiating effect of the leucine zipper domain. However, the increased α-helix content of our lactam-bridged GCN4 analogue, peptide br-C (Figure 1), made quantification of their DNA binding affinities by CD difference spectropolarimetry more uncertain. Furthermore, the standard, gel-based separation assays typically used for quantification of this binding reaction were also difficult to apply to these small, monomeric peptides under physiological salt conditions, presumably because of the high dissociation rates of their complexes with duplex DNA. Therefore, we have also chosen to develop a fluorescence-based

competitive displacement assay, in order to measure the binding of our helix-stabilized GCN4br analogue to duplex DNA. A helix-stabilized, NBD-labelled analogue of GCN4br, peptide NBD-br-C (Figure 1), was designed for use as the labelled ligand in this assay. Peptide NBD-br-C has the environmentally sensitive fluorescent NBD probe placed at the *N*-terminus of a truncated version of peptide br-C, so that the NBD group is located as close as possible to the consensus recognition sequence of the basic-region peptide without modification of these essential residues, or of any of the basic side-chain groups that may form salt bridges with the phosphodiester DNA backbone. The NBD group is also placed exactly two helical turns away from the central -AA- DNA-recognition sequence in GCN4br positions 13 and 14, so that it will be positioned facing towards the major groove of the duplex DNA in the bound, α-helical peptide. The synthesis and characterization of the binding of this fluorescent probe to CRE-containing duplex DNA (CRE-12, Figure 1) is now described, together with its use in fluorescence assays of DNA binding by GCN4br and its *C*-terminal helix-stabilized analogue, peptide br-C.

MATERIALS AND METHODS

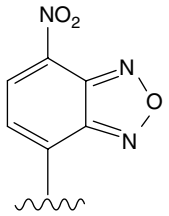
Peptide Synthesis

All peptides were synthesized using the established approach described by Felix and coworkers [25] for the solid-phase assembly of side-chain cyclized peptides. The peptide chain was assembled on a 4-methylbenzhydrylamine resin (Advanced ChemTech) at an initial substitution level of the *C*-terminal amino-acid derivative of about 0.25 meq/g, using Boc/Benzyl chemistry [27]. The following N^{α}-Boc protected L-amino acid derivatives were employed for peptide chain assembly: Arg(Tos)-OH, Asp(OFm)-OH, Glu(OBzl)-OH, Lys(Cl-Z)-OH, Ser(Bzl)-OH and Thr(Bzl)-OH, except that

A Peptides

GCN4br: N^{α}-Acetyl-DPAAL^{5}KRARN^{10}TEAAR^{15}RSRAR^{20}KLQRM^{25}KQLE-amide
Peptide br-C: N^{α}-Acetyl-(cyclo$^{25\text{-}29}$)-DPAAL^{5}KRARN^{10}TEAAR^{15}RSRAR^{20}KLQRK^{25}AAAD-amide
Peptide NBD-br-C: N^{α}-NBD-(cyclo$^{20\text{-}24}$)-KRARN^{5}TEAAR^{10}RSRAR^{15}KLQRK^{20}AAAD-amide

NBD:



B DNA

CRE-18: 5′-GCACATGACGTCATGTGC-3′
3′-CGTGTACTGCAGTACACG-5′

CRE-18S: 5′-GCACTAAGCGCTTAGTGC-3′
3′-CGTGATTCGCGAATCACG-5′

CRE-12: 5′-GCAATGACGAGC-3′
3′-CGTTACTGCTCG-5′

Figure 1 Peptide and oligonucleotide structures. (A) The structures of the peptides used in this study are indicated using the one-letter codes. The GCN4br sequence corresponds to yeast GCN4 residues 226–254 [6,40]. Residues in close contact with the major groove of the specific AP-1 and CRE recognition sites for this protein are underlined. Peptides br-C and NBD-br-C are derived from this structure, and both incorporate a lactam bridge linking the side chains of lysine and aspartic acid residues at their C termini. (B) The structures of the duplex DNAs used in this study are shown, with the CRE recognition site and half-site for GCN4 underlined in CRE-18 and CRE-12, respectively.

Lys(Fmoc)-OH was used in the appropriate bridging positions of peptides br-C and NBD-br-C (residues 25 and 20, respectively). Routine coupling reactions were performed in the presence of three equivalents each of the Boc amino-acid derivatives, HBTU, and HOBt, and six equivalents of DIEA. Side-chain cyclizations in the syntheses of peptides br-C and NBD-br-C were performed immediately after coupling each of the Boc-Lys(Fmoc)-OH residues. The Fmoc and OFm side-chain protecting groups on the Lys/Asp residue pairs were cleaved with 25% (v/v) piperidine in DMF for 20 min, and then cyclization was achieved by shaking the peptide resin in DMF with 1.1 equivalents each of HBTU, HOBt and DIEA for 4 h (peptide br-C) or 5 equivalents each of the reagents for 2 h (peptide NBD-br-C). After complete peptide chain assembly and N^{α}-Fmoc deprotection, the *N*-termini of GCN4br and peptide br-C were capped by acetylation with a 10-fold excess of acetic anhydride in the presence of 1.5 equivalents of DIEA for 1 h. The deprotected *N*-terminus of peptide NBD-br-C was capped by shaking the peptide resin in a minimum volume of DMF with 1.2 eq of NBD-F and 2.0 eq of DIEA for 30 min. The latter reaction, and all subsequent steps involving the peptide NBD-br-C were performed in dim light and/or with protection from ambient light conditions using aluminium foil covers.

Crude peptides were obtained by deprotection and cleavage from the solid support of 0.5 g aliquots of peptide-resin, by stirring in liquid HF (10 ml) in the presence of anisole (0.5 ml) at 0 °C for 2 h under nitrogen [27]. The peptide material was then washed with ether and extracted into aqueous acetic acid solution, lyophilized and purified to apparent homogeneity by reversed-phase HPLC on Dynamax C_{18} semi-preparative columns. The correct peptide structures were confirmed at the University of Michigan Protein Analysis Facility (Ann Arbor, MI) by amino acid analysis after hydrolysis in 6 N HCl at 110 °C for 24 h, and by identification of the correct parent $[M + H]^+$ ion (error less than 1 mass unit) by matrix-assisted laser-desorption time-of-flight mass spectrometry (GCN4br and peptide br-C) or by electrospray mass spectrometry (required for peptide NBD-br-C to avoid fragmentation of the parent ion).

The concentrations of all peptide stock solutions were determined by amino acid analysis, performed in triplicate as described above, with valine and phenylalanine added into the hydrolysis reactions as internal standards. Peptide NBD-br-C had an absorbance maximum at 468 nm in aqueous buffer containing 100 M NaCl and 10 mM NaH_2PO_4, titrated to pH 7.4 with NaOH, and the calculated extinction coefficient at 470 nm was 19 300 M^{-1} cm^{-1}.

Preparation of Oligonucleotides

The self-complementary oligonucleotides CRE-18 and CRE-18S were purchased from The Midland Certified Reagent Co. (Midland, TX), purified by reversed-phase HPLC and then converted to the sodium form by ion exchange, according to published methods [28]. Oligonucleotides corresponding to each strand of CRE-12 were synthesized and purified as previously described [29]. The concentrations of stock oligonucleotide solutions were determined from the calculated extinction coefficients at 260 nm and 25 °C [30], by extrapolation of the high-temperature linear portion of the UV melting curves.

Circular Dichroism Studies

Circular dichroism (CD) spectra were measured using an Aviv Model 62ds spectropolarimeter fitted with a Peltier temperature controller. Spectra were measured at 25 °C in a 1.0 mm pathlength cell, recording data points every 0.5 nm in the range 200–250 nm, using a 1.0 nm bandwidth and a signal averaging time of 1.0 s. Up to five wavelength scans were averaged to obtain a low signal-to-noise ratio. Blank spectra measured with the same cells under the same conditions were subtracted for each experiment. All peptide and DNA samples were prepared and studied in a buffer consisting of 100 mM NaCl, 10 mM NaH_2PO_4-NaOH, pH 7.4.

Fluorescence Studies

Fluorescence measurements were carried out on a Shimadzu RF-1501 spectrofluorophotometer. Temperature control was achieved using a Shimadzu constant-temperature cell holder connected to a circulating heating-cooling water bath. Excitation and emission bandwidths were both set at 10 nm, and all recordings were made on samples equilibrated at 25 °C in the water bath and/or the sample compartment. Emission spectra were recorded from 520 nm to 570 nm, using an excitation wavelength of 470 nm. For single wavelength readings, emission intensities at 540 nm were used.

All fluorescence studies were performed using the same sample buffer as for the CD studies, but with added bovine serum albumin to inhibit peptide binding to surfaces (1 mg/ml). All dissociation constants

are given as the mean $\pm \sigma$ determined from independent repeated experiments. The binding constant of NBD-br-C was determined by titrating a solution of 0.10 μM NBD-br-C with a solution containing a mixture of 10 μM CRE-12 and 0.1 μM NBD-br-C, and allowing the fluorescence reading to equilibrate for 2–7 min after each addition. Data were then fit to Eqn (1), which describes the binding of peptide NBD-br-C to CRE-12 to form a 1 : 1 complex. In this Eqn, $[\text{CRE-12}]_t$ and $[\text{NBD-br-C}]_t$ are the total concentrations of the DNA and peptide, respectively; $\Delta I = I_0 - I_{\text{CRE12}}$, the difference in peptide fluorescence intensity in the presence of CRE12 (I_{CRE12}) compared with its fluorescence intensity in the absence of CRE-12 (I_0); $\Delta I_{\max}$ is the maximum value of ΔI, corresponding to 100% of the peptide being bound to CRE12; and ${}^*K_D$ is the dissociation equilibrium constant for the DNA-bound NBD-br-C complex. Data for $[\text{CRE-12}]_t$ as a function of ΔI were fit to Eqn (1) using KaleidaGraph v3.0 software (Synergy Software, Reading, PA) run on an Apple Macintosh computer.

$$[\text{CRE-12}]_t = [\text{NBD-br-C}]_t(\Delta I/\Delta I_{\max}) + {}^*K_D/([\Delta I/\Delta I_{\max}] - 1) \quad (1)$$

For competitive displacement titrations, buffered solutions (3.0 ml) containing 0.10 μM NBD-br-C and either 0.09 μM or 0.15 μM CRE-12 were titrated with stock solutions of GCN4br or peptide br-C at concentrations of about 600 μM, and the fluorescence change, ΔI, due to competitive displacement of the bound NBD-br-C was plotted as a function of the total competing ligand concentration. Dilution effects due to the additions of competing ligand were less than 3% of the total volume and were ignored. Data were then analysed using the program Radlig v. 6.0 in the KELL program package from Biosoft (Ferguson, MO). This program uses input values for the concentrations of bound labelled ligand with a known dissociation constant for the receptor (${}^*K_D$) as a function of the concentration of total competing unlabelled ligand concentration in a competition assay, and generates solutions for the unknown dissociation constant of the competing ligand for the receptor, K_D, and the apparent receptor concentration, $B_{\max}$. To input the data from the fluorescence assays, the ΔI values were converted to concentrations of bound NBD-br-C, $[\text{NBD-br-C}]_b$, using Eqn (2), where $[\text{NBD-br-C}]_0$ is the concentration of the bound labelled ligand in the absence of competing peptide, and $\Delta I_{\max}$, is the maximal fluorescence change due to competitive displacement of the NBD-br-C from its CRE-12 binding sites.

$$[\text{NBD-br-C}]_b = [\text{NBD-br-C}]_0(\Delta I_{\max} - \Delta I)/\Delta I_{\max} \quad (2)$$

$[\text{NBD-br-C}]_0$ was calculated from the experimental value of ${}^*K_D = 1.41$ μM for NBD-br-C binding to CRE-12, which gave values of 5.64 nM for experiments where $[\text{CRE-12}]_t = 0.09$ μM, and 9.09 nM for experiments where $[\text{CRE-12}]_t = 0.15$ μM. The value of $\Delta I_{\max}$ for each experiment was initially estimated by visual extrapolation of the ΔI data, and was then adjusted in 1–2% increments until the optimal value giving the best Radlig fit to the data (minimal RMS deviation) was identified. The output values of K_D and $B_{\max}$ from repeated experiments with each competing peptide ligand were combined to calculate the mean $\pm \sigma$ values. The output values for $B_{\max}$ were always in good agreement with the known concentrations of $[\text{CRE-12}]_t$ used in the experiment, which served as a check on the curve fitting method and the binding stoichiometry. The K_D values were also in close agreement with those obtained by estimating IC_{50} values from the raw data and applying the Cheng-Prusoff equation [31].

RESULTS AND DISCUSSION

Circular Dichroism Studies of DNA Binding

The CD spectra of GCN4br and peptide br-C were compared in aqueous solution alone, and in the presence of the duplex DNAs CRE-18, CRE-18S and CRE-12. The DNA concentrations were adjusted so that the CRE-18 and CRE-18S concentrations were one half the molar concentrations of the peptides, and the CRE-12 concentrations were equimolar with the peptides. Under these conditions, the concentrations of the GCN4 CRE recognition half-sites in the peptide/CRE18 and peptide/CRE-12 mixtures were equal to the peptide concentrations. The resultant spectra are presented in Figure 2 as the difference spectra after subtraction of the spectra of the DNA measured separately.

A qualitative analysis of the peptide spectra in the absence of added DNA indicates that these peptides exist predominantly as mixtures of disordered and α-helical structure, as indicated by the minima in their spectra around 208 nm and 222 nm [32]. The greater intensity of these ellipticity minima in the

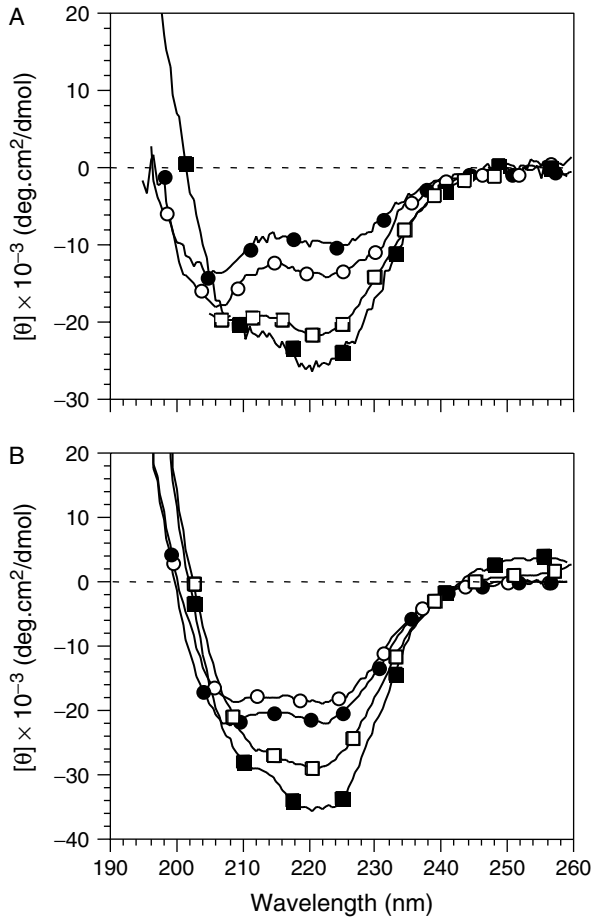


Figure 2 CD spectra of peptides and peptide–DNA mixtures. The CD spectra of (A) GCN4br (10 μM), and (B) peptide br-C (44 μM), in phosphate-buffered saline at 25 °C were measured alone (filled circles), or in the presence of 0.5 equivalents of CRE-18 duplex DNA (filled squares) or CRE-18S duplex DNA (open circles), or 1.0 equivalents of CRE-12 duplex DNA (open squares). The spectra of the peptide-DNA mixtures are presented as the difference spectra after subtracting the CD spectrum of the DNA alone.

peptide br-C spectrum indicates that, as expected, this peptide is helix-stabilized by the lactam-bridged segment at its *C*-terminal end, in comparison to the linear peptide GCN4br. A quantitative analysis of these spectra to evaluate the secondary structure contents of the peptides alone in aqueous solution was performed by fitting these curves to linear combinations of the standard spectra of Brahms and Brahms [32] using the program LINCOMB [33]. This analysis gives helix contents of 41% and 64% for GCN4br and peptide br-C, respectively (Table 1).

The difference CD spectra obtained from the various peptide–DNA mixtures indicate an increase in negative ellipticity in the spectral region from 205 nm to 235 nm in all cases, in comparison to the corresponding spectra of the peptides alone. This indicates that both GCN4br and peptide br-C interact with all of the duplex DNAs tested. However, much stronger effects were observed upon addition of CRE-18 or CRE-12, which contain the recognition half-sites for GCN4, than were observed upon addition of the scrambled CRE sequence in CRE-18S. Since this region of the CD spectrum is dominated by changes in the α-helical structure of the peptides, these results are indicative of a conformation-specific binding interaction of the peptides GCN4br and br-C with the CRE half-sites in CRE-18 and CRE-12, in which the α-helical conformation of these peptides is stabilized in these peptide–DNA complexes. This result is, therefore, consistent with prior observations of specific DNA binding by dimeric GCN4 basic region [18] and b-zip [10] peptides, which have shown that the partially disordered basic region peptides become α-helical upon binding into the major groove of their AP1 or CRE recognition sites in duplex DNA [8,9].

The weaker effects of adding CRE-18S, the duplex DNA having a scrambled CRE binding site sequence, on the CD spectra of GCN4br and peptide br-C are

Table 1 Helical Structure and DNA Binding by GCN4br and Peptide br-C

Peptide	$[\theta]_{222}$ (deg.cm^2/dmol)	% Helix[a]	Binding to CRE-12[b]		
			K_D (μM)	$B_{max}/[CRE]_t$	ΔG (kcal/mol)
GCN4br	−9,610	41	3.9 ± 0.5	0.92 ± 0.04	−7.38 ± 0.15
Peptide br-C	−17,216	64	0.65 ± 0.09	0.94 ± 0.05	−8.44 ± 0.08

[a] Estimated by least-squares fit of the CD spectra to linear combinations of the standard peptide spectra of Brahms and Brahms [32], using the program LINCOMB [33].
[b] $B_{max}/[CRE]_t$ estimates the apparent number of peptide binding sites per duplex DNA molecule, based on analysis of the data from competitive binding assays using the program Radlig from KELL v. 6.0 (see Materials and Methods).

an indication of specificity in the binding interaction of these monomeric GCN4 basic region peptides for DNA containing the CRE half-site: the peptide interactions with CRE-18S are either of significantly lower affinity compared to those with CRE-18 or CRE12, or the binding interactions with CRE-18S do not involve significant stabilization of α-helical structure. Recently, monomeric GCN4-derived peptides that were designed for helix stabilization by their incorporation into the pancreatic polypeptide fold have been shown by gel-shift assays to have a high differential affinity for their cognate DNA sequences over non-cognate DNA sequences under binding assay conditions containing physiological salt concentrations that are similar to those used here [23]. This suggests that our CD data are best explained by GCN4br and peptide br-C having a significantly lower affinity for the scrambled CRE site in CRE-18S than they have for the correct sequence CRE sites in CRE-18 and CRE-12.

CD studies of peptide NBD-br-C indicate that this peptide also exists as a mixture of α-helical and disordered structure in aqueous buffer at 25 °C, and that addition of CRE-12 increases the ellipticity minima at 208 and 222 nm that are indicative of the α-helical structure in the peptide (Figure 3). Therefore, the conformation-specific binding to the CRE half-site that was observed for peptide br-C is retained in its *N*-terminal truncated and NBD-labelled analogue, peptide NBD-br-C. This result, which is for a monomeric basic-region peptide, is consistent with the length requirements reported previously for DNA binding by disulphide-linked, GCN4 basic-region dimers: the disulphide-linked dimer of a peptide corresponding to GCN4br residues 6–27 (Figure 1), which is identical to the NBD-br-C sequence in residues 1–20, has been observed to bind specifically to CRE-site DNA by CD melting studies, which showed an enhancement of the ellipticity signal at 222 nm; binding for this peptide was not observed by gel-shift assay, and no binding by either method was reported for shorter dimeric peptides corresponding to GCN4br residues 5–20, or even 2–20 [19]. It is worth noting, however, that the importance of the NBD label in determining the present result for monomeric peptide NBD-br-C is unknown.

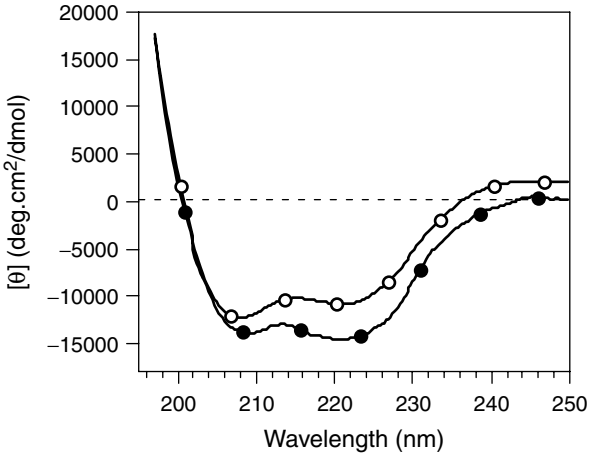


Figure 3 CD spectra of peptide NBD-br-C alone and mixed with CRE-12. The CD spectra of peptide NBD-br-C in phosphate-buffered saline at 25 °C were measured alone (open circles), or in the presence of an equimolar amount of CRE-12 duplex DNA (filled circles). The spectrum of the peptide-DNA mixture is presented as the difference spectrum after subtracting the CD spectrum of the CRE-12 DNA alone.

Fluorescence Assays of DNA Binding

Fluorescence excitation at 470 nm gave an emission spectrum for peptide NBD-br-C with a maximum at 540 nm. In the presence of CRE-12, the fluorescence of peptide NBD-br-C was quenched. Titration of peptide NBD-br-C with increasing concentrations of CRE-12 gave DNA concentration-dependent decreases in the peptide fluorescence indicative of saturable binding sites for the peptide on the DNA (Figure 4). A model of one peptide binding site per molecule of duplex CRE-12 DNA gave a good fit to these binding isotherms and a calculated dissociation constant of 1.41 ± 0.22 μM. Based on this result, a Job plot analysis [34] was designed to test the assumed 1 : 1 stoichiometry of the peptide:DNA complex. In this experiment, the fluorescence intensity of different mixtures of peptide and CRE-12 DNA at a constant total concentration (peptide plus duplex DNA) of 20 μM was plotted against the mole fraction of peptide. Extrapolation of the linear extreme portions of the curve then gave an apparent stoichiometry of 1.38 peptide molecules per duplex DNA molecule in the bound complex, from the mole fraction at the intercept (Figure 5). Within the error of this experiment, this result is in reasonable agreement with the stoichiometry of 1 : 1 that is assumed in calculating the dissociation constant for the complex formed by peptide NBD-br-C and CRE-12.

Based on the CD and fluorescence data described above, the designed peptide NBD-br-C appeared to be a suitable fluorescent-labelled ligand for the CRE half-site for use in competitive binding assays to determine dissociation constants in the low micromolar range for other peptides recognizing the same

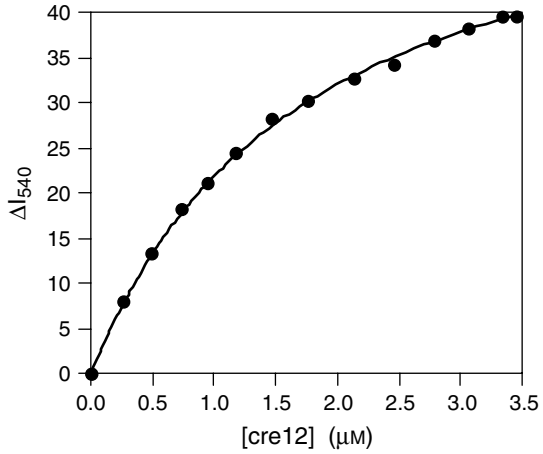


Figure 4 Binding isotherm for the peptide NBD-br-C complex with CRE-12. The decrease in fluorescence intensity (ΔI, arbitrary units) of peptide NBD-br-C (0.10 μM) in phosphate-buffered saline containing added BSA at 25°C is plotted as a function of the concentration of added CRE-12 duplex DNA (filled circles). Data were fit to an equation describing the binding equilibrium, as described in the text (solid line).

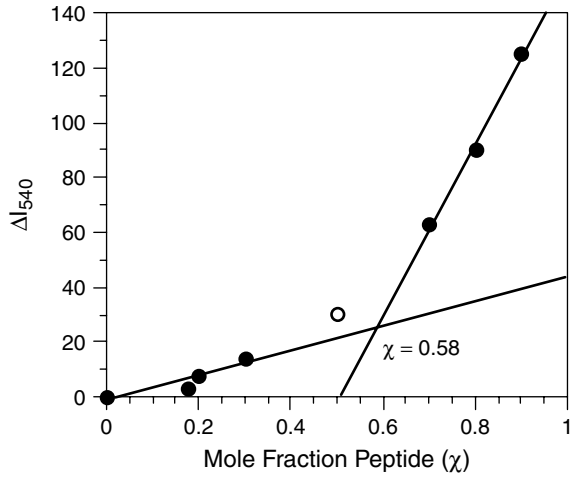


Figure 5 Stoichiometry of the peptide NBD-br-C complex with CRE-12. Peptide NBD-br-C and CRE-12 duplex DNA were mixed in phosphate-buffered saline at 25°C with different mole fractions, χ, of peptide and a constant total concentration (20 μM) of the two components. The fluorescence intensity at 540 nm (I, arbitrary units) is plotted against the mole fraction peptide (χ) to give a Job plot [34]. Linear extrapolations of the data on either side of the midpoint intercept at $\chi = 0.58$, corresponding to the apparent stoichiometry of the complex.

binding site. We have tested its utility for this function, using the unlabelled peptides GCN4br and br-C as competitive inhibitors of peptide NBD-br-C binding. Both of these peptides, when added to mixtures of peptide NBD-br-C and CRE-12 at constant concentrations, caused increases in the measured fluorescence intensity of peptide NBD-br-C to approximately the same plateau level. This indicates increasing displacement of the bound fluorescent-labelled peptide from its binding sites on CRE-12 with increasing concentrations of the competitive peptides (Figure 6). In control experiments performed in the absence of added CRE-12, essentially no changes in peptide NBD-br-C fluorescence were observed upon addition of the competing peptides, confirming the requirement for the DNA-bound peptide NBD-br-C to obtain these displacement curves. These experiments rule out possible artifactual effects due to displacement of peptide NBD-br-C bound to the BSA in the binding assay buffer, or to the quartz glass surfaces of the fluorescence cell (such binding *was* observed in buffers without added BSA), or direct effects of the peptides on the fluorescence of peptide NBD-br-C or its NBD label.

A comparison of the binding curves in Figure 6 shows that the helix-stabilized peptide br-C displaced DNA-bound peptide NBD-br-C at lower concentrations than did GCN4br, indicating that the affinity of peptide br-C for the CRE half-site is higher

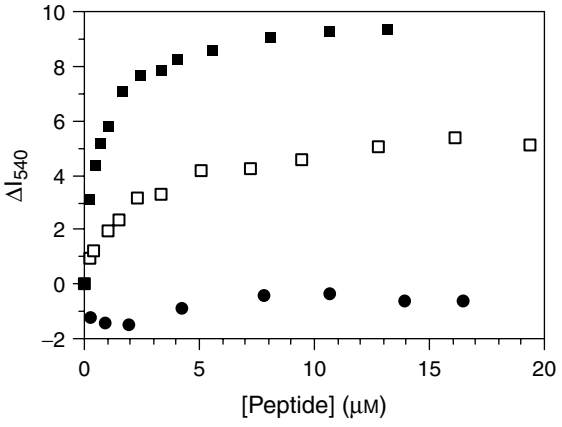


Figure 6 Competitive binding assays of peptide affinities for CRE-12 duplex DNA. Typical experiments are shown. Peptide NBD-br-C (0.10 μM) was incubated (a) in the presence of 0.15 μM CRE-12 and increasing concentrations of peptide br-C (filled squares), or (b) with increasing concentrations of GCN4br in the presence of 0.09 μM CRE-12 (open squares) or in the absence of any DNA (filled circles). The increase in fluorescence intensity at 540 nm (arbitrary units) is plotted as a function of the concentration of the competing peptides.

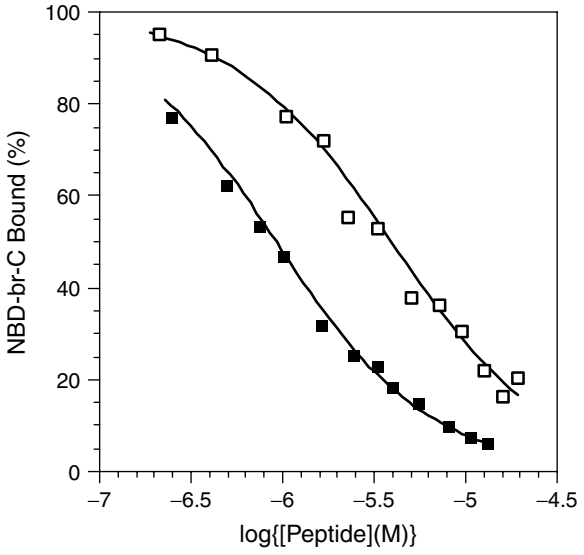


Figure 7 Curve-fitting analysis of the competitive binding assays of peptide affinities for CRE-12 duplex DNA. Analysis of the representative experiments from Figure 6 is shown. Solid lines indicate the fitted curves generated by Radlig analysis of the data from competition assays using increasing concentrations of peptide br-C (filled squares) or GCN4br (open squares). The fluorescence data from Figure 6 are presented as the % of the maximum NBD-br-C binding in the absence of competing peptide and plotted as a function of the competing ligand concentration (log scale).

than that of GCN4br. Curve fitting of the experimental data to a one-site model (Figure 7), using the experimentally determined dissociation constant for peptide NBD-br-C, gave dissociation constants of 0.65 ± 0.09 μM and 3.9 ± 0.5 μM for CRE-12 binding by peptide br-C and GCN4br, respectively (Table 1). The higher affinity of the helix-stabilized analogue, peptide br-C, is consistent with the principle used in designing this peptide as a ligand for the CRE site: lowering the unfavourable conformational entropy change associated with helix formation upon DNA binding [13,14] by prior stabilization of helix in the unbound peptide, will increase the affinity of GCN4br peptides for their recognition site in the major groove of the CRE half-site.

It is interesting to note that the free energy difference, $\Delta\Delta G$, corresponding to the differences between the K_D values for these two peptides (Table 1) is approximately 1.06 kcal/mol. We suggest that this $\Delta\Delta G$ value may be interpreted as a measure of the helix-stabilization achieved by substitution of the lactam-bridged amino acid sequence in peptide br-C for the native GCN4 sequence in GCN4br. The major groove recognition site in the GCN4br peptide sequence is centred around the Ala-Ala sequence corresponding to peptide br-C residues 13–14. Consideration of the crystal structure of the GCN4 bzip-CRE complex [9] indicates that the lactam bridge linking residues 25 and 29 is placed beyond this DNA-binding site and the surrounding ring of cationic residues that form salt bridges to the DNA backbone. This bridge is not, therefore, expected to alter peptide–DNA interactions directly. Previously, we have estimated a value of 0.4 kcal/mol per bridge for the helix stabilization due to substituting a lactam-bridge linkage of the Lys^{i}, Asp^{i+4} type for glutamine residues [35]. The higher value of 1.06 kcal/mol obtained here probably reflects the added helix-stabilizing effect of the Ala_3 sequence substituted for the native GCN4 sequence in the intervening bridged positions (residues 26–28), since alanine residues are also expected to be helix stabilizing relative to the native sequence [36,37].

There are two other reports of attempts at enhancing DNA-binding affinity in basic-region peptides through helix stabilization that we are aware of to date [23,38]. As in our study, these investigators have chosen to analyse the DNA binding of GCN4 basic-region peptides under physiological salt conditions, but helix stabilization was achieved through differing approaches. Lajmi *et al.* [38] chose to incorporate multiple alanine residues into the basic region of the bzip peptide in positions around the GCN4 residues that are essential for recognition. This produced dimerizing peptides that were stripped of their native basic residues. As a result, they obtained dimerized peptides that had enhanced helix contents of up to 71%. However, the loss of the salt bridging residues in the basic region lowered their affinities for a minimal AP-1 site duplex DNA (5′-TGACTCA-3′) significantly. The study of Zondlo and Schepartz [23] is more directly comparable to our own study, as it involved the design of helix-stabilized monomeric basic region peptides. These investigators stabilized the GCN4 recognition helix throughout its DNA recognition surface by incorporation of those residues from the GCN4 sequences that are essential for recognition and DNA-binding affinity into the α-helical segment of a PP-fold structural motif [41]. This resulted in enhancements in peptide binding affinity for CRE half-site DNA by a factor of 80–200 ($\Delta\Delta G = 2.4$–2.9 kcal/mol) in comparison to control peptides in which the helix stabilizing PP-fold was

lacking or disrupted. In comparison, the combination of *C*-terminal lactam-bridge and alanine substitutions that we have explored in peptide br-C has produced a more modest enhancement of DNA binding affinity. This suggests that the helix stabilization achieved in the GCN4 basic region through use of the PP fold was more extensive than that which we now report for peptide br-C, although additional direct peptide–DNA interactions may also have contributed to the stability of the PP-fold peptide–DNA complex. On the other hand, the *C*-terminal helix stabilization we have designed into peptide br-C is a better model for investigating the helix propagating role of the leucine-zipper dimer in native GCN4 and its potential role in stabilizing the GCN4–DNA complex. The correlation that we report between *C*-terminal helix stabilization in the isolated basic-region peptide from GCN4 with its affinity for the CRE half site indicates that helix propagation from the leucine zipper is, as predicted, a significant factor contributing to the affinity of native GCN4 for its cognate DNA. Furthermore, helix formation in the lactam-bridged *C*-terminal region of peptide br-C is likely to be less complete than that in the same region of the dimerized GCN4 bzip motif. Therefore, we can estimate that helix propagation from the leucine zipper may contribute significantly more than our $\Delta\Delta G$ value of 1 kcal/mol to the binding of each basic region in the GCN4 dimer to its recognition site in DNA. Recent studies characterizing the stability of GCN4 bzip dimer binding to half-site DNA support this conclusion [39].

CONCLUSIONS

We have demonstrated that GCN4br and its helix-stabilized analogue, peptide br-C, bind to duplex DNA in the micromolar concentration range, with a concomitant helix stabilization that is selective for duplex DNA containing the CRE recognition site or half-site for GCN4. Based on this initial result, we have successfully designed a GCN4br analogue, peptide NBD-br-C, that incorporates a fluorescent reporter group for DNA binding, and we have demonstrated the utility of this peptide as a reporter ligand in competitive binding assays by its use to determine the relative affinities of GCN4br and peptide br-C for the CRE half-site DNA, CRE-12. The results show that peptide affinity for the CRE half-site correlates with helix stabilization in the peptide ligand, as expected. Since the lactam-bridged region of peptide br-C should not interact directly with the DNA upon binding, the free energy difference in the stabilities of the complexes of GCN4br and peptide br-C with the CRE half site (1.06 kcal/mol) is considered to be a measure of the degree of helix stabilization achieved in peptide br-C relative to GCN4br. This free energy difference also provides a lower limit for the effect of helix initiation by the dimerized leucine zipper motif in GCN4 on the affinity of each basic region peptide for its CRE half-site. Competitive binding assays using the peptide NBD-br-C complex with CRE-12, should prove to be a useful system for quantitatively evaluating a wide variety of helix stabilizing modifications of peptides, or for investigating the energetic contributions of individual GCN4–DNA interactions. The design of similar assay systems for studies of other members of the bzip class of proteins also appears quite straightforward, following this model.

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