The leucine zipper (LZ) domain of the HY5 transcription factor from *Arabidopsis thaliana* has unique primary structural properties, including major occupation by the Leu residues as well as two buried polar residues in the α positions and a localized distribution of charged and polar residues in the first three heptad repeats. In this study, we solved the crystal structure of the HY5 LZ domain and show that the peculiarities in the primary sequence yield unusual structural characteristics. For example, the HY5 LZ domain exhibits a bipartite charge distribution characterized by a highly negative electrostatic surface potential in its N-terminal half and a nearly neutral potential in its C-terminal half. The LZ N-terminal region also contains two consecutive putative trigger sites for dimerization of the coiled coil. The HY5 LZ domain also contains two potential in its N-terminal half and a nearly neutral potential in its C-terminal half. The LZ N-terminal region also contains two consecutive putative trigger sites for dimerization of the coiled coil. In addition, two buried asparagines at α positions 19 and 33 in the HY5 LZ domain display distinct modes of polar interaction. Whereas Asn shows a conformational flip-flop, Asn is engaged in a permanent hydrogen bond network. CD spectropolarimetry and analytical ultracentrifugation experiments performed with versions of the HY5 LZ domain containing mutations in the α positions yielded further evidence that position α amino acid residues are crucial for achieving an oligomeric state and maintaining stability. However, a low correlation between position α amino acid preference, core packing geometry, and rotamer conformations suggests that the oligomeric state of the LZ domain is not governed entirely by known structural properties. Taken together, our results suggest structural factors conferring conformational integrity of the HY5 LZ homodimer that are more complicated than proposed previously.

A major challenge that faces protein chemists is the ability to predict the three-dimensional structures of proteins from their primary amino acid sequences. Because of its simplicity and periodicity, the coiled-coil motif is an ideal model system with which to investigate properties of the protein folding process. In terms of biological function, the coiled coil is one of the most common structural motifs and participates in many cellular processes through the formation of protein-protein interactions.

Among coiled-coil proteins, the basic leucine zipper (bZIP) proteins play crucial roles in the regulation of transcription. These proteins bind to their cognate DNA elements through their basic regions as they dimerize via their leucine zipper (LZ) domains. The LZ domain consists of an α-helix that contains a series of leucine residues on one face. Two LZ α-helices intertwine to produce a dimeric structure with a slight superhelical twist. The LZ domain consists of heptad sequence repeats, designated nearest neighbors, where positions α and δ create the interface between two LZ strands by engaging in hydrophobic interactions, and positions e and g flank the dimer interface by forming electrostatic interactions. The various amino acid positions of the LZ heptad repeat have been shown to play unique roles in determining the specificity (homodimeric) (1, 3–5), orientation (parallel or antiparallel) (6), oligomeric state (7), and stability of the LZ domain (3, 7).

The recently sequenced *Arabidopsis thaliana* genome (11) contains 75 genes that encode members of the bZIP transcription factor family (12). Compared with those of human bZIP proteins, the primary structures of *A. thaliana* LZ domains share unique characteristics, including longer heptad repeats and abundant Asn residues in position α (13). It has been suggested that these unique properties might specify LZ dimers in ways that differ from mammalian bZIP proteins. Dimerization of *A. thaliana* LZ domains was predicted to be specified by the unique distribution of Asn residues in position α, not by electrostatic interactions between positions e and g in human LZ motifs (13). The *Arabidopsis* bZIP protein HY5 is a positive regulator of photomorphogenesis (14). HY5 is constitutively nuclear localized to be involved in light regulation of the transcriptional activity of promoters containing the G-box light-responsive element (15). Interaction of HY5 with G-box elements is expected to be mediated by the C-terminal region of the protein, which contains a bZIP domain. The HY5 LZ domain shares common structural features with other *Arabidopsis* LZ motifs such as the presence of two Asn residues in position α (13). In addition, HY5 displays some primary structural features that differ from those of other *Arabidopsis* bZIP proteins: major occupation by leucines of the hydrophobic residues (three of 13).
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four) of position $a$ and localized distribution of the charged and polar residues in the first three heptad repeats. Thus, we hypothesized that classical notions of dimerization specificity and stability might not apply to HY5 LZ dimers. To investigate the structural basis for the conformational integrity of the HY5 LZ homodimer, we determined its crystal structure at 2.0 Å and characterized the functions of position $a$ amino acids using CD spectropolarimetry and analytical ultracentrifugation. This is the first LZ structure among the Arabidopsis LZ motifs.

**EXPERIMENTAL PROCEDURES**

**Peptide Expression and Purification**—N-terminally acetylated synthetic peptides were purchased from Peptron, Inc. (Daejeon, South Korea). The molecular mass and purity of each peptide were verified by mass spectrometry and analytical high performance liquid chromatography.

**Cloning and Mutagenesis**—To obtain the wild-type (WT) LZ domain of HY5 (amino acids 111–150), PCR was performed using, as a template, the A. thaliana gene that encodes full-length HY5. PCR fragments generated from the HY5 gene were inserted into the pGEX4T3 vector (Amersham Biosciences) between the BamHI and XhoI restriction sites.

For the production of mutant LZ domains, mutations were introduced using the QuickChange site-directed mutagenesis kit (Stratagene). Mutations were introduced by PCR using, as a template, the WT LZ domain-containing pGEX4T3-based vector described above. PCR was followed by digestion with DpnI to digest the methylated parental plasmid. The resulting DNA was transformed into XL10 competent cells. All mutant plasmid constructs were verified by DNA sequencing.

**Peptide Synthesis**—Peptide synthesis—N-terminally acetylated synthetic peptides were purchased from Peptron, Inc. (Daejeon, South Korea). The molecular mass and purity of each peptide were verified by mass spectrometry and analytical high performance liquid chromatography.

The selenomethionine (SeMet)-containing WT HY5 LZ domain was prepared from E. coli strain BL21 grown in M9 medium (16) supplemented with 2 mM MgSO$_4$, 0.4% (w/v) glucose, and 0.1 mM CaCl$_2$. When this culture reached $A_{600} \sim 0.8$ at 37 °C, 100 mg each of Leu, Ile, Val, Thr, Phe, and Lys and 50 mg of l- (+)-SeMet (Sigma) were added per liter of growth medium. The culture was incubated for an additional 20 min at 37 °C, and the cells were then induced to express the recombinant proteins by the addition of 1 mM isopropyl β-D-thiogalactopyranoside. Protein production was allowed to proceed for 4 h at 37 °C, after which the proteins were purified as described above. When the SeMet-containing version of the WT HY5 LZ domain was purified, 5 mM dithiothreitol was added to the buffers used for a native sample preparation. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry demonstrated $>95\%$ substitution of one Met residue with SeMet.

**CD Spectropolarimetry**—CD measurements were performed on a Jasco J-715 spectrometer with $\sim 100 \mu M$ protein samples in 5 mM sodium phosphate (pH 7.4) or 5 mM sodium acetate (pH 4.0) at the Mokpo National University Central Laboratory (Mokpo, South Korea) or at the Korea Basic Science Institute (Ochang, South Korea). A cuvette with a 1-mm path length was used. Each spectrum is the result of the averaging of five consecutive scans. A separate spectrum was generated for the buffer alone, and this spectrum was subtracted from spectra taken in the presence of protein. For thermal denaturation profiles, ellipticity at 222 nm (which allowed us to inspect the peptide backbone region) was measured for each protein construct over a linear temperature gradient of 5–90 °C at a constant scan rate of 1 °C/min. The reversibility of the thermal denaturation was determined by monitoring the return of the CD signal at 222 nm upon cooling from 90 to 5 °C immediately after the thermal unfolding experiments. The thermal transitions were $>95\%$ reversible for all recombinant proteins except HM12 and HM13 in 5 mM sodium acetate (pH 4.0). The helicity was estimated with the following equation: % $\alpha$-helicity = $-([\theta]_{222}^MRW + 2340)/30,300 \times 100$ (where $[\theta]_{222}^MRW$ is the mean residue ellipticity at 222 nm) (17).

**Sedimentation Equilibrium**—Sedimentation equilibrium experiments with the WT and mutant versions of the HY5 LZ domain was performed at 20 °C in a ProteomeLab XL-A analytical ultracentrifuge (Beckman Coulter, Inc.) using an An-60 Ti rotor and 6-channel centripieces in the Mokpo National University Central Laboratory. All peptides were dissolved in buffer containing 5 mM sodium phosphate (pH 7.4) or 5 mM sodium acetate (pH 4.0). Samples with an initial $A_{280}$ of $\sim 0.13$ ($\sim 100 \mu M$) were centrifuged at 30,000 and/or 35,000 rpm. Radial absorbance scans for 10 replicates were collected in continuous scan mode at 280 or 275 nm with a step size of 0.001 cm and a time interval of 4 h. Global fitting of data sets was performed with the program UltraScan Version 8.0 (18).

**Crystallization**—WT HY5 LZ crystals were grown at 23 °C by the hanging-drop vapor-diffusion method, mixing 7.5 mg/ml peptide with an equal volume of reservoir solution containing 0.1 M BisTris (pH 6.0), 0.1 M MgCl$_2$, and 25% (w/v) polyethylene glycol 3350. Crystals were equilibrated in cryoprotectant buffer containing reservoir buffer plus 20% (v/v) glycerol and flash-frozen in liquid nitrogen. The crystals belong to space group...
C2, with \( a = 48.4 \, \text{Å}, b = 24.5 \, \text{Å}, c = 76.3 \, \text{Å}, \alpha = \gamma = 90^\circ, \) and \( \beta = 100^\circ. \)

**Data Collection and Structure Determination**—The diffraction data were collected at the 4A and 6B beam lines of the Pohang Accelerator Laboratory and processed using the HKL package (see Table 1). The three-dimensional structure was determined by combining the three-wavelength anomalous dispersion data set from an SeMet-containing crystal and by single isomorphous replacement analysis using the SeMet crystal as a derivative. Two ordered selenium sites were identified from remote and native data sets using the program SAPI (19). Phasing and density modifications were carried out using MLPHARE (20) and RESOLVE (21). Ninety percent of the amino acid residues were placed by the automatic modeling procedure of RESOLVE. Iterative cycles of manual model building and refinement were carried out using the programs O (22) and CNS (23), respectively. All non-glycine amino acid residues were located in the most favored region of the Ramachandran plot calculated with the refined model.

**RESULTS**

**Overall Structure of the HY5 LZ Homodimer**—A peptide corresponding to the LZ fragment (residues 111–150) of Arabidopsis HY5 with additional N-terminal Gly and Ser residues was crystallized using the hanging-drop vapor-diffusion method. The HY5 LZ crystals belong to space group C2 and contain two monomers in the asymmetrical unit with a dimer formed around the crystallographic 2-fold symmetry axis. The HY5 LZ crystal structure was determined by multiwavelength anomalous dispersion methods using a peptide containing SeMet. Diffraction data were collected to 2.0 Å from crystals of both the SeMet-containing and native peptides. The structure was refined to a crystallographic R-factor of 24.3% with \( R_{\text{free}} = 29.6\% \) over a resolution range of 20.0 to 2.0 Å (Table 1). The current structure includes 42 residues (Fig. 1A) as well as 102 water molecules. The root mean square deviations of bond lengths and bond angles from the idea values were 0.007 Å and 1.24°, respectively (Table 1).

The HY5 LZ domain consists of a parallel, two-stranded, \( \alpha \)-helical coiled coil (Fig. 1B) with a diameter of \( \sim 18 \, \text{Å} \) and a length of \( \sim 60 \, \text{Å} \). The two \( \alpha \)-helices wrapped around one another with a left-handed superhelical twist. Residues 3–41 form an \( \alpha \)-helix that makes a 23.9° crossing angle. Because there are just 3.5 residues/\( \alpha \)-helical turn, each strand contained 11 turns. The average backbone dihedral angles for the helical region (residues 3–41) are \( \sim 66.5 \pm 8.6^\circ \) for \( \varphi \) and \( \sim 40.0 \pm 10.0^\circ \) for \( \psi \). These values are similar to but differ slightly from those measured for the yeast Gcn4-p1 (16–75° for \( \varphi \) and 42–72° for \( \psi \)) and human c-Jun (63–89° for \( \varphi \) and 42–13° for \( \psi \)) homodimers (24). As predicted by “knobs-into-holes” packing (25), the dimer interface is formed by the side chains of residues at positions \( a \) and \( d \) of one monomer and those at positions \( a' \) and \( d' \) of the other (Fig. 1C). This classical intertwined packing has been observed in the Gcn4-p1 (7) and c-Jun homodimers (24).

**Contributions of Electrostatic Interactions to the Stability and Orientation of the HY5 LZ Domain**—Positions \( e \) and \( g \) of the heptad repeat flank the dimer interface of the helices and are generally occupied by charged or polar residues (2, 26–28). Interhelical electrostatic interactions are formed between an amino acid in position \( g \) of a heptad repeat in one helix (\( g \)) and the following amino acid in position \( e \) of a heptad repeat in the other helix (\( e_{g+3} \)) of an LZ domain (29). These positions have been suggested to be involved in stabilizing or destabilizing the coiled coil (3, 7) and in the dimerization specificity of an LZ domain (1, 3–5) depending on whether the interhelical electrostatic interactions are attractive or repulsive. Although they

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**TABLE 1**

Data collection, phasing, and refinement statistics for the SeMet-containing HY5 LZ domain

|                      | Native | Selenium |
|----------------------|--------|----------|
| **Data collection**  |        |          |
| Space group          | C2     | C2       |
| Cell dimensions      |         |          |
| \( a, b, c (\text{Å}) \) | 48.3, 24.5, 76.5 | 48.4, 24.5, 76.3 |
| \( \alpha, \beta, \gamma \) | 90.0°, 100.0°, 90.0° | 90.0°, 100.0°, 90.0° |
| **Resolution (Å)**   | 2.0    | 2.0      |
| **R**\(_{\text{factor}}\) | 9.5 (39.2) | 5.6 (11.1) |
| **I/\text{m}**       | 12.8   | 45.5     |
| **Completeness (%)** | 97.7 (87.8) | 97.5 (95.4) |
| **Redundancy**       | 2.9    | 4.3      |
| **Refinement**       |        |          |
| Resolution (Å)       | 20.0–2.0 | 20.0–2.0 |
| No. reflections (work/test) | 5059/621 | 24.3/29.6 |
| \( R_{\text{work}}/R_{\text{free}} \) | 24.3/29.6 | 24.3/29.6 |
| No. atoms            | 364    | 364      |
| Protein              | 102    | 102      |
| Water                | 47.9   | 47.9     |
| r.m.s.d. deviations  | Bond lengths (Å)  | 0.007    |
| Bond angles          | 1.24°  |          |

*The highest resolution shell is shown in parentheses.

Root mean square.
Crystal Structure of the HY5 LZ Homodimer

![Figure 1](image_url)

**FIGURE 1. Structures of HY5 LZ homodimers.** A, primary structure of the HY5 LZ domain. Amino acids 111–150 of HY5 are renumbered as 3–42. Each group of seven residues corresponds to a heptad repeat, and the repeats are separated by spaces, starting with Leu\(^1\). The first residue of each heptad repeat corresponds to position \(a\). Heptad positions \(a, d, e,\) and \(g\) are colored blue, yellow, orange, and green, respectively. The N-terminal Gly-Ser residues shown in lowercase letters remained after removal of the N-terminal GST fusion protein by thrombin digestion. B, ribbon diagrams of the side (left) and top (right) views of the overall structure of the HY5 LZ dimer with the backbone atoms traced. Ribbon diagrams of strands A and B are shown in orange and purple, respectively. C, side views of HY5 LZ dimers with the backbone atoms represented by ribbons on which the interfacial side chains at positions \(a\) and \(d\) (upper) and their corresponding van der Waals surfaces (lower) are depicted. The side chains of the residues in positions \(a\) and \(d\) are colored pink and green, respectively. B and C were prepared with the program MOLMOL (58).

Contribute to the global stability of the coiled coil far less than do the energetic contributions of van der Waals packing effects of residues at positions \(a\) and \(d\) (30–32), salt bridges formed between the residues at positions \(e\) and \(g\) are still believed to be important for modulating both interhelical (29, 30, 33, 34) and intrahelical (30, 31, 35) stability.

On the basis of the interatomic distances in the final HY5 LZ structure, we predicted that five interhelical salt bridges occur at the dimer interface: Arg\(^{11}\)A–Glu\(^{16}\)B, Glu\(^{16}\)A–Arg\(^{11}\)B, Glu\(^{23}\)A–Lys\(^{18}\)B, Lys\(^{18}\)A–Arg\(^{25}\)B, and Arg\(^{37}\)A–Glu\(^{32}\)B (where A and B in parentheses refer to strands A and B) (Fig. 2A). In addition to the expected interhelical salt bridges between residues in positions \(e\) and \(g\), intrahelical salt bridges were also predicted to form between the residues in positions \(c\) and \(g\): Glu\(^{7}\)A–Arg\(^{11}\), Glu\(^{21}\)Lys\(^{18}\), and Glu\(^{21}\)A–Arg\(^{25}\) for strand A and Glu\(^{21}\)Lys\(^{18}\) and Glu\(^{21}\)A–Arg\(^{25}\) for strand B (Fig. 2A).

Ninety-one percent of positions \(b, c, e,\) and \(g\) of the HY5 LZ domain have either charged or polar residues across all of the heptad repeats. Specifically, in the first three repeats, all of the residues in positions \(c\) and \(e\) are negatively charged, whereas all of the residues in positions \(b\) and \(g\) are either positively charged or polar. Because of the localized charge distribution along each helix, 8 of 10 intra/interhelical ion pairs occur in the N-terminal half of the HY5 LZ domain. Accordingly, the occurrence of a negative charge-rich region in the N-terminal half of the helix gives rise to a highly negative electrostatic surface potential, whereas the C-terminal half shows low electrostatic potential (Fig. 2B). Electrostatic attractions between oppositely charged residues provide local charge compensation in three different regions around the positively charged residues Arg\(^{11}\), Lys\(^{18}/\text{Arg}^{25}\), and Arg\(^{37}\), which correspond to the N-terminal, central, and C-terminal domains of the helix, respectively. Of six positively charged residues in one strand, two are not involved in electrostatic interactions because of their positions in the helix and the orientation of their side chains. Specifically, the side chain of Lys\(^{13}\) stretches out to the exterior of the helix, thus avoiding the formation of a salt bridge with neighboring negatively charged residues, whereas Lys\(^{41}\) is not positioned near any negatively charged residues, thus rendering Lys\(^{41}\) incapable of forming an ion pair. Despite the net charge of +1 for the complete HY5 LZ domain, localized charge distributions allow the negatively charged N-terminal half of the helix to be distinguishable from the C-terminal half. This is the first LZ domain that shows such a localized charge distribution and thus a bipartite electrostatic surface potential.

In general, charges are sufficiently delocalized such that repulsive interactions do not interfere with the conformation (folding and orientation) of LZ domains (7, 32, 36). In the case of the HY5 LZ domain, however, a highly localized negative electrostatic potential seems to lead to unfavorable electrostatic repulsion in its N-terminal half. It is noteworthy that the negative charge-rich region in the N-terminal half consists of residues from same helix and that the distances between side chains of negatively charged residues in this helix are too far apart to cause electrostatic repulsion. Because of this lack of interhelical electrostatic repulsion, favorable interhelical electrostatic attractions between oppositely charged residues in positions \(e\) and \(g\) of the two helices can confer parallel orientation in the HY5 LZ domain. If the HY5 LZ dimer assumed an antiparallel conformation, electrostatic repulsion would occur between like charges in position \(e\) or \(g\), thus destabilizing the LZ dimer. This pattern of favorable interhelical electrostatic attractions between oppositely charged residues that occur in the midst of negatively charged surfaces without being disturbed by them is unique to the HY5 LZ domain.

The interatomic distances and angles between hydrogen donor and acceptor residues suggest the presence of hydrogen bonding interactions. Seven interhelical hydrogen bonds were predicted to be formed in the HY5 LZ domain (Fig. 2), which is more than the number found in any other naturally occurring LZ domain (7, 24). Three hydrogen bonds are formed between the residues in positions \(e\) and \(g\) on the surface of the coiled coil. Two buried hydrogen bonds between Asn residues are formed for Asn\(^{19}\) and Asn\(^{33}\) at the dimer interface, and two additional
interhelical hydrogen bonds are mediated by Asn$^{33}$ (this will be discussed in detail below). In addition to the expected hydrogen bonds that form between the carbonyl group of an i residue and the backbone amide group of the i + 4 residue of each helix, interactions between positions a/e, b/e, b/f, c/f, c/g, d/e, and f/g create a 15-intrahehelical hydrogen bond network that contributes to the intrahehelical stability. Interestingly, this interaction is mediated largely by position f, and all of these positions (three Asn residues, one Glu residue, and one His residue) play crucial roles in forming the intrahehelical hydrogen bond network. This is the first LZ domain for which position f was shown to be involved in such a hydrogen bond network.

In each of the HY5 LZ heptad repeats, positions b, c, and e–g are occupied by either a charged or polar residue, except for Met$^{35}$ and Ile$^{39}$ in positions c and g, respectively. Our results indicate that this primary sequence peculiarity gives rise to much more extensive intra/interhelical hydrogen bonding as well as more salt bridges than are found in other naturally occurring LZ domains. This unique structure results in high intra/interhelical stability and thus confers strong structural integrity upon the HY5 LZ domain.

Effects of Packing Angle Geometries and Rotamer Conformations on the Stability and Stoichiometry of the HY5 LZ Domain—Positions a and d of LZ domains are generally occupied by Leu, Ile, or Val residues, the side chains of which form a dimer interface along the superhelix. These residues at the dimer interface are packed according to the knobs-into-holes model that was first provided by Crick (25). According to this model, knobs in position a (n) in one helix fit into holes formed by residues in positions a (n), d (n), d (n − 1), and g (n − 1) in the other helix (where n refers to the number of the heptad repeat). Likewise, knobs in position d (n) in one helix fit into holes that are created by residues in positions a (n), a (n + 1), d (n), and e (n) in the adjacent helix. Harbury et al. (8) suggested previously that the residue types in positions a and d determine the oligomeric state of coiled coils. If positions a and d are occupied by (Ile/Val)-Leu, Ile-Ile, or Leu-(Ile/Val), as in Gcn4-p1, then the LZ domains can exist as dimers, trimers, or tetramers, respectively. The authors proposed that this correlation between the types of residues in positions a and d and the stoichiometry of the coiled coil is caused by the angles at which knobs orient with respect to their corresponding holes. Defined by the relative orientation of the C$^\alpha$–C$^\beta$ bond vectors and the peptide bond linking residues in positions d and e on the opposing helix (8), the core packing angle geometries are parallel (−30°) and perpendicular (−90°) for positions a and d in dimers, perpendicular and parallel for positions a and d in trimers, and acute (−60°) for both positions a and d in tetramers. As residues in positions a and d pack in specific geometries, these amino acids prefer the most favored rotamer conformation for α-helices. In the most favorable conformation for α-helices, Leu and Ile have rotamer angles of $\chi_1 \sim −60°$ and $\chi_2 \sim 180°$, and Val has a rotamer angle of $\chi_1 \sim 180°$, whereas in the less populated conformation, Leu and Ile have rotamer angles of $\chi_1 \sim −180°$ and $\chi_2 \sim 60°$, and Val has a rotamer angle of $\chi_1 \sim −60°$ (37). This hypothesis was further supported by thermodynamic studies in which Leu was shown to be energetically favored in position $d$, which requires a parallel packing geometry, whereas the β-branched amino acids were shown to be preferred in position a, which shows perpendicular packing in the LZ dimer (38).

As mentioned above, the HY5 LZ domain has a unique amino acid in position a, whereas position d is occupied by Leu, as is the case with other LZ domains. Three of four hydrophobic residues in position a are Leu, which is different from the general notion that the β-branched amino acids are preferred in position a in LZ dimers (8, 38). Surprisingly, most leucines in position a of the HY5 LZ domain display unfavorable rotamer angles for helices, as these residues still assume parallel packing geometries (Table 2). On the other hand, except for Leu$^{15}$, all Leu residues at position $d$ display the most favorable rotamer angles with perpendicular core packing angles, as predicted from other LZ structures. Leucine residues in both positions a and d exhibit different core packing geometries depending on the position of the heptad repeat (Fig. 3). These results imply that a certain amino acid type does not give rise to a specific...
Crystal Structure of the HY5 LZ Homodimer

TABLE 2
Rotamer and core packing angles of positions a and d in the HY5 LZ domain
All angles are shown in degrees. Core packing angles were calculated with the program SOCKET (60).

| Residue | Strand A | Packing angle | Strand B | Packing angle |
|---------|----------|---------------|----------|---------------|
|         | $\chi_1$ | $\chi_2$ |          | $\chi_1$ | $\chi_2$ |          |
| Position a |          |              |          |              |          |              |
| Leu$^5$ | 176.2    | 82.6         |          | $-163.8$ | 64.0 |          |
| Val$^{12}$ | 163.7    |              |          | $-70.3$ |       | 27.1 |
| Asn$^{19}$ | $-66.4$ | 152.3        | 31.4 | $-169.9$ | 81.6 | 30.4 |
| Leu$^{26}$ | $-125.8$ | 33.2         | 33.0 | $-137.6$ | $-34.6$ | 32.6 |
| Asn$^{33}$ | $-67.5$ | $-23.2$ | 35.2 | $-177.8$ | $-82.6$ | 35.1 |
| Leu$^{40}$ | $-135.8$ | $-161.1$ |          | $-73.8$ | 157.4 |          |
| Position b |          |              |          |              |          |              |
| Leu$^{8}$ | $-47.4$ | 176.9 | 80.6 | $-176.6$ | 43.5 | 94.6 |
| Leu$^{15}$ | $-92.0$ | $-75.7$ | 92.6 | $-77.8$ | 163.6 | 97.4 |
| Leu$^{22}$ | $-75.7$ | 174.2 | 98.1 | $-65.5$ | 176.3 | 88.4 |
| Leu$^{29}$ | $-70.4$ | 179.6 | 102.6 | $-76.3$ | 179.9 | 97.4 |
| Leu$^{36}$ | $-64.8$ | 177.3 | 104.1 | $-72.2$ | 176.3 | 90.8 |

FIGURE 3. Core packing geometries of leucine residues in the HY5 LZ domain. Helix cross-sectional views of the HY5 LZ domain through leucine residues illustrate that the position d residue Leu$^{26}$ (A) and position a residue Leu$^{26}$ (B) adopt perpendicular and parallel geometries, respectively. The $C^-C^\dagger$ bond vector of each knob (red) and the $C^-C^\dagger$ bond vectors at the base of the acceptor hole on the opposing helix (yellow) are indicated. The surface representation of the residues is shown. The figure was prepared with the program MOLMOL (58).

core packing geometry and vice versa, as suggested previously (8). The weak correlation of rotamer preferences and packing angle geometries has also been observed in the heterogeneous nuclear ribonucleoprotein C leucine zipper-like (CLZ) oligomerization domain (36). The CLZ tetramer has mixed packing geometries: $\beta$-branched amino acids have both parallel and perpendicular packing geometries, and Leu residues occupy both parallel and perpendicular packing angles. However, the CLZ tetramer has favorable rotamer angles at positions a and d. Thus, both the HY5 LZ and CLZ domains provide structural evidence for the existence of a weak correlation between rotamer preferences and packing angle geometries; indeed, the HY5 LZ domain has the classical core packing geometries of a dimer despite unfavorable rotamer angles at position a, whereas the CLZ domain has mixed packing geometries at positions a and d with preferred rotamer conformations.

It has been predicted that the unfavorable packing angles of Leu residues in position a might provide high packing energies around those regions. In fact, energy computation performed with the GROMOS96 implementation of the Swiss-PdbViewer (39) revealed that the torsion energies for Leu$^{26}$ and Leu$^{40}$ of the HY5 LZ domain are much higher than the average values of all residues (data not shown). Why would residues in the a positions of the HY5 LZ domain adopt energetically unfavorable rotamer conformations? If classical notions about the packing geometries and stoichiometry of the coiled coil are correct, then the HY5 LZ domain might assume unfavorable rotamer conformations to produce parallel packing geometries for position a at the expense of packing energies, resulting in the formation of a dimer. As described above for the CLZ domain, however, it is also possible that the HY5 LZ domain would form a dimer even if the rotamer conformations of position a residues were favorable and all Leu residues in position a displayed perpendicular packing geometries (because mixed geometries in positions a and d do not appear to affect the oligomeric state of a coiled coil). However, if the latter model is correct, then HY5 would not have paid such unfavorable, unnecessary energetic costs to form a dimer. Thus, the former model appears to be more plausible. From this observation, we drew the following conclusions: (i) a specific residue in position a or d is not always packed with a rotamer conformation that is favorable for dimer formation; (ii) core packing geometries are preferred over rotamer conformation in determining the stoichiometry of the HY5 LZ domain; and (iii) the oligomeric state of the HY5 LZ domain is not governed entirely by conformational preferences of positions a and d for particular packing geometries, which is also true for the CLZ domain (36).

Hydrophobic interactions in LZ domains are created by knobs-into-holes packing, in which side chains of the knobs in one helix interact with hydrophobic (part of) side chains of four hydrophobic or polar residues in the adjacent helix at the dimer interface. These four hydrophobic side chains include not only those of hydrophobic residues that are part of the complementary hole, but also hydrophobic parts of side chains extending from charged hole residues (e.g. position g or e hole residues for position a or d knob residues) (7). This structural design likely contributes to the considerable stability of the HY5 LZ dimer (7). Judging by the interatomic distances, the majority (77%) of residues in the knobs form hydrophobic interactions with the four hole residues in the neighboring helix. However, Leu$^{8}$ and Leu$^{15}$ in the d positions in each helix (strands A and B) create...
fewer hydrophobic interactions than expected. The hydrophobic side chain of Leu\(^8\) in strand A forms hydrophobic interactions only with the side chain of Leu\(^5\) and the hydrophobic part of the Glu\(^9\) side chain, both in strand B. Leu\(^8\) in strand B interacts with Leu\(^5\), Glu\(^9\), and Val\(^12\), all in strand A, but not with Leu\(^8\) in strand A. Leu\(^15\) in strand A, which is the only Leu residue with a rotamer conformation that is not preferred in position \(d\) (Table 2), forms hydrophobic interactions only with the hydrophobic parts of the Glu\(^{16}\) (strand B) and Asn\(^{19}\) (strand B) side chains and not with the hydrophobic residues Val\(^{12}\) (strand B) and Leu\(^{15}\) (strand B) (Fig. 4A). Val\(^{12}\) (strand B), which adopts a less favored rotamer angle (Table 2), lacks hydrophobic interactions with Leu\(^8\) (strand A) and Leu\(^{15}\) (strand A) and interacts only with Val\(^{12}\) (strand A) and Arg\(^{11}\) (strand A) (Fig. 4B). This configuration differs from that of the corresponding residues in Gcn4-p1 (Fig. 4C), which makes hydrophobic interactions (7).

These data show that, although the majority of hydrophobic residues at the dimer interface participate in hydrophobic interactions, it is possible that the non-ideal hydrophobic interactions contribute less to HY5 LZ stability than expected. In accord with this notion, the putative high packing energies caused by the unfavorable rotamer geometries of residues in the \(a\) positions, as discussed above, might provide an additional destabilizing effect on the HY5 LZ domain. On the other hand, the high packing energies imposed by unfavorable rotamer angles in the \(a\) positions might be compensated by the more densely packed Leu residues that reside in this position. This is because the side chains of Leu residues in position \(a\) contain more carbon atoms than do other \(\beta\)-branched amino acids. Thus, it is probable that the putative destabilizing effects caused by unfavorable rotamer conformations in position \(a\) and fewer hydrophobic interactions at the dimer interface might not reduce the stability of the HY5 LZ domain as much as one would expect.

Effects of Buried Polar Interactions on HY5 LZ Stability—The occurrence of a buried polar Asn residue in position \(a\) is a common feature of naturally occurring LZ domains (26, 40). In homodimers, two Asn residues, one from each monomer, form an interhelical hydrogen bond in order for two monomers to form a dimer (7, 41–43). Although this interaction is energetically less favorable than the hydrophobic interactions formed by \(\beta\)-branched amino acids in this position (8, 44, 45), Asn residues sacrifice some stability to facilitate the formation of specific dimers by destabilizing higher order oligomers (8–10). These Asn residues also determine the relative orientation of helices in a coiled coil (6).

The amino acid composition of HY5 is unique among other known bZIP proteins because it has two Asn residues at the \(a\) positions, one in a heptad repeat in the central region and the other in a heptad repeat at the C terminus; other LZ domains typically contain only a single Asn residue in one of the central repeats (26). As expected from other hydrogen bonds formed by position \(a\) Asn, Asn\(^{19}\) and Asn\(^{33}\) in both monomers form interhelical hydrogen bonds through their side chains, as illustrated in Fig. 5A. Interestingly, despite our high resolution electron density map, the results in Fig. 5A show that the Asn\(^{19}\) side chains around C\(^{\gamma}\) do not fit into the map very well, whereas those of Asn\(^{33}\) do. In addition, Asn\(^{19}\) displays a higher \(B\)-factor.

![Crystal Structure of the HY5 LZ Homodimer](image-url)
FIGURE 5. Buried polar interactions of position α asparagine residues in the HY5 LZ domain. A, the electron densities \((2F_o - F_c)\) contoured at 1.5σ of Asn\(^{19}\) (left) and Asn\(^{33}\) (right) from each helix strand are shown. Hydrogen bonds formed between the side chains of asparagines from each monomer are indicated by dashed green lines. B, shown is the extensive hydrogen bond network of Asn\(^{33}\) in the HY5 LZ domain. Interstrand and non-helical intrastrand hydrogen bonds are formed in and around Asn\(^{33}\). Hydrogen bonds are indicated by dashed green lines. Distances are expressed in angstroms. Ribbon diagrams of the residues from strands A and B are shown in orange and purple, respectively. C, the hexad structure of Asn\(^{33}\), which consists of Glu\(^{12}\)--Asn\(^{15}\)--Arg\(^{17}\)/Glu\(^{12}\)--Asn\(^{15}\)--Arg\(^{17}\) (upper), is contrasted with the corresponding regions that contain Lys\(^{18}\)--Asn\(^{19}\)--Glu\(^{23}\)/Lys\(^{18}\)--Asn\(^{19}\)--Glu\(^{23}\) (lower). The hexad structure of Asn\(^{33}\) is tightly packed, whereas that of Asn\(^{19}\) is loosely packed. Polar, negatively charged, and positively charged residues are colored yellow, red, and blue, respectively. The figure was prepared with the program Swiss-PdbViewer (39).
than does Asn\textsuperscript{33}. The B-factors for C\textsuperscript{\textalpha}, N\textsuperscript{\textbeta}, and O\textsuperscript{\textgamma} are 29–37 Å\textsuperscript{2} and 5–17 Å\textsuperscript{2} for Asn\textsuperscript{19} and Asn\textsuperscript{33}, respectively. The poor electron density and the relatively high temperature factors indicate that a high degree of structural flexibility exists in the region around Asn\textsuperscript{19}. It has been reported that the interfacial Asn\textsuperscript{29} side chain of the Jun LZ homodimer undergoes a conformational averaging process and thus flips around \( \chi_2 \) between two distinct conformations (9, 24). \(^{15}\)N NMR relaxation experiments further revealed that this increased mobility is confined to the Asn side chain, not to the backbone atoms (46). From these data, it has been proposed that this motional disorder might provide some entropic compensation for the enthalpically unfavorable desolvation that occurs at the hydrophobic dimer interface (46). In this context, it is tempting to speculate that the structural flexibility of the Asn\textsuperscript{19} side chains of the HY5 LZ dimer allows these residues to flip around C\textbeta–C\textgamma between different conformations. This ability to flip-flop might make the Asn\textsuperscript{19} side chain benefit thermodynamically. In contrast, Asn\textsuperscript{33} might lose large solvation free energy upon burial because of the absence of flip-flop.

In addition to the interhelical hydrogen bonds between Asn side chains, we found that there are additional inter/intrahelical hydrogen bonds mediated by the Asn\textsuperscript{33} residues in the HY5 LZ domain (Fig. 2A). The N\textsuperscript{\textbeta} of Asn\textsuperscript{33} (strand A) is engaged in an interhelical hydrogen bond with the backbone carbonyl of Leu\textsuperscript{29} in position \( d \) of strand B (Fig. 5B). Based on structural, amide exchange, and thermodynamic studies, such an interhelical interaction between an Asn side chain in position \( a \) and a Leu backbone in the preceding position \( d \) was first reported for the c-Myc/Max heterodimeric LZ domain (47). On the other hand, Asn\textsuperscript{33} (strand B) is involved in both the interhelical and intrahelical hydrogen bonds. The N\textsuperscript{\textbeta} of Asn\textsuperscript{33} in strand B forms a hydrogen bond with the O\textsuperscript{\textgamma} of Glu\textsuperscript{32} (strand A), and the O\textsuperscript{\textbeta} of the same Asn\textsuperscript{33} residue also interacts with the N\textsuperscript{\textalpha} of Arg\textsuperscript{37} in strand B, in addition to the helical hydrogen bond that exists between Asn\textsuperscript{33} (strand B) and Arg\textsuperscript{37} (strand B). Such an interhelical hydrogen bond between Asn in position \( a \) on one strand and the side chain of Glu in position \( g \) on the opposite strand has also been observed for the c-Jun/c-Fos heterodimer (48) and also might occur in the c-Myc/Max heterodimer (47).

Remarkably, in addition to the hydrogen bonds mediated by Asn\textsuperscript{33}, symmetrical interhelical hydrogen bonds are formed between the side chain amide groups of Arg\textsuperscript{37} in position \( e \) and the O\textsuperscript{\textbeta} of Glu\textsuperscript{32} in position \( g \) on the opposite strand within the dimer flanking region (Fig. 5B). This unusual arrangement of Glu\textsuperscript{32}–Asn\textsuperscript{33}–Arg\textsuperscript{37} from each monomer allows the formation of a unique “hexad structure,” in which positively and negatively charged residues are located on either side of the two Asn residues (one from each monomer) such that an extensive hydrogen bond network is formed (Fig. 5C, upper). In the case of Asn\textsuperscript{19}, however, the corresponding residues (Lys\textsuperscript{18}, Asn\textsuperscript{19}, and Glu\textsuperscript{23} in positions \( g, a, \) and \( e \), respectively) are not able to form such interactions because they are loosely packed and thus cannot make hydrogen bonding interactions with each other (Fig. 5C, lower). A unique amide tetrad formed by Gln/Asn/Gln’–Asn’ (the prime means the residues from the opposite strand) of the Max helix-loop-helix LZ homodimer is predicted to form a symmetrical structure (49), which is different from the asymmetrical orientation of Asn in position \( a \) in Gcn4–p1 (7) and c-Jun (24). According to the molecular models proposed for the Gln/Asn/Gln’–Asn’ tetrad of the Max homodimer (49), the Asn side chains of the Max LZ domain might not be involved in a conformational exchange process, in contrast to the Asn side chains in the Jun homodimer (9, 24). This is because the Asn residues in the Max LZ domain are able to form permanent hydrogen bonds between symmetrical side chains (49). As discussed above, we predicted that Asn\textsuperscript{33} of the HY5 LZ domain forms permanent hydrogen bonds rather than undergoing such a conformational averaging process. This is a tempting hypothesis not only because Asn\textsuperscript{33} is well fitted to the electron density map and has a low B-factor value, but also because Asn\textsuperscript{33} has been shown to be part of the permanent, interconnected hydrogen bond network formed by the hexad of residues.

In summary, whereas Asn\textsuperscript{19} is predicted to flip-flop around the C\textbeta–C\textgamma vector, Asn\textsuperscript{33} is expected to be an integral part of an extensive hydrogen bond network. The former residue might gain thermodynamic benefit by entropic compensation for the loss of enthalpy that occurs with the burying of the polar Asn residue at the dimer interface. The latter structure can be either stabilizing because permanent hydrogen bonds similar to those in the Max LZ domain contribute to its stability (49) or destabilizing because such a large hydrogen bond network at the dimer interface is energetically unfavorable. The data presented here do not allow us to make a final determination of how these Asn residues contribute to the stability of the HY5 LZ domain.

Effects of Position \( a \) Mutations on the Stability and Stoichiometry of the HY5 LZ Domain—To further assess the effects of position \( a \) (particularly the Asn residues) on the stability and oligomeric state of the HY5 LZ domain, a variety of HY5 LZ domain mutant peptides (Table 3) were subjected to biophysical studies by CD spectropolarimetry and analytical ultracentrifugation.

Fig. 6 shows the thermal denaturation profiles of the HY5 LZ domain mutant peptides, which were produced by monitoring changes in the CD signals at 222 nm with increasing temperature. Thermal unfolding can be classified into three different profiles on the basis of thermal cooperativity and the presence of plateaus. The thermal profiles shown in Fig. 6A display both thermal cooperativity and the presence of a plateau; those in Fig. 6B demonstrate cooperativity, but do not exhibit a plateau; and those in Fig. 6C exhibit neither cooperativity nor a plateau.

Mutation of Val\textsuperscript{12}, the only \( \beta \)-branched amino acid among the residues in position \( a \), to Leu (peptide HM1) caused a slight decrease in the \( T_m \) of the HY5 LZ domain from 60.5 to 56.5 °C. The observed decrease in the \( T_m \) indicates that the Val\textsuperscript{12}–to-Leu change had a destabilizing effect on HY5 LZ stability (Fig. 6A). However, when the position \( a \) Leu residues were mutated to either Val (HM2) or Ile (HM3), the mutant HY5 LZ domain was destabilized relative the WT domain, and Val residues in these positions were more destabilizing than were Ile residues (Fig. 6A). These results indicate that neither the \( \beta \)-branched amino acids (Val and Ile) nor Leu is energetically preferred in position \( a \). In other words, any of the three residues can be preferred in this position. Thus, it is likely that the ability of a
specific amino acid in position a to stabilize the HY5 LZ domain is dependent on the position of a heptad repeat. This is inconsistent with the generally accepted notion that the β-branched amino acids are energetically preferred in position a (38). The oligomeric state of HM1 and HM3 is dimeric, as is the case for the WT peptide (Table 4). Taken together, these results indicate that HY5 shows only a weak preference of amino acid type in the a positions, which is consistent with the findings discussed above, and that the amino acid composition of position a in the HY5 LZ domain is optimized in terms of thermal stability of the dimer.

To investigate further the effect of mutating position a Asn residues to hydrophobic amino acids on the stability of the HY5 LZ domain, biophysical studies were carried out for a series of Asn mutants. Fig. 6B demonstrates the thermal denaturation of peptides containing a single Asn residue change (Asn19 or Asn33) to either Val or Leu. Assuming that a rightward shift of the thermal profile of a peptide indicates an increase in stability, the Asn-to-Val mutations yielded peptides (HM4 and HM5) that formed more stable LZ domains than did the Asn-to-Leu mutations. Thus, Val is more stabilizing than Leu in the buried polar a positions of the HY5 LZ domain. In the same way, the thermal profiles of LZ domains formed from Asn33 mutant peptides (HM5 and HM8) were shifted rightward and were therefore more stable than those formed with Asn19 mutant peptides (HM4 and HM7). These findings indicate that the HY5 LZ domain is more stabilized when Asn33 is replaced by hydrophobic residues than when Asn19 is substituted with hydrophilic residues. Furthermore, we concluded that Asn33 is more destabilizing than Asn19 in the WT HY5 LZ domain. Interestingly, mutation of both Asn residues to hydrophobic ones (HM6 and HM9) caused linear patterns of thermal denaturation with identical slopes (Fig. 6C). An additional substitution of Leu for the remaining Val residue in position a in HM9 (HM10) also showed a linear, similarly sloped thermal denaturation pattern. The oligomeric state of all Asn mutant peptides containing hydrophobic amino acid substitutions (HM4–9) was observed to be trimeric as determined by analytical ultracentrifugation (Table 4). This is consistent with the previous finding that Asn residues in position a specify the formation of dimers by destabilizing higher order oligomers (8–10). The HM10 peptide, which has Leu residues in both positions a and d, was also shown to form trimers, consistent with similar observations made for Gcn4-pLL (8). Taken together, these results indicate that even a single amino acid change in position a can switch the oligomeric state of the HY5 LZ domain from dimeric to trimeric and (ii) highlight the importance of the effect of packing in the hydrophobic core on the oligomeric state.

When Asn residues in position a were mutated to charged residues (Asp), the thermal denaturation patterns of the mutant peptides (HM11 and HM12) (Fig. 6A) were totally different compared with the mutant peptides in which the Asn residues were changed to hydrophobic residues (Fig. 6B). The HM11 and HM12 LZ domains showed cooperative thermal unfolding, but were far less stable than the WT peptide. Interestingly, the far-UV CD spectrum of HM13, in which two position a Asn residues were mutated to Asp (Table 3), displayed a single negative band at ~200 nm (Fig. 7A), which is indicative of an unstructured conformation, and that of HM11 exhibited a pattern similar to that of HM13, except that it had an increased signal at 222 nm and less of a signal at ~200 nm (Fig. 7B).

In contrast, the CD spectrum of HM12 was characterized by double minima at 208 and 222 nm (Fig. 7C), suggesting the typical α-helical conformation. However, the HM12 CD spectrum did not show evidence of interacting α-helices, as indicated by the observed θ208/θ222 ratio of 0.93 (33, 50, 51), compared with a value of 1.02 for the WT HY5 LZ domain (which is a typical value for an interacting coiled coil) (33, 50, 51). The unstructured properties of HM11 and HM13 might have arisen because electrostatic repulsions between the negatively charged side chains of the Asp residues destabilized the HY5 LZ domain when ionized at neutral pH. As expected, when the pH was decreased to 4, at which the Asp side chains were not ionizable, the far-UV CD spectra of HM11 and HM13 indicated the presence of α-helical conformations (Fig. 7, A and B) and the spectrum of HM12 changed to a pattern indicative of the presence of interacting α-helices (θ222/θ208 = 1.16) (Fig. 7C).

The α-helices were calculated to be 25.9, 84.6, and 12.8% for HM11, HM12, and HM13 at pH 7.4, respectively, and 57.6, 93.7, and 23.6% for HM11, HM12, and HM13 at pH 4.0, respectively. The stabilization of Asp mutants at acidic pH was further supported by the thermal unfolding pattern, in which the CD signals of HM11 and HM12 were shifted to the right compared with those measured at neutral pH (Fig. 6A). This pH-depend-
ent folding of Asn-to-Asp mutants has also been observed for a similar Gcn4-p1 mutant (52).

Even if HM11, HM12, and HM13 are stabilized at low pH by eliminating the electrostatic repulsions between Asp residues, the \( \alpha \)-helical contents of these mutants are still much lower than that of the WT peptide (95% \( \alpha \)-helicity), as indicated by the 222 nm signal, and these mutant peptides also show less thermal stability than the WT peptide (Fig. 6A). There are two possible reasons for the instability of Asp mutants at acidic pH. Salt bridges mediated by Asp and Glu in other positions might not be formed at pH 4 because these two residues are not ionizable at this pH. Reduced intra- and/or interhelical electrostatic interactions at low pH might impart more destabilizing effects on the stabilities of HM11, HM12, and HM13 than expected. The other possibility is the elimination of the energetically favorable contribution of the hydrogen bonds between Asn side chains. Even though the existence of a polar residue at the dimer interface has been shown to be energetically unfavorable compared with hydrophobic residues (8, 44, 45), interhelical hydrogen bonds between Asn side chains might provide an energetically favorable contribution to HY5 LZ stability. This hypothesis is supported by thermodynamic studies on Gcn4-p1 Asn mutants showing that buried polar residues (Asn) contribute to Gcn4-p1 stability by forming interstrand hydrogen bonds between the Asn side chains (53). The effects described in both hypotheses might affect the stability of Asn-to-Asp mutants at acidic pH. Taking into account the fact that hydrophobic interactions and hydrogen bonds are the major forces that maintain the structures of globular proteins, rather than electrostatic interactions, the latter hypothesis seems more reasonable. In other words, interhelical hydrogen bonds between Asn side chains might make an energetically favorable contribution to HY5 LZ stability.

**DISCUSSION**

Even though coiled coils seem to present simple structural motifs, they are more complex and versatile than they appear. Despite the existence of large numbers of structural studies on coiled-coil proteins, new features continue to be discovered in addition to the classical coiled coils such as Gcn4-p1 (7) and c-Jun (24). The *Arabidopsis* HY5 LZ protein appears to form such a coiled coil, as it possesses several intriguing structural characteristics.

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**TABLE 4**

| Peptides | Calculated molecular mass* | Observed molecular mass** | Observed/calculated molecular mass |
|----------|---------------------------|---------------------------|-----------------------------------|
| WT       | 4958                      | 9935                      | 2.0                               |
| HM1      | 4972                      | 10,880                    | 2.2                               |
| HM2      | 4813                      | ND                        | ND                                |
| HM3      | 4858                      | 9445                      | 1.9                               |
| HM4      | 4943                      | 14,200                    | 2.9                               |
| HM5      | 4943                      | 15,010                    | 3.0                               |
| HM6      | 4957                      | 14,420                    | 2.9                               |
| HM7      | 4957                      | 15,040                    | 3.0                               |
| HM8      | 4957                      | 15,140                    | 3.1                               |
| HM9      | 4956                      | 14,630                    | 3.0                               |
| HM10     | 4970                      | 15,380                    | 3.1                               |
| HM11     | 4959                      | 8853 (9873)               | 1.8 (2.0)                         |
| HM12     | 4959                      | 7805 (12850)              | 1.6 (2.6)                         |
| HM13     | 4960                      | 7116 (8847)               | 1.4 (1.8)                         |

* Molecular mass of each peptide monomer calculated from its primary sequence.
** Molecular mass of each peptide experimentally determined by analytical ultracentrifugation.

* Numbers in parentheses are for peptides dissolved in 5 mM sodium acetate (pH 4).
Bipartite Characteristics of the HY5 LZ Domain

The HY5 LZ domain has unique bipartite characteristics, the first reported among the LZ motifs. Divided by the third heptad repeat of the HY5 LZ domain, there are the N-terminal half (amino acids 5–25), which has a highly negative surface potential (Fig. 2B), and the neutral C-terminal half (amino acids 26–42).

Previously, Kammerer et al. (54, 55) hypothesized that a particular 13-amino acid sequence serves as a “trigger site” that is indispensable for the oligomerization of coiled coils. The trigger site is separate from the heptad repeats and is thought to be an autonomous folding unit that first folds unfolded polypeptide chains to form monomers and then mediates dimerization of the coiled coil during the folding process (54). According to the proposed consensus trigger sequence, it has been predicted that the HY5 LZ domain contains two possible trigger sites, SELENRVKDLENK (amino acids 6–18) and KDLENKNSELER (amino acids 13–25) (the underlined residues diverge from the consensus trigger site). As the second to last amino acid in the trigger sequence of kinesin is also an Asn residue and because this element has been shown to be a functional trigger site (54), the first putative trigger site of the HY5 LZ domain is likely to be functional. The second one also might function as a trigger sequence because one-amino acid inconsistencies have been shown to be tolerable in other proteins (54).

It is interesting that the trigger sequences overlap with the N-terminal half of the HY5 LZ domain, which displays the bipartite structural characteristics described above. Because the trigger sequence is an autonomous folding unit, this region might need to behave in a structurally independent manner relative to the rest of the coiled coil. Thus, it is quite reasonable to postulate that the bipartite characteristics of the HY5 LZ domain can be ascribed to the presence of the consecutive trigger sites that cover the N-terminal half of the protein. The enrichment of charged residues in the trigger sites of the HY5 LZ domain may be necessary for the intrahelical stability of the monomeric trigger helices.

As it has been suggested that the intrahelical electrostatic interactions between charged residues within these sites are important for the stability of a monomeric helix (35, 56), the electrostatic interactions within these sequences may be important for maintaining the intrahelical stability of the HY5 LZ domain. The intrahelical ion pair formed between Glu7 (strand A) and Arg11 (strand A) within the trigger sequence of the HY5 LZ domain (Fig. 2A) may contribute to its intrahelical stability, as does the salt bridge between Glu22 and Arg25 within the trigger site of Gcn4 (35, 57). Also, the unique intrahelical hydrogen bonding interactions mediated by position f residues in the HY5 LZ domain are within the trigger sites (Fig. 2) and are expected to provide an additional contribution to its intrahelical stability.

Different Modes of Polar Interactions of Asparagines 19 and 33—The two asparagine residues buried in the dimer interface of the HY5 LZ domain show different modes of hydrogen bonding interactions. Asn19 might be involved in a conformational averaging process (Fig. 5A, left), whereas Asn33 might form a permanent hydrogen bond (right) by building a unique hexad structure around it (Fig. 5, B and C).

Asn33 was shown to be more destabilizing in the context of the HY5 LZ domain compared with Asn19 (Fig. 6B) probably because the ability of Asn19 to flip-flop confers a thermodynamic benefit by an enthalpic-entropic compensation (46). On the other hand, the N19D mutant (HM11) showed pH-dependent folding, whereas the N33D mutant (HM12) was affected much less by pH changes in terms of thermal stability (Fig. 6A) and secondary structure (Fig. 7). As for Asn19, the interhelical hydrogen bond it forms is the only hydrogen bond in the HY5 LZ domain; thus, Asn19 is very important for stability in this region of the HY5 LZ domain. Because the stability of the buried polar region at Asn19 is dependent on the hydrogen bonding

FIGURE 7. Far-UV CD spectra of HM13 (A), HM11 (B), and HM12 (C). All spectra were recorded at 5 °C with ~100 μM samples dissolved either in 5 mM sodium phosphate (pH 7.4) (closed symbols) or in 5 mM sodium acetate (pH 4.0) (open symbols). [θ]_{222}, mean residue ellipticity; deg, degrees.
of Asn\textsuperscript{19}, one expects that the N19D mutant would be very sensitive to electrostatic repulsion between like charges and to neutralization by acidic pH. In contrast, because Asn\textsuperscript{33} forms a hydrogen-bonded hexad structure around itself, this residue might be less sensitive to replacement with Asp. Our results support these hypotheses and suggest that buried polar residues require favorable packing to form hydrogen bonds with neighboring residues and thus maintain the local structural stability of the polar dimer interface.

Taken together, our results indicate that two buried Asn residues in different modes of hydrogen bonds contribute to HY5 LZ stability in different ways: the hydrogen bond of Asn\textsuperscript{19} ben-

...to have parallel rather than perpendicular packing geometries (Fig. 3 and Table 2). This results in the Leu residues at positions a and d having parallel and perpendicular core packing geometries, respectively, thus allowing formation of the HY5 LZ dimer. Hence, dimer specificity is conferred at the expense of unfavorable energies of position a Leu residues. However, HY5 LZ stoichiometry is not solely regulated by core packing geometries and rotamer angles. Two buried Asn residues were found to play important roles in specifying HY5 LZ dimers as evidenced by mutation studies (Table 4), which is consistent with a previous finding of the roles of buried polar residue in determin-

...in the HY5 LZ domain by forming their characteristic hydrogen bonds. In addition to the hydrophobic interactions and van der Waals packing effects at the dimer interface that are known to be major forces in maintain globular fold of proteins, a large number of salt bridges and hydrogen bonds may contribute to intra/interhelical HY5 LZ stability. The HY5 LZ domain has larger numbers of such interactions than do other known LZ homodimers (7, 24). Remarkable intrahelical hydrogen bonds mediated mostly by the f positions and salt bridges within the putative trigger sites may contribute to the intrahelical stability of the HY5 LZ domain, and its interhelical stability may be promoted by interhelical hydrogen bonds and electrostatic attractions between positions e and g’ (Fig. 2A).

Implications—The ability to design proteins that have the desired globular folds is a very challenging task for protein chemists. Even simple structural motifs such as the coiled coil are so sophisticated that we are not fully aware of the structural components that specify structural integrity. By solving the crystal structure of the HY5 LZ domain, we were able to further characterize its versatile and unique structural components. Thus, these new findings will help scientists gain a richer understanding of the contribution of complex structural features to the conformational integrity of coiled coils and thus design novel protein folds. Furthermore, we have presented the first LZ structure among Arabidopsis bZIP proteins. The unique structural features of the HY5 LZ domain may be related to its peculiar functions in plants. However, the functional relevance of these structures has yet to be elucidated.

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