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Structural determinants of Cys_2His_2 zinc fingers

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Two mutants of the zinc finger peptide Xfin-31 (Ac-YKCGLCERSFVEKSALSRHQRVHKN-$CONH_2$) containing alterations to the conserved hydrophobic core have been constructed and their zinc-bound structures investigated by ^1H NMR techniques. In the first (Xfin-31B) a double mutation R8F/F10G places the conserved core aromatic residue at position 8 rather than position 10. In the second (Xfin-31C), Phe-10 is replaced by Leu. A qualitative analysis of ^1H chemical shifts, NOE connectivities and coupling constants indicates that the global folds of both mutants are similar to that of the wild-type protein. However, amide exchange rates suggest that the F10L mutant is much less stable than either the wild-type or the R8F/F10G mutant.

Zinc finger; 2D NMR; Protein stability; Supersecondary structure

1. INTRODUCTION

The Cys_2His_2 zinc finger motif represents an important and ubiquitous class of eukaryotic nucleic acid binding proteins [1–3]. Zinc finger peptides from many differing systems have been investigated by NMR techniques [4–13]. Although some discussion prevails on the consensus structure for Cys_2His_2 fingers, it is apparent that nearly all Cys_2His_2 fingers examined to date contain a length of helix packed against a β-hairpin and are stabilized by the formation of a hydrophobic core.

In this paper, we describe ^1H NMR studies on 2 mutant fingers related to finger 31 of Xfin [14], a domain representative of the consensus sequence for Cys_2His_2 fingers [4,5]. These mutant fingers were designed to probe the determinants of structure in Cys_2His_2 fingers, in particular, the importance of the highly conserved aromatic residue that composes part of the core. The first mutant, Xfin-31B, contains a double mutation (R8F/F10G) which switches the conserved aromatic residue from position 10 to position 8 (see Fig. 1 for numbering scheme). This switch is observed in finger 6 from TFIIIA [15], *Drosophila melanogaster* hb [16] and in the alternating even fingers from the human male-associated protein ZFY [10–12]. In the second mutant finger, Xfin-31C, Phe-10 is replaced by Leu, a change which is observed in the zinc finger proteins TRS-1 from *Trypanosoma brucei* [17], and XlcOF and XlcGF from *Xenopus laevis* [18].

Analysis of chemical shift patterns and NOE data from these mutant fingers indicates that the native structure is remarkably tolerant of these mutations since, in the presence of zinc, both mutants adopt a global fold closely similar to that of the wild-type finger. However, amide exchange rates indicate that the F10L mutant is significantly less stable than either the wild-type or R8F/F10G double mutant fingers.

Abbreviations: NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; 2QF-COSY, double quantum filtered correlation spectroscopy; TOCSY, total correlation spectroscopy.

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2. MATERIALS AND METHODS

2.1. *Peptide synthesis and sample preparation*

Xfin-31B (Ac-YKCGLCEFSGVEKSALSRHQRVHKN-$CONH_2$) and Xfin-31C (Ac-YKCGLCERSLVEKSALSRHQRVHKN-$CONH_2$) were synthesized and purified as previously described [4], except that each residue was double-coupled and capped. Samples were prepared for NMR as 5 (Xfin-31B) or 4 mM (Xfin-31C) solutions in either 90% H_2O/10% D_2O or 100% D_2O at pH 5.5 and also at pH 6.3 for Xfin-31C (uncorrected for deuterium isotope effects). Free peptide was dissolved in degassed water (300 μl) and 1.2 eq. zinc chloride added as a 100 mM solution in D_2O. The sample was titrated to pH 5.5 with μl amounts of 0.1 M NaOH and heated to 70°C for 5 min, then cooled to 25°C. The sample volume was adjusted to 450 μl to produce a final proportion of 90% H_2O/10% D_2O.

2.2. *NMR measurements*

^1H NMR spectra were recorded at 278 and 298 K using Bruker AMX-500 and AM-500 spectrometers equipped with digital phase shifting hardware. Data were processed on a CONVEX C240 computer using a modified version of the FTNMR software provided by Dr. Dennis Hare (Hare Research, Woodinville, WA).

Two-dimensional NMR spectra were recorded in the phase-sensitive mode with quadrature detection in the ω_1 dimension using time-proportional phase incrementation [19,20]. Spectra were acquired with the carrier set on the ^1H_2O and residual HOD frequency. For most experiments, 512 t_1 values were usually acquired with either 48 or 64

scans per t_1 increment. Double quantum-filtered COSY (2QF-COSY) spectra were acquired using the standard pulse sequence and phase cycling [21]. Phase-sensitive NOESY spectra were acquired using standard methods [22] with 100, 150, 200 and 300 ms mixing periods. TOCSY experiments were performed according to the method of Rance [23] using a DIPSI-2 sequence for isotropic mixing at a spin-locking field strength of ca. 10 kHz. $^3J_{HN\alpha}$ coupling constants were obtained from 2QF-COSY experiments at 298 K.

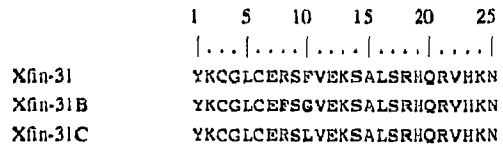

Fig. 1. Sequences of wild-type, Xfin-31, and mutants, Xfin-31B and Xfin-31C. Mutated residues are shown in bold.

3. RESULTS AND DISCUSSION

3.1. *Xfin-31B*

The 1D ^1H NMR spectrum of mutant Xfin-31B (R8F/F10G) in the presence of zinc displays the wide chemical shift dispersion characteristic of a folded zinc finger [4,5]. Complete sequence-specific resonance assignments were obtained for Xfin-31B using standard 2D homonuclear techniques. Spin-system assignments were made from 2QF-COSY and TOCSY experiments in 90% H_2O/D_2O and 100% D_2O. Sequence specific assignments were made by standard methods [24] and are listed in Table 1.

Fig. 2a shows the patterns of sequential and medium-range NOE connectivities identified for Xfin-31B. It is apparent from these NOEs that the secondary structure of Xfin-31B closely resembles that of wild-type Xfin-31 (compare with Fig. 2 in ref. [4]). Thus, strong $d_{NN}(i,i+1)$ NOEs in residues 13–24, together with medium range $d_{\alpha N}(i,i+3)$ and $d_{\alpha\beta}(i,i+3)$ NOEs are consistent with the presence of helical structure in this region. Furthermore, the presence of $d_{\alpha N}(i,i+4)$ NOEs towards the N-terminal residues of this region, contrasting with $d_{\alpha N}(i,i+2)$ NOEs in the C-terminal residues suggests that the helix begins as α-helix but terminates as 3_{10}-helix, as in the wild-type zinc finger [4,5]. Evidence for the presence of N-terminal β-structure in Xfin-31B is provided by a strong $d_{\alpha\alpha}$ NOE detected between Lys-2 and Ser-9, and by a number of other cross-strand NOEs. NOEs are also observed between the side-chain of Tyr-1 and residues 11–13.

Long-range NOEs suggest that the global fold of Xfin-31B is similar to that of Xfin-31 (Fig. 3a, and see Fig. 3 in ref. [4]). However, in Xfin-31B the Phe-8 side-chain contacts the side-chains of Ala-15, Leu-16 and His-19 (Fig. 3a). By contrast, Arg-8 in the wild-type does not exhibit these NOEs [4]. The small $^3J_{HN\alpha}$ coupling constant of 3.8 Hz in Phe-8 Xfin-31B (compared with 8.2 Hz in Arg-8 for wild-type) suggests that the R8F/F10G mutations may have introduced a local distortion in the polypeptide backbone compared with the wild-type structure. With one or two notable excep-

Table 1

^1H chemical shifts of the zinc-complexed Xfin-31B mutant (R8F/F10G) at 278 K and pH 5.5

	NH	$C^\alpha H$	$C^\beta H$	$C^\gamma H$	$C^\delta H$	$C^\varepsilon H$	$C^\zeta H$
Tyr-1	8.60	4.35	2.53, 2.83		6.91	6.77	
Lys-2	8.36	4.64	1.611, 1.811	1.34, 1.43	1.71, 1.48	2.90	7.45
Cys-3	8.81	4.22	2.77, 3.38				
Gly-4	8.82	3.81, 4.23					
Leu-5	9.44	4.41	0.65, 0.89	1.333	0.54, 0.66		
Cys-6	7.74	4.86	3.23, 3.29				
Glu-7	8.50	4.30	1.96, 2.17	2.21, 2.31			
Phe-8	9.02	3.95	2.05, 2.62		6.70	6.93	7.02
Ser-9	7.19	4.68	3.29, 3.37				
Gly-10	8.62	3.30, 4.37					
Val-11	8.86	4.09	2.16	0.94, 0.95			
Glu-12	7.39	4.60	1.95, 2.16	2.23			
Lys-13	8.65	3.09	1.28, 1.50	1.04	1.49	2.85	7.58
Ser-14	8.84	4.13	3.87				
Ala-15	7.31	4.24	1.76				
Leu-16	7.42	4.39	1.53, 2.14	1.62	0.93, 1.06		
Ser-17	8.37	4.26	3.87				
Arg-18	7.90	3.97	1.78, 1.86	1.47	3.12, 3.22	7.28	
His-19	7.64	3.90	2.23, 2.99		6.64	7.53	
Gln-20	8.03	3.55	2.16, 2.22	2.72		7.52, 7.27	
Arg-21	7.08	3.95	1.79	1.71	3.13	7.22	
Val-22	7.74	3.78	1.75	0.23, 0.48			
His-23	7.10	4.68	3.00, 3.16		6.46	7.85	
Lys-24	7.76	4.20	1.72, 1.78	1.36	1.59	2.88	7.55
Asn-25l	8.50	4.61	2.70, 2.77				

The chemical shifts are referenced to H_2O at 4.96 ppm and are accurate to ± 0.01 ppm (± 0.03 ppm for geminal protons separated by <0.1 ppm).

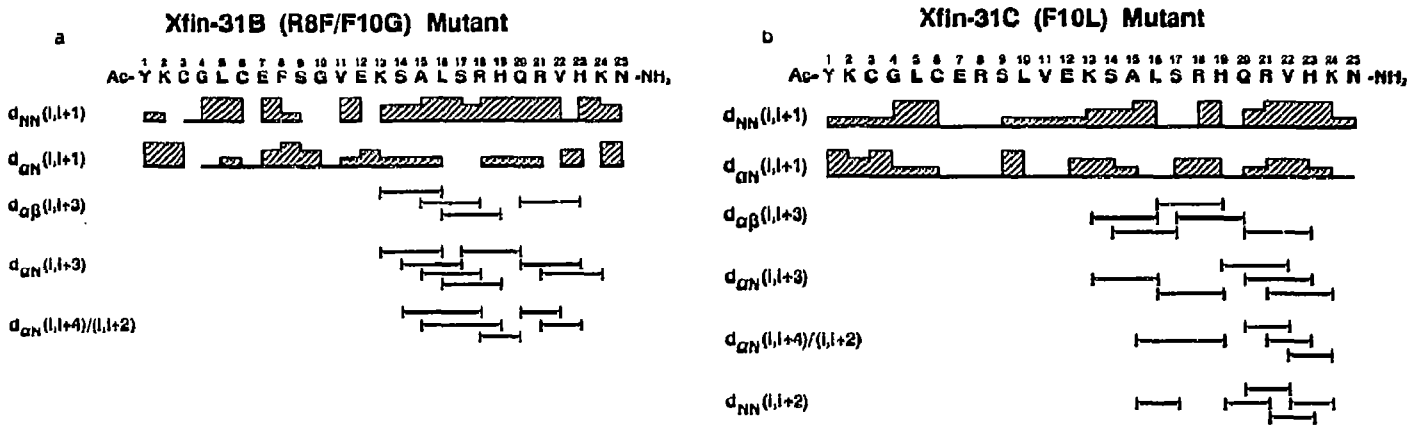

Fig. 2. Summary of sequential and medium-range NOE connectivities for (a) Xfin-31B with 150 ms mixing time and (b) Xfin-31C with 200 and 300 ms mixing times. Sequential ($i,i+1$) NOEs are represented by lines or hatched blocks and the height of the block is a qualative measure of NOE intensity in a NOESY spectrum at 278 K and pH 5.5. Medium-range ($i,i+2$), ($i,i+3$), and ($i,i+4$) NOEs are represented by bars connecting the appropriate residues.

tions, the pattern of chemical shifts in Xfin-31B resembles that of the wild-type Xfin-31. The downfield shift of Leu-5 NH, the upfield shift of Lys-13 $C^{\alpha}H$ and the upfield shift of Val-22 $C^{\gamma}H$ are consistent with a common global fold [25]. Several backbone chemical shifts are perturbed from values observed in the wild-type. The upfield resonance shift of Ser-9 $C^{\alpha}H$ (0.45 ppm) relative to that in the wild-type presumably is associated with the change in position of the adjacent aromatic residue. The largest perturbation involves Leu-16 $C^{\alpha}H$, which is shifted downfield by 1.2 ppm compared with the wild-type. In Xfin-31, the Phe-10 side-chain shields Leu-16 $C^{\alpha}H$; displacing the Phe side-chain in Xfin-31B (and removing it entirely in Xfin-31C) both serve to eliminate this ring current effect.

3.2. *Xfin-31C*

The ^1H NMR spectrum of mutant Xfin-31C (F10L) in the presence of zinc exhibits upfield-shifted methyl resonances and widely dispersed amide and aromatic proton resonances. Both these observations strongly indicate that this mutant, in which the hydrophobic core is composed solely of aliphatic residues, is folded in the presence of zinc. Complete sequence-specific assign-

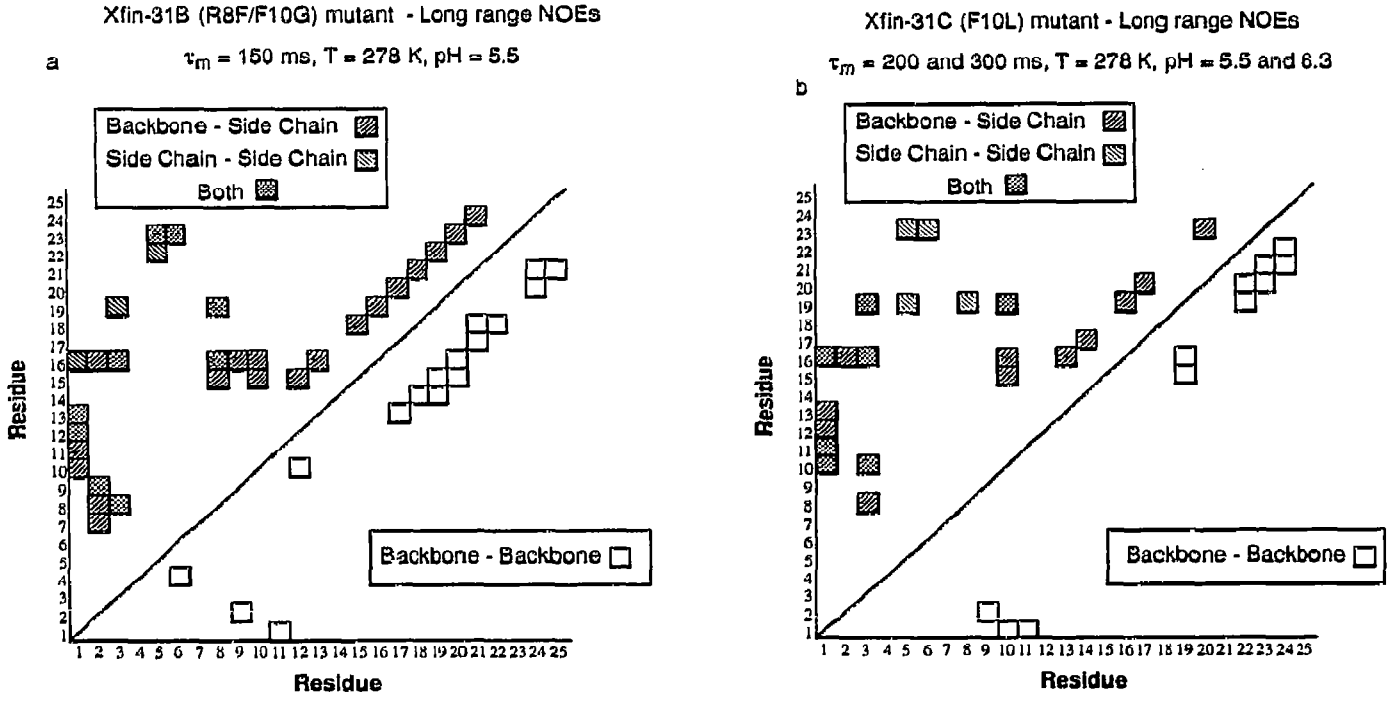

Fig. 3. Summary of long-range and backbone NOE connectivities observed for (a) Xfin-231B and (b) Xfin-31C.

ments were obtained for Xfin-31C by the techniques described previously. The chemical shift fingerprint of Xfin-31C closely resembles that of the wild-type and Xfin-31B fingers and is consistent with a common global fold [25]. Sequential and medium-range NOE connectivities establish the presence of β-hairpin and helix which begins as α-helix and ends as 3_{10}-helix (Fig. 2b). NOEs between the Leu-10 side-chain and the side-chains of residues 15, 16 and 19 (Fig. 3b) are entirely consistent with the packing of Leu-10 in the hydrophobic core.

Although these data show that Xfin-31C adopts a similar fold to the wild-type, the stabilities of the mutant fingers differ markedly. At pH 5.5, Xfin-31B is >95% folded. In comparison, at the same pH, Xfin-31C contains <90% folded protein; the protein is >95% folded at pH 6.3. Amide exchange rates were determined by the acquisition of 1D NMR data for several hours following dissolution of the lyophilized sample in 100% D_2O. In both wild-type and Xfin-31B, the amide protons internal to the β-sheet and on the inner face of the helix which packs against the hydrophobic core exchange relatively slowly over a period of hours [26]. However, all amide protons of Xfin-31C exchanged within minutes of addition of D_2O at 278 K. The increase in internal flexibility and/or destabilization of the folded structure due to the F10L mutation suggests that the presence of an aromatic residue in the hydrophobic core is an important stabilizing force in zinc finger peptides. Destabilization of this mutant may be due to inefficient packing of the hydrophobic core and/or the absence of specific aromatic-aromatic interactions [10,27] between Phe-8 (Xfin-31B) or Phe-10 (wild-type) and the His-19 aromatic sidechain.

Kochoyan et al. [10,11] and Omichinski et al. [12] have shown that synthetic peptides derived from ZFY-6T ($FxCx_2CxFx_7Lx_2Hx_4H$) and EBP-1 ($FxCx_2Cx$-$FxFx_5Lx_2Hx_5H$), respectively, fold in the presence of zinc and that their global folds are similar to that of Xfin-31 [5] ($FxCx_2Cx_3Fx_5Lx_2Hx_3H$). Since the Xfin-31B mutant ($FxCx_2CxFx_7Lx_2Hx_3H$) also folds and adopts a stable tertiary conformation in the presence of zinc, neither the spacing between histidine ligands, nor placing the conserved core Phe at position 8 or 10, appears crucial in determining whether the finger folds. In almost all Cys_2His_2 zinc finger peptides, the highly conserved hydrophobic residue at position 8 or 10 is aromatic [28]; our data clearly demonstrate that the structure is able to tolerate an aliphatic residue as part of the hydrophobic core, although at the cost of reduced thermodynamic stability.

4. CONCLUSION

Complete ^1H resonance assignments of two zinc finger mutants Xfin-31B (R8F/F10G) and Xfin-31c (F10L) have been determined by two-dimensional ^1H NMR spectroscopy. Both mutants fold in the presence

Table II

^1H chemical shifts of the zinc-complexed Xfin-31C mutant (F10L) at 278 K and pH 5.5

	NH	$C^\alpha H$	$C^\beta H$	$C^\gamma H$	$C^\delta H$	$C^\epsilon H$	$C^\zeta H$
Tyr-1	8.70	4.33	2.60, 2.86		6.95	6.75	
Lys-2	8.46	4.54	1.56, 1.67	1.23, 1.34	1.48, 1.53	2.875	7.50
Cys-3	8.77	4.18	2.52, 3.21				
Gly-4	8.85	3.84, 4.07					
Leu-5	9.18	4.35	0.77, 0.81	1.38	0.66, 0.81		
Cys-6	7.72	4.84	3.05, 3.29				
Glu-7	8.47	4.19	2.03, 2.19	2.16, 2.27			
Arg-8	8.48	4.05	1.28, 1.46	1.38, 1.61	2.86, 2.99	7.25	
Ser-9	8.37	4.93	3.49				
Leu-10	8.81	4.79	1.60, 1.79	1.50	0.59, 0.71		
Val-11	8.52	3.87	2.13	0.93, 0.96			
Glu-12	7.44	4.67	1.94, 2.15	2.26			
Lys-13	8.71	3.19	1.31, 1.49	1.09, 1.31	1.45, 1.49	2.82	7.58
Ser-14	8.95	4.14	3.87				
Ala-15	7.11	4.17	1.58				
Leu-16	7.84	4.14	1.37, 2.08	1.59	0.87, 0.94		
Ser-17	8.21	4.28	3.92				
Arg-18	7.84	4.01	1.87, 1.82	1.53, 1.78	3.24, 3.13	7.28	
His-19	8.19	4.32	3.21, 3.52		7.03	7.74	
Gln-20	8.21	3.69	2.19, 2.24	2.72, 2.85			
Arg-21	7.32	4.00	1.76, 1.84	1.61, 1.67	3.17	7.24	
Val-22	7.64	3.83	1.89	0.46, 0.63			
His-23	7.14	4.64	3.02, 3.18		6.44	7.89	
Lys-24	7.82	4.21	1.75, 1.82	1.37	1.61	2.92	7.57
Asn-25	8.46	4.61	2.70, 2.79				

The chemical shifts are referenced to H_2O at 4.96 ppm and are accurate to ± 0.01 ppm (± 0.03 ppm for geminal protons separated by < 0.1 ppm).

of zinc with similar secondary structures and global folds to that found in the wild-type Xfin-31. The sensitivity of chemical shift to changes in local environment suggest that Phe-8 and Phe-10 do not occupy identical positions in the core. Mutant Xfin-31C (F10L) demonstrates that the highly conserved aromatic residue at position 10 can be mutated to an aliphatic hydrophobic residue without loss of structural integrity although the structure is significantly destabilized. Calculations of the three-dimensional structure of both mutants are currently underway in this laboratory.

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