The Basic Helix-Loop-Helix Domain of the Aryl Hydrocarbon Receptor Nuclear Transporter (ARNT) Can Oligomerize and Bind E-box DNA Specifically*

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The arylic hydrocarbon receptor nuclear transporter (ARNT) is a basic helix-loop-helix (bHLH) protein that contains a Per-Arnt-Sim (PAS) domain. ARNT heterodimerizes in vivo with other bHLH PAS proteins to regulate a number of cellular activities, but a physiological role for ARNT homodimers has not yet been established. Moreover, no rigorous studies have been done to characterize the biochemical properties of the bHLH domain of ARNT that would address this issue. To begin this characterization, we chemically synthesized a 56-residue peptide encompassing the bHLH domain of ARNT (residues 90–145). In the absence of DNA, the ARNT-bHLH peptide can form homodimers in lower ionic strength, as evidenced by dynamic light scattering analysis, and can bind E-box DNA (CACGTG) with high specificity and affinity, as determined by fluorescence anisotropy. Dimers and tetramers of ARNT-bHLH are observed bound to DNA in equilibrium sedimentation and dynamic light scattering experiments. The homodimeric peptide also undergoes a coil-to-helix transition upon E-box DNA binding. Peptide oligomerization and DNA affinity are strongly influenced by ionic strength. These biochemical and biophysical studies on the ARNT-bHLH reveal its inherent ability to form homodimers at concentrations supporting a physiological function and underscore the significant biochemical differences among the bHLH superfamily.

The arylic hydrocarbon receptor nuclear transporter (ARNT) protein belongs to the basic-helix-loop-helix Per-Arnt-Sim (bHLH PAS) family of transcriptional regulator proteins. These functionally oligomeric proteins are important for cell cycle and developmental regulation and for sensing and responding to environmental conditions (1, 2). ARNT shows high sequence homology to other bHLH motifs of this family (3, 4), particularly at residues known to contact DNA (5–10). In general, bHLH domains bind a consensus DNA element, the so-named E-box (CANNTG), and are required for oligomerization. PAS domains, which are found in all kingdoms, are involved in protein-protein interactions and ligand/inducer binding, acting as environmental sensors (1). PAS-containing proteins typically have two such conserved, repeated domains that are separated by a spacer region. PAS domains are not always contiguous with the bHLH DNA binding domain.

ARNT heterodimerizes in vivo with other bHLH PAS proteins, including the aryl hydrocarbon receptor (AHR) and hypoxia-inducible factor 1α (HIF1α), to form activated DNA binding complexes (11). Formation of the AHR/ARNT heterodimer requires the binding of polycyclic and halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin, which are known exogenous ligands for AHR and mediate carcinogenesis via AHR-ARNT complex activation (reviewed in Ref. 12). The resulting AHR/ARNT complex binds an atypical E-box DNA sequence, TNGCGTG, thereby activating the transcription of a number of target genes by direct interaction with general transcription factors, such as transcription factor IIB (13). Among the activated genes are CYP1A1 and CYP1A2, cytochrome氧化ases that metabolize polycyclic and aromatic compounds to electrophilic derivatives, which are the ultimate chemical agents that attack DNA. The AHR/ARNT heterodimer also activates transcription of the mdr1 multidrug transporter, albeit indirectly (14). The HIF1α/ARNT heterodimer (HIF1) senses the oxygen tension in cells and, under hypoxic conditions, activates the transcription of a number of genes, the promoters of which contain the E-box sequence, TACGTGC. Transcription is activated by formation of a HIF1-CREB (cAMP-response element-binding protein)/ATF1-(p300/CBP (CREB-binding protein) complex on the cognate DNA (15). Among the genes regulated are erythropoietin, vascular endothelial growth factor, glycolytic enzymes, tyrosine hydroxylase, inducible nitric-oxide synthase, and heme oxygenase-1, all of which allow the cell to cope with lower oxygen levels (reviewed in Ref. 16). HIF1 also plays a role in iron homeostasis by its activation of the ceruloplasmin gene (17). This HIF1-mediated response has been found to be crucial for angiogenesis and solid tumor formation (18–20). Neither AHR or HIF1α homodimers nor AHR/HIF1α heterodimers have been observed.

In contrast to the in vivo importance of AHR/ARNT and HIF1α/ARNT heterodimers, the biological relevance of ARNT homodimers is unclear. No physiological role for ARNT homodimers has yet been defined, and in vitro coimmunoprecipitation studies have been unable to detect the homodimeric ARNT complex (21). However, in vivo reporter gene assays have demonstrated that putative ARNT homodimers can acti-
vate transcription via E-box binding (3, 22), and a preferred DNA binding site has been identified that contains the E-box sequence (23). It is expected that ARNT homodimers would bind to the CACGTG E-box as do other canonical bHLH proteins, whereas ARNT in one of its heterodimeric complexes would bind one half-site of an asymmetric consensus binding site, with the heterodimeric partner binding the other, nonconsensus half-site. As demonstrated for the AHR/ARNT heterodimer, ARNT is located on the GTG E-box half-site, and AHR is situated on the less restrictive (A/C)(G/T)(A/T) non-E-box half-site (24).

The structures of several bHLH domain-containing peptides bound to DNA have been determined by x-ray crystallography, including MAX, USF, MyoD, Pho4, E47, and SREBP1 (5–10).

From these structures, it has been determined that the basic region is helical, and its residues make the primary contacts to the DNA, whereas the helix-loop-helix region is largely responsible for dimerization. Additionally, synthetic peptides of several bHLH domains have been characterized biochemically and biophysically, of which the Deadpan bHLH was perhaps the most rigorously investigated (25–30). However, these latter studies do not address the biochemical and biophysical properties of the bHLH-PAS family members, in particular ARNT.

To delineate the structural mechanism of transcription regulation by ARNT either as a homodimer or an AHR/ARNT or HIF1α/ARNT heterodimer, we have undertaken a series of biophysical and biochemical studies on a chemically synthesized, 56-residue peptide that encompasses the ARNT-bHLH domain. Specifically, we have used circular dichroism (CD) to determine the extent to which the peptide is folded in both the presence and absence of DNA and equilibrium sedimentation to determine its oligomerization state. Furthermore, using fluorescence anisotropy, we have determined the binding affinity of this peptide for E-box DNA under a variety of experimental conditions. As a complement to our sedimentation equilibrium experiments, we conducted dynamic light scattering studies to evaluate the oligomerization state and monodispersity of the peptide at higher concentrations. Unexpectedly, significant biochemical differences from other bHLH proteins such as Deadpan and Tal were found. Moreover, the data provide evidence that the bHLH of ARNT can form homodimers, which might have biological relevance.

**EXPERIMENTAL PROCEDURES**

**Solid Phase Peptide Synthesis**

A peptide encompassing residues 90–145 of ARNT (ARNT-bHLH) was synthesized using Fmoc (N-[9-fluorenylethoxycarbonyl]) chemistry on a Milligen/Biosearch peptide synthesizer. Cleavage and deprotection reactions were carried out in trimethylsilane bromide, ethanediol, m-cresol, thionisol, and trifluoroacetic acid for 1 h at 0 °C under a nitrogen blanket to ensure that cysteine residues remained protected after removal of the triyl protection groups. The cleaved peptide was filtered through a medium-sintered glass filter to separate it from resin. The peptide was then washed with trifluoroacetic acid. The filtrate and washings were combined, and all liquid was evaporated using a rotary evaporator. The peptide was precipitated with diethyl ether and filtered through a medium-sintered glass funnel. The peptide was dried under a stream of nitrogen, dissolved in 10% acetic acid, and lyophilized. The peptide was then dissolved in 0.1% trifluoroacetic acid and a linear 60-min gradient of mobile phase of 0.1% trifluoroacetic acid and a linear 60-min gradient of 0–100% acetonitrile. Chromatography runs were recorded with a diode array detector and analyzed using Millennium 2000 software (Waters). The peptide eluted at 73% acetonitrile. Fractions from 70 to 74% were pooled and rechromatographed. The purity of the peptide was ascertained by mass spectrometry (data not shown).

**Circular Dichroism**

Circular dichroism experiments were performed with a Jasco-J500 instrument. An E-box-containing oligonucleotide, ARNDNA (5’-GGCTCAGCTGAGGAGCGCAG-3’), was purchased from Oligos, Etc. This sequence was chosen to contain the consensus RTCAAGCTGAGQ sequence determined to be recognized by ARNT using a site affinity and amplification assay (23). The oligonucleotide, which has an unpaired 5’ guanosine upon duplex formation, was suspended from the lyophilized pellet in 10 mM sodium cacodylate, pH 6.5, such that the final concentration of single-stranded oligonucleotide was 2.0 μM. The concentration was calculated using data that were provided by Oligos, Etc. for each strand. The palindromic strands were annealed by heating to 90 °C, followed by slow cooling to 25 °C. Complete annealing was confirmed by high pressure liquid chromatography and gel electrophoresis (data not shown). The final concentration of duplex DNA was determined by measuring the absorbance of the solution at 260 nm and using the equation \( A \) = \( csd \), where \( A \) is the absorbance at 280 nm, \( c \) is the known extinction coefficient for double-stranded DNA, 0.02 μg·cm⁻¹·μmol⁻¹, and \( s \) is the length path through the cuvette, 1 cm. The molecular weight of the duplex oligonucleotide (13.0 kDa) was subsequently used to calculate the molar concentration of ARNDNA. The CD spectra of both 100 μM ARNT-bHLH peptide, calculated for a monomer, and 50 μM duplex ARNDNA in Buffer A (50 mM NaCl or 50 mM NaF, 20 mM Tris, pH 7.4) were measured from 260 to 180 nm at 20 °C in a 0.01-cm cell. The subtraction of the buffer NaCl had no effect on the spectra (data not shown). The spectrum of 100 μM ARNT containing 50% trifluoroethanol (TFE) or 50 μM duplex ARNDNA oligonucleotide was also measured. The final reported spectra are averages of 10 runs. To measure any changes in peptide secondary structure upon binding DNA, the CD difference spectrum was calculated by subtracting the spectrum of ARNDNA alone from that of the ARNDNA/ARNT mixture. The concentration of the peptide solution was verified by amino acid analysis. The secondary structures were analyzed using the variable selection method (32).

**DNA Binding at Lower Salt Concentrations; Fluorescence Polarization**

Fluorescence polarization experiments were done with a PanVer a Beacon fluorescence polarization system (PanVera Corp.). 5’-Fluoresceinated oligonucleotides corresponding to ARNDNA and the Escherichia coli purF operator (Oligos, Etc.) (ARNDNA, 5’-F-GCTAGTCA-CGTTGACGGCAGCCTCGTGATCGATGACCTGACGCG-3’; purF 5’-F-AAAAGAAAAACGTTTGCAACCCCATGACAAAAAGTTTTTTCTT-3’) were self-annealed in 10 mM sodium cacodylate, pH 6.5, by heating to 80 °C followed by flash cooling to form a stem-loop structure with the E-box motif (underlined) or purF operator motif, respectively, at the center of the stem. Oligonucleotide concentrations were calculated as described for ARNDNA used in CD experiments. Binding was assayed in a 1-ml volume at 25 °C. Unless otherwise noted in the text, the components of each binding experiment were 2 nM fluoresceinated oligonucleotide (13.0 kDa) and 1.0 μg/ml poly(d[IF]-Cl) in Buffer A (50 mM NaCl or NaF, 20 mM Tris, pH 7.4). Poly(d[IF]-Cl) (Sigma) was included as a control for nonspecific DNA binding. It is expressed in μg/ml rather than molar to reflect the fact that the exact length of the poly(d[IF]-Cl) molecules is not discrete but averages between 1200 and 3000 base pairs. In one set of experiments, the amount of poly(d[IF]-Cl) included was varied to 0.0, 0.1, or 1.0 μg/ml. After each addition of peptide, samples were incubated in the Beacon instrument at 25 °C for 30 s before a measurement was taken. The 30-s incubation allowed equilibrium to be reached. The multipolarization (\( P \) × 10⁻³ where \( P \) is polarization) at each titration point represents the average of eight measurements integrated over 6 s. Samples were excited at 490 nm, and emission was measured at 530 nm.

The data of each binding isotherm were analyzed by curve fitting using SigmaPlot software (Jandel Corp.). Because the calculated dissociation constants were all greater than 20 nM and the experimental DNA concentration was 10-fold less than this value, it was assumed that the concentration of protein bound to DNA was negligible in comparison with the total protein concentration. Therefore, the following equation could be applied (33),

\[
P = (P_{\text{bound}} - P_{\text{free}}) [\text{ARNT}] / (K_d + [\text{ARNT}]) + P_{\text{free}} \quad \text{(Eq. 1)}
\]

where \( P \) is the polarization measured at a given total concentration of peptide ([ARNT]), \( P_{\text{free}} \) is the initial polarization of the free DNA, and \( P_{\text{bound}} \) is the maximum polarization of specifically bound DNA. Nonlinear least squares analysis was used to determine \( P_{\text{free}}, P_{\text{bound}}, \) and \( K_d \).
DNA Binding at Higher Salt Concentrations; Fluorescence Anisotropy

Anisotropy studies at higher salt concentrations (Buffer B; 150 mM NaCl, 100 mM Tris, pH 7.4) were done with an SLM 8000 spectrophotometer with T optics at 25 °C. The sample was excited at 480 nm, and the parallel and perpendicular polarization components of fluorescein emission were measured at 520 nm. Except for the buffer, the components in the assay were the same as those used in measuring anisotropy in lower salt buffer. After each addition of peptide, the solution was incubated for 1 min to attain equilibrium. Each titration point is an average of 12 measurements, each integrated over 30 s. Data were analyzed using Scientist (MicroMath). Anisotropy was measured as the function of concentration of ARNT-bHLH (μM) added to the binding reaction, and the data were fit using a two-step binding model that follows. Anisotropy, A, is related to polarization, P, by A = 2P(3P - 1)(P)^(-1) (Ref. 33).

Step 1; Cooperative Binding of ARNT-bHLH to ARNDNA—The equilibrium model that best describes the initial phase of the higher salt binding isotherm is two ARNT-bHLH monomers binding to the ARNDNA duplex, i.e., 2 ARNT-bHLH monomers + ARNDNA ↔ ARNT-bHLH·ARNDNA + ARNT-bHLH ↔ ARNT-bHLH·ARNDNA. The equation to describe this cooperative binding is as follows,

\[ v_1 = \frac{C_{\text{ARNT}}}{1 + K_{\text{ARNT}}} \]  
(2.1)

where \( v_1 \) is the average number of bound ARNT-bHLH molecules per molecule of ARNDNA, \( C \) is the peptide concentration (μM), and \( C_{\text{ARNT}} \) is the concentration at half-saturation of the cooperative binding event. \( P \), the Hill coefficient, is the average number of interacting sites on ARNDNA, which is two. \( v_1 \) is also the measure of cooperativity in a binding event.

Step 2; Noncooperative Oligomerization of ARNT-bHLH Dimer on ARNT-bHLH Dimer-ARNDNA—The following equilibrium model describes the second phase of binding: 1 ARNT-bHLH dimer-ARNDNA + 1 ARNT-bHLH dimer ↔ 2 ARNT-bHLH dimers-ARNDNA. In noncooperative binding, all binding sites are equivalent and independent of each other. The binding curve is a rectangular hyperbola (34) and is described by the equation,

\[ v_2 = K[\text{ARNT}] / (1 + K[\text{ARNT}]) \]  
(2.2)

where \( v_2 \) is the average number of ARNT-bHLH dimers bound to the ARNT-bHLH dimer-ARNDNA complex formed in the first step. \( K \) is the association constant of the noncooperative event, and [ARNT] is the concentration of free ARNT-bHLH monomers. At high concentrations (>25 μM), ARNT-bHLH is dimeric. In this step of binding, only the dimer-bound DNA is available for further binding of ARNT.

Dynamic Light Scattering

Dynamic light scattering studies were done using a DynaPro-801 instrument (Protein Solutions, Inc.). All solutions were filtered through 0.1-μm filters (Whatman) to remove aggregated peptide and other particulates. Scattering of the ARNT-bHLH peptide was analyzed at concentrations of 5.0 and 10.0 mg/ml (0.78 and 1.56 mM ARNT-bHLH monomer, respectively) in its storage buffer (40 mM KCl, 2 mM dithiothreitol, 0.4 mM EDTA, 5% glycerol, and 20 mM Tris, pH 7.4). Peptide-DNA experiments were performed by mixing 1.56 mM ARNT-bHLH with DNA at concentrations of 5.0 and 10.0 mg/ml (0.78 and 1.56 mM ARNT-bHLH monomer, respectively) in its storage buffer. After each addition of peptide, the solution was incubated for 1 min to attain equilibrium. Each titration point is an average of 12 measurements, each integrated over 30 s. Data were analyzed using Scientist (MicroMath). AutoPro 4.0 PC software (Protein Solutions, Inc.) was used for experimental data fitting. Weight average molecular weight for a single species model was calculated using the formula.

\[ C = C_0 + (C_M - C_0) \exp\left(-\frac{v}{p+1}\right) \]  
(2.3)

where \( v \), the distance from the meniscus to the axis of the rotor, \( r \) is the distance between each point along the concentration gradient and the isodensity axis, and \( C_0 \) and \( C_M \) are the concentrations at points \( r \) and \( r_M \), respectively. \( v \), \( r \), and \( T \) are angular velocity, partial specific volume, density, buoyancy, gas constant, and temperature, respectively.

RESULTS

Polypeptide Synthesis and Circular Dichroism—We synthesized a 56-aminoacyl residue polypeptide that corresponds to the bHLH DNA binding domain of the ARNT protein as determined by amino acid sequence homology with other bHLH proteins (Refs. 5–10, Fig. 1). CD spectra were measured to determine the secondary structure content of the ARNT-bHLH peptide and to observe any changes in secondary structure upon binding to DNA. CD spectra were also taken in the absence of TFE. TFE increases helicity of polypeptides by selectively destabilizing solvent-amide group interactions. Compact conformations such as α-helices, which maximize intramolecular polypeptide backbone hydrogen bonding and lessen solvent exposure are favored (36). Medium-sized peptides with an intrinsic tendency to assume a helical conformation in water show an increase in helicity upon the addition of TFE. Hence, the CD spectra of the peptide with and without 50% TFE were measured to confirm that the synthetic peptide had the ability to adopt a helical structure. In both cases, spectra were obtained with strong maxima at 190 nm and double minima at 200–210 and 222 nm, characteristic of α helices. The amplitude of the spectrum for ARNT-bHLH with TFE was approximately three times that of peptide in buffer alone (Fig. 2A). Secondary structure analysis using the variable selection method showed that the helicity increased from 12.8 to 68.0% upon the addition of TFE (Table 1). The predicted maximum helicity attainable by the peptide is 82.1%. This maximum is calculated by assuming that the basic region, helix 1, and helix 2 (Fig. 1) would be completely α-helical, as observed in the crystal structures of other bHLH peptides bound to DNA.
The bHLH family of transcription factors binds to specific DNA sequences primarily as dimers and tetramers (37). bHLH transcription factors are known to recognize cognate DNA by means of an intrinsically flexible basic region that forms an α helix upon binding to cognate E-box DNA. The helix-loop-helix region is involved in dimerization and might undergo structural transitions as well, albeit smaller. Therefore, the CD spectrum of ARNT-bHLH peptide and duplex ARNDNA in 2:1 stoichiometry (monomer peptide-DNA) was measured, and the CD spectrum of ARNDNA alone was subtracted from this to obtain a difference spectrum representing peptide structure in the presence of DNA (Fig. 2B and Table I). The secondary structure analysis showed a 5-fold increase in the helical content of the peptide (from 12.8 to 65.0%) and a 3-fold decrease (40.1 to 12.9%) in the random coil structure in the presence of ARNDNA. The percentage of turn also decreased 3-fold, from 39.2 to 12.5%. The random coil-to-helix transition correlates with the increased helical content and decreased random coil content observed on association of ARNT-bHLH with the ARNDNA oligonucleotide. Crystal structures of bHLH peptides bound to cognate DNA have shown that the DNA is not appreciably bent as a result of peptide binding. Oligonucleotide base stacking is a major contributor to optical activity in the 260–300-nm range. No change in the optical activity was seen in this range on binding ARNT-bHLH, indicating that there is no gross change induced in the structure of the oligonucleotide (data not shown).

Fluorescence Anisotropy—We used a fluorescence anisotropy binding assay to measure the equilibrium binding affinity of the ARNT-bHLH peptide for oligonucleotides with and without the E-box sequence. Fluorescence anisotropy is a straightforward technique for directly measuring macromolecular interaction in solution (reviewed in Ref. 33), and hence, the effects of a number of variables can be tested readily. The technique is based on the observation that the rotational motion of a fluoresceinated oligonucleotide is slowed by peptide binding, thus increasing the measured anisotropy (or polarization) of the DNA. It is assumed that peptide binding to the DNA is directly proportional to the increase in anisotropy, if the temperature and viscosity of the solution are constant. This is confirmed by the dynamic light scattering and equilibrium sedimentation studies reported later in which no large, nonspecific aggregate particles were observed.

**Specificity of DNA Binding**—The specificity of the ARNT peptide for E-box DNA was determined by comparing the binding isotherms of peptide and E-box DNA (ARNDNA) to peptide

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**Table I**

| Polypeptide | % H | % A | % P | % T | % O |
|-------------|-----|-----|-----|-----|-----|
| ARNT        | 12.8| 11.4| 3.4 | 39.2| 40.1|
| ARNT + 50% TFE | 68.0| 0.5 | 2.5 | 18.7| 14.2|
| ARNT + DNA (2:1) | 65.0| 3.2 | 3.1 | 12.5| 12.9|

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**Fig. 1** Primary sequence alignment of selected bHLH domains. GenBank™ accession numbers for each, as well as the percent identity to the ARNT-bHLH, in parentheses, are: ARNT P53762 (100), AHR P41738 (21), HIF1α NP_001521 (21), USF Q07965 (25), MAX P28574 (27), PHO4 P07270 (26), MYOD P15172 (27), MYC P01106 (29), DPN Q26263 (28), E47 B31492 (25), SREBP1 P36956 (33), ARNT, AHR, and HIF1α are members of the bHLH-PAS family. Identical residues are boxed in black, and homologous residues are boxed in gray. The first and last residue number of the bHLH domain is given at the beginning and end of each sequence. Crystal structures have been solved for USF (6), MAX (5), PHO4 (8), MYOD (7), E47 (9), and SREBP1 (10). These structures reveal similar three-dimensional folds of bHLH domains. Alignments were performed using ClustalW, and this figure was generated using Boxshade.

**Fig. 2** Circular dichroism spectra of ARNT and ARNT-DNA. A, ARNT peptide alone (solid line) and in 50% TFE (dotted line). B, ARNT peptide (solid line) and the difference spectrum (dotted line) obtained by subtracting ARNDNA spectrum from that of ARNDNA:ARNT complex (1:2 DNA-peptide monomer ratio). The ARNT:ARNDNA spectrum was carried out in 50 mM NaF, 20 mM Tris, pH 7.4.

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**Characterization of the bHLH of ARNT**

| BASIC | HELIX 1 | LOOP | HELIX 2 |
|-------|---------|------|---------|
| ARNT  | 90      | CA2E | TRRNRA | DLYHV |
| AHR   | 41      | CA2E | TRRNRA | DLYHV |
| HIF1α | 18      | CA2E | TRRNRA | DLYHV |
| USF   | 192     | CA2E | TRRNRA | DLYHV |
| MAX   | 24      | CA2E | TRRNRA | DLYHV |
| PHO4  | 251     | CA2E | TRRNRA | DLYHV |
| MYOD  | 110     | CA2E | TRRNRA | DLYHV |
| MYC   | 356     | CA2E | TRRNRA | DLYHV |
| DPN   | 41      | CA2E | TRRNRA | DLYHV |
| E47   | 72      | CA2E | TRRNRA | DLYHV |
| SREBP1| 323     | CA2E | TRRNRA | DLYHV |

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- **H**, helix; **A**, anti-parallel β sheet; **P**, parallel β sheet; **T**, turn; **O**, other aperiodic structures. Spectra were taken in 50 mM NaF, 20 mM Tris, pH 7.4.
and noncognate DNA (E. coli purF operator) in 50 mM NaCl, 20 mM Tris, pH 7.4. The resulting binding curves are shown in Figs. 3, A and B. The calculated equilibrium dissociation constant, \( K_d \), is 56.2 ± 11.7 nM for the ARNT-bHLH-ARNDNA complex. The \( K_d \) for purF operator DNA was calculated to be 1.91 ± 0.73 \( \mu \text{M} \).

Effects of Lower Concentrations of Monovalent and Divalent Salts on DNA Binding—To characterize DNA binding by the ARNT-bHLH peptide further, the buffer and salt concentrations were varied for a series of ARNT-bHLH peptide titrations into ARNDNA. Representative curves are shown in Figs. 3 and 4. The best binding was observed in the solution containing 50 mM NaCl or 50 mM NaF and 20 mM Tris HCl, pH 7.4 (Fig. 3A and 4A). In some binding experiments, NaF was used in place of NaCl because it is a more suitable salt for spectroscopic studies that utilize far UV wavelengths, such as circular dichroism. The affinity of the ARNT-bHLH for ARNDNA was not changed by this substitution, with \( K_d \) values equal to 56.2 ± 11.7 nM in 50 mM NaCl and 57.2 ± 4.8 nM in 50 mM NaF. We found that the addition of 10 mM MgCl₂ to the solution containing 50 mM NaF and 20 mM Tris HCl, pH 7.4, also had no effect on binding (\( K_d = 56.4 ± 13.9 \text{ nM, data not shown} \)).

Effects of Higher Concentrations of Salt on DNA Binding—Anisotropy measurements of ARNT-bHLH binding to F-ARNDNA in higher salt conditions (150 mM NaCl, 100 mM Tris, pH 7.4) resulted in a complex biphasic curve (Fig. 3C). The first phase of the curve has sigmoidal shape, implying cooperative binding. Such cooperative binding of ARNT-bHLH to ARNDNA is evident to \( -23 \text{ \mu M} \). The peptide concentration at half-saturation, was determined to be 11.7 ± 0.1 \( \mu \text{M} \). The value of the Hill coefficient, \( P \), is 1.6 ± 0.1 and indicates that ARNT-bHLH monomers dimerize cooperatively on ARNDNA. The second phase of the binding curve is able to be fit by a rectangular hyperbola, implying noncooperativity. This binding mode has a dissociation constant, \( K_d \), of about 20 \( \mu \text{M} \). However, we cannot elucidate the molecular complexes formed during this second binding event, i.e. peptide tetramerization or a second bHLH dimer binding to DNA cannot be discerned from this analysis.

Effects of Poly(d[I-C]) on Equilibrium Binding—As a control for DNA binding specificity, we included 1.0 \( \mu \text{g/ml} \) poly(d[I-C]) in all fluorescence polarization experiments. The use of poly(d[I-C]) ensures that any DNA binding observed at low concentrations of peptide is specific because the high concentration of poly(d[I-C]) offers a huge excess of nonspecific binding sites (on the order of 10 \( \mu \text{m} \)) compared with the ARNDNA (2 nM). To determine the effects of poly(d[I-C]) on the measured equilibrium binding constants, additional experiments were conducted in which the poly(d[I-C]) was either removed (0.0 \( \mu \text{g/ml} \)) or at a concentration diminished 10-fold (0.1 \( \mu \text{g/ml} \)). The lower salt binding buffer, containing 50 mM NaF and 20 mM Tris HCl, pH 7.4, was used in each binding experiment. The resulting binding isotherms are shown in Fig. 4. The equilibrium dissociation constants were \( K_d(0) = 30.0 ± 4.3 \), \( K_d(0.1) = 48.1 ± 12.1 \), and \( K_d(1.0) = 57.2 ± 4.8 \text{ nM} \). Thus, the ARNT-bHLH binding affinity decreases less than 2-fold as poly(d[I-C]) concentration is increased. The very weak dependence of the \( K_d \) of the ARNT-bHLH for ARNDNA on the concentration of poly(d[I-C]) contrasts sharply with the DNA binding characteristics of the bHLH peptide of Deadpan (29). Whereas we observe less than a 2-fold increase in \( K_d \) (30–57 nM for 0.0 to 1.0 \( \mu \text{g/ml} \) poly(d[I-C])), respectively), Winston et al. (29) report a 15-fold increase (2.5–37 nM (14.8-fold) for 0.0–0.6 \( \mu \text{g/ml} \) poly(d[I-C]), respectively). The fold differences for the Deadpan peptide would be expected to be even greater at 1.0 \( \mu \text{g/ml} \) poly(d[I-C]).

Dynamic Light Scattering—To determine the oligomeric state of the complex observed in the lower salt fluorescence polarization experiments, we carried out dynamic light scattering studies on high concentrations of free peptide and peptide-DNA mixtures. Dynamic light scattering allows the assessment of the oligomeric state as well as the “dispersity” of a macromolecule or its complex solution (35). Dispersity is the degree to which the particles in a solution are the same size. The results of the dynamic light scattering experiments on the ARNT-bHLH in the presence and absence of ARNDNA are given in Table II. At 5.0 mg/ml (0.78 mM), the ARNT-bHLH peptide (6.5 kDa/monomer) forms a dimer with an apparent molecular weight of 11.1 ± 1.5. At 10.0 mg/ml (1.56 mM), the peptide is also a dimer with apparent molecular weight 13.5 ± 1.0, which is nearly identical to the calculated molecular weight. When solutions of 1 mM duplex ARNDNA oligonucleotide (13.0 kDa) and 15 mM ARNT-bHLH are mixed, the average molecular weight of the components in solution is 20.3 ± 4.5. This is consistent with a population containing free peptide dimers (13.0 kDa), free duplex DNA (13.0 kDa), and dimeric peptide-DNA complexes (26.0 kDa). The lower molecular weight average is expected under our experimental conditions because of the molar excess of DNA (0.5 mM) to peptide dimers (~0.4 mM). It is not possible to accurately resolve two species that differ only 2-fold in molecular weight using this method. However, the change we see upon addition of DNA to the peptide indicates that one peptide dimer binds one duplex DNA molecule at these concentrations. Moreover, the aggregation of components...
is not significant as judged by the standard deviations of the measured diffusion coefficients (Table II).

Equilibrium Sedimentation Studies—To investigate the effects of lower peptide concentrations and the higher salt environment on the peptide-DNA oligomerization state, a series of sedimentation studies were done. Using 2.5 μM peptide monomer and 1.25 μM duplex DNA, the analysis yielded complexes with an apparent molecular weight of 19.9 ± 0.2 (Table III). This value corresponds to either one ARNT monomer (6.5 kDa) bound to ARNDNA (13.0 kDa) or to the average molecular weight of two species, a 26-kDa complex of one 13-kDa ARNT-bHLH homodimer and one ARNT-bHLH dimer bound to DNA. Under the conditions of 2:1 ARNT-bHLH monomer:ARNDNA stoichiometry at 2-fold higher concentrations (5 μM peptide and 2.5 μM DNA), a 23.0 ± 0.1-kDa species was observed. This is likely the average molecular mass of two species, a 26-kDa complex of one 13-kDa ARNT-bHLH dimer bound to one 13-kDa ARNDNA duplex and the peptide dimer and ARNDNA alone, where the fraction of the 26-kDa species is higher. At higher concentrations (10 μM peptide and 5 μM DNA), a 39.0 ± 0.2-kDa species was observed. Because the absorption wavelength was 280 nm, it was not possible to determine whether this was a tetramer bound to DNA (39 kDa) or a mixture of aggregated states. To differentiate peptide-DNA complexes from free peptide or DNA, fluorescein-labeled ARNDNA was employed, and the absorbance of the sample was monitored at 494 nm, a wavelength at which only the fluorescein labeled deoxyribonucleotide absorbs. At a 4:1 ARNT-bHLH monomer:ARNDNA stoichiometry (40 μM:10 μM, respectively), a complex with a molecular mass of 43.3 ± 2.1 kDa was observed. This is consistent with an ARNT-bHLH tetramer bound to ARNDNA.

DISCUSSION

ARNT is the common heterodimeric partner of a number of bHLH-PAS family members and, thus, plays an essential role in many important pathways (reviewed in Ref. 2). Among these, the AHR-mediated pathway is essential for the xenobiotic response (12), and the HIF-mediated pathway controls the hypoxic response and, subsequently, hypoxia-mediated apoptosis (16, 38). Both pathways are involved also in the development and progression of cancer, where the AHR pathway is the target of action of carcinogenic compounds found in cigarette smoke and Agent Orange (39), and the HIF-mediated hypoxic response is critical for the formation and growth of solid tumors (18–20). A chromosomal translocation resulting in the production of a TEL-ARNT fusion protein, which contains the N-terminal domain of TEL and almost all of ARNT including the bHLH domain, has been described (40). This protein contributes to leukemogenesis, likely via dysregulation of ARNT-mediated pathways (40).

To understand the biochemical and hence, physiological, function of these members of the bHLH-PAS family, we synthesized a 56-residue peptide corresponding to the bHLH domain of ARNT. We have characterized this domain biophysically and biochemically to ascertain its ability to form a homodimer and bind E-box DNA and compared it to other bHLH domains. Although there are similarities between these bHLH domains in their CD spectra and oligomerization properties, there are also distinct differences, particularly in their DNA binding properties. This is notable, especially, given the high degree of amino acid sequence similarity across the bHLH family of proteins and the near structural identities of those members, the structures of which are known. However, none of the bHLH peptides studied to date has been a member of the bHLH domain family. We have characterized this domain biophysically and biochemically to ascertain its ability to form a homodimer and bind E-box DNA and compared it to other bHLH domains. Although there are similarities between these bHLH domains in their CD spectra and oligomerization properties, there are also distinct differences, particularly in their DNA binding properties. This is notable, especially, given the high degree of amino acid sequence similarity across the bHLH family of proteins and the near structural identities of those members, the structures of which are known. However, none of the bHLH peptides studied to date has been a member of the bHLH domain family. In light of these studies, the biochemical and biophysical differences between ARNT and bHLH proteins such as Deadpan (29) indicate that subtle, but significant, differences exist within the bHLH super family.

As anticipated, the α helical content of the ARNT-bHLH peptide increases dramatically in the presence of DNA or TFE. Our CD studies have shown that the peptide obtains its maximum helicity, 68.0%, in TFE. A nearly identical helicity, 65.0%, is seen upon its binding to specific DNA. This can be explained largely by the coil-to-helix transition of the flexible basic region and helix 1, which together account for 50% of the length of the peptide. The α-helical content in the presence of DNA is significantly lower than the predicted maximum helicity, 82.1%, and that observed for other bHLH proteins, e.g. MyoD-E47, 80%, and Mash-1, 85% (25, 27). This difference can be ascribed to either our overestimation of the predicted helical content of the ARNT-bHLH protein or helical fraying. Our experimental result, however, is similar to that reported for...
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Dpn, i.e. 63% α-helical content in the presence of DNA. Although the α helicity of the ARNT-bHLH peptide alone is significantly lower (12.8%) than that observed with other bHLH peptides, e.g. MyoD-E47 and Mash-1 (both 50%), these differences may be explained by different methods used in CD data analysis. Alternatively, the homodimeric bHLH domain of ARNT may be intrinsically more flexible, a property that might be required for its biological function. Further studies on the bHLH domains of ARNT/AHR and ARNT/HIF1α heterodimers will be required to address these questions.

Our DNA binding studies indicate that the ARNT-bHLH homodimer is able to bind the E-box (CACGTCG) specifically and with high affinity (56 nM) but not other unrelated DNA sequences. Using fluorescence anisotropy, we have determined DNA far less readily than the Dpn-bHLH. In further contrast, DNA indicates that the ARNT-bHLH peptide binds nonspecifically with high affinity (56 nM) but not other unrelated DNA sequences. Using fluorescence anisotropy, we have determined

| Protein-DNAa | Average molecular weight | Wavelength (nm) | Sedimenting species |
|--------------|--------------------------|-----------------|--------------------|
| 2.5 μM ARNT-bHLH + 1.25 μM ARNDNA | 19.9 ± 0.2 | 280 | ARNT-DNA or DNA + 2 ARNT-DNA |
| 5 μM ARNT-bHLH + 2.5 μM ARNDNA | 23.0 ± 0.1 | 280 | 2 ARNT-DNA + DNA + ARNT-DNA + 2 ARNT |
| 10 μM ARNT-bHLH + 5 μM ARNDNA | 39.0 ± 0.2 | 280 | 4 ARNT-DNA |
| 40 μM ARNT-bHLH + 10 μM ARNDNAa | 43.3 ± 2.1 | 494 | 4 ARNT-DNA |

aAll references to ARNT-bHLH and ARNT are based on monomeric peptide, and all references to DNA correspond to double-stranded ARNDNA oligonucleotide.

ARNDNA used in this experiment was fluorescein labeled.

The buffer was 100 mM Tris, pH 7.4. The NaCl concentration was 150 mM in all experiments.

A. Lower salt binding

B. Higher salt binding

C. Higher salt binding, [ARNT] > 23 μM

FIG. 5. Proposed model for ARNT-bHLH binding to cognate DNA under lower and higher salt conditions. A, In lower salt solution (50 mM NaCl, 20 mM Tris, pH 7.4), ARNT-bHLH is predominantly found as a dimer, which is able to bind cognate DNA with high affinity. In higher salt conditions (150 mM NaCl, 100 mM Tris, pH 7.4), ARNT-bHLH binds ARNDNA in a two-step process. B, step 1: below 23 μM ARNT-bHLH, one peptide monomer binds first to the DNA followed by cooperative recruitment of a second monomer to the DNA. C, step 2: above 23 μM, ARNT-bHLH is present largely as dimers, and one dimer binds to the preformed 2 ARNT-bHLH monomer-ARNDNA complex.

The higher salt binding mode differs from the high affinity DNA binding of ARNT-bHLH in lower salt concentrations and with the DNA binding of other bHLH proteins (37). However, two simple mechanisms by which two dimers of the ARNT-bHLH bind ARNDNA can be proposed. In one, the increase in peptide structure caused by binding to DNA might allow the formation of a four-helix bundle-like tetramer. This is unlikely, because similar oligomerization phenomena should be expected at lower salt concentrations, and such binding is not observed under that condition (Table II). In the second, a dimer of dimers on the DNA could be stabilized by interactions between the loop regions of each homodimer. Protein-protein cross-linking and peptide sequencing should clarify the issue of tetramerization. It is possible that the sensitivity we see to ionic strength is a result of salt competition with dimerization surfaces that are more hydrophilic than observed for other bHLH domains. In-
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deed, a theoretical model of the HIF1α/ARNT bHLH heterodimer (42) places several hydrophilic residues at or near the interface between the domains, unlike the MyoD bHLH interface, which is largely hydrophobic (7).

The sedimentation data presented here differ from those studies done on the bHLH of MyoD, which was shown to be dimeric and tetrameric in solution but bound DNA only as a dimer (25). However, it is important to note that not every homodimeric bHLH-containing protein is able to bind DNA. A peptide corresponding to the bHLH domain of Tall was shown to homodimerize in solution, but no DNA binding was detected (30). However, Tall was able to bind DNA as a heterodimer with the bHLH of E47.

In conclusion, we have carried out biophysical and biochemical studies on the oligomerization and DNA binding properties of the bHLH domain of ARNT that suggest homodimeric ARNT could be a viable transcription regulator in vivo. The ARNT-bHLH does homodimerize, as evidenced by our dynamic light scattering studies. We observe high affinity binding of ARNT-bHLH dimers to a specific DNA site at lower ionic strength and the induction of a coil to helix transition in the presence of DNA. At higher ionic strength, the ARNT-bHLH cooperatively dimerizes on ARNDNA, and at even higher concentrations of peptide, an additional ARNT-bHLH dimer is able to bind to the dimeric peptide-duplex DNA complex. This cooperativity may have a biological significance as it was seen in salt concentrations thought to be present in the cell (43). However, the biological significance of ARNT-bHLH tetramerization is unclear since the cellular concentration of ARNT has not been established. Furthermore, the PAS-A domain, which is critical for the heterodimerization of AHR and ARNT and is not present in the bHLH peptide, could play a significant negative or positive role in ARNT homodimerization (44). Comparison of the sensitivity to ionic strength or other solution parameters of ARNT homodimers with ARNT heterodimers (e.g. ARNT-AHR or ARNT-HIF1α) may yield information regarding the transcription regulation activity of each of these complexes. Further biochemical and structural studies to characterize the ARNT-AHR and ARNT-HIF1α interactions will be required to understand the mechanisms of ARNT heterodimerization and its attendant gene regulation.

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