DNA bending by bHLH charge variants

Robert J. McDonald¹, Jason D. Kahn² and L. James Maher, III³,*

¹Medical Scientist Training Program, Mayo Clinic College of Medicine, Rochester, MN 55905, USA, ²Department of Chemistry and Biochemistry, University of Maryland, College Park, MD 20742-2021, USA and ³Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN 55905, USA

Received June 29, 2006; Revised July 13, 2006; Accepted July 14, 2006

ABSTRACT

We wish to understand the role of electrostatics in DNA stiffness and bending. The DNA charge collapse model suggests that mutual electrostatic repulsions between neighboring phosphates significantly contribute to DNA stiffness. According to this model, placement of fixed charges near the negatively charged DNA surface should induce bending through asymmetric reduction or enhancement of these inter-phosphate repulsive forces. We have reported previously that charged variants of the elongated basic-leucine zipper (bZIP) domain of Gcn4p bend DNA in a manner consistent with this charge collapse model. To extend this result to a more globular protein, we present an investigation of the dimeric basic-helix–loop–helix (bHLH) domain of Pho4p. The 62 amino acid bHLH domain has been modified to position charged amino acid residues near one face of the DNA double helix. As observed for bZIP charge variants, DNA bending toward appended cations (away from the protein:DNA interface) is observed. However, unlike bZIP proteins, DNA is not bent away from bHLH anionic charges. This finding can be explained by the structure of the more globular bHLH domain which, in contrast to bZIP proteins, makes extensive DNA contacts along the binding face.

INTRODUCTION

The DNA double helix is a relatively stiff biopolymer with a persistence length of ~150 bp. Over this length the average deflection of the helix axis is one radian under physiological salt conditions and in the absence of proteins (1–3). In contrast, very long DNA molecules spontaneously collapse to conformations whose end-to-end distances are proportional to the square root of the contour length (4). These characteristics imply that DNA is locally stiff yet globally flexible. Fundamental cellular processes such as nucleosome-mediated compaction (5), assembly and regulation of transcriptional machinery (6–8), and genetic recombination (9,10) require energetically unfavorable DNA deformation. Protein binding must provide the energetic driving force required for sharp DNA bending. We are interested in how asymmetric electrostatic interactions between proteins and DNA contribute to the generation of DNA-bending forces (3,11–16). Protein-mediated DNA bending is a crucial element in many essential biological processes, yet the contributions of electrostatic effects remain poorly understood.

Previous results from our laboratory support the idea that simple DNA-binding proteins can be engineered to induce DNA bending in opposite directions by positioning positive or negative charges asymmetrically on one face of the DNA duplex (13). Considerable efforts are still focused on understanding how neighboring cations and the phosphate diester backbone affect the stiffness of DNA (17–23). These findings support the proposals of Rich, Manning and others who theorized that charge–charge repulsions between neighboring phosphate diesters are at least partially responsible for the intrinsic rigidity of DNA (24–26).

The basic-leucine zipper (bZIP) domain of the Saccharomyces cerevisiae transcription factor Gcn4p (Figure 1A) has been studied extensively as a biophysical model because of its simple secondary structure and orthology to the mammalian oncogenes Fos and Jun (27–33). The bZIP domain of Gcn4p is a 57 amino acid peptide sufficient for homo-dimerization and sequence-specific DNA binding (34). In the absence of DNA, the basic region of free bZIP peptides remains relatively disordered (35,36), whereas at high-protein concentration the leucine zipper region is natively folded into an alpha-helical coiled-coil. DNA binding potentiates folding, and crystal structures suggest that the basic region adopts a continuous alpha-helical structure when bound to DNA (34,37). Previous efforts in our laboratory have shown that the bZIP domain is adaptable as a scaffold for charge variations, while preserving the ability of the modified bZIP peptides to bind DNA (13,15,16).

Electrophoretic assays have been widely used to detect DNA bending because mobility in native gels is related to DNA shape (38–40). Our own electrophoretic studies using DNA phasing probes were influenced by the work
of Schepartz (30,41) and Kerppola (29,33,42) and have suggested a role for electrostatics in DNA bending. For example, the bZIP domain of transcription factor Gcn4p was used as a scaffold to position charged amino acids near one face of the DNA duplex to explore if local charges induce DNA bending (Figure 1A). When bound to DNA, charged residues on Gcn4p have the potential to create laterally asymmetric charge distributions that appear to modulate inter-phosphate repulsions in a manner consistent with predictions (3,11,24,25,43,44). Specifically, DNA bends toward cationic charges that asymmetrically reduce phosphate repulsions, and away from anionic charges that enhance these repulsions.

DNA bending interpretations in electrophoretic phasing assays involving bZIP proteins can be controversial due to concerns that observed mobility changes could reflect changes in the shape, flexibility and/or gel-matrix interactions of the elongated peptide structure (28,33,45,46). In particular, DNA cyclization kinetics experiments have suggested that certain bZIP proteins do not bend DNA (46,47). Our previous work on the effect of appending globular domains onto bZIP peptides showed that apparent charge-dependent DNA bending was preserved in these more symmetrical complexes but was not detected by minicircle binding experiments (15,16). We reconciled the results by proposing that the bending potential for bZIP–DNA complexes is quite flat, so that different average geometries are observed with different methods (15). In the present work, we study a relatively globular protein, the basic helix–loop–helix (bHLH) domain of yeast Pho4p (Figure 1B and C), as an alternative scaffold for positioning charges asymmetrically near DNA. Charge variants of Pho4p provide an independent opportunity for exploration of DNA bending by laterally asymmetric charge interactions and of protein–DNA complex flexibility.

Pho4p, a 312 amino acid transcription factor in S.cerevisae, belongs to the PHO gene family, which encodes >30 proteins responsible for regulation of exogenous inorganic phosphate uptake (48,49). At high exogenous phosphate, Pho4p is rendered inactive through phosphorylation by CDK complex orthologs, Pho80p–Pho85p (50). In phosphate-poor media Pho4p is hypophosphorylated and imported into the nucleus, where it acts together with Pho2p to activate genes required for phosphate scavenging (51) by specific recognition of the E-box sequence, 5'-CACGTG (Figure 1D) (52,53). The C-terminal bHLH domain of Pho4p, the domain responsible for both selective DNA binding and homodimerization, includes a basic residue-rich region followed by a HLH fold (54–57). The X-ray co-crystal structure of the 62 amino acid Pho4p bHLH bound to DNA has been solved at 2.8 Å (58). The resulting model (Figure 1B) shows that the loops of the bHLH dimers play a critical role in conferring stability and specificity to Pho4p DNA binding through extensive protein-phosphodiester backbone contacts.

Here we show that, as seen for the bZIP domain of Gcn4p, charge variants of the bHLH domain of Pho4p appear to induce DNA bending toward appended cations. Unlike bZIP peptides, anionic charge variants of the bHLH peptide do not cause DNA bending in the opposite direction. We propose that DNA bending toward the bound protein is prevented by DNA contacts made by the loop region of the bHLH dimer.
MATERIALS AND METHODS

Plasmids

Pho4p expression plasmid pJ1080, derived from a pET21d (Novagen), encodes the 62 amino acid bHLH domain of Pho4p and was the gift of D. Wemmer (59). Site-directed mutagenesis using standard methods was used to generate plasmids encoding the bHLH\textit{SHORT} Pho4p charge variants Pho4p and was the gift of D. Wemmer (59). Site-directed (Novagen), encodes the 62 amino acid bHLH domain of

pellets were frozen at

4°C before centrifugation (5000 g, 4 5 min, 4°C) and further purified on a gravity flow SP sepharose column (Pharmacia Biotech, Sweden). After loading, the cationic exchange column was washed with 40 ml steps of lysis buffer supplemented with increasing salt concentrations (0.5, 1.0, 1.5 and 2.0 M NaCl). Eluted fractions containing the bHLH peptides (1.0 M NaCl for bHLH\textit{SHORT} and 1.5 M NaCl for bHLH\textit{LONG}) were concentrated using 5000 molecular weight cutoff centrifugal concentrators (VivaSpin 20; VivaScience) and then purified by HPLC [C18 preparative reverse phase column (250 21.2 mm), Beckman 127P System Gold, buffer A: 0.1% trifluoroacetic acid (TFA), buffer B: 80% CH3CN, 0.1% TFA, gradient 10–70% buffer B over 50 min]. Peptide purity was assessed by ESI GC-MS to be >95%. Purified peptides were lyophilized for storage.

bHLH-DNA-binding affinity studies

DNA probes for binding affinity assays containing either an E-box (5′-CGGCGGACGTCGCG; E-box underlined) or CRE (5′-CGATGCATGACGCTACG; CRE underlined) sequence were prepared by annealing a single-complementary oligonucleotide to generate duplexes with 3′ recessed termini. The annealed probe (400 pmole) was end-labeled using 20 U of the Klenow fragment of \textit{E.coli} DNA polymerase I and 10 μCi each of [\textit{α}-32P]dGTP and dCTP (3000 Ci/mmol) in 70 mM Tris–HCl, pH 7.6, 10 mM MgCl2 and 5 mM DTT for 45 min at 37°C, followed by incubation with 5 mM each of dGTP and dCTP for 15 min to polymerize all termini. Radiolabeled probes were purified from unincorporated nucleotides using spin columns (Chromaspin TE-10; Clontech) and diluted for binding affinity assays. bHLH samples were dissolved in water and concentrations were determined by the method of Pace et al. (60) followed by dilution into binding buffer [1× phosphate-buffered saline containing 5% glycerol and 0.025% NP-40]. Binding reactions were performed by incubation of peptide dilutions (10 pM to 1 μM) with annealed radiolabeled DNA probes (100 PM) in a total volume of 20 μl of binding buffer supplemented with 100 μg/ml BSA. Binding reactions were incubated on ice for 30 min before analysis on native polyacrylamide gels (12% acrylamide; 29:1 acrylamide/bisacrylamide) in 0.5× TBE buffer run for 750 V-h at 22°C. Dried gels were analyzed using a STORM 840 scanner (Amersham). Equilibrium dissociation constant (\textit{Kd}) values were estimated by measuring the fractional occupancy (\textit{θ}) of the radiolabeled duplex [\textit{D}] as a function of the bHLH concentration [\textit{P}]. The \textit{Kd} was estimated with nonlinear least squares fitting to Equation 1, using Kaleidagraph™ software (Synergy Software).

\[
\theta = \frac{(D_{\text{total}} + K_d + [P]_{\text{total}}) - \sqrt{(D_{\text{total}} + K_d + [P]_{\text{total}})^2 - 4(D_{\text{total}}[P]_{\text{total}})}}{2D_{\text{total}}}.
\]

Circular dichroism spectroscopy

CD spectra were recorded using a Jasco 810 spectropolarimeter. Far UV-CD spectra were obtained in the continuous mode, collecting measurements every 1 nm with an averaging time of 5 s at 295 K between 200–260 nm. bHLH samples were 10 μM in 0.2 cm path length cells. DNA probes containing the E-box sequence (underlined) were generated by annealing the single self-complementary oligonucleotide (5′-CGCGCGCACGTGCGCGCGCG) at 500 μM. Spectra were acquired in binding buffer with either 10 μM free bHLH peptides or bHLH complexes with E-box DNA at a molar ratio of 1:1.1. All baseline buffer and nucleic acid contributions to ellipticity were subtracted after spectral collection. Mean residual ellipticity (\textit{θ}_{MRE}, deg cm²/dmol) was calculated using the relation:

\[
\textit{θ}_{MRE} = \frac{\textit{θ}_{mdeg}}{10 \times lcn^2},
\]

where \textit{θ}_{mdeg} is the ellipticity in mdeg, \textit{l} is the path length in cm, \textit{c} is the molar peptide concentration and \textit{n} is the...
number of residues in the peptide. Alpha-helical estimates of bHLH peptides were based upon the previously published equation (61):

$$\%\text{Helix} = \left(\frac{\Theta_{\text{MRE}} - \Theta_0}{\Theta_{100}}\right) \times 100,$$

where $\Theta_{\text{MRE}}$ is the mean residual ellipticity at 222 nm. For $\Theta_0$ and $\Theta_{100}$ representing 0 and 100% helicity, values of $-2340$ and $-30300$ (deg cm$^2$/dmol) were used.

**Electrophoretic phasing assays of DNA bending**

DNA probes for phasing analyses were generated and radiolabeled by PCR from derivatives of plasmids pDP-AP-1-21, pDP-AP-1-23, pDP-AP-1-25, pDP-AP-1-26, pDP-AP-1-28 and pDP-AP-1-30 (30) that had been modified through site-directed mutagenesis of the original AP-1 binding site to generate E-box-binding sites suitable for bHLH binding. DNA probes were incubated with sufficient peptide for $\sim$50% mobility shift in 20 µl reactions containing binding buffer and 100 µg/ml BSA. Reactions were incubated on ice for 30 min before native PAGE (6% acrylamide; 29:1 m/m mobility of each set of DNA probes according to:

$$\text{migration distance for free and bound DNA probes were incubated with sufficient peptide for } \sim 50\% \text{ mobility shift in 20 } \mu \text{l reactions containing binding buffer and 100 } \mu \text{g/ml BSA. Reactions were incubated on ice for 30 min before native PAGE (6\% acrylamide; 29:1 m/m mobility of each set of DNA probes according to:}$$

Values of $\mu_{\text{rel}}$ were plotted against the spacer distance (bp spacing between the center of the E-box site and the center of the proximal A$_5$-tract) for fitting to the phasing function:

$$\mu_{\text{rel}} = \frac{\mu}{\mu_{\text{avg}}},$$

where $\mu_{\text{rel}}$ is the normalized mobility, $\mu$ is the measured mobility of each probe and $\mu_{\text{avg}}$ is the average mobility of a group of phasing probes sharing a common bound peptide.

Values of $\mu_{\text{rel}}$ were plotted against the spacer distance (bp spacing between the center of the E-box site and the center of the proximal A$_5$-tract) for fitting to the phasing function:

$$\mu_{\text{rel}} = \left(\frac{A_{\text{PH}}}{2}\right)\cos[2\pi(S - S_T)/P_{\text{PH}}] + 1,$$

where $\mu_{\text{rel}}$ is the normalized mobility of the species, $S$ is the spacer length (bp), $P_{\text{PH}}$ is the phasing period (10.5 bp/turn), $S_T$ is the trans spacer length (predicted length in bp where peptide-induced and A$_5$-tract bends are in trans), and $A_{\text{PH}}$ the amplitude of the phasing function. The value of $A_{\text{PH}}$ was approximated by least squares curve fitting using KaleidaGraph. The bend angle, $\alpha_B$, was then estimated from the empirical relation:

$$\tan(k\alpha_B/2) = \left(\frac{A_{\text{PH}}}{2}\right)/[\tan(k\alpha_C/2)],$$

where $\alpha_C$ is the reference bend angle of the phased array of A$_5$ tracts (54°), and $k$ is an empirically determined constant (~1.09 in this case).

**DNA bending energy predictions**

The $\Delta\Delta G^0$ contributions to the overall process of protein–DNA complex formation, defined as $\Delta G^0(\text{variant}) - \Delta G^0(\text{SAA})$, were estimated from experimental data or else were proposed as reasonable guesses that rationalize experimental observations. The individual $\Delta\Delta G^0$ contributions that come from the experiment were estimated as follows:

For protein folding, the folding reaction is considered to be two-state, with the equilibrium constant calculated as follows:

$$K_{\text{fold}} = \frac{[\text{Native}]}{[\text{Unfolded}]} = \frac{f_{\text{native}}}{1 - f_{\text{native}}} = \frac{(\% \text{ helix/42\%})}{1 - (\% \text{ helix/42\%})},$$

where $f_{\text{native}}$ is the fraction of folded protein. The relative free energy for folding is calculated from the ratio of equilibrium constants:

$$\Delta\Delta G^0 = -RT \ln \left(\frac{K_{\text{fold}}(\text{variant})}{K_{\text{fold}}(\text{SAA})}\right).$$

The free energy cost of DNA bending is calculated as described previously (64). The equation assumes a persistence length of 150 bp:

$$\Delta\Delta G^0_{\text{bend}}(\text{cal/mol}) = 14\frac{\Theta^2}{L},$$

where the bend angle $\Theta$, in degrees, is evenly distributed over $L$ base pairs. For example, the free energy cost of 600 cal/mol shown in Figure 5 corresponds to a bend of 24.5° over 14 bp.

The total of all the $\Delta\Delta G^0$ contributions associated with protein–DNA complex formation is given by the observed dissociation constants:

$$\Delta\Delta G^0_{\text{total}} = RT \ln \left(\frac{K_{\text{dis}}(\text{variant})}{K_{\text{dis}}(\text{SAA})}\right).$$

**RESULTS AND DISCUSSION**

**Experimental design**

The native bHLH domain of Pho4p (Figure 1B and C), representing residues 248–312 from the wild-type sequence (59), was modified to laterally position asymmetric charges near one face of the DNA. bHLH$_{\text{SHORT}}$ peptides were generated by insertion of three additional residues (XXX, Figure 1B and C) N-terminal to the basic domain to position charge modifications near the phosphodiester backbone of DNA. This strategy placed charged residues at positions similar to those studied previously using the bZIP domain of Gcn4p (Figure 1A and B) (13). Comparison of the crystal structures shows that the bHLH peptide, despite its longer primary sequence, folds into a more globular structure (Figure 1A and B). The bHLH$_{\text{LONG}}$ peptide group, while positioning charges in a manner identical to bHLH$_{\text{SHORT}}$, was predicted from studies of bZIP peptides (unpublished data) to stabilize folding by addition of 12 N-terminal residues (Figure 1C). The sequences of the CRE and E-box binding sites for Gcn4p and Pho4p, respectively, are shown in Figure 1D.

**Binding affinities of bHLH charge variants**

Before assessing their abilities to bend DNA, the DNA-binding affinities of Pho4p bHLH charge variants were determined by protein titrations against a constant concentration of an 18 bp DNA duplex containing the E-box-binding site (Figure 1D), and were assayed by electrophoretic mobility
shift (Figure 2A). The fits shown in Figure 2B (bHLHSHORT) and 2C (bHLHLONG) were used to estimate apparent dissociation constants ($K_d$) for each of three charge variants for both peptide lengths (Table 1).

The data for both short and long bHLH peptides show that the neutral charge variant has the highest affinity for the E-box DNA, followed by the anionic form (Table 1). For both peptides, the cationic bHLH displayed 3- to 4-fold lower affinities for DNA (Table 1). The weaker binding affinities of cationic (KKK) charge variants were initially puzzling, as positive charges near the DNA surface might be expected to promote protein–DNA interactions. It is noteworthy that the appendage of the 12 N-terminal residues in bHLHLONG peptides reduces the differences in DNA binding affinities among the charge variants (Figure 2B and C and Table 1). Control experiments verified that all peptides retained proper DNA sequence specificity in binding assays (data not shown).

### Folding of free and DNA-bound bHLH charge variants

To test if the observed binding affinity changes were due to changes in the coupling between protein folding and DNA binding, we characterized the folding of bHLH charge variants using circular dichroism (CD) spectroscopy (65). Changes in protein conformation induced upon DNA binding can be detected as spectral changes in the far UV CD region between 210 and 230 nm, a region largely unaffected by DNA, but useful in observing protein secondary structure. Under conditions comparable to those used in binding studies, far-UV CD data were collected for both free peptide and DNA-peptide complexes to determine the structural effects of charge insertion and DNA binding.

The far-UV CD spectra of the free bHLH peptides (10 μM) are shown in Figure 3. Based on the molar ellipticity values at...

| $K_d$ (nM)$^a$ | SAA | EEE | KKK |
|----------------|-----|-----|-----|
| bHLHSHORT      | 3 ± 2 | 15 ± 2 | 67 ± 7 |
| bHLHLONG       | 10 ± 2 | 19 ± 2 | 55 ± 6 |

$^a$ $K_d$ values were estimated through curve fitting to Equation 1 as described in Materials and Methods. Mean and standard deviations were obtained from at least three independent measurements.
222 nm (Q222) and published interpretations (66), the helical content of unbound bHLHSHORT charge variants was calculated to be 34, 40 and 16% for SAA, EEE and KKK, respectively. Helical content values of 32, 37 and 27% were observed for the bHLHLONG peptide charge variants SAA, EEE and KKK, respectively (Table 2). The results for the neutral charge variant (SAA) are consistent with the expected helical content of the peptides (free bHLHSHORT: 43%; free bHLHLONG: 37%) based upon the number of bHLH residues expected to be helical when not bound to DNA. For free bHLHSHORT peptides, insertion of cationic residues (KKK) destabilizes folding whereas addition of anionic residues (EEE) stabilizes folding (Figure 3A and Table 2). These effects are significantly reduced in bHLHLONG peptides, demonstrating the strong stabilizing effect the 12 amino acid leader segment. We have observed similar effects for corresponding charge variants of the bZIP domain of Gcn4p (data not shown). The ratio of molar ellipticity at 205 nm to the ellipticity at 222 nm, diagnostic for alpha helix content, confirms that unbound bHLH charge variants remain partially unfolded. Partial unfolding is consistent with a previous report that noted a stabilizing effect of buffer cation composition (66).

Upon addition of 1.1 molar equivalents of cognate E-box DNA to the bHLH charge variant peptides, alpha-helical content was increased in all cases (Figure 3 and Table 2). For the bHLHSHORT peptides, comparison of the results for the cationic variant (KKK) to the neutral (SAA) and anionic (EEE) variants suggests that the cationic variant remains incompletely alpha-helical, despite a clear conformational change upon DNA binding (Figure 3A and Table 2). In contrast, bHLHLONG peptides displayed similar folding enhancements in the presence of DNA (Figure 3B and Table 2). In all cases, the random-coil minimum at 207 nm shifted (by 5 nm) to 212 nm and was less prominent relative to the alpha-helical signature with a Q212:Q222 ratio of ~0.9 (66). This change is consistent with an increase in alpha-helical structure and a reduction in the random-coil folded state. The calculated helical content of the neutral bHLH:DNA complex is again consistent with predicted results in both long and short bHLH peptides. These findings confirm reports of far-UV CD spectral changes upon bHLH binding to DNA (66).

Our data highlight the importance of electrostatic effects in the folding of the bHLH domain. Placement of additional negative charges near the peptide N-terminus enhances the intrinsic helical content of the bHLH fold whereas additional positive charge reduces helical content. These results are consistent with the expected electrostatic interactions between charged residues near the terminus of an alpha helix and the intrinsic helix macrodipole (67,68), which are favorable for negatively charged residues near the N-terminus and unfavorable for positively charged residues. Our CD results show that cationic bHLH charge variant samples the unfolded state much more often than the other variants, explaining the ~20-fold decrease in binding affinity for the E-box DNA in the case of the bHLHSHORT cationic variant. The reduction in the charge-dependent variation of folding stabilities of the bHLHLONG peptide group further supports this notion, as the inserted charges are placed further from the amino terminus, and so the electrostatic–macrodipole interaction is greatly reduced leading to more uniform folding stabilities and presumably binding affinities. The reduction in binding affinity seen for the SAA variant and the lack of the expected increase in affinity upon improved folding of the KKK variant may be due to steric interference between the extended helix and the DNA or to loss of contacts formed previously by less structured DNA tails.

DNA bending by Pho4p charge variants

We employed a well-established electrophoretic DNA phasing assay that permits quantitative estimation of both the magnitude and direction of DNA bending. Our goal was to determine how asymmetric charge interactions at the Pho4p bHLH binding site (E-box) can lead to changes in observed DNA bending. Figure 4A illustrates the phasing probe design based on constructs originally developed by Schepartz (30) and Crothers (40). These ~300 bp DNA duplexes contain a
Table 2. CD and derived alpha helical content for free and DNA-bound bHLH peptides

|            | SAA | EEE | KKK | SAA:DNA | EEE:DNA | KKK:DNA |
|------------|-----|-----|-----|---------|---------|---------|
| bHLH_SHORT MRE\(^a\) | -12,050 | -14,400 | -6350 | -21,100 | -25,200 | -11,950 |
| bHLH_SHORT helicity (%)\(^b\) | 34  | 40  | 16  | 62  | 76  | 33  |
| bHLH_LONG MRE\(^a\) | -12,000 | -13,800 | -10,500 | -21,800 | -24,000 | -20,800 |
| bHLH_LONG helicity (%)\(^b\) | 32  | 37  | 27  | 64  | 71  | 61  |

\(^a\)Mean Residual Ellipticity (MRE) is calculated as described in Materials and Methods (Equation 2).

\(^b\)Helical content, expressed as a fraction of the total number of residues, was determined as described in Materials and Methods (Equation 3). In the case of this bHLH_SHORT peptide, the maximum helical content of the free peptide (Helix 1 + Helix 2 + loop helix) is predicted to be 28 of 65 residues (~43%). In the case of bHLH_LONG peptide, the maximum helical content of the free peptide (Helix 1 + Helix 2 + loop helix) is predicted to be 28 of 75 residues (~37%). The maximum helical content of the bHLH:DNA peptide (Helix 1 + Helix 2 + loop helix + basic region) is predicted to be 43–47 of 65 residues (~66–72%) for bHLH_SHORT and 48–52 of 75 residues (~64–69%).

central binding site for Pho4p (E-box) variably spaced from an array of three phased A5-tracts that serves as a curved reference. This spacer distance is varied in the five phasing probes by a total of 10 bp (in 2–3 bp increments) in order to generate unique helical phasings between the phased A5 tract and the E-box. DNA probes in which the phased A5 tract and protein-induced bend occur on the same helical face (cis) will migrate more slowly due to a large composite helical deflection, whereas DNA probes in which the deformations lie on opposite helical faces (trans) will migrate more rapidly as the two deformations tend to cancel.

Results of electrophoretic phasing experiments for bHLH peptides are shown in Figure 4B and C. Probe migration rates were normalized, fit to a phasing function and plotted against spacer length (Figure 4D). The calculated bend angles (Table 3) show that the free E-box site has little intrinsic bending (estimated bend angle of 1°–2° toward the major groove). bHLH binding bends the E-box site slightly, with
Table 3. E-box bending using bHLH charge variants

| E-box   | SAA:DNA | EEE:DNA | KKK:DNA |
|---------|---------|---------|---------|
| bHLH<sub>SHORT</sub> bend angle<sup>1</sup> | 1.2 ± 0.8° | 5.6 ± 1.1° | 4.1 ± 1.1° | 28.7 ± 3.7° |
| bHLH<sub>LONG</sub> bend angle<sup>1</sup> | 1.5 ± 0.6° | 4.2 ± 0.7° | 2.6 ± 1.5° | 25.5 ± 2.8° |
| Bend direction<sup>2</sup> | Major | Minor | Minor | Minor |

<sup>1</sup>Bend angle estimates are generated from Equations 2–4 as described in Materials and Methods. Mean and standard deviations were obtained from three independent measurements.

<sup>2</sup>Bend direction from Equations 4–6 refers to the DNA groove that is narrowed by bending, relative to a reference frame at the center of the E-box sequence.

The neutral SAA:DNA complex showing a DNA bend toward the minor groove of 4°–5° (a 5°–7° difference from the free E-Box DNA; Table 3). These findings are consistent with previous crystallographic studies that show very little DNA bending in the bHLH:DNA complex (58). In contrast to the neutral variants, the cationic charge variants (KKK) showed a strong apparent bend, estimated at 26°–29°, toward the minor groove at the center of the E-box. This amounts to ∼24° of net bending relative to the SAA:DNA complex. These data are consistent with previous reports for cationic bZIP charge variants based on electrophoretic phasing assays (13, 15, 63). Both the anionic bHLH charge variants (EEE) caused little DNA bending, yielding minor-groove directed bends of 3°–4° (Table 3). This result contrasts with previous observations for bZIP charge variants, where approximately equal but opposite DNA bending (anionic: major groove; cationic: minor groove) was observed for opposite charges.

Why does the bHLH scaffold permit DNA bending in only one direction? Since the DNA-binding affinities and specificities of the neutral (SAA) and anionic (EEE) bHLH variants are comparable, the lack of DNA bending by bHLH (EEE) is presumably not due to misfolding or loss of DNA-binding activity. Inspection of the crystal structures of both bZIP and bHLH suggests that the inability of the anionic charge variant of bHLH to bend DNA toward the major groove is explained by the bulky loop residues of the bHLH, which are known to be in contact with DNA and involved in enhancing DNA binding and specificity (58). The Pho4p bHLH loop directly contacts DNA on the major groove face of the E-box. Unlike the case for bZIP peptides, this presents a considerable steric obstacle to DNA bending in that direction.

To explicate the linkages among charged bHLH peptide folding, DNA bending and changes in protein–DNA contacts, we sketch a plausible free energy balance model in Figure 5. Figure 5A compares DNA bending by bHLH charge variants (top) and bZIP charge variants (bottom), illustrating the steric hindrance that prevents DNA from binding toward the bHLH protein. Figure 5B rationalizes the bending, folding and binding affinity results coherently in terms of ∆ΔG° contributions (relative free energies for protein–DNA complex formation) in the EEE and KKK complexes relative to the SAA complex. The total of all the ∆ΔG° contributions relative to the SAA state must equal the observed ∆ΔG° for bending, which is about +1.8 kcal/mol for bHLH<sub>SHORT</sub> and +1.0 kcal/mol for bHLH<sub>LONG</sub>. The description below uses measured or calculated ∆ΔG° contributions where possible, as described in Materials and Methods. Otherwise, the values are chosen to be reasonable and to rationalize experimental observations. The point of the analysis is not to claim that these values are correct but simply to list some of the contributions that must be considered and to show that small free energy changes can rationalize DNA bending by the KKK variant, lack of binding by the EEE variant, and the experimentally determined ∆ΔG° for bHLH:DNA complex formation.

The elements of the model are as follows:

(i) The KKK variant disrupts folding, which decreases the available binding free energy relative to SAA because more of the binding free energy must be coupled to folding and/or because contacts made by the N-terminal tail are lost. This free energy cost is larger for the bHLH<sub>SHORT</sub> variant because it does not fold as well. The free energy cost for folding can be estimated from the CD data by assuming that the EEE peptide is 95% folded and calculating the equilibrium constants for folding. The free energy cost shown for disrupting Helix 1 contacts is not known from experiment.

(ii) The free energy needed to bend DNA by ∼25° over ∼15 bp is small, only ∼0.6 kcal/mol [assuming a persistence length of 150 bp; (7)], and the bending is paid for by improved contacts with the N-terminal positive charges, again chosen arbitrarily to rationalize the existence of bending while being consistent with measured energetics.

(iii) The improved Helix 1 contacts in the KKK variants cause some distortion of contacts on the loop side, which costs binding free energy. The net effect is that as long as the sum of DNA bending energy and the loss of loop contacts is less than the free energy gained from electrostatic contacts, then the bent state will be a free energy minimum.

(iv) For the EEE variants, the small negative ∆ΔG° expected from enhanced protein folding is opposed by unfavorable steric contacts made by the N-terminal helix and/or unfavorable EEE–DNA electrostatic interaction, leading to a slight decrease in binding affinity that is again smaller for the bHLH<sub>LONG</sub> variants. As discussed above, DNA bending is not observed because the free energy cost of compressing the protein–DNA interface is greater than the free energy gain from decreasing electrostatic repulsion by bending away from Helix 1.

Attempts to test some of these hypotheses directly using bHLH variants with either extensions of helix 1 or substituting residues with less bulky side-chains in the loop region were unsuccessful because these peptides failed to bind DNA (data not shown). These results are consistent with the model in Figure 5, although not conclusive. The essential result is that it is understandable how DNA bending can occur only with the KKK charge variant, even though the KKK peptide binds DNA less tightly than the SAA and EEE peptides: Contacts between the loop region and the DNA are disrupted by the apparent bending away from the KKK variants, and the disruption is reflected in the decreased binding affinity of the KKK variants. Electrostatic repulsion or structural changes destabilize the binding of EEE variants but cannot induce bending.
Summary

The work presented here supports the interpretations of previous experiments suggesting that electrostatic effects can cause DNA bending. Gel-based DNA bending assays have been compared previously to other DNA bending assays such as DNA cyclization and minicircle binding, with variable results (15,45,46). There has been concern that discrepancies might be explained by the elongated shape of bZIP peptides bound to DNA. The use of the more globular bHLH domain of Pho4p complements our previous elongated bZIP phasing assay results as it demonstrates that electrostatic effects in DNA bending are observed in the context of a less elongated, more globular peptide complex. We also observed electrostatic effects on DNA bending when we previously appended globular domains to the N-termini of bZIP peptides (15). Taken together, these results argue against the concern that electrophoretic phasing assay results are dominated by peptide shape. The difference in bending behavior between bHLH and bZIP charge variants likely results from a difference in how peptides pack against DNA, as the simple helical structure and lack of bulky domains on the bZIP peptide permits DNA bending in both directions, whereas the larger bHLH domain only permits bending away from the protein.

Our findings support the electrostatic collapse model of DNA bending and are consistent with (but do not prove) the somewhat controversial notion that DNA phosphate diester charges contribute significantly to DNA stiffness (3,11,25,26,44,69,70). Complementary approaches to these gel-based assays are currently under investigation.

ACKNOWLEDGEMENTS

We thank D. Wemmer for the Pho4p bHLH expression plasmid, J. Cave for the BL21 expression strain, A. Schepartz for the original pDP-AP1 phasing plasmids, M. Ramirez-Alvarado for instrument access and useful discussion, and G. Mer for technical discussion. The technical support of J. Zimmerman is acknowledged. We thank N. Becker, K. Riley and E. Smith for comments on the manuscript. This work was supported by the Mayo Foundation and NIH grants GM53620 (J.D.K.) and GM054411 (L.J.M). Funding to pay the Open Access publication charges for this article was provided by the Mayo Foundation.

Conflict of interest statement. None declared.

REFERENCES

1. Hagerman,P.J. (1988) Flexibility of DNA. Annu. Rev. Biophys. Chem., 17, 265–286.
2. Maher,L.J., III (1998) Mechanisms of DNA bending. Curr. Opin. Chem. Biol., 2, 688–694.
3. Williams,L.D. and Maher,L.J., III (2000) Electrostatic mechanisms of DNA deformation. Annu. Rev. Biophys. Biomol. Struct., 29, 497–521.
4. Cantor,C. and Schimmel,P. (1980) Biophysical Chemistry Part III: The Behavior of Biological Molecules. W.H. Freeman and Company, NY.
5. Lugr,K., Mader,A.W., Richmond,R.K., Sargent,D.F. and Richmond,T.J. (1997) Crystal structure of the nucleosome core particle at 2.8 A resolution. Nature, 389, 251–260.
6. Schultz,S.C., Shields,G.C. and Steitz,T.A. (1991) Crystal structure of a CAP–DNA complex: the DNA is bent by 90 degrees. Science, 253, 1001–1007.
7. Kahn,J.D. and Crothers,D.M. (1993) DNA bending in transcription initiation. Cold Spring Harb. Symp. Quant. Biol., 58, 115–122.
8. Parkinson,G., Wilson,C., Gunasekera,A., Ebright,Y.W., Ebright,R.E. and Berman,H.M. (1996) Structure of the CAP-DNA complex at 2.5 angstroms resolution: a complete picture of the protein–DNA interface. *J. Mol. Biol.*, 260, 395–408.

9. Goodman,S.D. and Nash,H.A. (1989) Functional replacement of a protein-induced bend in a DNA recombination site. *Nature*, 341, 251–254.

10. Paul,T.T., Haykinson,M.J. and Johnson,R.C. (1993) The nonspecific DNA-binding and -bending proteins HMG1 and HMG2 promote the assembly of complex nucleoprotein structures. *Genes Dev.*, 7, 1521–1534.

11. Strauss,J.K. and Maher,L.J., III (1994) DNA bending by asymmetric phosphate neutralization. *Science*, 266, 1829–1834.

12. Strauss,J.K., Prakash,T.P., Roberts,C., Switzer,C. and Maher,L.J. (1996) DNA bending by a phantom protein. *Chem. Biol.*, 3, 671–678.

13. Strauss-Soukop,J.K. and Maher,L.J., III (1998) Electrostatic effects in DNA bending by GCN4 mutants. *Biochemistry*, 37, 1060–1066.

14. Tomky,L.A., Strauss-Soukop,J.K. and Maher,L.J., III (1998) Effects of asymmetric neutralization of phosphate groups. *Biochemistry*, 37, 8277–8288.

15. Hardwidge,P.R., Kahn,J.D. and Maher,L.J., III (2002) Dominant effect of protein charge rather than protein shape in apparent DNA bending by engineered bZIP domains. *Biochemistry*, 41, 8777–8782.

16. Hardwidge,P.R., Wu,J., Williams,S.L., Parkhurst,K.M., Parkhurst,L.J. and Maher,L.J., III (2002) DNA bending by bZIP charge variants: a unified study using electrophoretic phasing and fluorescence resonance energy transfer. *Biochemistry*, 41, 7732–7742.

17. Soto,A.M., Kankia,B.I., Dande,P., Gold,B. and Marky,L.A. (2002) Incorporation of a cationic aminopropyl chain in DNA hairpins: thermodynamics and hydration. *Nucleic Acids Res.*, 29, 3638–3645.

18. Gold,B. (2002) Effect of cationic charge localization on DNA structure. *Biopolymers*, 65, 173–179.

19. Kosikov,K.M., Gorin,A.A., Lu,X.J., Olson,W.K. and Manning,G.S. (2003) Is a small number of charge neutralizations sufficient to bend nucleosome core DNA onto its superhelical ramp? *Proc. Natl Acad. Sci. USA*, 100, 1763–1767.

20. Koo,H.S., Drak,J., Rice,J.A. and Crothers,D.M. (1990) Determination of the extent of DNA bending by an adenine-thymine tract. *Biochemistry*, 29, 4227–4234.

21. Paolella,D.N., Liu,Y., Fabian,M.A. and Schepartz,A. (1997) Electrostatic mechanism for DNA bending by bZIP proteins. *Biochemistry*, 36, 10033–10038.

22. Kerppola,T.K. and Curran,T. (1995) The transcription activation domain of Fos and Jun induce DNA bending through electrostatic interactions. *EMBO J.*, 16, 2907–2916.

23. Rich,A. (1978) Localized positive charges can bend double helical nucleic acid. *FEBS Lett.*, 51, 71–81.

24. Podesta,A., Indrirey,M., Broglio,D., Manning,G.S., Milani,P., Guerra,R., Finzi,L. and Dunlap,D. (2005) Positively charged surfaces increase the flexibility of DNA. *Biophys. J.*, 89, 2558–2563.

25. Hagerman,P.J. (1996) Do basic region-leucine zipper proteins bend their DNA targets... does it matter? *Proc. Natl Acad. Sci. USA*, 93, 9993–9996.

26. Saitani,A. and Crothers,D.M. (1998) Structural and hydration effects for the incorporation of a cationic 3-aminopropyl chain into DNA. *Nucleic Acids Res.*, 30, 3171–3180.

27. Range,K., Mayaen,E., Maher,L.J., III and York,D.M. (2005) The contribution of phosphate-phosphate repulsions to the free energy of DNA bending. *Nucleic Acids Res.*, 33, 1257–1268.

28. Williams,S.L., Parkhurst,L.K. and Parkhurst,L.J. (2006) Changes in DNA bending and flexing due to tethered cations detected by fluorescence resonance energy transfer. *Nucleic Acids Res.*, 34, 1028–1035.

29. Mizrabaev,D. and Rich,A. (1979) Asymmetric lateral distribution of unshielded phosphate groups in nucleosomal DNA and its role in DNA bending. *Proc. Natl Acad. Sci. USA*, 76, 1118–1121.

30. Manning,G.S., Ebralidise,K.K., Mizrabaev,A.D. and Rich,A. (1989) An estimate of the extent of folding of nucleosomal DNA by laterally asymmetric neutralization of phosphate groups. *J. Biomol. Struct. Dyn.*, 6, 877–889.

31. Manning,G.S. (2003) Is a small number of charge neutralizations sufficient to bend nucleosome core DNA onto its superhelical ramp? *J. Am. Chem. Soc.*, 125, 15087–15092.

32. Oakley,M.G. and Dervan,P.B. (1990) Structural motif of the GCN4 DNA binding domain characterized by affinity cleaving. *Science*, 248, 847–850.

33. Kerppola,T.K. and Curran,T. (1991) DNA bending by Fos and Jun: the flexible hinge model. *Science*, 254, 1210–1214.

34. Kerppola,T.K. and Curran,T. (1993) Selective DNA bending by a variety of bZIP proteins. *Mol. Cell. Biol.*, 13, 5479–5489.

35. Paolella,D.N., Palmer,C.S. and Schepartz,A. (1994) DNA targets for certain bZIP proteins distinguished by an intrinsic bend. *Science*, 264, 1130–1133.

36. Suckow,M., Madan,A., Kisters-Woike,B., von Wilcken-Bergmann,B. and Muller-Hill,B. (1994) Creating new DNA binding specificities in the yeast transcriptional activator GCN4 by combining selected amino acid substitutions. *Nucleic Acids Res.*, 22, 2198–2208.

37. Kerppola,T. and Curran,T. (1995) Transcription. Zen and the art of Fos and Jun. *Nature*, 373, 199–200.

38. Kerppola,T.K. (1996) Fos and Jun bend the AP-1 site: effects of probe geometry on the detection of protein-induced DNA bending. *Proc. Natl Acad. Sci. USA*, 93, 10117–10122.

39. Ellenberger,T.E., Brandl,C.J., Struhl,K. and Harrison,S.C. (1992) The GCN4 basic region leucine zipper binds DNA as a dimer of uninterrupted alpha helices: crystal structure of the protein–DNA complex. *Cell*, 71, 1223–1237.

40. Weiss,M.A., Ellenberger,T., Wobbe,C.R., Lee,J.P., Harrison,S.C. and Struhl,K. (1990) Folding transition in the DNA-binding domain of GCN4 on specific binding to DNA. *Nature*, 347, 575–578.

41. Metzloio,S.I., Paolella,D.N. and Schepartz,A. (1997) The role of a basic amino acid cluster in target site selection and non-specific binding of bZIP peptides to DNA. *Nucleic Acids Res.*, 25, 2967–2972.

42. Konig,P. and Richmond,T.J. (1993) The X-ray structure of the GCN4-bZIP bound to ATF/CREB site DNA shows the complex depends on DNA flexibility. *J. Mol. Biol.*, 233, 139–154.

43. Koo,H.S. and Crothers,D.M. (1988) Calibration of DNA curvature and a unified description of sequence-directed bending. *Proc. Natl Acad. Sci. USA*, 85, 1763–1767.

44. Kaffman,A., Herskowitz,I., Tjian,R. and O'Shea,E.K. (1994) The transcriptional regulation of the PHO82-pho4 locus controlling the synthesis of repressible acid phosphatase in Saccharomyces cerevisiae. J. Bacteriol., 176, 2298–2305.

45. Hagerman,P.J. (1996) Do basic region-leucine zipper proteins bend DNA targets... does it matter? *Proc. Natl Acad. Sci. USA*, 93, 1130–1133.

46. Sitlani,A. and Crothers,D.M. (1996) Electrostatic gel electrophoresis. *Biochemistry*, 35, 621–702.

47. Murre,C., McCaw,P.S. and Baltimore,D. (1989) A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. *Cell*, 56, 777–783.

48. Berger,B., Legrain,M., Gilliquet,V. and Hilger,F. (1990) The yeast regulatory gene PHO4 encodes a helix–loop–helix motif. *Yeast*, 6, 451–454.

49. Littlewood,T.D. and Evan,G.I. (1995) Transcription factors 2: helix–loop–helix. *Protein Profile*, 2, 621–702.
57. Shao, D., Creasy, C.L. and Bergman, L.W. (1998) A cysteine residue in helixII of the bHLH domain is essential for homodimerization of the yeast transcription factor Pho4p. *Nucleic Acids Res.*, 26, 710–714.
58. Shimizu, T., Tounoto, A., Ibara, K., Shimizu, M., Kyogoku, Y., Ogawa, N., Oshima, Y. and Hakoshima, T. (1997) Crystal structure of PHO4 bHLH domain–DNA complex: flanking base recognition. *EMBO J.*, 16, 4689–4697.
59. Cave, J.W., Kremer, W. and Wemmer, D.E. (2000) Backbone dynamics of sequence specific recognition and binding by the yeast Pho4 bHLH domain probed by NMR. *Protein Sci.*, 9, 2411–2423.
60. Chen, Y., Yang, J.T. and Chau, K.H. (1974) Determination of the helix and beta form of proteins in aqueous solution by circular dichroism. *Biochemistry*, 13, 3350–3359.
61. Kerppola, T. (1994) DNA bending specificity among bZIP family proteins. In Conaway, R. and Conaway, J. (eds), *Transcription: Mechanisms and Regulation*. Raven Press, Ltd, NY, pp. 387–424.
62. Lacroix, E., Viguera, A.R. and Serrano, L. (1998) Elucidating the folding problem of alpha-helices: local motifs, long-range electrostatics, ionic-strength dependence and prediction of NMR parameters. *J. Mol. Biol.*, 284, 173–191.
63. Mills, J.B. and Hagerman, P.J. (2004) Origin of the intrinsic rigidity of DNA. *Nucleic Acids Res.*, 32, 4055–4059.