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Secondary Structure of a Leucine Zipper Determined by Nuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: Previous work has shown that a synthetic peptide corresponding to the leucine zipper region of the yeast transcriptional activator GCN4 forms a stable dimer of α-helices and that the helices are oriented in a parallel manner. Two-dimensional nuclear magnetic resonance spectroscopy (NMR) is used here to demonstrate that the helix is continuous for at least 32 of the 33 residues in the peptide. The results also indicate that the dimer is symmetric. It is therefore unlikely that the interdigitation model for the structure of leucine zippers is correct, since interdigitation of leucine residues in a parallel dimer would lead to an asymmetric structure. The data are consistent with a coiled-coil structure.

Landschulz et al. (1988) proposed the leucine zipper as a hypothetical, α-helical, dimeric structure found in some DNA-binding transcriptional activator proteins. Leucine zipper sequences are characterized by a region of ~30 residues containing a periodic repeat of leucines at every seventh position. Recently, several research groups [e.g., Kouzarides and Ziff (1988), Schuermann et al. (1989), Landschulz et al. (1989), Turner and Tjian (1989), Gentz et al. (1989), and Ransone et al. (1989)] have obtained evidence that the leucine zipper regions of some proteins are important for homo- and heterodimerization, as originally proposed (Landschulz et al., 1988).

Structural studies of proteins containing leucine zippers have not been reported yet, although synthetic peptides corresponding to the isolated leucine zippers of GCN4, Fos, and Jun are found to form very stable dimers in aqueous solution. These dimers are helical as determined by circular dichroism spectroscopy (O'Shea et al., 1989a,b), again consistent with the leucine zipper hypothesis (Landschulz et al., 1988).

In the original leucine zipper model, leucine side chains in adjacent helices are interdigitated like teeth in a zipper (Landschulz et al., 1988). The argument can be made, however, that structure in the dimer does not involve interdigitation of leucine side chains but rather that leucine zippers are short coiled coils. First, disulfide cross-linking studies indicate that the orientation of α-helices in the dimer is parallel (O'Shea et al., 1989a): the parallel orientation is rare in protein structures [for review, see Richardson (1981)] with the exception of coiled coils [for reviews, see Talbot and Hodges (1982) and Cohen and Parry (1986)]. Second, several of the leucine zipper sequences contain an additional heptad repeat of hydrophobic side chains (O'Shea et al., 1989a) that corresponds, together with the leucine heptad repeat, to the 4-3 hydrophobic pattern characteristic of coiled-coil sequences (Hodges et al., 1972; McLachlan & Stewart, 1975).

We report here two-dimensional proton NMR[1] studies of a 33-residue peptide, corresponding to the leucine zipper of the yeast transcriptional activator GCN4 (Jones & Fink, 1982;

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[1] Abbreviations: NMR, nuclear magnetic resonance; 2D NMR, two-dimensional NMR; NOE, nuclear Overhauser enhancement; DQF-COSY, double quantum filtered two-dimensional *J*-correlated spectroscopy; NOESY, two-dimensional nuclear Overhauser enhancement spectroscopy; TOCSY, two-dimensional total correlation spectroscopy; ppm, parts per million; FAB, fast atom bombardment; HPLC, high-pressure liquid chromatography; T_m, temperature at the midpoint of the thermal unfolding transition.

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Table I

residue	chemical shift (ppm)[a] NH	$C^{\alpha}H$	$C^{\beta}H$
acetyl-Arg1	8.47	4.08	1.81
Met2	8.61	4.07	2.08
Lys3	7.83	4.14	1.84
Gln4	7.78	4.13	2.22
Leu5	8.23	4.08	1.34, 2.13
Glu6	8.56	3.86	2.28
Asp7	8.73	4.39	2.68, 2.85
Lys8	8.03	4.23	2.03, 2.13
Val9	8.51	3.42	2.27
Glu10	7.75	4.00	2.18
Glu11	8.24	4.11	2.28, 2.30
Leu12	8.74	4.01	1.27, 2.09
Leu13	9.05	4.06	1.46, 1.96
Ser14	7.78	4.36	4.09
Lys15	8.33	4.21	1.94
Asn16	8.87	4.39	2.75, 3.28
Tyr17	8.26	4.39	3.15, 3.28
His18	7.93	4.38	3.44
Leu19	8.66	3.98	1.33, 2.16
Glu20	8.88	3.94	2.01, 2.19
Asn21	7.77	4.44	2.76, 2.84
Glu22	8.04	4.39	2.02, 2.16
Val23	8.68	3.39	2.12
Ala24	7.78	4.01	1.49
Arg25	7.94	3.98	2.13
Leu26	8.47	3.98	1.32, 1.97
Lys27	9.01	3.82	1.83
Lys28	7.38	4.13	1.90, 1.97
Leu29	7.49	4.19	1.62, 2.05
Val30	7.79	4.08	2.24
Gly31	7.88	3.90, 4.08	
Glu32	7.96	4.38	1.77
Arg33	7.99	4.13	1.70, 1.83

[a] All chemical shifts were obtained at 20 °C and are expressed in ppm relative to internal [$^2H_4$]trimethylsilyl propanoate [assumed δ = −0.014 ppm (DeMarco, 1977)].

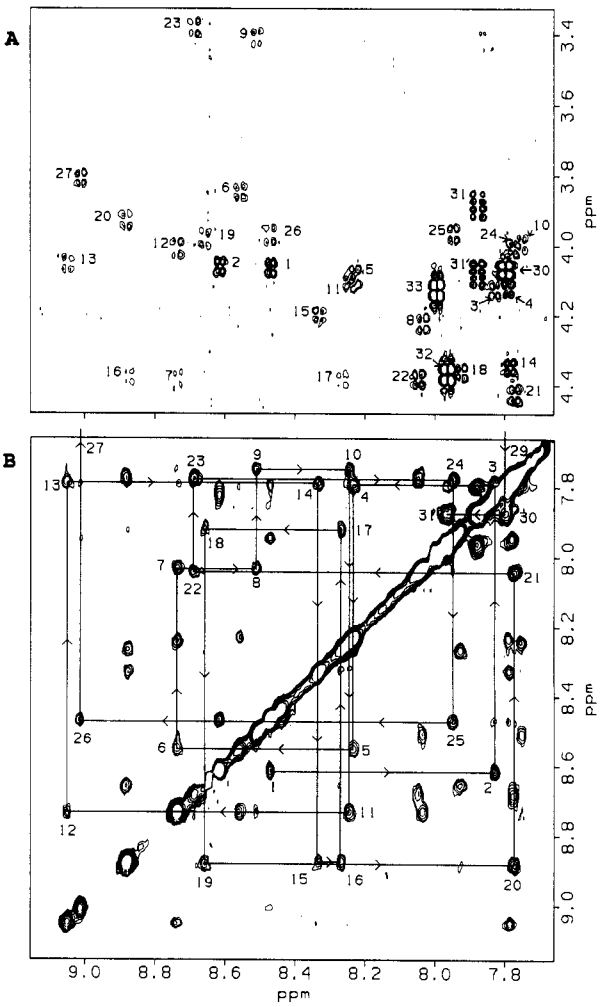

FIGURE 1: 2D NMR spectra of GCN4-p1 indicate that it is a symmetric dimer of helices. These spectra were collected at 20 °C. (A) DQF-COSY spectrum (amide–α region). (B) NOESY spectrum (amide–amide region) with a continuous stretch of sequential cross peaks from residue 1 to residue 32, indicating that the backbone is helical over this range. The arrows indicate sequential NH_i–NH_{i+1} NOEs, and the labels indicate the value of i. For clarity, cross peaks to residues 28 and 29 are not shown, and the contour plot has been made at a high level so that NH_i–NH_{i+2} cross peaks are not visible.

Hinnebusch & Fink, 1983). The results confirm that the peptide is helical and indicate that at least 32 of the 33 residues in the peptide participate in helix formation. We pay particular attention to differences between the interdigitation model and the coiled-coil model of dimerization, because this distinction has implications for the prediction of functional leucine zippers from primary amino acid sequence.

Materials and Methods

The synthesis and purification of the peptide used here, GCN4-p1, has been described (O'Shea et al., 1989a). The identity of the HPLC-purified peptide was confirmed by FAB mass spectrometry (O'Shea et al., 1989a).

The NMR samples contained ~10 mM peptide in 10% D_2O/90% H_2O at pH 5.0. Spectra were recorded at 20, 30, and 35 °C on a General Electric GN500 (Oregon) or a Bruker AM500 spectrometer (Fox Chase Cancer Center, Philadelphia, PA). The DQF-COSY spectra (Shaka & Freeman, 1983; Rance et al., 1983) were collected as 410 t_1 × 2048 t_2 complex points. TOCSY spectra (Braunschweiler & Ernst, 1983; Davis & Bax, 1985), with 38–100-ms mixing periods and a MLEV-17 pulse train, were measured as 450 t_1 × 2048 t_2 complex points. NOESY spectra (Macura & Ernst, 1980) with 5–300-ms mixing times were collected as 300 t_1 × 2048 t_2 complex data points. The spectra were apodized with a 20° phase-shifted sine bell and zero-filled to 2048 × 2048 real data points. The sweep width was 5 kHz in both dimensions. The resonance from H_2O was suppressed by continuous irradiation except during the acquisition period (Zuiderweg et al., 1986). The recycle time after acquisition was 1.5 s. The two-dimensional NMR data were processed with the FTNMR software package, kindly provided by Dr. Dennis Hare, Hare Research Inc., Woodinville, WA. For measurements of NOE cross-saturation rates, the intensities of the cross peaks were determined as volumes and corrected for the volume measured at a 5-ms mixing time.

Results

Figure 1A shows the NH–$C^{\alpha}H$ fingerprint region of the DQF-COSY proton spectrum of GCN4-p1 at pH 5.0. The resonances were assigned from DQF-COSY, TOCSY, and NOESY spectra collected at 20, 30, and 35 °C (Table I). Resonance assignments were made by a main-chain-directed approach (Englander & Wand, 1987) using the NOE connectivities depicted in Figure 2. The spin systems of Gly-31 and Ala-24 were unique reference points for the assignments. The most striking pattern of NOESY cross peaks was found between amide protons on adjacent residues (d_{NN}) (Wüthrich et al., 1984). With only two exceptions, all d_{NN} connectivities could be determined unambiguously at one or more temperatures. Only the Lys-3 to Gln-4 and the Glu-32 to Arg-33 connectivities were ambiguous because at all temperatures they involved cross peaks that were close to the diagonal.

The NMR spectra of GCN4-p1 show only one resonance for each proton in the peptide (e.g., Figure 1), suggesting

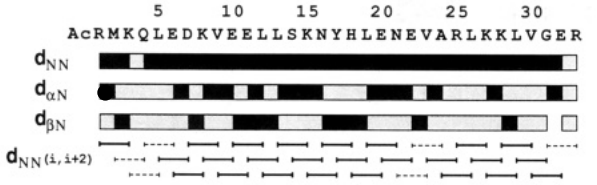

FIGURE 2: NOEs between amide protons of GCN4-p1 indicate that it is a helix for at least 32 out of 33 residues. These and other connectivities were used in the assignment procedure. The connectivities between the NH of residue $i + 1$ and either the NH, $C^{\alpha}H$, or $C^{\beta}H$ of residue i are denoted as d_{NN}, $d_{\alpha N}$, and $d_{\beta N}$, respectively. Dark bars indicate that NOESY cross peaks could be assigned unambiguously (i.e., definitive assignment to a unique pair of protons and no possibility of overlap with other resonances) at one or more temperature: 20, 30, or 35 °C. Light bars and dotted lines indicate that the NOESY cross peaks were observed but could not be assigned unambiguously. The ends of the lines for $d_{NN}(i,i+2)$ indicate the residues involved in these NOEs. None of the connectivities depicted in this figure are unambiguously absent.

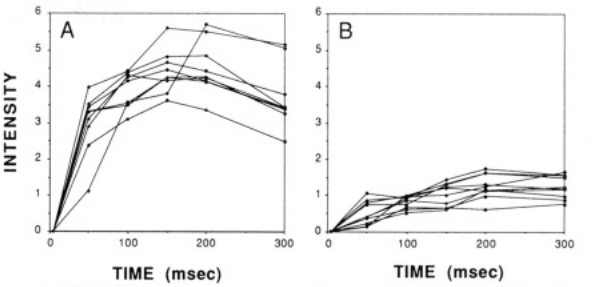

FIGURE 3: NOE intensity measurements as a function of mixing time indicate that d_{NN} is uniformly shorter than $d_{NN}(i,i+2)$, as expected for a helical backbone conformation. (A) The NH_i–NH_{i+1} cross-peak intensities build up more rapidly and to a greater extent as mixing times are increased than (B) the NH_i–NH_{i+2} cross peaks.

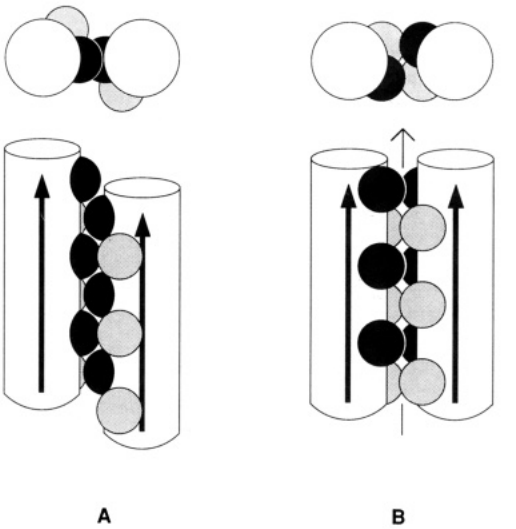

FIGURE 4: Two models for the structure of the leucine zipper can be distinguished on the basis of symmetry. Schematic representations of the two models are shown in top and side views. Parallel helices are represented by white cylinders with arrows showing orientation. The leucine heptad repeat is represented by dark spheres, and the alternate hydrophobic repeat is represented by light spheres. (A) The interdigitated model has the leucine side chains interdigitated at the dimer interface (Landschulz et al., 1988). For parallel helices, this structure will be asymmetric. (B) The coiled-coil model places both members of the 4-3 hydrophobic repeat (see text) at the dimer interface. This structure is symmetric with respect to an axis in the middle of the interface (thin arrow). Although the helices in a coiled coil are supercoiled, they are depicted here as straight cylinders for simplicity.

strongly that the dimer is symmetric.

The NOESY spectra indicate helical secondary structure in at least 32 of the 33 residues of GCN4-p1. In a helix the distance between amide protons on adjacent residues is <3.6 Å (Wüthrich et al., 1984). Thus, a characteristic feature of helices is the presence of strong NOESY cross peaks arising from adjacent amide protons (d_{NN}). Figure 1B shows a continuous series of amide–amide cross peaks in the NOESY spectrum of GCN4-p1, starting with the cross peak between residues 1 and 2 and ending with the cross peak between residues 31 and 32. Although the cross peak between residues 3 and 4 cannot be assigned unambiguously, both residues have strong cross peaks to flanking residues and are therefore included in Figure 1B. The probability of correctly identifying helical secondary structure on the basis of d_{NN} increases greatly as the segment length increases (Wüthrich et al., 1984). The continuous stretch of 32 strong d_{NN} cross peaks in the NOESY spectrum of GCN4-p1 provides conclusive evidence for stable helical structure in the peptide dimer.

In continuous stretches, NOEs between amide protons spaced *two* residues apart are uniquely indicative of helical structure. In general, NOEs are not observed when interproton distances are >5 Å. The distance between amide protons at residues i and $i + 2$ [$d_{NN}(i,i+2)$] is 4.2 Å in an ideal α-helix but greater than 6.5 Å in β-structures. In turns, $d_{NN}(i,i+2)$ can be short, but not in continuous stretches (Wüthrich et al., 1984). As depicted in Figure 2, extensive unambiguous $d_{NN}(i,i+2)$ connectivities are observed throughout essentially the entire length of the peptide. As expected for a helix, the NH_i–NH_{i+1} NOE intensities increase more rapidly and to a greater extent than the NH_i–NH_{i+2} NOEs (Figure 3).

Another class of characteristic helical connectivities (Wüthrich et al., 1984) is $d_{\alpha N}(i,i+3)$. These could not be determined unambiguously because there is low chemical shift dispersion among the α-protons. However, there was no evidence that any of the $d_{\alpha N}(i,i+3)$ connectivities were missing in any of the NOESY spectra recorded at three temperatures.

Discussion

Two models for the structure of the leucine zipper have been proposed, both based on a dimer of helices (Figure 4). The first model suggests that the leucine side chains are interdigitated at the dimer interface (Landschulz et al., 1988). The second model proposes that leucine zipper sequences fold as parallel coiled coils (O'Shea et al., 1989a).

Given that the helix dimer is parallel (O'Shea et al., 1989a), the two structural models can be distinguished by NMR because they predict different symmetry properties. Whereas a coiled coil is symmetric about the dimerization axis (Crick, 1953), a parallel dimer of helices with interdigitated side chains is not (Figure 4). NMR is a sensitive probe of the local environment of side-chain protons. In an asymmetric dimer, different environments within each monomer will usually result in two resonances for each proton in the peptide. The 2D NMR spectra of GCN4-p1 show only one resonance for each proton, suggesting strongly that the dimer is symmetric. The possibility that only one resonance per proton in the peptide is observed due to fast exchange on the NMR time scale between different asymmetric conformations cannot be excluded formally. However, this is very unlikely because dimerization and unfolding are in slow exchange on the NMR time scale at the T_m (data not shown). Moreover, the folded peptide dimer is very stable (O'Shea et al., 1989a), and exchange between fragments of GCN4 and the intact protein dimer takes days at 4 °C (Hope & Struhl, 1987).

An implication of coiled-coil folding of leucine zippers is that the prediction of coiled coils from amino acid sequence considers the hydrophobicity of the alternate (i.e., non-leucine)

position in the 4-3 repeat whereas the interdigitation model does not (Figure 4). Previous studies demonstrating that GCN4-p1 is a parallel dimer of helices (O'Shea et al., 1989a) also support the coiled-coil structural model. However, structural features of the coiled-coil leucine zipper, such as the detailed backbone conformation of the intertwining helices, the hydrophobic packing at the dimer interface, and postulated electrostatic interactions between side chains (O'Shea et al., 1989b), remain to be determined. We have not addressed these issues here because of symmetry constraints and limited side-chain NMR resonance dispersion.

Finally, when bound to DNA, the GCN4 dimer axis of symmetry is likely to be perpendicular to the DNA helix axis, because the recognition site for GCN4 is palindromic (Hill et al., 1986) and the leucine zipper is parallel (O'Shea et al., 1989a). These constraints imply that GCN4 contains a new type of DNA-binding structural motif [for reviews of other DNA binding motifs and their symmetry properties, see Pabo and Sauer (1984), Struhl (1989), and Brennan and Matthews (1989)]. A hypothetical structure for leucine zipper DNA-binding domains has been described (Vinson et al., 1989). The peptide studied here corresponds to the C-terminal half of the 60-residue DNA-binding domain of GCN4 (Hope & Struhl, 1986, 1987). We anticipate that the work described here will facilitate NMR structural studies of leucine zipper DNA-binding domains.

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