Leucine Is the Most Stabilizing Aliphatic Amino Acid in the **d** Position of a Dimeric Leucine Zipper Coiled Coil

Jaideep Moitra, Lászlo Szilák, Dmitry Krylov, and Charles Vinson*

Building 37, Room 4D06, Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892

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ABSTRACT: The energetic contribution of seven amino acids in the **d** position of a dimeric leucine zipper coiled coil structure was measured by determining the thermal stability. The **d** position contains the conserved leucines found in the leucine zipper. We used a natural bZIP protein as our host−guest system that remains dimeric when a single **d** position is mutated. We have determined the thermal stability, monitored by circular dichroism, of 14 proteins which indicate that alanine is 4.6 kcal mol^{-1} per residue less stabilizing than leucine. The similarly sized amino acid isoleucine is 2.9 kcal mol^{-1} per residue less stabilizing than leucine, suggesting that leucine is well-packed. Model building indicates that the β-branched amino acids isoleucine and valine in the **d** position produced interhelical clashes between the $C\gamma 2$ methyl groups when placed in the favored rotamer conformation. The stabilization by leucine in different **d** positions is context-dependent; it varies by over 2 kcal mol^{-1} in the two positions examined. The order of stabilization is L, M, I, V, C, A, and S. Cysteine in the **d** position can form a disulfide bond which stabilizes the coiled coil.

Protein mutagenesis studies suggest that hydrophobic amino acids in the protein interior contribute to protein stability via two separate mechanisms (Pace, 1995; Pace et al., 1996; Shortle et al., 1990; Eriksson et al., 1992; Serrano et al., 1992; Lee & Vasmatzis, 1997). The first mechanism, termed the hydrophobic effect, is the burying of a hydrophobic amino acid in the protein interior, effectively removing it from the solvent. The magnitude of this effect can be measured by determining the transfer free energy of an amino acid from water to a hydrophobic solvent (Fauchere & Pliska, 1983). The second mechanism is van der Waals interactions between the amino acid side chain of interest and other hydrophobic amino acids in the protein interior. This has been referred to as "packing". Typically, mutagenesis studies indicate that the hydrophobic effect contributes 0.5 kcal mol^{-1} per methylene, while well-packed methylenes can contribute approximately twice as much (1.0 kcal mol^{-1} per methylene) to protein stability (Pace et al., 1996).

We have examined the **d** position of a dimeric leucine zipper coiled coil to explore the contribution of different hydrophobic amino acids to protein stability. The leucine zipper is a name coined to describe the dimerization domain (Landschultz et al., 1988) of the bZIP class of sequence-specific transcription factors (Vinson et al., 1989). The dimerization domain of bZIP proteins has a protein sequence suggesting an amphipathic α-helical structure with a near invariant heptad repeat of leucines (Alber, 1992; Baxevanis & Vinson, 1993; Lupas, 1996). X-ray crystal studies have demonstrated that the leucine zipper structure is a dimeric parallel coiled coil (O'Shea et al., 1991; Ellenberger et al., 1992), with the holes-and-knobs packing of the α-helices as predicted by Crick (1953) over 40 years ago. The X-ray structure indicates that the **d** position leucine interacts intrahelically with neighboring hydrophobic amino acids in the **a** position and interhelically with the **d** position on the opposite helix. Amino acids in the **e** and **g** positions, typically long charged amino acids, lie across the top of the **d** position with the methylenes essentially burying the **d** position (Alber, 1992). Analyses of coiled coil proteins, found primarily in the structural proteins of the cell, indicate that a variety of hydrophobic amino acids can occupy the **d** position and produce the characteristic amphipathic helices (Cohen & Parry, 1990), unlike the near invariance of leucine in the **d** position of bZIP transcription factors.

Several groups have explored the contribution of leucine in the **d** position of the leucine zipper of bZIP proteins to protein stability by examining DNA binding. These results demonstrate that leucine in the **d** position produces the tightest DNA binding (Landschultz et al., 1989; Kouzarides & Ziff, 1988). Genetic selection procedures have demonstrated that leucine and also other hydrophobic amino acids can function in the **d** position (Hu et al., 1990). Several groups have used a structure−function type analysis to explore the function of leucine in the **d** position using synthetic peptides. Using the leucine zipper from the yeast transcription factor GCN4, Kim's and Abler's groups have mutated all **d** position leucines to isoleucine or valine. The result is a change in the oligomerization of the peptide. Instead of forming a dimer, the peptides form trimers or tetramers (Harbury et al., 1993, 1994). Hodges' group, using another peptide system modeled after tropomyosin, explored the importance of leucine in the **d** position, and did not find a change in oligomerization but did find that leucine was 0.26 kcal mol^{-1} more stable than isoleucine (Zhu et al., 1993) and 1.5 kcal mol^{-1} more stable than alanine (Zhou et al., 1992a,b).

We have performed a structure−function analysis of the second and fifth **d** positions of the leucine zipper of a bZIP

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* Corresponding author. Telephone: (301) 496-8753. Fax: (301) 402-3095. E-mail: Vinsonc@dc37a.nci.nih.gov.

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domain. Seven proteins containing different aliphatic amino acids in the fifth **d** position remained dimeric when assayed by analytical ultracentrifugation. This has allowed us to determine that alanine is 4.6 kcal mol^{-1} per residue less stabilizing than leucine. The similarly sized amino acid isoleucine is 2.9 kcal mol^{-1} per residue less stabilizing than leucine. The order of stabilization is L, M, I, V, C, A, and S. This suggests that the reason leucine is so conserved in the leucine zipper region of bZIP proteins is because of the dramatic contribution it confers on dimerization stability.

MATERIALS AND METHODS

Host−Guest System. The sequence of the 96-amino acid host−guest protein is ASMTGGQQMGRDP-LEE-KVFVP-DEQKDEKYWTRRKK*N*NVAAKRS*R*DARRLKENQ **I**TI-RAAF ***L***EKENTA **L**RTEVAE **L**RKEVGR **X**RNIVSK **Y**ETRYGPL. The **d** positions of the leucine zipper coiled coil are shown in bold, and the position mutated in this study is presented as an **X**. The italicized ***L*** has been altered to A in the **d**$_2$A series of proteins. The invariant asparagine and arginine in the basic region are italicized. The first 16 amino acids are from ϕ10 (Studier & Moffatt, 1986) and a cloning linker, and the remaining 80 amino acids are the C terminus of VBP (Iyer et al., 1991), the chicken equivalent of mammalian TEF (Fonjallaz et al., 1996). These 80 amino acids contain the entire VBP bZIP region of the protein and are able to bind to DNA as a dimer in a sequence-specific manner (Vinson et al., 1993).

Protein Expression and Purification. Proteins were synthesized in *Escherichia coli* using the phage T7 expression system (Studier & Moffatt, 1986). Four hundred milliliter bacterial cultures at an optical density of 0.6 at 600 nm were induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 2 h. Cells were recovered by centrifugation, resuspended in 6 mL of lysis buffer [50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM benzamidine, 1 mM dithiothreitol (DTT), and 0.2 mM phenylmethanesulfonyl fluoride (PMSF)], frozen, thawed, and gently brought to 1 M KCl by the addition of 2 mL of 4 M KCl. The samples were centrifuged at 30 000 rpm in a Beckman Ty50.2Ti rotor, and the supernatant was isolated. The isolated supernatant was then heated to 65 °C for 10 min and centrifuged, and the supernatant was again isolated. The proteins were dialyzed to 20 mM Tris-HCl (pH 8.0), 100 mM KCl, 1 mM EDTA, and 1 mM DTT and loaded onto a heparin−agarose column. The column was washed with lysis buffer containing 100 mM KCl, followed by a 300 mM KCl wash, and eluted with buffer containing 1 M KCl. The samples were then dialyzed to 12.5 mM potassium phosphate (pH 7.4), 150 mM KCl, and 1 mM EDTA, with or without 1 mM DTT for 48 h with a change of buffer after 24 h. The proteins were 98% pure as assayed by SDS−PAGE (Laemmli, 1970).

Construction of Mutant Proteins. Amino acid substitution mutants were introduced into VBP by the four-primer PCR mutagenesis method (Ho et al., 1989). DNA sequencing was performed on double-stranded templates using the Sanger dideoxynucleotide method (Sanger et al., 1977).

Equilibrium Sedimentation. Equilibrium sedimentation measurements were performed using a Beckman XL-A Optima Analytical Ultracentrifuge equipped with absorbance optics and a Beckman An-60Ti rotor. Samples were loaded at three concentrations, 10, 20, and 40 μM (0.1, 0.2, and 0.4 OD at 280 nm), into a six-hole centerpiece and spun at 25 000 rpm for 24 h at 10 °C. Twenty data sets for three concentrations were jointly fit for a singular molecular mass. Some calculations assumed a monomer−dimer equilibrium. Compositional partial specific volumes for the proteins were calculated according to Zamyatnin (1984).

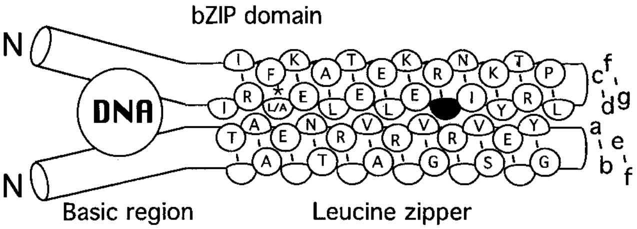

FIGURE 1: Schematic of a bZIP protein binding to DNA. The N terminus, basic region, and leucine zipper are shown. The coiled coil region is presented, without the supercoiling, with the relative placement of the seven amino acids in the coiled coil heptad represented by circles. Identified are the amino acids in the VBP leucine zipper region which extends to the natural C terminus. The black circle is the host−guest position which has been changed to seven different amino acids in this study, the **d**$_2$L**d**$_5$X series. The asterisk (*) marks the **d** position labeled L or A which was mutated to alanine to make the **d**$_2$A**d**$_5$X series of protein. To the right is the identification of the **a**−**g** positions of the coiled coil.

Circular Dichroism Experiments. All samples were in 12.5 mM potassium phosphate (pH 7.4), 150 mM KCl, and 1 mM EDTA. DTT (1 mM) was added to the sample just before thermal melting, and the sample was preheated to 65 °C for 5 min. Protein concentrations and T_m values were calculated as described before (Krylov et al., 1994) and converted to ΔG(37 °C) using a ΔC_p of −0.96 kcal mol^{-1} °C^{-1} calculated from a T_m vs ΔH plot for all the proteins used in this study. All thermal melts of reduced protein were reversible. The fitting of the oxidized **d**$_2$L**d**$_5$C protein was carried out assuming a monomolecular unfolding model.

RESULTS

Host−Guest System. We have used the native bZIP domain of vitellogenin binding protein (VBP) (Iyer et al., 1991; Krylov et al., 1994), the chicken homolog of the mammalian protein TEF (Fonjallaz et al., 1996), to investigate the contribution of aliphatic amino acids in the **d** position of the leucine zipper coiled coil to protein thermal stability. The protein sample is 96 amino acids long; the 80 C-terminal amino acids contain the bZIP domain of VBP. The bZIP domain is a long bipartite α-helix when bound to DNA. The N-terminal half consists of basic amino acids and is essential for sequence-specific DNA binding. The C-terminal half is the leucine zipper dimerization domain and consists of an amphipathic helix with a heptad repeat of leucines. Figure 1 is a schematic of the bZIP protein used for this study. The fifth **d** position, which is shown in black, is the position where we placed seven amino acids (L, M, I, V, C, A, and S; the **d**$_2$L**d**$_5$X series). To confirm our results, we used the same series of seven amino acids in a different leucine zipper background where the second **d** position contains an alanine (the **d**$_2$A**d**$_5$X series). This allowed us to validate the results in a separate experimental setting. Table 1 presents the nomenclature used to describe the 14 proteins used in this study.

Previous circular dichroism experiments with the VBP bZIP domain (Krylov et al., 1994) have shown a change in ellipticity, measured at 222 nm, from an α-helical structure

Table 1: Nomenclature of Protein Variants[a]

coiled	defgabc	defgabc	defgabc	defgabc	defgabc	defgabc-
coil	1	2	3	4	5	
proteins	leucine zipper protein sequence					
d$_2$L**d**$_5$L	ITIRAAF	LEKENTA	LRTEVAE	LRKEVGR	LRNIVSK	YETRYGPL-
d$_2$L**d**$_5$C*		L			C	
d$_2$L**d**$_5$M		L			M	
d$_2$L**d**$_5$I		L			I	
d$_2$L**d**$_5$V		L			V	
d$_2$L**d**$_5$A		L			A	
d$_2$L**d**$_5$S		L			S	
d$_2$A**d**$_5$L		A			L	
d$_2$A**d**$_5$C		A			C	
d$_2$A**d**$_5$M		A			M	
d$_2$A**d**$_5$I		A			I	
d$_2$A**d**$_5$V		A			V	
d$_2$A**d**$_5$A		A			A	
d$_2$A**d**$_5$S		A			S	

[a] The **a**−**g** positions within a coiled coil and the numbering of the **d** positions of the leucine zipper are shown. The amino acid sequence of the VBP leucine zipper is presented. The proteins are named according to the amino acids in the second and fifth **d** positions. The first series of mutants has leucine in the second position and seven different residues in the fifth **d** position (**d**$_2$L**d**$_5$X). The series is repeated with alanine in the second **d** position (**d**$_2$A**d**$_5$X). *Wild type VBP is **d**$_2$L**d**$_5$C with a lysine in the fifth **e** position.

at low temperatures to nonhelical structure at higher temperatures, a result also seen for the bZIP domain of GCN4 (Thompson et al., 1993). Analytical ultracentrifugation experiments at different temperatures revealed a change in oligomerization from a dimer at low temperatures to a monomer at higher temperatures. The combination of analytical ultracentrifugation and circular dichroism data indicates that there is a transition between α-helical dimers at low temperatures and nonhelical monomers at high temperatures (Krylov et al., 1994). The parental VBP protein, **d**$_2$L**d**$_5$C, has a melting temperature (T_m) of 50 °C in a buffer of 12.5 mM phosphate, 150 mM KCl, 1 mM EDTA, and 1 mM DTT, the same T_m's observed for the bZIP proteins GCN4 (Thompson et al., 1993), C/EBP (Krylov et al., 1995), and the heterodimer Fos/Jun (Olive et al., 1997). This T_m allowed us to mutate amino acids in the **d** position of the leucine zipper region but have the T_m's remain in a range where we were able to identify clear and reversible low- and high-temperature base lines ($T_m \approx 20-75$ °C). This feature permitted us to calculate the Gibbs free energy (ΔG) for the variant proteins.

The VBP leucine zipper contains a cysteine in the fifth **d** position (Figure 1), which we suggest is part of the α-helical coiled coil structure. The ellipticity of our sample indicates that 60% of the protein is α-helical, which we suggest extends from the C terminus into the basic region. This **d** position is flanked by valines in both adjoining **a** positions, a canonical amino acid for the **a** position of leucine zippers (Cohen & Parry, 1990; Hurst, 1995). There is an E ↔ R electrostatic pair in the **g** ↔ e′ ($i,i' + 5$) position, suggesting that the **d** position is covered by methylenes from these long amino acids as predicted from the X-ray crystal structure of the GCN4 and Fos/Jun leucine zipper (O'Shea et al., 1991; Ellenberger et al., 1992; Glover & Harrison, 1995). We produced seven proteins differing only in the amino acid in the **d** position in the fifth heptad (**d**$_2$L**d**$_5$X), where we introduced L, M, I, C, V, A, and S, primarily aliphatic amino acids.

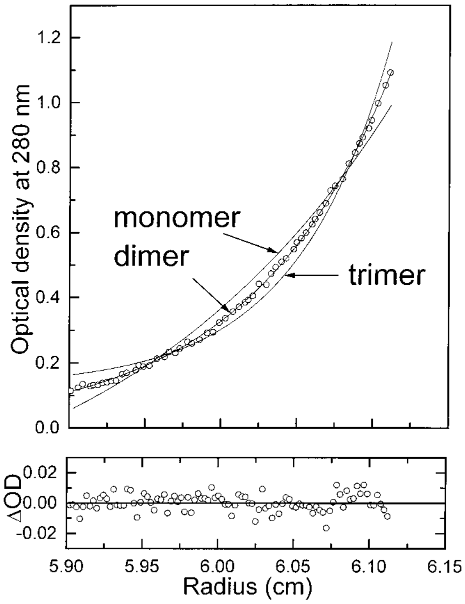

FIGURE 2: Sedimentation equilibrium determination of the molecular mass of **d**$_2$L**d**$_5$I at 10 °C in 12.5 mM potassium phosphate (pH 7.4), 150 mM KCl, and 1 mM EDTA. Theoretical curves for the monomer, dimer, or trimer are plotted as solid lines. The data are plotted as circles which fit onto the dimer curve. The bottom panel shows the residual plots of fitting the experimental data to a dimer model. No systematic error is evident. The dimer molecular mass is 22 532 Da, and the calculated mass is 22 200 Da.

Figure 2 presents the analytical ultracentrifugation data for the isoleucine protein **d**$_2$L**d**$_5$I. The raw data are shown with the theoretical fits for the monomer, dimer, and trimer presented. At the bottom of the figure are the residuals of the data fit to the calculated dimeric molecular mass. The molecular mass of a dimeric **d**$_2$L**d**$_5$I is 22 532 Da which agreed with the mass of 22 200 Da calculated from the analytical ultracentrifugation data. Table 2 presents the analytical ultracentrifugation data for the seven proteins of the **d**$_2$L**d**$_5$X series. All are dimeric, suggesting that, in the host−guest system we are using, mutating a single amino acid in the fifth **d** position does not modulate oligomerization. This allows us to determine the contribution of individual amino acids relative to alanine to the stability of dimeric leucine zippers ($\Delta\Delta G_A$).

Thermal Stability of the Variant Proteins. The thermal stability (T_m) and calculated thermodynamic parameters (ΔG and ΔH) are presented for the 14 variant proteins, as determined by thermal denaturation monitored by circular dichroism at 222 nm (Table 2). Figure 3 presents the thermal denaturation curves for the seven proteins in the **d**$_2$L**d**$_5$X series, whose T_m's ranged from 70.4 to 41.9 °C, a range of 9.7 kcal mol^{-1}. They all have similar low-temperature ellipticities (θ), indicating that the mutations did not dramatically alter the α-helical nature of the dimeric sample. The **d**$_2$A**d**$_5$X series had a T_m range from 59.6 to 17.0 °C, which represents a range of 9.6 kcal mol^{-1}. The thermal denaturations are fit to a model which assumes a linear low-temperature sloped base line and a horizontal high-temperature base line; the length of the base lines used in the calculations is noted by the length of the fitted curve (Figure 3). The slope of the low-temperature base line is

Table 2: Thermodynamic and Biophysical Characterization of Protein Variants[a]

protein	amino acid	T_m (°C)	$\Delta H(T_m)$ (kcal mol^{-1})	ΔG(37 °C) (kcal mol^{-1})	$\Delta\Delta G_A$ for the dimer (kcal mol^{-1})	$\Delta\Delta G_A$ for the monomer (kcal mol^{-1})	MW (kDa)
$\mathbf{d}_2$L$\mathbf{d}_5$L	Leu	70.4 ± 0.02	−113 ± 0.7	−18.7	−9.2	−4.6	21.1
$\mathbf{d}_2$L$\mathbf{d}_5$M	Met	55.6 ± 0.02	−102 ± 0.4	−13.5	−4.0	−2.0	18.7
$\mathbf{d}_2$L$\mathbf{d}_5$I	Ile	55.5 ± 0.02	−90 ± 0.3	−12.8	−3.3	−1.65	22.2
$\mathbf{d}_2$L$\mathbf{d}_5$V	Val	49.6 ± 0.03	−103 ± 0.8	−11.7	−2.2	−1.1	17.3
$\mathbf{d}_2$L$\mathbf{d}_5$C	Cys	49.5 ± 0.02	−95 ± 0.4	−11.4	−1.9	−0.95	21.9
$\mathbf{d}_2$L$\mathbf{d}_5$A	Ala	43.0 ± 0.03	−81 ± 0.4	−9.5	0.0	0.0	21.7
$\mathbf{d}_2$L$\mathbf{d}_5$S	Ser	41.9 ± 0.02	−83 ± 0.3	−9.0	+0.5	+0.25	15.0
$\mathbf{d}_2$A$\mathbf{d}_5$L	Leu	59.6 ± 0.03	−100 ± 0.6	−14.5	−9.0	−4.5	
$\mathbf{d}_2$A$\mathbf{d}_5$M	Met	44.6 ± 0.04	−72 ± 0.5	−9.4	−3.9	−1.95	
$\mathbf{d}_2$A$\mathbf{d}_5$I	Ile	43.8 ± 0.5	−65 ± 0.4	−9.1	−3.6	−1.8	
$\mathbf{d}_2$A$\mathbf{d}_5$V	Val	37.4 ± 0.08	−60 ± 0.5	−7.8	−2.3	−1.15	
$\mathbf{d}_2$A$\mathbf{d}_5$C	Cys	36.8 ± 0.04	−61 ± 0.3	−7.7	−2.2	−1.1	
$\mathbf{d}_2$A$\mathbf{d}_5$A	Ala	26.4 ± 0.05	−60 ± 0.3	−5.5	0.0	0.0	
$\mathbf{d}_2$A$\mathbf{d}_5$S	Ser	17.0 ± 0.7	−41 ± 0.9	−4.9	+0.6	+0.3	

[a] Thermodynamic parameters for unfolding were determined by reversible thermal denaturation using circular dichroism at 222 nm to monitor α-helical content. The buffer is 12.5 mM potassium phosphate (pH 7.4), 150 mM KCl, and 1mM EDTA. The two cysteine-containing proteins were preheated to 65 °C in the presence of 1 mM DTT. The table is organized into two parts; the top seven proteins contain a leucine in the second **d** position, and the bottom seven proteins contain an alanine in the second **d** position. Within each section, proteins are organized in terms of thermal stability. The following parameters are presented: melting temperature and the error from the fitting procedure (T_m), dimerization van't Hoff enthalpy at $T = T_m$ and the error from the fitting procedure [$\Delta H(T_m)$], dimerization free energy extrapolated to $T = 37$ °C [ΔG(37 °C)], stability relative to the alanine protein dimer ($\Delta\Delta G_A$), stability relative to the alanine protein monomer ($\Delta\Delta G_A$), and molecular mass determined by sedimentation equilibrium (MW). All the samples reported here have a similar ellipticity at low temperatures, suggesting that they are dimeric before thermal denaturation and the mutation does not cause a drastic change in the α-helical structure.

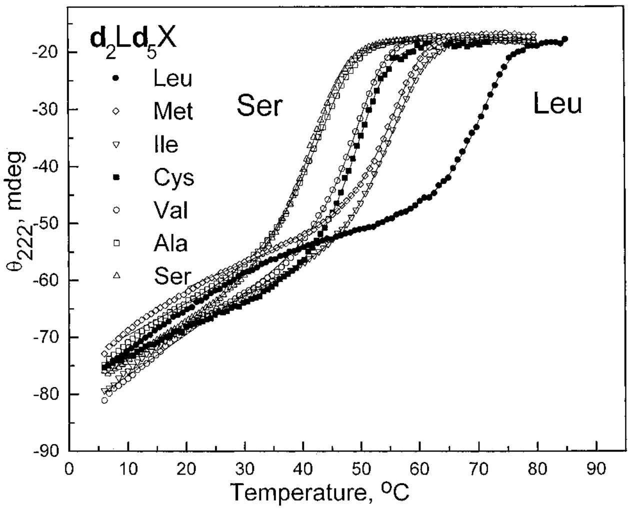

FIGURE 3: Thermal denaturation of proteins. CD thermal melting curves for the $\mathbf{d}_2$L$\mathbf{d}_5$X series where the X position contains one of the following amino acids: L, M, I, C, V, A, or S. The line through each of the seven labeled curves is a fitted curve assuming a two-state transition model. Note that the initial base line ellipticity for each of the curves is similar, indicating that the mutagenesis did not alter the gross α-helical structure of the sample.

assumed to reflect a change in α-helical signal with increased temperatures. We suggest that at low temperatures, the basic region is partially α-helical, and it is the unfolding of the basic regions that contributes to the steep base line below 40 °C. The low-temperature base line for $\mathbf{d}_2$L$\mathbf{d}_5$L, a protein with a high T_m, is fit well by the following function: $a + b(1 - e^{-kT})$. When this function was used to describe the low-temperature base line in the fitting procedure, ΔG(37 °C) for the $\mathbf{d}_2$L$\mathbf{d}_5$X series is lower by 0.1−0.4 kcal mol^{-1}.

Mutating leucine to alanine was destabilizing by 4.6 kcal mol^{-1}, when calculated per monomer or individual amino acid. Using alanine as a base line, valine and cysteine are −1 kcal mol^{-1} more stabilizing. Isoleucine and methionine, which contain larger hydrophobic side chains, are 1.65 and 2.0 kcal mol^{-1} more stabilizing than alanine, respectively.

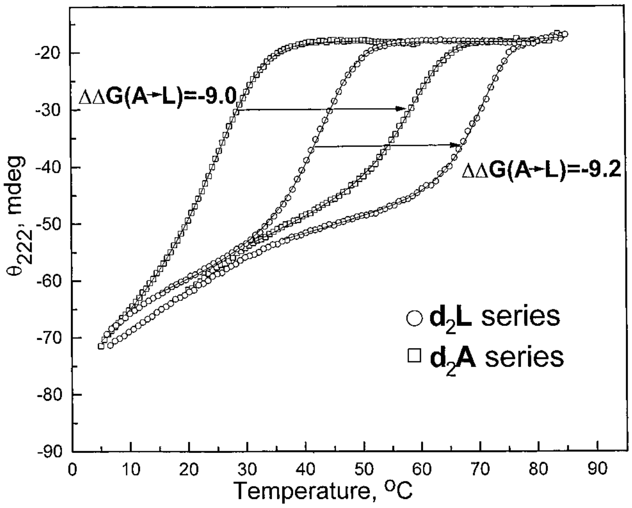

FIGURE 4: Thermal denaturation of both the $\mathbf{d}_2$L$\mathbf{d}_5$X (circles) and $\mathbf{d}_2$A$\mathbf{d}_5$X (squares) series. The thermal denaturation of the most stable proteins of each series ($\mathbf{d}_2$L$\mathbf{d}_5$L, $T_m = 70$ °C; $\mathbf{d}_2$A$\mathbf{d}_5$L, $T_m = 60$ °C) is compared against the alanine mutants ($\mathbf{d}_2$L$\mathbf{d}_5$A, $T_m = 43$ °C; $\mathbf{d}_2$A$\mathbf{d}_5$A, $T_m = 26$ °C).

There is a rough correlation between larger hydrophobic side chains being more stabilizing. The clear outlier in this general trend is leucine, being 2.95 kcal mol^{-1} more stabilizing than isoleucine even though the side chains of leucine and isoleucine contain the same number of methylenes.

The $\mathbf{d}_2$A$\mathbf{d}_5$X series of proteins, whose T_m's ranged from 59.6 to 17.0 °C, produced the same differences in stability between corresponding mutants as those obtained from the $\mathbf{d}_2$L$\mathbf{d}_5$X series. Figure 4 compares the energetic consequences of an alanine or leucine in the $\mathbf{d}_5$ position for both the $\mathbf{d}_2$L and $\mathbf{d}_2$A series. The difference in stability ($\Delta\Delta G$) is 9.2 kcal mol^{-1} for the $\mathbf{d}_2$L series and 9.0 kcal mol^{-1} for the $\mathbf{d}_2$A series. This indicates that the absolute T_m of our proteins is not confounding our results and argues for the

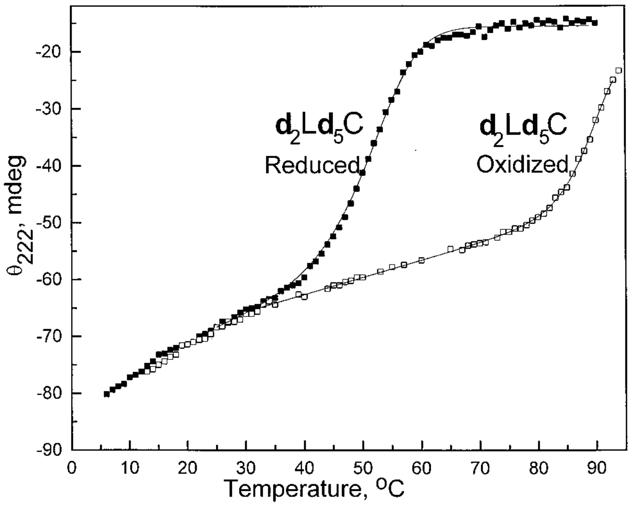

FIGURE 5: Thermal denaturation of either reduced or oxidized **d**$_2$L**d**$_5$C. The line through each curve is a fitted curve as described in Materials and Methods. Note that the initial base line ellipticity for each of the curves is similar, indicating that forming the disulfide did not alter the gross α-helical structure of the sample.

validity of the thermodynamic calculations. We can also conclude that the possible structural perturbation from the leucine to alanine mutation in the **d** position of the second heptad of the leucine zipper does not influence the energetic contribution of individual amino acids in the fifth **d** position.

The oxidation of the cysteine in the **d** position (**d**$_2$L**d**$_5$C) does not affect the ellipticity of the sample, but it does dramatically increase the thermal stability from 50 to around 94 °C (Figure 5). Again, the inflection of the low-temperature base line is seen for the oxidized **d**$_2$L**d**$_5$C protein, which we attribute to a nonlinear change in ellipticity with temperature. Calculations indicate that, for the **d**$_2$L**d**$_5$L protein to have a T_m of 94 °C, the concentration would have to be 400 mM. Hodges' group has also shown that oxidizing the **d** position increases the stability of the coiled coil structure (Zhou et al., 1993).

DISCUSSION

We have used the bZIP domain of VBP, which is capable of sequence-specific DNA binding, to comprehensively examine the contribution of seven amino acids in the **d** position to leucine zipper thermal stability. Crystal structures show that amino acids in the **d** position of leucine zipper proteins are buried in the hydrophobic interhelical interface (Alber, 1992; O'Shea et al., 1991). This position would therefore be expected to contribute to protein stability through the hydrophobic effect and van der Waals interactions with neighboring residues. Our data show that leucine is the most favored amino acid in this position, being 2.9 kcal mol^{-1} more stabilizing that the similarly sized amino acid isoleucine, indicating that leucine is uniquely suited to hydrophobically packing in the **d** position of a coiled coil.

We have analyzed the data by examining the energetic contributions from three forces: the hydrophobic effect, α-helical propensities, and packing or van der Waals interactions. The hydrophobic effect should be invariant for a particular amino acid provided it is completely buried in the folded state. The energetic contribution of the hydrophobic effect we used was from *n*-octanol to water partition coefficients (Fauchere & Pliska, 1983). This is termed the transfer free energy ($\Delta G_{\text{transfer}}$). The second term is the α-helix-forming propensity which reflects the inherent tendency of an amino acid to be in an α-helix (O'Neil & DeGrado, 1990). The remaining energy reflects the packing of the side chain with its neighbors. We have subtracted the α-helix propensity from our measured protein stability of the mutant proteins to determine the contribution to the stability of the different amino acids in the **d** position, relative to alanine (Table 3). The relative magnitude of these three forces is different. The transfer free energy varies from 0.05 kcal mol^{-1} for serine to −2.46 kcal mol^{-1} for isoleucine, the same general degree of magnitude seen for the packing free energy which we calculate varies from 0.19 kcal mol^{-1} for cysteine to −2.85 kcal mol^{-1} for leucine. The variation in helix-forming propensity is much smaller, varying from 0.0 for alanine to the 0.63 for valine. The energetic dependence between helix propensity and packing is not clear, but our data indicate that the magnitudes of the observed effects are too great to be accounted for solely by helix propensities.

Several investigators have examined the contribution of hydrophobic amino acids to protein stability by replacing large amino acids with smaller ones. The energy difference between leucine or isoleucine and alanine (Shortle et al., 1990; Eriksson et al., 1992; Serrano et al., 1992; Pace et al., 1996) is 4−5 kcal mol^{-1}, with both amino acids contributing similarly to protein stability. This is twice what would be expected for transfer free energies (representing hydrophobicity) alone. This additional energy has been attributed to van der Waals interactions between the amino acid side chains and neighboring amino acids in the protein interior.

Matthews' group determined the crystal structures of their mutants in phage T4 lysozyme and found that the cavity size resulting from deletion of a side chain correlated with the contribution of the amino acid to protein stability (Eriksson et al., 1992). The larger the cavity, the greater the energetic cost of the mutation. Extrapolation of their results to a zero cavity size gave an energy of 1.9 kcal mol^{-1} which is similar to the transfer free energy for a leucine to alanine mutation (Eriksson et al., 1992). This represents a situation in which the protein rearranges to accommodate the absence of the leucine side chain. A protein which showed no rearrangement when the leucine was mutated to alanine contained the biggest cavity. In this case, the alanine protein was 5.0 kcal mol^{-1} less stable than the leucine protein.

The contribution of 5.0 kcal mol^{-1} relative to alanine for a leucine side chain in a protein that undergoes no structural rearrangement is similar to the value of 4.6 kcal mol^{-1} that we observe for a leucine to alanine change in the fifth **d** position of VBP. This suggests that the leucine zipper structure does not rearrange to close the cavity created by the leucine to alanine mutation. This is not particularly surprising, since rearrangement of the leucine zipper would require energetically unfavorable deformation of the α-helices comprising the coiled coil dimer.

The β-branched amino acids isoleucine and valine display significantly less packing energy than leucine, although they are of similar sizes. One difference between these amino acids is that the β-branched amino acids are conformationally constrained in α-helices; there is only one preferred orientation for the side chain (Ponder & Richards, 1987). Molecular modeling indicates that the favored rotamer conformation for both isoleucine (−,t) and valine (t) in the **d** position

Table 3: Estimation of the van der Waals Contacts for Amino Acids in the **d** Position[a]

amino acid	$\Delta\Delta G_A$ for the monomer (kcal mol^{-1})	$\Delta\Delta G_{helix}$ (kcal mol^{-1})	$\Delta\Delta G_A - \Delta\Delta G_{helix}$ (kcal mol^{-1})	$\Delta\Delta G_{transfer}$ (kcal mol^{-1})	$\Delta\Delta G_{packing}$ (kcal mol^{-1})
Leu	−4.6	0.15	−4.75	−2.32	−2.85
Met	−2.0	0.27	−2.27	−1.68	−1.01
Ile	−1.65	0.54	−2.19	−2.46	−0.25
Val	−1.1	0.63	−1.73	−1.66	−0.49
Cys	−0.95	0.54	−1.49	−2.10	0.19
Ala	0.0	0.0	0.0	−0.42	0.0
Ser	0.25	0.42	−0.17	0.05	−0.64

[a] Thermodynamic parameters were taken for the $\mathbf{d}_2$L$\mathbf{d}_5$X series. $\Delta\Delta G_A$ is the free energy of unfolding for a single amino acid in the monomer relative to alanine. $\Delta\Delta G_{helix}$ is the helical propensity of amino acids relative to alanine (O'Neil & DeGrado, 1990). $\Delta\Delta G_{transfer}$ is the change in the solvent transfer free energy relative to alanine (Fauchere & Pliska, 1983). The difference between $\Delta\Delta G_A$ and the sum of $\Delta\Delta G_{transfer}$ and $\Delta\Delta G_{helix}$ is defined as the $\Delta\Delta G_{packing}$.

produces a steric clash between the Cγ2 methyl groups from the two amino acids on opposite helices. The Cγ2 atoms were 2.02 Å away from each other. The energetic cost of changing the rotamer conformation to avoid a steric clash may account for part of the 2.9 kcal mol^{-1} difference in energy between leucine and isoleucine.

Hodges' group has examined the energetic contribution of hydrophobic amino acids in the **d** position to coiled coil stability, using a 35-amino acid long peptide modeled after tropomyosin. In their system, replacing a leucine with alanine decreases stability 1.5 kcal mol^{-1} (Zhou et al., 1992a,b), significantly less than the 4.6 kcal mol^{-1} we observe. On the basis of Matthews' data, the result with the Hodges' model tropomyosin peptide could be attributed to transfer free energy (1.9 kcal mol^{-1}). The heptad repeat is (L−E−A−L−E−G−K)$_n$ from **a** to **g**. The peptide contains a glycine in the **f** position, which might make the α-helices more flexible, allowing the peptide to rearrange and close the cavity created by the leucine to alanine mutation. The loss of stability caused by the mutation was slightly greater (2.0 kcal mol^{-1}) when the peptide was cross-linked and therefore somewhat more constrained (Zhou et al., 1992a,b). Additionally, they observe that leucine is only 0.15 kcal mol^{-1} more stabilizing that isoleucine while we observe a 3.0 kcal mol^{-1} difference (Zhu et al., 1993). A similarity between the two data sets is seen in the isoleucine and valine amino acids. We observe that isoleucine is 0.55 kcal mol^{-1} more stable, while Hodges reports a similar value of 0.72 kcal mol^{-1} (Zhu et al., 1993). The dramatic disparity between our results and those of Hodges suggests that the structural assumption that we are examining the same **d** position is probably wrong. The **d** position examined by Hodges may reflect a position more akin to the second **d** position we observe in VBP.

Comparing the $\mathbf{d}_2$A$\mathbf{d}_5$X series and the $\mathbf{d}_2$A$\mathbf{d}_5$X series of proteins allows us to compare leucine or alanine in two structurally different **d** positions. In the fifth **d** position, the difference in stability between a leucine and alanine ($\mathbf{d}_2$L$\mathbf{d}_5$L and $\mathbf{d}_2$L$\mathbf{d}_5$A) is 4.6 kcal mol^{-1}. In this case, leucine is completely buried and presumably well-packed. In the second **d** position, the difference in stability ($\mathbf{d}_2$L$\mathbf{d}_5$X and $\mathbf{d}_2$A$\mathbf{d}_5$X, where X is any amino acid) is only 2.1 kcal mol^{-1}. This implies that, in this position, either the leucine is not as well-packed or the protein rearranges to compensate for the mutation. An important structural difference between the second and fifth **d** positions examined in this study is in the flanking **a** positions. The fifth **d** position is flanked in the **a** position by two valines which interact with the **d** position in the X-ray structure. In contrast, the second **d** position is flanked by an alanine and asparagine. The loss of an *i,i*+3 leucine−valine interaction has been shown experimentally (Padmanabhan & Baldwin, 1994) and theoretically (Creamer & Rose, 1995) to be energetically costly. This is likely to result in a lower packing energy for the second **d** position leucine. Additionally, the presence of the asparagine may disrupt the 0.5 kcal mol^{-1} coupling energy for the interhelical **g**↔**e**′ interaction between R and E across the hydrophobic interface. The methylenes of the R and E may be less well-packed with the leucine. Alternatively, the fewer structural constraints in this heptad may also allow some structural rearrangement.

VBP contains a single cysteine in the **d** position near the C terminus of the leucine zipper region. This cysteine can be oxidized by raising the T_m by 46 °C to form more stable dimers than the reduced protein. Oxidized and reduced proteins display identical helicity at low temperatures. A disulfide bond between cysteines in the **d** position has previously been shown not to disrupt the helix-forming capacity of coiled coils (Zhou et al., 1993). The biological occurrence of this disulfide is an intriguing question. The formation of the disulfide could stabilize the binding to DNA and regulate gene expression. Alternatively, the presence of the disulfide could regulate the exchange of leucine zippers that are able to heterodimerize when reduced. Using our system, which is stabilized by disulfide bond formation, we can address these biological issues.

The question of the specificity of dimerization being controlled by amino acids in the **d** position is an interesting one. At present, only amino acids in the **e** and **g** positions (Lupas, 1996; Krylov et al., 1994) are known to control specificity of leucine zipper dimerization.

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