The solution structure of the c-Jun leucine zipper domain has been determined to high resolution using a new calculation protocol designed to handle highly ambiguous sets of interproton distance restraints. The domain comprises a coiled coil of parallel α-helices in which most of the hydrophobic residues are buried at the highly symmetrical dimer interface; this interface extends over 10 helical turns and is the most elongated protein domain solved to date using NMR methods. The backbone fold is very similar to that seen in crystal structures of the GCN4 and J-un-Fos leucine zippers; however, in contrast with these crystal structures, the J un leucine zipper dimer appears to be devoid of favorable intermolecular electrostatic interactions. A polar asparagine residue, located at the dimer interface, forms the sole point of asymmetry in the structure; furthermore, the side chain of this residue is disordered due to statistical averaging. This residue, which is highly conserved in the leucine zipper family of transcription factors, provides a destabilizing influence that is likely to facilitate the rapid exchange of zipper strands in vivo.

c-Jun is a transcriptional activator that binds to specific DNA sites either as a homodimer or as a heterodimer with the c-Fos protein (1). Together, these proteins play a pivotal role in the regulation of cell growth and differentiation; their role in transducing afferent growth signals into specific genetic responses means that they represent a critical nexus between the transduction of afferent growth signals into specific genetic responses and the downstream events that control the specificity, affinity, and kinetics of leucine zipper formation. Structural information of the type provided by NMR or x-ray crystallography not only provides a molecular framework for understanding the way in which leucine zippers mediate gene transcription, but also paves the way for rational engineering of dominant-negative leucine zippers or other molecules that might block the activity of bZIP oncoproteins (4). In this study, we have used NMR spectroscopy to determine the solution structure of the c-Jun leucine zipper in order to better understand the interactions that control the affinity and kinetics of c-Jun homo- and heterodimers. We show that the dimer appears to be devoid of favorable intermolecular electrostatic interactions and that two key residues at the dimer interface might play an important role in stabilizing the leucine zipper in order to facilitate the exchange of zipper strands in vivo.

EXPERIMENTAL PROCEDURES

NMR—NMR studies were carried out on both an 86-residue synthetic J un leucine zipper homodimer (5) and a uniformly 15N-labeled 92-residue homodimer produced by bacterial overexpression (rJ unLZ) (6). rJ unLZ differs from synthetic J unLZ by the addition of three non-native residues (Glu-Ser-Met) at the N-terminus of each monomer (see Fig. 1A); these residues were not included in the structure calculations. The monomers were covalently linked via a disulfide bond formed between the cysteine residues located in the N-terminal linker regions (shown in reverse type in Fig. 1A).

Structure Calculations—Structure calculations were performed using X-PLOR Version 3.1 (7). Initially, a subset of 271 unambiguous intramonomer distance restraints (8) were used in combination with 31 hydrogen bond and 33 φ dihedral angle restraints (9) to calculate a family of 50 monomer structures using the nmr/random.inp and nmr/dgsa.inp protocols (10). The weight on the NOE term was increased 3-fold to improve convergence (11).

Dimer structures were calculated by employing a new protocol (MDSA-SCC-REFMR-1.0 (11)) that uses the dynamic assignment method (12) to resolve the ambiguous distance restraints obtained from the spectra of symmetric multimers. For each of the 50 monomer structures, a second monomer structure was generated from the first one by rotating the coordinates 180° around the long axis of the molecule to produce the initial set of dimer structures. These structures were then refined using the complete set of 1334 distance restraints.

Dimer symmetry was enforced using the two-constraint approach proposed by Nilges (12). The noncrystallographic symmetry restraint applies a force to keep the monomers superimposable, while a second global symmetry term allows the structure to evolve its own axis of symmetry during refinement; it was necessary to calculate the global symmetry potential using a randomized atom selection in order to avoid artificial local minima close to the trivial solution (superimposed monomers) (11). Since the previously demonstrated formation of a hydrogen bond between the Asn-291 residues is inconsistent with a symmetrical arrangement of their side chains (9), the symmetry terms were turned off for these residues. A HEPTAD constraint term, which forces the geometric centers of each symmetry-related heptad repeat to...
be within 10.4 Å (13), was used to ensure that the two monomers aligned in parallel; this constraint increases confidence without influencing the final structures (11).

Calculation of the dimer structure proceeded in three stages: searching, annealing, and energy minimization. Since the starting monomer structures were relatively accurate, the noncrystallographic symmetry term was maintained with a high weight factor throughout the protocol, thus keeping the two monomers very nearly superimposable from the outset and constraining them to move in a cooperative manner. In the initial high temperature search stage, the initial atomic velocities were assigned randomly on the basis of a Maxwellian distribution at 2000 K. In this stage, nonbonded interactions were only calculated between α-carbon atoms; the repel nonbonded potential was used with a scale factor (s) of 1.2 and an initial weighting factor (wUNAMBIG) of 0.025. The following X-PLOR nonbonded parameters were used: CUTNB = 100 Å, TOLERANCE = 45 Å, and NBXMOD = 4. All other atoms were free to occupy overlapping regions of space, constrained only by the bond, angle, planar, NOE, and symmetry terms. Hydrogen bond restraints and unambiguous distance restraints were restrained using the square-well function for the NOE potential with an initial weight of wUNAMBIG = 0.16, while the "soft" NOE potential with an initial weight of wUNAMBIG = 0.05 was used for all ambiguous restraints. The search phase comprised 100 ps of simulated dynamics. In the second stage, the annealing stage, the repel potential was turned on for all atoms, and the system was cooled from 2000 to 100 K in decrements of 50 K, with 1.3 ps of dynamics/decrement; initial parameters were s = 0.9, CUTNB = 45 Å, TOLERANCE = 0.5 Å, NBXMOD = 3, WPLNAR = 0.1, WHETPET = 0.1, and wUNAMBIG = 0.00075. After each decrement, the weights were rescaled by constant factors to reach the following values at 100 K: s = 0.8, WPLNAR = 1.0, WUNAMBIG = 1.5, WPLNAR = 1.0, WPLNAR = 1.0, and wUNAMBIG = 1.0. The final stage consisted of 500 cycles of Powell minimization using these weights and nonbonded parameters. The theoretical and computational principles underlying this structure calculation protocol are described elsewhere (11); the X-PLOR calculation protocol is freely available.²

Intermonomer Mean Force Potentials—The intermonomer mean force potentials (MFPs) were calculated using the program PROSA (14). The value for each residue was obtained by subtracting the MFP calculated for each monomer from that calculated for the dimer. The mean intermonomer MFP was then obtained by taking the average over both monomers and over all seven structures.

² The X-PLOR calculation protocol is freely available from http://www.nmr.embl-heidelberg.de/nilges/.

Deposition of Coordinate and Restraint Files—The NMR restraints used for structure calculations (R1 UNMR) and the coordinates of the ensemble of seven refined JunLZ structures (1JUN) have been deposited with the Brookhaven Protein Data Bank.

RESULTS

Tertiary Structure of JunLZ—A new structure calculation protocol (11), which was specifically designed to handle highly symmetric multimers, was applied to 50 starting monomer structures to yield an ensemble of seven JunLZ dimer structures that had the correct topology and that adequately satisfied the geometric and experimental terms in the force field. The convergence rate of this protocol is low because of the large number of monomer NOE constraints (a mixture of intra- and intermonomer NOE s (11)) that must be handled during the structure calculation procedure; this situation arises because the dimer interface extends along the entire dyad symmetry axis. Nevertheless, the seven lowest energy structures had good covalent geometry (mean r.m.s. differences from ideal bond lengths and bond angles of 0.010 ± 0.0001 Å and 0.18 ± 0.01°, respectively) and an absence of bad nonbonded contacts. Furthermore, the structures displayed no violations of bounds > 0.05 Å or of angles or improper angles > 5°, and —95% of all pairs of non-glycine (φ and ψ) dihedral angles lie within the "most favored" region of a Ramachandran plot (15). The average backbone dihedral angles for the helical region (Arg-276–Val-312) are —63 ± 10° for φ and —42 ± 13° for ψ, which correspond exactly to those measured from the crystal structure of the GCN4 leucine zipper (GCN4-LZ) (16) and those reported for "typical" α-helical peptides (17). The seven structures superimpose over the region Leu-280–Leu-308 (see Fig. 2a) with an average r.m.s. difference of 0.97 Å for all atoms and 0.56 Å for the backbone atoms only.

Fig. 2 shows that the JunLZ dimer comprises a parallel coiled coil of α-helical strands that wrap around one another with a slight left-handed superhelical twist. The dimer interface is formed by the hydrophobic side chains of residues in the α- and d-positions of one monomer packing side-by-side against the α- and d'-residues, respectively, of the other monomer as...
predicted by Crick’s “knobs-into-holes” model (Fig. 1B) (18). The superhelical twist, which can be seen most clearly in Fig. 3, a cross-sectional view of 1JUNLZ through the Val-305 residues illustrating that a-position residues pack in a parallel orientation (24), such that a Cα-Cβ bond vector (red) representing the “knob” of the interfacial residues is almost parallel to the Cα-Cα vector (yellow) at the base of the acceptor hole on the opposing helical strand. b, a cross-sectional view of 1JUNLZ through the Leu-301 residues illustrating that b-position residues pack in a perpendicular orientation (24), such that the Cα-Cα-Cβ bond vector is almost perpendicular to the Cα-Cα vector at the base of the acceptor hole on the opposing helix. In both diagrams, the continuation of the helix backbone on either side of the Cα-Cα vector is displayed in magenta.

The superhelical twist, which can be seen most clearly in Fig. 2b, enables an extensive interface to be maintained in which each interfacial residue is surrounded by four others, thus maximizing the buried hydrophobic surface area (≈960 Å²) and enhancing the stability of the dimer (16).

The backbone atoms of 1JUNLZ superimpose onto the leucine zipper of Max (19) with an r.m.s difference of 2.3 Å, suggesting a substantial difference between the coiled coils of bHLH-ZIP and bZIP proteins. In contrast, the crystal structure of GCN4-LZ (15) superimposes onto the backbone atoms of the homologous region of the lowest energy 1JUNLZ structure with an r.m.s difference of 0.9 Å. Similar superpositions of a segment of 1JUNLZ bounded by the first and fifth leucine residues onto each of two crystallographically distinct structures of a cJun-c-FosAP-1 DNA complex (20) give an r.m.s difference of 1.0 Å. These r.m.s differences are within the expected accuracy of ≈1 Å for NMR structures (21), indicating that the backbone fold of 1JUNLZ is essentially identical to these crystal structures. Thus, as demonstrated previously for GCN4 (22, 23), the tertiary fold of the cJun leucine zipper appears to be largely unaltered when it binds to DNA.

Disposition of Residues at Dimer Interface—As in the x-ray crystal structure of GCN4-LZ (16), all interfacial valine residues (Val-284, Val-305, and Val-312) in the 1JUNLZ dimer adopt the most preferred rotamer conformation (χ1 = 180°); the mean χ1 dihedral angle (averaged over the six valine residues in all seven structures) is 177°. The interfacial leucine residues adopt conformations close to that of the most preferred rotamer (χ1 = −60° and χ2 = 180°); the mean dihedral angles of the leucine side chains are χ1 = −86° and χ2 = 156°, similar to those observed in the crystal structure of GCN4-LZ (χ1 = −69° and χ2 = 155°) (16). The interfacial Asn-291 side chain has no fixed orientation, indicative of the conformational averaging process described previously (9).

The a- and d-layers at the dimer interface exhibit different types of knobs-into-holes packing. According to the definitions introduced by Harbury et al. (24), the d-position Leu residues pack in a “perpendicular” orientation, whereby a vector formed by the Cα-Cβ bond of each leucine knob packs approximately perpendicular to the Cα-Cα vector at the base of the hole into which it packs on the adjacent helix (Fig. 3b). In contrast, the a-position residues pack in a “parallel” orientation, such that the Cα-Cα-Cβ bond vector lies almost parallel to the Cα-Cα vector at the base of the acceptor hole on the opposing helical strand (Fig. 3a). Thus, the Leu side chains at the d-positions point into the dimer interface, whereas the side chains at the a-positions point away from the interface and make side-by-side van der Waals contacts.

**DISCUSSION**

Dimer Symmetry Problem—Two- and three-dimensional NMR spectra of 1JUNLZ revealed a single set of resonances corresponding to the primary structure of the monomer (5, 6),
thus indicating that the dimer is highly symmetrical. Determination of the structures of symmetric dimers from NMR data, especially those with extensive interfaces, is difficult because of the problem of distinguishing between intra- and intermolecular connectivities in multidimensional spectra displaying NOEs (12). Coiled coils represent perhaps the worst case scenario in this respect as the dimer interface extends over the entire length of the domain, and the helical nature of the individual strands minimizes the number of long-range NOEs and maximizes the number of ambiguous NOEs.

Thus, a special calculation protocol was developed (see “Experimental Procedures”) so that the inherently ambiguous distance restraints could be used to determine a high resolution J unLZ structure without resorting to asymmetric isotopic labeling (25). This protocol has enabled us to calculate the first high resolution solution structure of a leucine zipper domain. The backbone fold of the solution structure of J unLZ (Fig. 2) is very similar to the crystal structures of the GCN4 and J un-Fos leucine zipper domains.

Intermolecular Electrostatic Interactions—While it has been previously demonstrated that preferential formation of J un-Fos heterodimers over either homodimer results largely from the relief of unfavorable interhelical electrostatic repulsion in the Fos homodimer (26), the contribution of intermolecular ion pairs and salt bridges to global stability and dimerization specificity in two-stranded coiled coils is less clear. It has been suggested that g-e' and g-e' electrostatic interactions (see Fig. 1B) contribute favorably to coiled-coil stability (27, 28). However, recent NMR solution studies have revealed that the interhelical salt bridges seen between Lys-15' and Glu-20 and between Glu-22 and Lys-27 in the crystal structure of GCN4-LZ (16) do not appear to contribute significantly to dimer stability in solution (29, 30).

The solution structure of the J unLZ homodimer has no intermolecular salt bridges. The only potentially favorable e-g' electrostatic interaction in J unLZ is that between Arg-276 and Glu-281 at the N-terminal end of the zipper; however, the closest distance between the charged moieties on these side chains is ~10.3 Å (averaged over both strands of all seven structures), precluding the formation of an ion pair. On the other hand, the N-C atoms of Lys-283 and Lys-288 are separated on average by only 6.7 Å, and this distance is <4.7 Å in two of the seven structures. This repulsive eg electrostatic interaction may contribute to the preferential formation of J un-Fos heterodimers over either of the homodimers as Lys-283 and Lys-288 make favorable electrostatic interactions with Glu-173 and Glu-168 of c-Fos, respectively, in the crystal structure of the c-Jun-c-FosAP-1 DNA complex (20).

Role of Conserved Interfacial Asn Residue—The J unLZ homodimer is completely symmetrical except for Asn-291. This a-position Asn residue is highly conserved in the leucine zipper domains of the dystrophin and utrophin families (31) as well as in the bZIP and bHLH-ZIP families of transcription factors. We have previously shown (9) that the two a-position Asn residues form a buried hydrogen bond between their side chain NH2 and CO groups, as noted for the equivalent Asn in GCN4-LZ (16). However, we showed that this is a dynamic interaction in solution, with each Asn acting alternately as hydrogen bond donor and acceptor (9). This motional averaging results in the side chain conformations of Asn-291 being substantially less well defined in the solution structure than those of other interfacial side chains (see Fig. 2a); the mean residue r.m.s difference for Asn-291 is 0.55 Å compared with 0.20 and 0.23 Å for the adjacent interfacial Leu residues.

The melting temperatures of rj unLZ and GCN4-LZ are increased substantially when this Asn residue is replaced by Leu (9, 24), indicating that the Asn residue destabilizes the coiled coil. However, 15N NMR relaxation measurements on rj unLZ suggest that there is no decrease in backbone rigidity in the vicinity of Asn-291 (32). Thus, the instability conferred by the conserved polar Asn residue probably results largely from its unfavorable desolvation at the hydrophobic dimer interface rather than being a consequence of its motional disorder in solution; indeed, this motional averaging might provide some entropic compensation for the enthalpically unfavorable desolvation that occurs when the Asn residue is buried at the hydrophobic dimer interface (32).

An important role of the conserved a-position Asn residue is to impose specificity for dimer structure; mutation of this residue to Leu in GCN4-LZ (24), the synthetic leucine zipper heterodimer ACID-p1/BASE-p1 (33), and rj unLZ (9) leads to the formation of higher order oligomers. However, the destabilizing influence of Asn-291 may also contribute significantly to fast strand exchange rates, which would facilitate rapid reassociation of J un and Fos monomer pairs in vivo; it has been estimated, for example, that the half-time for J un-Fos strand exchange is <10 s at 298 K (34). In stark contrast, a model leucine zipper in which all a- and d-positions are filled by Leu residues has a strand exchange half-time of 30 min at 298 K (35), but this is reduced to ~1 s when a single a-position Leu residue is replaced by either an Asn or Ala residue (35).

Asn and Ala residues fill consecutive a-positions in the J un coiled coil, and hence, they are likely to contribute significantly to fast strand displacement in vivo. MFPs were introduced by Sippl (14) as a means of examining the sequence context of interfacial interactions. In the current context, calculation of residue-by-residue MFPs from the family of rj unLZ solution structures provides a means of estimating the extent of the destabilization caused by the Ala and Asn residues compared with other interfacial residues. Fig. 4 shows that, while the intermonomer mean force potentials (14) calculated for most interfacial residues are significantly negative, indicating stabilization of the dimer (14), those calculated for the Asn and Ala residues are either substantially positive (Asn-291) or only marginally negative (Ala-298), indicating that they are highly destabilizing relative to other interfacial residues. As discussed above, the instability conferred by Asn-291 appears to arise predominantly from its enthalpically unfavorable desolvation. The a-position Ala residues in J unLZ are destabilizing relative to β-branched residues such as Val and Thr because their side
Solution Structure of the c-Jun Leucine Zipper

...chains are too short to make significant side-by-side van der Waals contact (see Fig. 3).

While Jun-Fos dimerization and DNA binding are rapid (half-times < 10 s) (34), dissociation of protein monomers from DNA is very slow (half-time > 16 h) (34). Hence, complex formation in vivo may favor those dimers that form most rapidly rather than those formed with highest affinity. The conserved a-position Asn residue in the leucine zipper family of transcription factors, along with other local factors such as the interfacial Ala residue in c-Jun, may serve to ensure rapid in vivo exchange of zipper strands, thus causing bZIP dimerization in vivo.

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