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Resonance assignments, secondary structure and topology of leukaemia inhibitory factor in solution

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Summary

The chemical shift assignments and secondary structure of a murine–human chimera, MH35, of leukaemia inhibitory factor (LIF), a 180-residue protein of molecular mass 20 kDa, have been determined from multidimensional heteronuclear NMR spectra acquired on a uniformly ^{13}C,^{15}N-labelled sample. Secondary structure elements were defined on the basis of chemical shifts, NH-C$^{\alpha}$H coupling constants, medium-range NOEs and the location of slowly exchanging amide protons. The protein contains four α-helices, the relative orientations of which were determined on the basis of long-range, interhelical NOEs. The four helices are arranged in an up-up-down-down orientation, as found in other four-helical bundle cytokines. The overall topology of MH35-LIF is similar to that of the X-ray crystallographic structure for murine LIF [Robinson et al. (1994) *Cell*, **77**, 1101–1116]. Differences between the X-ray structure and the solution structure are evident in the N-terminal tail, where the solution structure has a *trans*-Pro^{17} compared with the *cis*-Pro^{17} found in the crystal structure and the small antiparallel β-sheet encompassing residues in the N-terminus and CD loop in the crystal structure is less stable.

Introduction

Leukaemia inhibitory factor (LIF) is a glycoprotein that was identified and purified on the basis of its ability to induce differentiation in murine myeloid leukaemic M1 cells (Hilton et al., 1988a,b). LIF is produced in a diverse array of cell types (Gough et al., 1992) and is a highly pleiotropic cytokine, exerting a range of actions on a number of cell types, including hepatocytes, adipocytes, megakaryocytes, neuronal, muscle and embryonic stem cells, and osteoblasts (Hilton, 1992). The main biological activities attributable to LIF in vitro are the induction of proliferation and differentiation in various progenitor cells, such as myoblasts, and megakaryocytes. In addition, it suppresses differentiation in embryonic stem cells, allowing these cells to be manipulated genetically (Hilton, 1992). LIF also affects the function of a variety of terminally differentiated cell types such as neurons and adipocytes, and induces acute phase protein production in hepatocytes (Hilton, 1992). Despite having multiple activities, LIF appears to be absolutely necessary only for embryo implantation. Male mice with the gene for LIF knocked out are apparently normal, whereas the corresponding female mice are infertile and the deficiency can be corrected by injection of purified LIF (Stewart et al., 1992). In contrast, the LIF receptor is essential for nor-

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Abbreviations: CNTF, ciliary neurotrophic factor; CSI, chemical shift index; CT-1, cardiotrophin-1; EPO, erythropoietin; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; hLIF, human LIF; HSQC, heteronuclear single-quantum coherence spectroscopy; IL, interleukin; IFN, interferon; LIF, leukaemia inhibitory factor; M-CSF, macrophage colony stimulating factor; mLIF, murine LIF; NOE, nuclear Overhauser enhancement; NOESY, NOE spectroscopy; OSM, oncostatin M; PRL, prolactin; SCF, stem cell factor; TOCSY, total correlation spectroscopy.

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mal function, and mice lacking this gene have multiple placental, skeletal, neural and metabolic disorders which lead to perinatal death (Ware et al., 1995).

Members of the four-helical bundle family of cytokines to which LIF belongs are not only highly pleiotropic but also have overlapping functions (Metcalf, 1992), due in part to their sharing of receptor subunits. Many of the receptors for this family of cytokines are heterodimeric, and for high affinity binding they require both a unique α-chain and a shared β-chain. In the case of the LIF receptor, the β-chain is the signal transducing protein gp130, which is shared with receptors for oncostatin M (OSM), interleukin-6 (IL-6), ciliary neurotrophic factor (CNTF), IL-11 (Miyajima et al., 1992a) and cardiotrophin (CT-1) (Pennica et al., 1995a). Interaction of the cytokine with its specific α-chain is the first step in the signal transduction pathway (Miyajima et al., 1992b) and the execution of its biological function. LIF also shows sequence similarity to the cytokines with which it shares the gp130 β-chain. It is most closely related to CT-1 and OSM, with sequence identities of 24% and 22%, respectively, for the murine molecules (Pennica et al., 1995b), and has lower degrees of sequence identity with IL-11 (18%), CNTF (17%) and IL-6 (17%) (Bazan, 1991; Pennica et al., 1995b).

The diversity of biological targets of the haemopoietic growth factors has led to their investigation as therapeutic agents. G-CSF and GM-CSF are currently being used to accelerate neutrophil (Demetri and Griffin, 1991) or platelet recovery after bone marrow transplantation or cancer chemotherapy (Davis and Morstyn, 1991; Grant and Heely, 1992), their beneficial effects being derived from their ability to induce the differentiation and proliferation of progenitor cells. With its range of biological functions and activity against a variety of target cells, LIF also has potential therapeutic applications. For example, its ability to induce the growth and differentiation of nerve cells indicates that it is potentially useful in the treatment of peripheral nerve damage (Cheema et al., 1994). The receptor subunit gp130, which is shared amongst the IL-6 family of cytokines, is a potential target for antagonists, which could be used in treating certain cancers and autoimmune diseases associated with the overproduction of these cytokines (Peppard et al., 1996). A knowledge of the structures of the cytokines and their receptor complexes will open the way for the design of cytokine antagonists (Debets and Savelkoul, 1994) or agonists directed towards specific tissues.

Despite the low sequence identity amongst the four-helical bundle cytokines, there are common features of their three-dimensional structures. They have been divided into two classes on the basis of their sequences and tertiary structures, as reviewed by Sprang and Bazan (1993), Nicola (1994) and Mott and Campbell (1995). The short-chain molecules include IL-2, IL-3, IL-4, IL-5, IL-7, IL-9, IL-13, IL-15, GM-CSF, M-CSF, SCF and IFN-γ, while the long-chain molecules include IL-12, EPO, G-CSF, LIF, OSM, IL-6, CNTF, IL-11, GH, PRL, IFN-α, IFN-β, IL-10 and CT-1. The tertiary structures of representatives of both the short- and long-chain families of cytokines have been determined by X-ray crystallography and/or NMR spectroscopy. The two classes of cytokines share a common motif consisting of four helices (referred to as the A, B, C and D helices) arranged in an antiparallel, up-up-down-down fashion, with long loops between the A and B helices and the C and D helices. The short-chain molecules are differentiated from their long-chain counterparts by the lengths of their helices, which span only 15 or so residues, compared with about 25 in the long-chain class, and by the secondary structure elements in the long (AB and CD) loops, the short-chain molecules typically having a small β-sheet, and the long-chain molecules having α-helices (which in the case of IFN-γ can be considered to form a fifth long helix). Some of these cytokines (IL-5, IL-10, IFN-γ, M-CSF) are also dimers. While there has been no NMR-derived structure reported for a long-chain cytokine, preliminary NMR studies have been reported on GH (Abildgaard et al., 1992), IL-6 (Morton et al., 1995; Nishimura et al., 1996), hLIF (Smith et al., 1994) and a LIF chimera (Maurer et al., 1994), and resonance assignments have been reported for OSM (Hoffman et al., 1996). NMR structures of the short-chain molecules IL-2 (Mott et al., 1995), IL-3 (Feng et al., 1996), IL-4 (Powers et al., 1992,1993; Smith et al., 1992; Redfield et al., 1994) and G-CSF (Zink et al., 1994) have been determined, and the secondary structure has been reported for the related molecule IFN-γ (Grzesiek et al., 1992). X-ray structures have been published for GM-CSF (Diederichs et al., 1991; Walter et al., 1992b), M-CSF (Pandit et al., 1992), G-CSF (Hill et al., 1993), IL-4 (Walter et al., 1992a; Wlodawer et al., 1992), IL-2 (McKay, 1992), IL-5 (Milburn et al., 1993), GH (De Vos et al., 1992), IL-10 (Walter and Nagabhushan, 1995; Zdanov et al., 1995), CNTF (McDonald et al., 1995) and the related interferons IFN-β (Senda et al., 1992) and IFN-γ (Ealick et al., 1991). Backbone dynamics, as measured by ^{15}N relaxation measurements, have been characterised for IL-4 (Redfield et al., 1992), G-CSF (Werner et al., 1994; Zink et al., 1994) and IL-3 (Feng et al., 1996). These measurements show that residues in the long loops between the helices are relatively mobile compared to those in the helical bundle.

The structures of three complexes of four-helical cytokines with their receptors have also been determined by X-ray crystallography. Two class-1 receptor complexes have been solved, viz. GH bound to its own receptor (De Vos et al., 1992) and to the PRL receptor (Somers et al., 1994). A structurally related class-2 receptor, the IFN-γ receptor, which lacks the characteristic WS motif found in the class-1 receptors (Bazan, 1990), has been solved with IFN-γ bound (Walter et al., 1995). The mode of

binding of IFN-γ was shown to be somewhat different from that of GH to its receptor.

We have undertaken an investigation using NMR spectroscopy of the solution structure and dynamics of LIF. Mature LIF has three disulphide bonds (Nicola et al., 1993) and is heavily N-glycosylated, with an apparent molecular mass range of 32–62 kDa in the glycosylated state and approximately 20 kDa in the non-glycosylated form. LIF has been expressed in both *Escherichia coli* as a non-glycosylated form and in yeast as a hyperglycosylated protein with a molecular mass range of 250–450 kDa. Both the deglycosylated and the hyperglycosylated forms retain their biological activity in vitro (Gearing et al., 1987; Hilton, 1992), indicating that the glycosylation state is not important for interaction with its receptor. The expression levels of human LIF (hLIF) in *E. coli* grown on minimal media proved to be too low for the efficient incorporation of isotopic labels, which are necessary for the sequence-specific resonance assignment of large proteins. However, a murine–human chimera of LIF, MH35-LIF, expressed at high levels (Owczarek et al., 1993; Layton et al., 1994) and could be labelled with ^{13}C and ^{15}N by growth on minimal media containing $^{13}CH_3{}^{13}COONa$ and $^{15}NH_4Cl$ as the sole carbon and nitrogen sources, respectively (Maurer et al., 1994). MH35-LIF consists of residues 1–47 and 83–180 of murine LIF (mLIF) and residues 48–82 of hLIF, except for residues 107, 112, 113, 155 and 158, which are from hLIF (Fig. 1). It has 85% sequence identity with hLIF and 93% identity with mLIF but, most importantly, it has essentially the same biological activity as hLIF (Layton et al., 1994). Human LIF binds both the human and murine receptors with high affinity, while the murine molecule binds more strongly to the murine receptor than to the human receptor (Owczarek et al., 1993; Layton et al., 1994). As preliminary NMR data indicated that the structure of the chimera was indistinguishable from that of hLIF (Maurer et al., 1994), we are using this molecule to determine the 3D structure of LIF in solution. In this paper we report the sequential resonance assignment, secondary structure and topology of MH35-LIF.

Materials and Methods

Sample preparation

The cloning and expression of LIF and various murine–human chimeras have been described previously (Owczarek et al., 1993). Sample preparation and preliminary NMR investigations of MH35-LIF have also been described (Maurer et al., 1994). MH35-LIF labelled with ^{13}C and ^{15}N was prepared by growing *E. coli* in minimal media supplemented with $^{13}CH_3{}^{13}COONa$ as the sole carbon source and $^{15}NH_4Cl$ as the nitrogen source. A 40 ml culture for inoculation was grown overnight on unlabelled complete medium and used to inoculate minimal media after the cells had been separated from the unlabelled media by centrifugation. The cells were grown in 2 l shaker flasks containing 400 ml of labelling media and induced with IPTG. MH35-LIF was expressed as a GST fusion protein and the GST was removed by thrombin cleavage. The MH35-LIF released from the fusion protein was purified in a two-step process involving a cation-exchange step followed by reverse-phase HPLC, and assayed as described earlier (Owczarek et al., 1993). The yield for ^{15}N-MH35-LIF was 18.5 mg from a total fermentation volume of 2.8 l, and for double-labelled MH35-LIF 10.5 mg was obtained from a total volume of 1.6 l. The efficiency of isotope incorporation was determined to be 93% for ^{15}N-MH35-LIF and 96% for $^{13}C,^{15}N$-MH35-LIF using mass spectrometry.

NMR samples were prepared by dissolving lyophilised protein in 90% H_2O / 10% D_2O or D_2O without the addition of buffer. The concentrations were 2 mM for ^{15}N-labelled MH35-LIF and 1.2 mM for double-labelled MH35-LIF, and the pH was adjusted to 4.4 with NaOD or DCl as required, with no correction for the deuterium isotope effect.

NMR spectroscopy

Preliminary details have been described in Maurer et al. (1994). Spectra were recorded at 40 °C on Bruker AMX-500 and -600 spectrometers equipped with triple-resonance probes. Quadrature detection in the indirectly

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                10         20         30         40         50         60
Human   SPLPITPVNATCAIRHPCHNNLMNQIRSQLAQLNGSANALFILYYTAQGEPFPNNLDKLC
Murine  ..................G......KN............S............VE...
MH35    ..................G......KN............S..............

                70         80         90         100        110        120
Human   GPNVTDFPPFHANGTEKAKLVELYRIVVYLGTSLGNITRDQKILNPSALSLHSKLNATAD
Murine  A..M....S..G.....T.......M.A..SA..T.......V...T.V..QV.....I.
MH35    ........................M.A..SA..T.......V.....V.........I.

               130        140        150        160        170        180
Human   ILRGLLSNVLCRLCSKYHVGHVDVTYGPDTSGKDVFQKKKLGCQLLGKYKQIIAVLAQAF
Murine  VM............N..R......PPV..H.D.EA..R.........T...V.S.VV...
MH35    VM............N..R......PPV..H.D.E............T...V.S.VV...
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Fig. 1. Amino acid sequences of human LIF, murine LIF and MH35-LIF. Only the differences from human LIF are shown.

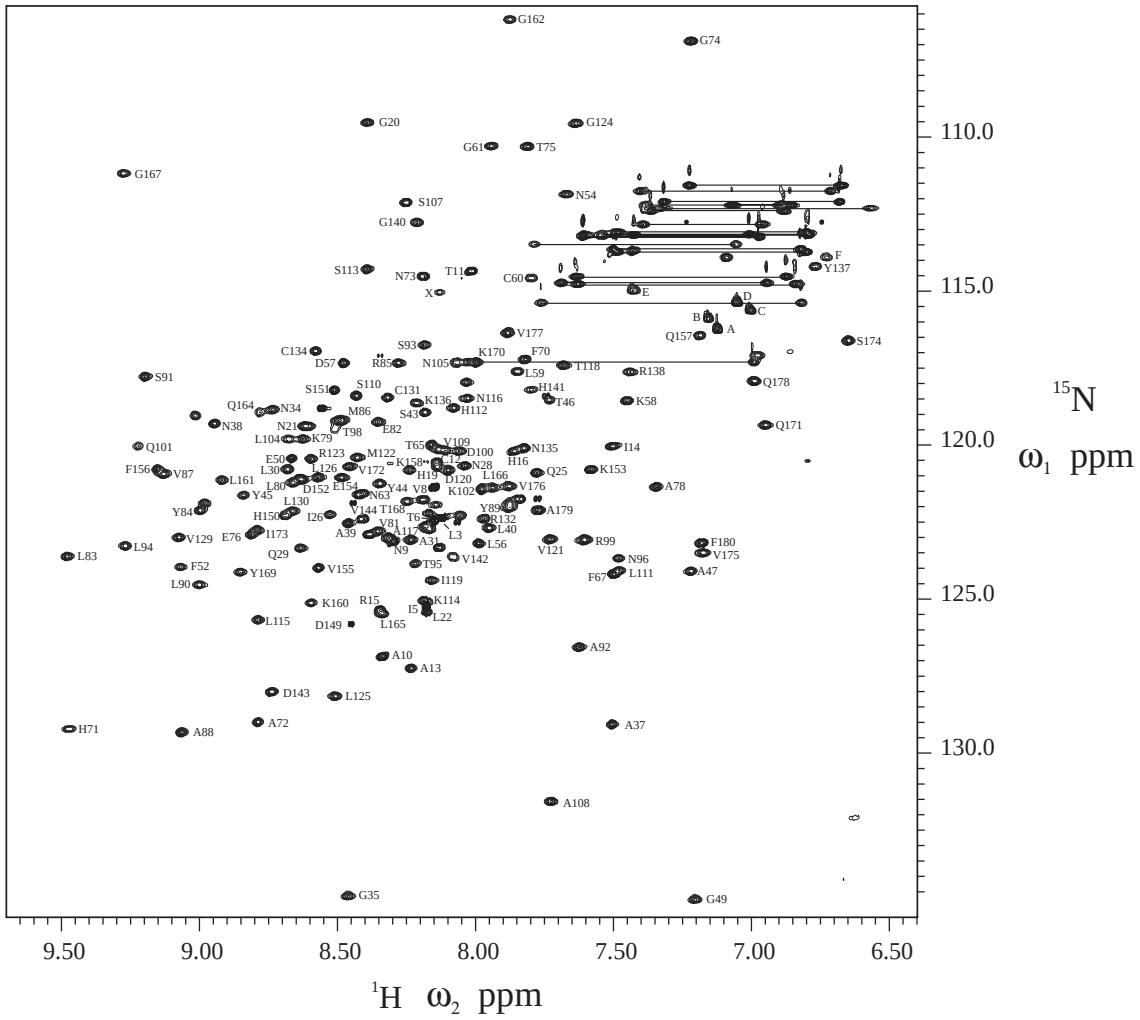

Fig. 2. Contour plot of the 600 MHz 2D ^{1}H-^{15}N-HSQC correlation spectrum on uniformly ^{15}N-labelled MH35-LIF showing the assigned ^{15}N to N<u>H</u> correlations. The spectrum was acquired on a 2 mM sample at pH 4.4 and 40 °C in 90% H_2O / 10% D_2O. G35 and G49 are folded. Bars connect side-chain NH_2 resonances. Folded arginine N^{ε}-H^{ε} resonances are lettered A–F. X designates Q48 $N^{\varepsilon}H$, whose coupling partner is not shown. Q29 $N^{\varepsilon}H$ resonances are beneath the lowest intensity contour shown here.

detected dimensions was achieved using either the TPPI method (Marion and Wüthrich, 1983) or the States-TPPI method (Marion et al., 1989b). Water suppression was achieved using low-power presaturation, high-power purge pulses (Messerle et al., 1989) or pulsed field gradients using the WATERGATE procedure (Piotto et al., 1992). Isotropic mixing was accomplished with MLEV (Levitt et al., 1982), DIPSI-2 (Rucker and Shaka, 1989) or DIPSI-3 (Shaka et al., 1988) mixing schemes. ^{15}N or ^{13}C decoupling during acquisition was achieved with a GARP1 pulse sequence (Shaka et al., 1985).

The following experiments were acquired on uniformly ^{15}N-enriched MH35-LIF: a two-dimensional (2D) ^{1}H-^{15}N-HSQC correlation spectrum (Bodenhausen and Ruben, 1980; Otting and Wüthrich, 1988) with purge-pulse water suppression (Messerle et al., 1989), a 3D ^{1}H-^{15}N-TOCSY-HSQC spectrum with a 30 ms isotropic mixing time (Marion et al., 1989a), 3D ^{1}H-^{15}N-NOESY-HSQC (Marion et al., 1989a) and ^{1}H-^{15}N-HMQC-NOESY-HMQC (Frenkiel et al., 1990; Ikura et al., 1990) spectra, each acquired with a 150 ms NOESY mixing period. NH-C^{α}H coupling constants were determined from an HNHA spectrum (Vuister and Bax, 1993). Slowly exchanging NH protons were identified by recording a series of ^{1}H-^{15}N-HSQC spectra after dissolution of lyophilised ^{15}N-MH35-LIF in D_2O (Smith, 1996; Yao et al., to be published).

The triple-resonance experiments CT-HNCA (Kay et al., 1990; Grzesiek and Bax, 1992), HN(CO)CA (Bax and Ikura, 1991), HNCO (Kay et al., 1990), HCCH-TOCSY (Bax et al., 1990), HCCH-COSY (Ikura et al., 1991) and ^{13}C-edited NOESY-HSQC (Muhandiram et al., 1993) were acquired on a ^{13}C,^{15}N-labelled sample of MH35-LIF at pH 4.4 and 40 °C. A 2D ^{1}H-^{13}C-CT-HSQC spectrum was acquired according to the procedure of Vuister and Bax (1992).

Spectra were processed using UXNMR (Bruker AG, Karlsruhe, Germany) or PROSA (Güntert et al., 1992). Linear prediction was used to extend the heteronuclear

dimensions to improve their digital resolution (Olejniczak and Eaton, 1990). Analysis of the spectra was accomplished using either AURELIA (Neidig et al., 1995) or XEASY (Bartels et al., 1995). The ^{15}N and ^{13}C chemical shifts were referenced indirectly to liquid NH_3 at 0 ppm and to TMS at 0 ppm, respectively, using the $^{15}N/^{1}H$ frequency ratio (Live et al., 1984; Wishart et al., 1995) or the $^{13}C/^{1}H$ ratio (Bax and Subramanian, 1986; Wishart et al., 1995).

Results and Discussion

Resonance assignments

NMR spectra of large molecules are generally characterised by intrinsically broad resonance line widths, arising from their relatively long rotational correlational times (Wüthrich, 1986), and large numbers of resonances, leading to problems of signal overlap. LIF, with a molecular mass of 20 kDa and mainly α-helical structure (Robinson et al., 1994), suffers from both these problems, thus requiring the use of $^{13}C,^{15}N$ doubly labelled protein for resonance assignments. With 21 leucines, 17 valines and no tryptophans in the amino acid sequence, there is also a high degree of spin-system redundancy in MH35-LIF. Procedures for making resonance assignments for molecules of molecular mass greater than ~10 kDa, with the application of isotopic labelling and heteronuclear NMR techniques, are now well established (Bax and Grzesiek, 1993; Bax, 1994), but in order to facilitate the spectral analysis of MH35-LIF it was also helpful to acquire data at an elevated temperature, where sharper resonances were observed. As it had been determined previously (Maurer et al., 1994) by 1D NMR spectroscopy that the structure of LIF was stable up to approximately 67 °C, a suitable temperature for spectral acquisition was determined to be 40 °C. There appeared to be no significant effect on the structure over the temperature range 25–53 °C, as judged by the chemical shifts measured in a series of ^{1}H-^{15}N correlation spectra acquired over this temperature range, although during the overall period of the NMR data acquisition some minor spectral changes were noted. The most obvious was a splitting of the aromatic CH resonances of His^{150}. This was not associated with any significant perturbation of the structure of MH35-LIF, as monitored by the lack of change in its ^{15}N-HSQC spectrum over the corresponding time period.

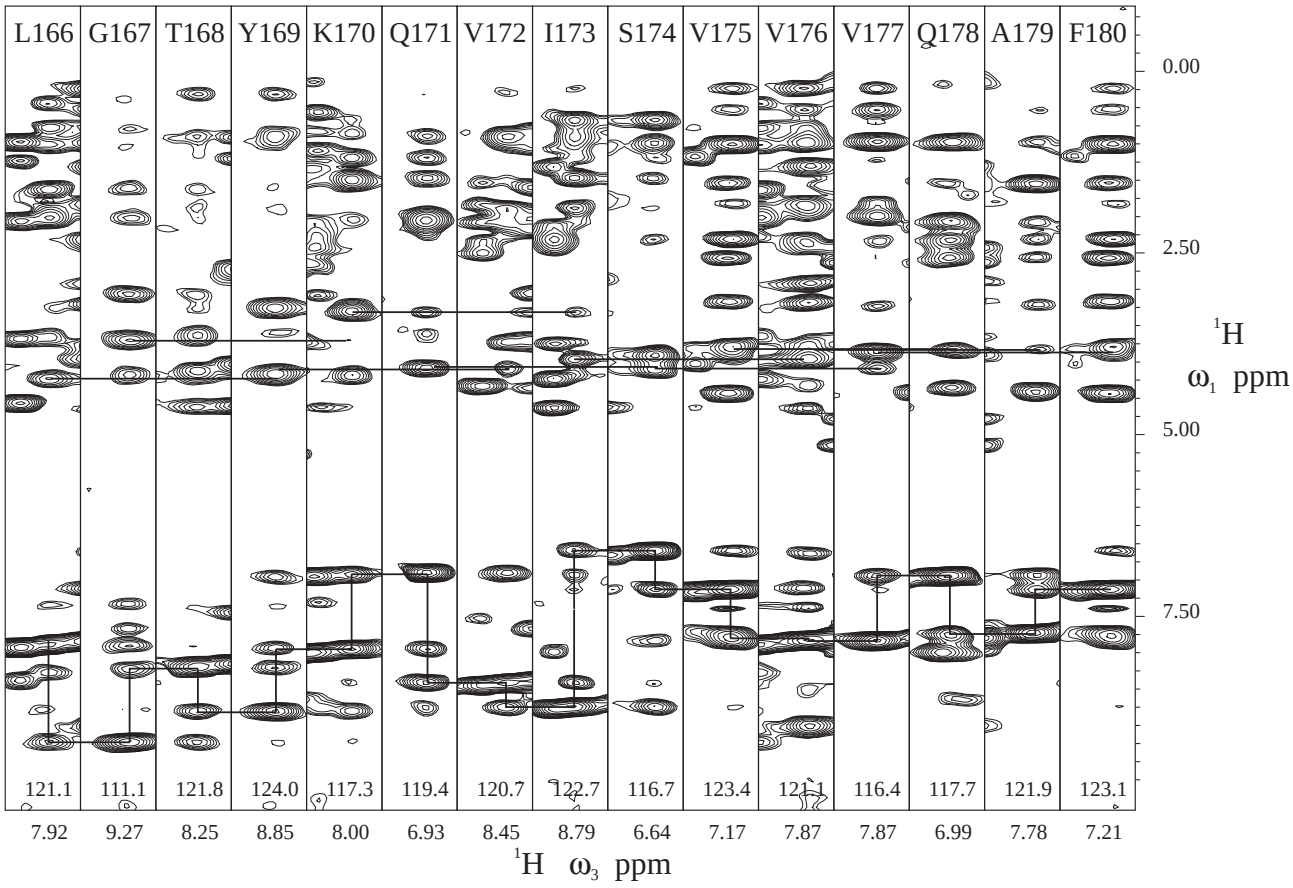

Fig. 3. Strip plots from a 3D NOESY-HSQC spectrum of ^{15}N-MH35-LIF showing the sequential assignment of residues Leu^{166} to Phe^{180} in the D-helix. The strong $d_{NN}(i,i+1)$ NOEs and the weak $d_{\alpha N}(i,i+3)$ and $d_{\alpha N}(i,i+4)$ NOEs observed are indicative of an α-helix. The $C^{\alpha}H$ 167-N170-NH170 cross peak is very weak at this plot level.

TABLE 1
CHEMICAL SHIFT ASSIGNMENTS FOR MH35-LIF AT pH 4.4 AND 40 °C

Residue	^{15}N	HN	CO	H^{α}	C^{α}	H^{β}	C^{β}	Other ^{1}H	Other ^{13}C,^{15}N
S1				4.82	54.8	3.90, 3.82	61.5		
P2								H^{δ} 3.86, 3.74	C^{δ} 48.9
L3	122.4	8.18		4.59	51.2	1.56			
P4								H^{δ} 3.83, 3.64	C^{δ} 48.7
I5	125.1	8.18							
T6	122.5	8.21		4.65	57.7	4.15	67.9	H^{γ} 1.23	C^{γ} 19.7
P7								H^{δ} 3.61, 3.28	C^{δ} 49.3
V8	121.4	8.16	175.8	4.10		2.04		H^{γ} 0.97	
N9	123.0	8.30	174.6	4.78	51.1	2.87, 2.79		H^{δ} 7.63, 6.84	N^{δ} 114.8
A10	126.6	8.33	180.0	4.51	50.3	1.62	18.7		
T11	114.3	8.03	176.8	4.57	59.3	4.29	68.3	H^{γ} 1.19	
C12	120.8	8.14	174.5	4.63		3.20, 2.94			
A13	127.4	8.23	177.1	4.37	51.0	1.40	16.5		
I14	120.1	7.50	176.2	4.15	58.9	1.87	36.9	H^{γ} 1.46, 1.19; H^{δ} 0.87	C^{γ} 25.4; $C^{\gamma 2}$ 15.5; C^{δ} 11.4
R15	125.7	8.34	175.7	4.16		1.71		H^{γ} 1.58; H^{δ} 3.10; H^{ε} 7.16	
H16	120.2	7.87		5.02	51.3	3.29, 3.15	27.1	$H^{\delta 2}$ 7.27; $H^{\varepsilon 1}$ 8.61	
P17				4.45	60.6			H^{δ} 3.79, 3.58	C^{δ} 48.8
C18			174.6						
H19	120.6	8.13	174.0	4.77	53.2	3.26, 2.98	26.4	$H^{\delta 2}$ 7.35; $H^{\varepsilon 1}$ 8.63	
G20	109.7	8.40	181.3	4.00, 3.93	42.8				
N21	119.7	8.61		4.62	53.1	3.10, 2.68		H^{δ} 7.68, 6.94	N^{δ} 114.8
L22	125.4	8.18		3.91		1.47			
M23									
N24	121.0	8.50		3.93	54.2				
Q25	120.8	7.77		4.10	57.6	2.53, 2.38	32.6	H^{ε} 7.22, 6.68	N^{ε} 111.6
I26	122.2	8.53		3.99	63.5	1.93	36.6	$H^{\gamma 2}$ 0.43; H^{δ} 0.75	$C^{\gamma 2}$ 13.4; C^{δ} 12.1
K27	120.0	8.13	177.9	4.06	55.9	1.98, 1.83	39.4	H^{γ} 1.74	
N28	120.8	8.03	178.6	4.53	54.5	3.02, 2.90	35.9	H^{δ} 7.50, 6.82	N^{δ} 113.6
Q29	123.6	8.63		4.07	57.7	2.38	33.1	H^{γ} 2.52; H^{ε} 7.37, 5.35	C^{γ} 33.5; N^{ε} 107.6
L30	120.8	8.68	179.0	3.93	56.4	1.86, 1.35	40.8	H^{γ} 1.79; H^{δ} 1.05, 0.87	C^{γ} 25.1; C^{δ} 24.1, 21.6
A31	122.9	8.23		4.14	53.4	1.61	15.9		
Q32	121.5	8.20		4.10	57.5	2.40, 2.22	26.7	H^{γ} 2.58, 2.40; H^{ε} 6.18	C^{γ} 32.2
L33	122.8	8.17	179.4	4.11	55.9	2.03, 2.03	38.2	H^{γ} 2.09; H^{δ} 1.05, 0.85	C^{γ} 24.5; C^{δ} 20.8, 25.2
N34	118.9	8.74		4.21	55.2	2.80, 2.47		H^{δ} 7.76, 6.82	N^{δ} 115.4
G35	104.8	8.46		4.11, 4.08	44.4				
S36	113.0	7.54	175.7	4.89	56.2	4.15, 4.00	63.6		
A37	129.0	7.51	179.7	4.35	54.4	1.25	17.0		
N38	119.4	8.95	177.0	4.61	54.9	2.87, 2.87		H^{δ} 7.64, 6.87	N^{δ} 114.5
A39	122.4	8.46		4.10	53.0	1.49	16.6		
L40	122.8	7.96		4.63	57.0	2.21	31.4	H^{δ} 1.15, 0.91	
F41	125.7	8.18		3.88		3.25, 2.94		H^{δ} 7.74; H^{ε} 7.74; H^{ζ} 7.40	C^{ε} 130.2; C^{ζ} 128.4
I42	119.7	8.63		2.99	62.1	1.54	37.0	$H^{\gamma 1}$ 1.98, 1.18; $H^{\gamma 2}$ 0.61; H^{δ} 0.89	$C^{\gamma 1}$ 27.2; $C^{\gamma 2}$ 15.0; C^{δ} 12.4
S43	118.9	8.17	176.4	3.87	60.1	3.63, 3.33			
Y44	121.1	8.35	178.9	3.95	59.8	3.36, 2.69	36.5	H^{δ} 6.69; H^{ε} 6.69	C^{δ} 130.2; C^{ε} 116.8
Y45	121.7	8.84	178.0	4.02	59.9	3.06, 2.62	35.1	H^{δ} 6.20; H^{ε} 6.66	C^{δ} 132.9; C^{ε} 116.4
T46	118.6	7.72		3.85	64.2	4.04	67.1	H^{γ} 1.14	C^{γ} 19.7
A47	124.2	7.21		4.12	51.6	1.25	16.7		
Q48	113.9	7.08		3.85	53.9	2.00		H^{γ} 2.60; H^{ε} 8.13, 5.93	N^{ε} 115.1
G49	105.1	7.19	172.7	4.35, 3.89	42.0				
E50	120.1	8.66		4.41	54.0	2.11, 2.03		H^{γ} 2.30	
P51				5.00	62.0	2.32, 2.13	27.1	H^{δ} 3.68, 3.47	C^{δ} 47.7
F52	124.0	9.08		3.99		3.69, 2.73		H^{δ} 7.48; H^{ε} 7.27; H^{ζ} 7.19	C^{ζ} 129.2
P53			177.2	3.98				H^{δ} 3.48	
N54	111.8	7.68	175.1	4.87	51.3	2.93, 2.69	36.8	H^{δ} 7.38, 6.89	N^{δ} 112.4
N55	121.8	7.99	174.9	5.22	50.7	2.74, 2.53	38.0	H^{δ} 8.03, 6.99	N^{δ} 117.3
L56	123.4	7.99	179.8	3.80	56.9	1.69, 1.35	40.5	H^{γ} 1.53; H^{δ} 0.48, 0.14	C^{γ} 24.4; C^{δ} 22.9, 21.9
D57	117.2	8.46	177.7	4.29	55.1	2.81, 2.48	37.3		
K58	118.4	7.44	178.9	4.30	55.7	1.80	31.8	H^{γ} 1.53, 1.40; H^{δ} 1.69	C^{γ} 23.1; C^{δ} 26.7; C^{ε} 40.0
L59	117.4	7.85	175.6	4.50	53.6	1.73, 1.59	42.7	H^{γ} 1.64; H^{δ} 0.84, 0.26	C^{γ} 25.1; C^{δ} 20.9, 23.6
C60	114.8	7.78	178.9	4.93	52.9	3.97, 3.68			
G61	110.1	7.95		4.28, 3.76	42.8				

TABLE 1 (continued)

Residue	^{15}N	HN	CO	H^{α}	C^{α}	H^{β}	C^{β}	Other ^{1}H	Other ^{13}C,^{15}N
P62				4.55	60.5	2.12, 1.93		H^{γ} 1.93; H^{δ} 3.60, 3.52	C^{δ} 47.7
N63	121.4	8.41		4.42		2.82			
V64	120.5	8.13	177.3	4.30	55.7	1.83	36.9	H^{γ} 0.91, 0.89	C^{γ} 15.6, 11.1
T65	120.0	8.17	174.1	4.10	62.8	4.21	66.6	H^{γ} 1.29	C^{γ} 19.9
D66	121.6	8.19	174.1	4.08	51.8				
F67	124.1	7.49		4.13	54.4	3.03, 2.95		H^{δ} 7.48; H^{ϵ} 7.26; H^{ζ} 6.70	C^{δ} 131.0
P68								H^{δ} 2.30, 1.64	C^{δ} 47.9
P69				4.50					
F70	117.3	7.84	173.6	4.50	56.4	2.88, 2.71	40.1	H^{δ} 7.07; H^{ϵ} 7.34; H^{ζ} 7.00	C^{δ} 130.2; C^{ζ} 126.9
H71	129.1	9.50	174.6	4.60	53.5	3.22, 3.13		$H^{\delta 2}$ 7.36; $H^{\epsilon 1}$ 8.37	
A72	128.8	8.79	176.2	3.63	52.7	1.47	16.7		
N73	114.3	8.20	175.8	4.80	50.4	2.93, 2.81	35.2		
G74	106.8	7.22	177.4	4.18, 3.75	42.8				
T75	110.2	7.82	174.4	4.34	59.8	4.70	68.7	H^{γ} 1.38	C^{γ} 20.1
E76	122.8	8.81		3.84	59.2	2.20		H^{γ} 2.37	
K77	117.8	8.04		3.16	58.8	2.82, 2.69	39.8		
A78	121.2	7.35	176.1	3.93	53.3	1.39	17.0		
K79	119.3	8.63		3.92	57.8	2.09, 1.96		H^{ϵ} 3.23	
L80	121.1	8.66	175.8	4.40	50.9	1.60, 1.44	41.4	H^{δ} 0.64	
V81	123.0	8.35	178.6	3.56	66.2	2.22		H^{γ} 1.05, 0.99	C^{γ} 19.4
E82	119.3	8.34	181.5	4.13	58.2				
L83	123.5	9.47	177.7	4.30	56.5	2.42, 1.87	41.1	H^{γ} 1.54; H^{δ} 0.87, 0.73	C^{γ} 21.2
Y84	122.0	8.99	177.2	3.64	60.9	3.33, 3.16	36.2	H^{δ} 6.75; H^{ϵ} 6.75	C^{δ} 130.6; C^{ϵ} 116.8
R85	117.3	8.28	178.3	3.87	58.8	2.05, 1.92	29.9	H^{γ} 1.76, 1.55; H^{δ} 3.07; H^{ϵ} 7.39	C^{γ} 23.9; C^{δ} 39.5
M86	119.4	8.50		3.77	57.6	2.41, 2.26	32.0	H^{ϵ} 1.48	
V87	120.8	9.12	177.8	3.67	64.7	2.07	28.9	H^{γ} 0.96	C^{γ} 22.4
A88	129.1	9.06	180.5	3.93	54.3	1.39	16.3		
Y89	121.9	7.86	179.7	4.08	59.3	3.26, 2.98	36.8	H^{δ} 6.67; H^{ϵ} 6.33; H^{η} 9.44	C^{δ} 130.2; C^{ϵ} 115.4
L90	124.7	8.99	178.0	3.76	56.3	2.31, 1.96	41.4	H^{δ} 1.04, 0.98	
S91	117.8	9.19	177.6	3.88	60.6	3.79			
A92	126.8	7.62	180.6	4.14	53.4	1.49	16.8		
S93	116.8	8.17	177.0	4.09	60.8	3.63, 3.63			
L94	123.0	9.27	180.7	4.05	56.5	1.87, 1.25	39.1	H^{γ} 1.64; H^{δ} 0.64, 0.55	C^{γ} 26.4; C^{δ} 22.1, 23.5
T95	123.7	8.21	176.0	3.82	66.3	4.42	65.9	H^{γ} 1.22	C^{γ} 19.0
N96	123.9	7.50		4.42	54.6	3.02, 2.90	36.3	H^{δ} 7.38, 6.89	N^{δ} 112.2
I97	121.3	8.20		4.08					
T98	119.5	8.48	175.9	3.76	66.5	4.26	66.3	H^{γ} 1.18	C^{γ} 19.5
R99	122.9	7.59	179.4	4.23	57.8	1.98, 1.85		H^{γ} 1.50; H^{δ} 3.20; H^{ϵ} 7.00	
D100	119.6	8.06	179.4	4.52	55.3	3.05, 2.51	36.3		
Q101	120.0	9.23	178.4	3.77	57.0	2.04		H^{γ} 2.34	
K102	123.3	8.04		3.59	57.4	1.76	29.9	H^{γ} 1.34, 0.84; H^{δ} 1.47, 1.41; H^{ϵ} 2.82	C^{γ} 22.9; C^{δ} 26.7
V103	116.8	6.97	178.2	3.95	62.5	2.21	30.6	H^{γ} 1.09	C^{γ} 20.0
L104	119.8	8.69	166.1	4.17	55.1	1.59, 1.44	41.8	H^{γ} 1.95; H^{δ} 0.96, 0.82	C^{γ} 24.6; C^{δ} 21.4, 23.5
N105	116.8	8.07		5.37	49.5	2.82, 2.65	37.6	H^{δ} 7.79, 7.06	N^{δ} 113.5
P106			174.5	3.97		2.55		H^{γ} 2.09; H^{δ} 3.69, 3.37	C^{δ} 48.7
S107	112.6	8.26	175.6	4.59	56.8	4.11, 3.99	61.4		
A108	131.7	7.73	177.9	4.63	49.1	1.28	14.5		
V109	120.8	7.98	178.5	3.76	63.3	2.14	29.5	H^{γ} 1.05, 1.05	C^{γ} 18.7
S110	118.1	8.44	176.8	4.27	58.5	3.92	60.2		
L111	123.7	7.42	179.4	4.19	56.1	2.37	25.2	H^{γ} 1.12; H^{δ} 1.16, 1.05	C^{γ} 26.5; C^{δ} 26.2, 21.7
H112	118.8	8.08		4.30	57.0	3.31, 3.18	28.2	$H^{\delta 2}$ 6.66; $H^{\epsilon 1}$ 7.72	
S113	114.0	8.38	176.5	4.32	60.9	4.10			
K114	125.1	8.19	181.2	4.24	58.0	1.95	31.5	H^{γ} 1.43; H^{δ} 1.59; H^{ϵ} 2.96	C^{δ} 27.2
L115	125.5	8.79	178.5	3.87	57.1	1.86, 1.64	39.6	H^{δ} 0.89	C^{δ} 20.4
N116	118.6	8.03	177.7	4.34	54.8	2.84, 2.79		H^{δ} 7.34, 6.59	N^{δ} 112.0
A117	122.9	8.30	180.9	4.25	53.3	1.58	16.5		
T118	117.2	7.66	175.8	3.88	66.1	4.42	66.2	H^{γ} 1.11; OH 5.42	C^{γ} 19.3
I119	124.2	8.15	177.8	3.38	64.5	1.91	36.1	H^{γ} 1.74; $H^{\gamma 2}$ 0.85; H^{δ} 0.75	C^{γ} 28.5; $C^{\gamma 2}$ 15.5; C^{δ} 12.2
D120	120.8	8.09	180.2	4.41	56.1	2.86, 2.73	38.1		
V121	123.4	7.73	178.6	3.77	64.9	2.24	29.3	H^{γ} 1.16, 0.94	C^{γ} 20.9, 20.2
M122	120.4	8.43	179.3	3.76		1.68			

TABLE 1 (continued)

Residue	^{15}N	HN	CO	H^{α}	C^{α}	H^{β}	C^{β}	Other ^{1}H	Other ^{13}C,^{15}N
R123	120.3	8.59	179.0	3.91	58.3	1.97, 1.86	27.7	H^{γ} 1.59; H^{δ} 3.21; H^{ε} 7.05	C^{γ} 26.2; C^{δ} 41.2
G124	109.4	7.63		3.82, 3.80	45.3				
L125	128.4	8.49	178.9	4.45	56.1	2.06, 1.40	40.2	H^{γ} 1.48; H^{δ} 1.00, 0.85	C^{γ} 25.5; C^{δ} 23.6, 26.3
L126	120.9	8.57	177.4	3.81	56.9	1.96, 1.52	39.5	H^{γ} 1.72; H^{δ} 0.59, 0.29	C^{γ} 24.5; C^{δ} 23.4, 22.2
S127	113.5	7.42		4.32	59.4	3.98, 3.98	60.6		
N128	121.8	7.87	177.7	3.99	54.2	2.51		H^{δ} 7.07, 6.86	N^{δ} 112.2
V129	122.9	9.08	178.0	3.58	65.6	2.44	29.9	H^{γ} 1.29, 1.10	C^{γ} 21.8, 22.5
L130	122.0	8.66	179.2	4.07	56.5	1.72	39.5	H^{γ} 1.72; H^{δ} 1.12, 0.91	C^{γ} 25.1; C^{δ} 24.5, 20.8
C131	118.5	8.31		4.19	54.4	3.61, 3.04	38.9		
R132	121.8	7.97		4.14	56.7	1.79		H^{δ} 2.96; H^{ε} 6.73	
L133	120.9	8.89	179.2	4.29		1.73	41.0	H^{γ} 1.32; H^{δ} 0.64	
C134	116.5	8.58		4.73	54.2	3.06, 2.53	36.9		
N135	120.2	7.83	176.0	4.49	53.9	2.91, 2.81	35.8	H^{δ} 7.61, 6.97	N^{δ} 113.3
K136	118.5	8.22	177.2	4.33	55.2	1.53	31.0	H^{γ} 0.57, 0.41; H^{δ} 1.34; H^{ε} 3.10	C^{γ} 22.7; C^{δ} 26.7; C^{ε} 40.0
Y137	114.3	6.75	174.3	4.81	56.5	3.36, 3.10	36.6	H^{δ} 7.24; H^{ε} 6.84	C^{δ} 131.0; C^{ε} 116.0
R138	117.6	7.44		4.27	55.0	1.84, 1.77	28.2	H^{γ} 1.63, 1.59; H^{δ} 3.20	C^{γ} 25.2; C^{δ} 41.1
V139	117.1	7.36	175.1	4.03	60.9	1.93	31.0	H^{γ} 0.82, 0.77	C^{γ} 19.7, 18.5
G140	112.6	8.22	173.7	4.02, 4.02	44.4				
H141	118.2	7.82	171.4	4.82	53.3	3.30, 3.18	28.4	$H^{\delta 2}$ 7.09; $H^{\varepsilon 1}$ 8.62	
V142	123.8	8.10	173.9	3.94	59.4	1.42	32.6	H^{γ} 0.94, 0.41	C^{γ} 20.3, 19.3
D143	127.8	8.42	173.2	4.78	52.5	2.68, 2.53	39.0		
V144	122.3	8.42		3.75	57.2	2.20		H^{γ} 1.04, 1.04	
P145								H^{δ} 3.29, 3.29	
P146									
V147									
P148			176.2	4.33					
D149	125.6	8.45		4.75	51.1	2.87, 2.78			
H150	122.2	8.70	175.7	5.02	52.6	3.24, 3.24	27.0	$H^{\delta 2}$ 7.24; $H^{\varepsilon 1}$ 8.53	
S151	118.3	8.51	174.1	4.21	59.0	3.95, 3.95	61.1		
D152	121.1	8.64	176.1	4.69	51.9	2.82, 2.70	37.5		
K153	120.5	7.58	176.2	4.40	53.3	1.96, 1.88		H^{γ} 1.58	
E154	120.9	8.50	177.8	4.38	54.3	2.16	34.6	H^{γ} 2.42	
V155	124.1	8.58	177.9	3.54	66.3	2.24	30.0	H^{γ} 1.25, 1.14	C^{γ} 20.8, 19.8
F156	120.5	9.17	177.7	4.31	59.9	3.48, 3.09	37.4	H^{δ} 7.38; H^{ε} 7.18; H^{ζ} 7.03	C^{δ} 130.1; C^{ε} 128.4; C^{ζ} 127.2
Q157	116.4	7.18	178.9	3.99	56.6	2.20		H^{γ} 2.34	
K158	120.8	8.24		3.83	59.6	1.88, 1.77			
K159	123.0	8.11	181.3	3.90	57.0	1.75			
K160	125.1	8.59	177.7	4.28	56.5	1.82			
L161	122.3	8.99	180.6	4.16	56.4	1.73, 1.38	38.9	H^{γ} 1.79; H^{δ} 0.86, 0.55	C^{γ} 25.1; C^{δ} 21.0, 22.6
G162	106.0	7.88		3.76, 3.76	45.0				
C163	123.0	7.61		3.79	57.5	3.74, 2.30			
Q164	118.7	8.78		4.17	56.7	2.08, 2.08		H^{γ} 2.27, 2.27	
L165	125.2	8.39	180.0	4.37	56.5	2.11, 1.93	38.8	H^{γ} 1.93; H^{δ} 0.91, 0.49	C^{γ} 26.1; C^{δ} 22.5, 22.6
L166	121.1	7.92	179.8	4.30	56.5	2.12	40.2	H^{γ} 1.72; H^{δ} 1.10, 0.83	C^{γ} 25.6; C^{δ} 22.2
G167	111.1	9.27	176.1	3.79, 3.13	45.3				
T168	121.8	8.25	175.3	3.67	65.7	4.16	66.0	H^{γ} 0.38	C^{γ} 19.0
Y169	124.0	8.85	175.1	4.24	57.0	3.34, 3.29	36.5	H^{δ} 7.03; H^{ε} 6.55; H^{η} 9.53	C^{δ} 130.6; C^{ε} 114.5
K170	117.3	8.00	177.0	3.41	57.1	1.63, 1.54		H^{γ} 1.33, 1.27; H^{δ} 1.49; H^{ε} 2.55	
Q171	119.4	6.93	179.4	4.13	56.1	2.07			
V172	120.7	8.45		3.81	63.9	1.97	30.5	H^{γ} 1.05, 0.95	C^{γ} 19.6, 20.3
I173	122.7	8.79	175.3	4.04	58.7	1.54	36.2	H^{γ} 1.34, 1.21; $H^{\gamma 2}$ 0.74; H^{δ} 0.87	C^{γ} 26.7; $C^{\gamma 2}$ 17.8; C^{δ} 11.3
S174	116.7	6.64	176.1	4.19	58.7	4.01, 3.95	61.1		
V175	123.4	7.17	179.1	3.85	63.4	2.40	30.2	H^{γ} 1.10, 1.06	C^{γ} 19.8, 19.9
V176	121.1	7.87	178.4	3.30	63.9	1.91	29.6	H^{γ} 0.60, 0.32	C^{γ} 20.7, 19.8
V177	116.4	7.87	176.6	3.93	63.6	2.08	29.6	H^{γ} 1.05	C^{γ} 18.7
Q178	117.7	6.99	176.2	4.46	54.7	2.09		H^{γ} 2.34	
A179	121.9	7.78	176.0	4.51	50.3	1.41	17.8		
F180	123.1	7.21		4.52	57.9	3.23, 2.66	40.2	H^{δ} 7.44; H^{ε} 7.44; H^{ζ} 7.20	C^{ε} 130.6; C^{ζ} 128.0

For methylene and aromatic protons two chemical shifts are indicated when two proton resonances are assigned; a single entry indicates it was not established whether the resonances were degenerate.

A 2D ^{1}H-^{15}N-HSQC spectrum of MH35-LIF acquired at 40 °C and pH 4.4 in 90% H_2O / 10% D_2O is shown in Fig. 2. The spectrum shows 141 well-resolved resonances of the anticipated 166 main-chain NH resonances and 20 side-chain NH_2 resonances from the 23 expected from 15 asparagine and 8 glutamine residues. This 2D spectrum shows that the majority of resonances had sufficient chemical shift dispersion to allow the chemical shift assignment to rely mainly on ^{15}N-edited techniques. 3D ^{15}N-TOCSY-HSQC and ^{15}N-NOESY-HSQC spectra were used in the conventional fashion (Markley and Kainosho, 1993) for assignment. Generally, a poor transfer of magnetisation during the TOCSY transfer stages was observed for MH35-LIF due to the combination of its mainly α-helical structure, with attendant small coupling constants, and its relatively short T_2 values. Proton TOCSY mixing periods were therefore kept short (typically ≤30 ms) and cross peaks were either weak or not observed for protons beyond $C^{\beta}H$ in the side chain in spectra reliant on a proton TOCSY step for magnetisation transfer. A ^{15}N-3D-HMQC-NOESY-HMQC (Frenkiel et al., 1990; Ikura et al., 1990) was useful in resolving cases where there was NH overlap in the ^{15}N-NOESY-HSQC. The α-helix is characterised by a series of strong d_{NN} NOEs and relatively weak $d_{\alpha N}$ NOEs (Wüthrich, 1986), and Fig. 3 shows a representative region of the sequential assignment from the ^{15}N-NOESY-HSQC spectrum which exhibits the strong sequential d_{NN} and $d_{\alpha N}(i,i+3)$ NOEs corresponding to the expected α-helical nature of MH35-LIF.

Experiments with a uniformly ^{13}C,^{15}N-labelled sample of MH35-LIF were performed to establish scalar connectivities. The backbone sequential connectivities were determined using a combination of CT-HNCA (Grzesiek and Bax, 1992) and HN(CO)CA (Bax and Ikura, 1991) experiments. In the HNCA spectra, each N^{H} potentially has a connectivity both to the intraresidue C^{α} and the preceding C^{α}, although the sequence was broken by proline residues. The HN(CO)CA spectrum identified the interresidue connectivity, while the HNCA spectrum identified both the intra- and interresidue connectivities and hence could detect degenerate resonances from adjacent residues. The ^{13}CO frequencies were obtained from an HNCO spectrum (Kay et al., 1990). As this spectrum connected each ^{13}CO with the preceding NH resonance, each ^{13}CO resonance could be assigned sequentially with the aid of the HNCA/HN(CO)CA pair. The sequence-specific assignment process relied on a combination of HNCA and HN(CO)CA spectra, which connected residues along the backbone through their mutual J-coupling, while the ^{15}N-edited TOCSY and NOESY spectra pair gave information on the side chain and through-space connectivities. The spectra used in combination gave complementary information. The sequential assignment was confirmed using a conventional 2D NOESY spectrum acquired with a good signal-to-noise ratio and 512 increments in ω_1. This was also useful in assigning resonances not observable in the isotope-edited spectra. For example, Tyr^{89}, Thr^{118} and Tyr^{169} had slowly exchanging side-chain OH protons which were observed in the 2D spectrum.

Side-chain resonances were assigned using 3D HCCH-COSY (Ikura et al., 1991) and HCCH-TOCSY (Bax et al., 1990) experiments. The 2D ^{13}C-CT-HSQC experiment of Vuister and Bax (1992) was also useful as it had good resolution in ω_1 due to the constant-time nature of the acquisition, which effectively removed ^{13}C-^{13}C coupling in ω_1. This experiment also had the advantage that the cross peaks were edited according to the number of coupling partners (those ^{13}C nuclei with an odd number of ^{13}C coupling partners appearing positive relative to those with an even number). Thus, the threonine C^{β}-$C^{\beta}H$ and glycine C^{α}-$C^{\alpha}H$ cross peaks had intensities opposite in sign to those of the C^{α}-$C^{\alpha}H$ cross peaks of all the other amino acids, making them readily distinguishable. The combined knowledge of the chemical shifts of the C^{α} and C^{β} resonances gave information on the residue type (Grzesiek and Bax, 1993). The resonance assignments of MH35-LIF are summarised in Table 1.

Very few $J_{NH\text{-}C^{\alpha}H}$ coupling constants in large molecules can be determined directly from DQF-COSY spectra due to the mutual cancellation in antiphase cross peaks caused by the relatively broad line widths. It was necessary to measure these coupling constants by using intensity ratios between the diagonal and cross peaks in an HNHA spectrum (Vuister and Bax, 1993), which are dependent upon the sizes of the coupling constants.

A 2D homonuclear NOESY spectrum proved useful in finding slowly exchanging OH and side-chain NH_2 protons. Earlier work (Maurer et al., 1994) had shown that there was one tyrosine residue undergoing slow aromatic ring flipping (Wüthrich, 1986). This was assigned to Tyr^{89}, which also had a slowly exchanging OH proton. This OH had a long-range NOE connectivity to the $C^{\delta}H$ aromatic resonance of Phe^{67}. Tyr^{169} had a slowly exchanging OH group which had long-range NOE connectivities to the $C^{\alpha}H$ of Leu^{33} and Ala^{37}, as well as medium-range NOEs to the $C^{\gamma}H$ and $C^{\delta}H$ methyl protons of Ile^{173}, consistent with Tyr^{169} being in an α-helix. The NOE connectivities observed for the OH protons of Tyr^{89} and Tyr^{169} were consistent with the crystal structure of mLIF (Robinson et al., 1994). Thr^{118} had a slowly exchanging OH proton with an NOE connectivity to the $C^{\gamma}H$ of Val^{121}, consistent with its position in a helix.

Secondary structure of MH35-LIF

The secondary structure of a protein can be established from a number of NMR-determined parameters, including chemical shift differences from random coil values (Wishart and Sykes, 1994), the magnitude of NH-$C^{\alpha}H$ coupling constants and the pattern of short- and medium-

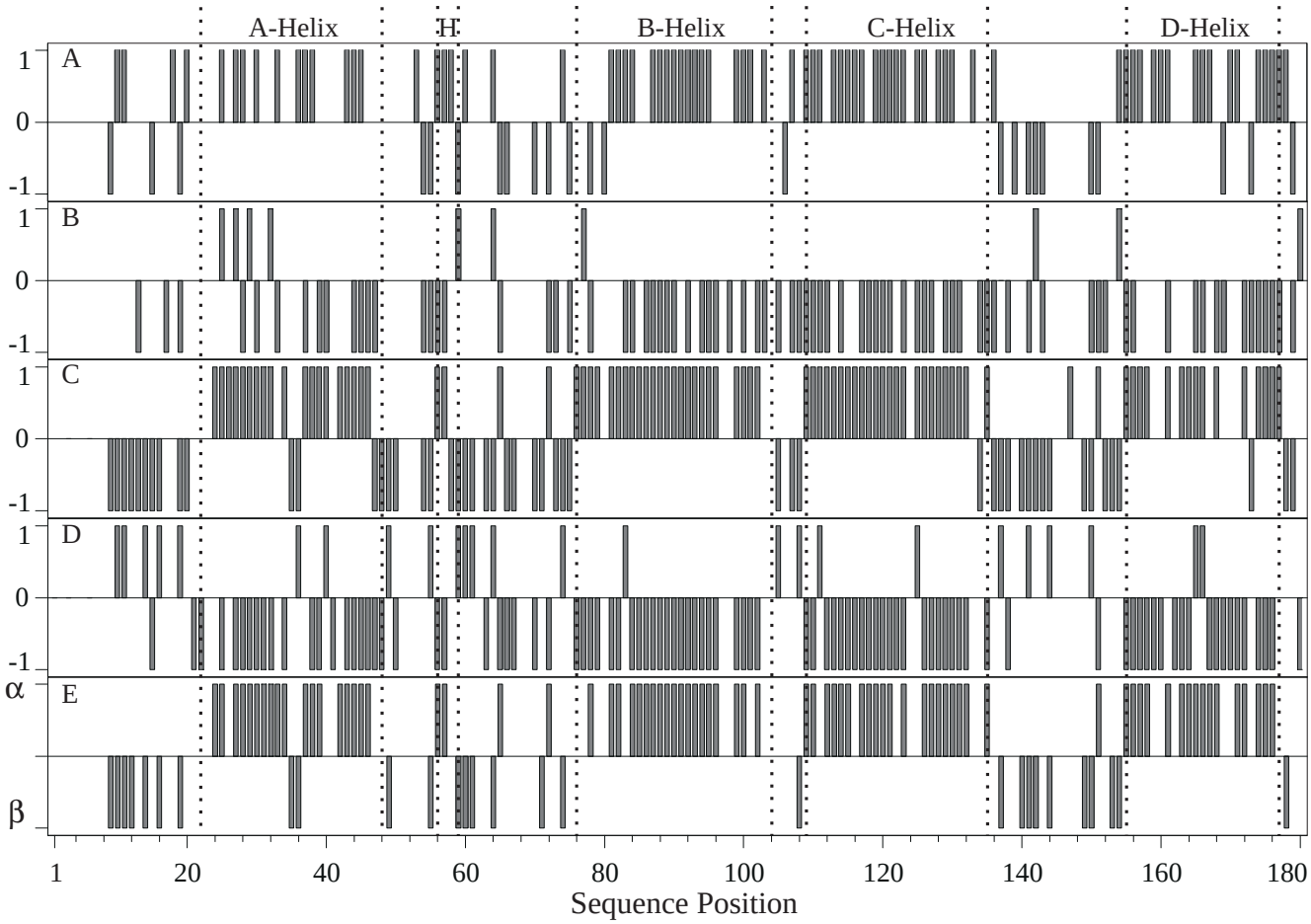

Fig. 4. Chemical shift indices of MH35-LIF. (A) CO, (B) C^{β}, (C) C^{α} and (D) $C^{\alpha}H$ resonances. The consensus plot is shown in (E), with the limits of the helices according to the X-ray structure indicated as vertical dashed lines and the helix in the AB loop indicated by H.

range NOEs (Wüthrich, 1986). The deviation of chemical shifts from their random coil positions, particularly for C^{α}, C^{β}, CO and $C^{\alpha}H$ resonances, is highly indicative of the secondary structure (Dalgarno et al., 1983; Spera and Bax, 1991; Wishart et al., 1991), and the chemical shift index (CSI) (Wishart and Sykes, 1994) is a convenient method of determining the elements of secondary structure from chemical shifts, although it is not designed to replace structure determination based on NOE data. Figure 4 summarises the chemical shift information from ^{13}C,^{15}N-labelled MH35-LIF. The series of −1 indices for the $C^{\alpha}H$ resonances and +1 for the C^{α} resonances indicates the presence of a helix. The consensus plot in Fig. 4E shows the limits of the helices according to the CSI, as well as the limits of the helices determined from the X-ray crystal structure. Large ring current effects on the chemical shifts are not expected for MH35-LIF as there are no tryptophans present in the sequence.

The sequential assignments and associated NMR data for MH35-LIF are summarised in Fig. 5. The pattern of NOEs is consistent with the presence of four α-helices (Wüthrich, 1986). Based on the pattern of short- and medium-range NOEs, the locations of small $J_{NH\text{-}C^{\alpha}H}$ coupling constants and slowly exchanging backbone amide protons, and the deviations of chemical shifts from their random coil values, the A-helix encompasses residues 22–49, the B-helix 75–105, the C-helix 109–135 and the D-helix 154–180. There is also a short irregular helix involving residues 55–60. This description of the secondary structure is in excellent agreement with that of the crystal structure of mLIF (Robinson et al., 1994), where the limits of the helices were 22–48, 76–104, 109–135, 155–177 and 56–59 for the A, B, C, D and short helices, respectively, as indicated in Fig. 5.

The exchange rates of the NH protons are valuable in determining which amide protons participate in stable hydrogen bonds and/or are shielded from the solvent. In the NH exchange data depicted in Fig. 5, the open circles denote NH protons that have exchanged within 48 h of dissolution of the sample in D_2O and the filled circles denote those that were still present after 48 h at 37 °C and pH 4.4. All the other NH protons were not observed in the initial ^{1}H-^{15}N-HSQC spectrum. The exchange data showed that residues in the loops between the helices had relatively rapidly exchanging NH protons compared with residues in the helices, indicating that these residues were

more mobile and on average more solvent-exposed. The exchange rates of amide protons near the ends of the helices were faster than those from the centres of the helices, reflecting 'helix fraying' at the ends (Rohl and Baldwin, 1994). In the short irregular helix in the AB loop, all NH protons were in intermediate or fast exchange. There were regions of fast exchange within the A-helix (Fig. 5) and there was a series of $d_{\alpha N}(i,i+2)$ and $d_{NN}(i,i+2)$ NOEs and a change in the chemical shift indices (Fig. 4) in this region, which indicate that the helix is not in a regular α-helical conformation. This coincides with a kink in the A-helix of the X-ray structure of mLIF centred around Ser^{36}, where the normal hydrogen bonding pattern is disrupted (Robinson et al., 1994). A similar irregularity in the expected chemical shift deviations for an α-helix has been observed for OSM (Hoffman et al., 1996) and is attributed to distortion of the α-helix conformation. There is a less pronounced kink in the D-helix of the crystal structure centred at Ser^{174}, but there is no obvious effect on the NH exchange rates in this part of the molecule (Fig. 5).

Capping interactions at the termini of helices are thought to play a role in helix stabilisation (Presta and Rose, 1988) by providing hydrogen bonding partners for at least some of the first four amide protons and the last four carbonyl oxygens in the helix. Interactions at the N-termini of helices have been termed N-capping boxes, and consist of the three N-terminal residues in the helix and the residue immediately preceding the helix, termed the N-cap. The side chain of the N-cap residue forms a hydrogen bond with the backbone amide of the third residue from the N-terminal end of the helix and a reciprocal hydrogen bond exists between the side chain of the third helical residue and the N-cap amide (Harper and Rose, 1993). The deviations of the C^{α} and C^{β} chemical shifts from their random coil positions at the N-terminus of helices provide a means of detecting the presence of a capping box (Gronenborn and Clore, 1994). In the present study the B, C, D helices and the helix in the AB loop show the distinctive pattern of chemical shift deviations for the C^{α} resonances, where the N-cap residue shows a negative deviation and the three N-terminal residues of the helix show a positive deviation from their respective random coil positions (not all resonances in the N-terminus of the A-helix nor all C^{β} resonances at the N-termini of the helices have been assigned). Although the patterns of deviations in chemical shifts apparently fit the N-capping box motif, the N-terminal residues of the C-helix do not fit the sequence pattern required for a side-chain hydrogen bond between the N-cap and the third residue from the end of the helix.

Topology of MH35-LIF

The X-ray structure of mLIF (Robinson et al., 1994) shows a flexible N-terminal tail reflected in the absence of electron density for the first eight residues of the mol-

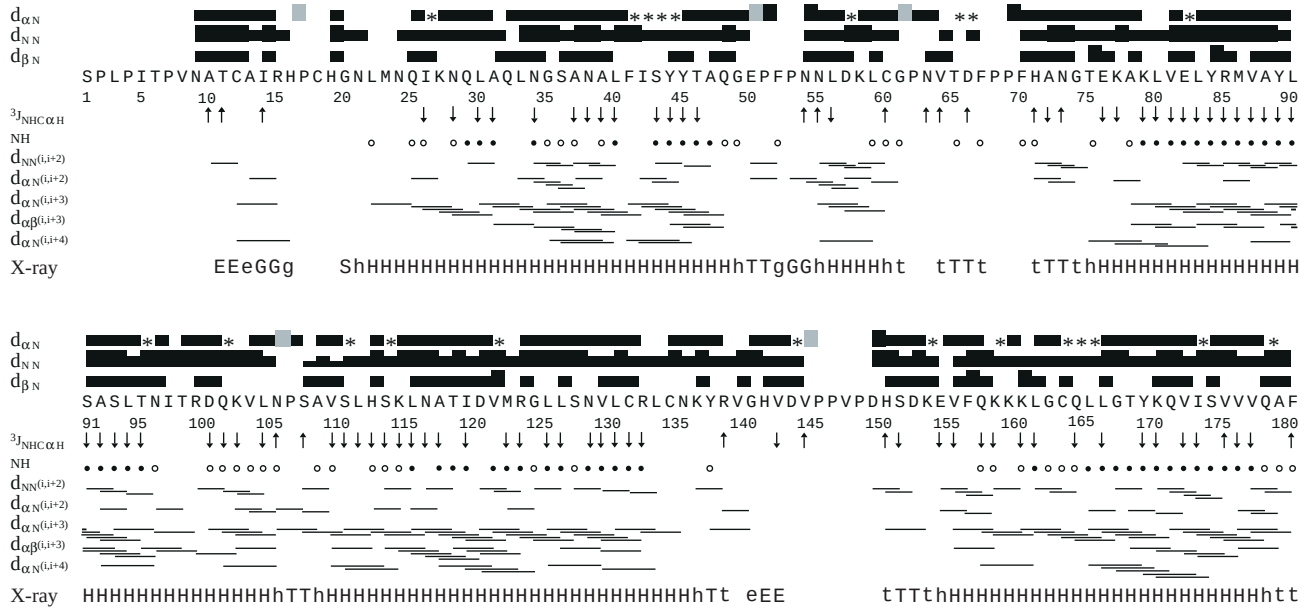

Fig. 5. Summary of NMR data on MH35-LIF. The bars represent NOE connectivities. The heights of the sequential connectivity bars are indicative of the relative intensities of those NOEs (* represents resonance overlap). $J_{NH\text{-}C^{\alpha}H}$ coupling constants, as determined from an HNHA spectrum, are depicted as arrows: ↓, $J_{NH\text{-}C^{\alpha}H} \leq 5$ Hz; ↑, $J_{NH\text{-}C^{\alpha}H} \geq 8$ Hz. The circles represent slowly exchanging NH protons at 37 °C and pH 4.4. Open circles represent NH protons that have exchanged within 48 h and filled circles those that are still present after this period; where there is no symbol the NH is in relatively fast exchange with the solvent. The secondary structure of mLIF (Robinson et al., 1994) is represented on the bottom line according to the symbolism of PROCHECK (Laskowski et al., 1993): H, helix; E, sheet; G, 3_{10}-helix; T, hydrogen-bonded turn; S, bends. Lowercase letters refer to extensions of the aforementioned secondary structure.

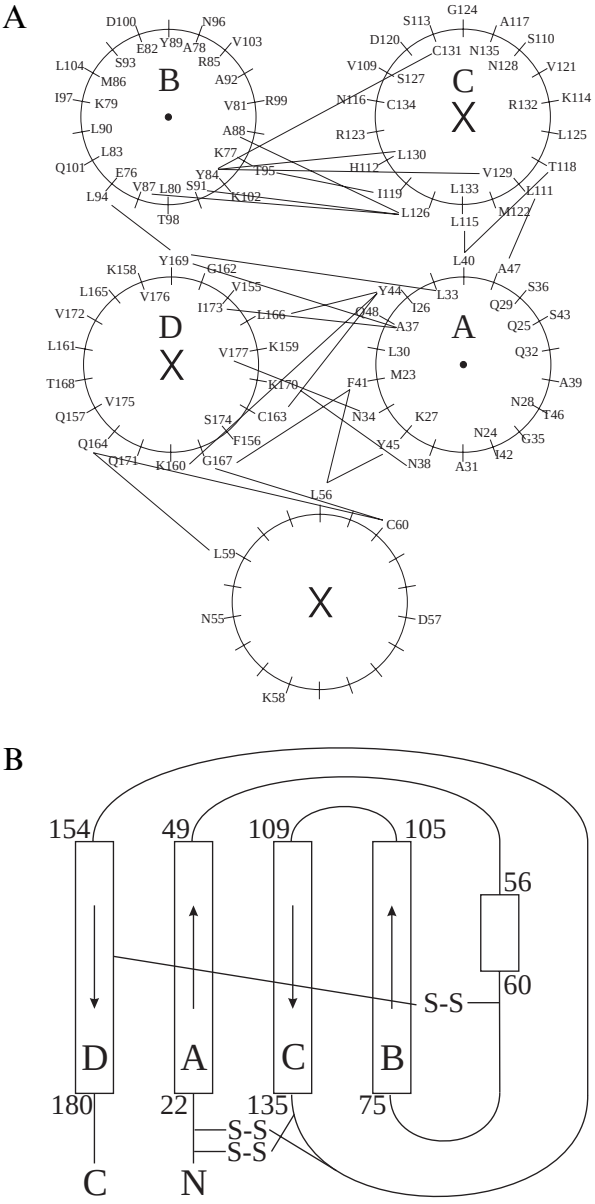

Fig. 6. (A) Long-range interhelical NOEs for MH35. Helices A and B are viewed from the C-terminal end, while helices C, D and the short helix in the AB loop are viewed from the N-terminal end. Each line may represent more than a single NOE. (B) Schematic diagram of LIF topology based on the NMR data. The molecular topology consists of four α-helices arranged in an up-up-down-down manner interconnected by long loops between the A and B helices and the C and D helices. The limits of the helices according to NMR-derived parameters are indicated.

ecule; similarly, the first five residues of MH35-LIF are difficult to assign in the NMR spectrum due to a lack of interresidue NOEs and the presence of three prolines in the N-terminal tail, making it likely that there will be multiple conformers in this region due to cis–trans isomerism. The crystal structure of mLIF shows a four-helical bundle structure with the helices arranged in an up-up-down-down manner and an additional small helix in the AB loop. Preliminary calculations of the solution structure from our current NMR data show the same tertiary fold and, as discussed above, the helices occur in the same regions of the amino acid sequence. The long-range, interhelical NOEs that define the overall tertiary fold are summarised in Fig. 6A. A comparison of the pattern of these long-range interhelical NOEs with those of IL-4 (Redfield et al., 1991; Powers et al., 1992), IL-2 (Mott et al., 1995) and IL-3 (Feng et al., 1995) shows that there is a similar distribution of long-range NOEs amongst these molecules. This similarity, coupled with the secondary structural information from medium-range NOEs, NH-$C^{\alpha}H$ coupling constants and exchange data outlined above, demonstrates that the tertiary folds for these molecules are similar, with the helix topology shown in Fig. 6B.

The only apparent deviations from the X-ray structure are in the N-terminal tail. Pro^{17} has a cis peptide bond in the crystal structure, while the solution structure has a trans peptide bond in this position as determined by the presence of a characteristic $C^{\alpha}H(i–1)$ $C^{\delta}H(i)$ NOE (Wüthrich, 1986) between residues 16 and 17 and the absence of a $C^{\alpha}H$-$C^{\alpha}H$ NOE. Analysis of the crystal structure using the program PROCHECK (Laskowski et al., 1993) reveals that there is a short β-sheet encompassing residues 10–12 in the N-terminal tail and residues 140–142 in the CD loop. This small section of antiparallel β-sheet does not appear to be present in the solution structure of MH35-LIF, as indicated by the lack of slowly exchanging NH protons and the absence of a contiguous set of residues in this region with the requisite large coupling constants, although the CSI analysis is consistent with sheet-like structure in this region (Figs. 4 and 5).

Conclusions

In summary, we have reported the almost complete assignment of the ^{1}H, ^{15}N and ^{13}C resonances of MH35-LIF. On the basis of our NMR data, the secondary structure in the solution state is consistent with the X-ray structure for mLIF and other related members of this family. NOE data, coupling constants, chemical shift values and hydrogen exchange data indicate that the helices are well defined in solution, while the long loops between the A and B helices and the C and D helices are more flexible. Furthermore, the long-range NOEs observed for MH35-LIF are consistent with an up-up-down-down, four α-helical bundle structure. There are only minor differences between the secondary structure of MH35-LIF in solution and that of mLIF in the crystal, and these are concentrated in the flexible N-terminal tail. The NMR data presented in this paper have defined the secondary structure and overall topology of MH35-LIF. Additional NOEs are now being determined as the basis for the calculation of a high-resolution structure of this protein in solution.

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References

Abildgaard, A., Jørgensen, A.M.M., Led, J.J., Christensen, T., Jensen, E.B., Junker, F. and Dalbøge, H. (1992) *Biochemistry*, **31**, 8587–8596.

Bartels, C., Xia, T.-H., Billeter, M., Güntert, P. and Wüthrich, K. (1995) *J. Biomol. NMR*, **6**, 1–10.

Bax, A. and Subramanian, S.J. (1986) *J. Magn. Reson.*, **67**, 565–569.

Bax, A., Clore, G.M., Driscoll, P.C., Gronenborn, A.M., Ikura, M. and Kay, L.E. (1990) *J. Magn. Reson.*, **87**, 620–627.

Bax, A. and Ikura, M. (1991) *J. Biomol. NMR*, **1**, 99–104.

Bax, A. and Grzesiek, S. (1993) *Acc. Chem. Res.*, **26**, 131–138.

Bax, A. (1994) *Curr. Opin. Struct. Biol.*, **4**, 738–744.

Bazan, J.F. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 6934–6938.

Bazan, J.F. (1991) *Neuron*, **7**, 197–208.

Bodenhausen, G. and Ruben, D.J. (1980) *Chem. Phys. Lett.*, **69**, 185–189.

Cheema, S.S., Richards, L., Murphy, M. and Bartlett, P.F. (1994) *J. Neurosci. Res.*, **37**, 213–218.

Dalgarno, D.C., Levine, B.A. and Williams, R.J.P. (1983) *Biosci. Rep.*, **3**, 443–452.

Davis, I. and Morstyn, G. (1991) *Semin. Hematol.*, **28**, 25–33.

Debets, R. and Savelkoul, H.F.J. (1994) *Immunol. Today*, **15**, 455–458.

Demetri, G.D. and Griffin, J.D. (1991) *Blood*, **78**, 2791–2808.

De Vos, A.M., Ultsch, M. and Kossiakoff, A.A. (1992) *Science*, **255**, 306–312.

Diederichs, K., Boone, T. and Karplus, P. (1991) *Science*, **254**, 1779–1782.

Ealick, S.E., Cook, W.J., Vijay-Kumar, S., Carson, M., Nagabhushan, T.L., Trotta, P.P. and Bugg, C.E. (1991) *Science*, **252**, 698–702.

Feng, Y.Q., Klein, B.K., Vu, L., Aykent, S. and McWherter, C.A. (1995) *Biochemistry*, **34**, 6540–6551.

Feng, Y.Q., Klein, B.K. and McWherter, C.A. (1996) *J. Mol. Biol.*, **259**, 524–541.

Frenkiel, T., Bauer, C., Carr, M.D., Birdsall, B. and Feeney, J. (1990) *J. Magn. Reson.*, **90**, 420–425.

Gearing, D.P., Gough, N.M., King, J.A., Hilton, D.J., Nicola, N.A., Simpson, R.J., Nice, E.C., Kelso, A. and Metcalf, D. (1987) *EMBO J.*, **6**, 3995–4002.

Gough, N.M., Willson, T.A., Stahl, J. and Brown, M.A. (1992) In *Polyfunctional Cytokines LIF and IL6* (Ed., Widdows, I.C.), CIBA Foundation Symposium 167, Wiley, New York, NY, U.S.A., pp. 24–46.

Grant, S.M. and Heely, R.C. (1992) *Drugs*, **43**, 516–560.

Gronenborn, A.M. and Clore, G.M. (1994) *J. Biomol. NMR*, **4**, 455–458.

Grzesiek, S. and Bax, A. (1992) *J. Magn. Reson.*, **96**, 432–440.

Grzesiek, S., Döbeli, H., Gentz, R., Garotta, G., Labhardt, A.M. and Bax, A. (1992) *Biochemistry*, **31**, 8180–8190.

Grzesiek, S. and Bax, A. (1993) *J. Biomol. NMR*, **3**, 185–204.

Güntert, P., Dötsch, V., Wider, G. and Wüthrich, K. (1992) *J. Biomol. NMR*, **2**, 619–629.

Harper, E.T. and Rose, G.D. (1993) *Biochemistry*, **32**, 7605–7609.

Hill, C.P., Osslund, T.D. and Eisenberg, D. (1993) *Proc. Natl. Acad. Sci. USA*, **90**, 5167–5171.

Hilton, D.J., Nicola, N.A., Gough, N.M. and Metcalf, D. (1988a) *J. Biol. Chem.*, **263**, 9238–9243.

Hilton, D.J., Nicola, N.A. and Metcalf, D. (1988b) *Anal. Biochem.*, **173**, 359–367.

Hilton, D.J. (1992) *Trends Biochem. Sci.*, **17**, 72–76.

Hoffman, R.C., Moy, F.J., Price, V., Richardson, J., Kaubisch, D., Frieden, E.A., Krakover, J.D., Castner, B.J., King, J., March, C.J. and Powers, R. (1996) *J. Biomol. NMR*, **7**, 273–282.

Ikura, M., Bax, A., Clore, G.M. and Gronenborn, A.M. (1990) *J. Am. Chem. Soc.*, **112**, 9020–9022.

Ikura, M., Kay, L.E. and Bax, A. (1991) *J. Biomol. NMR*, **1**, 299–304.

Kay, L.E., Ikura, M., Tschudin, R. and Bax, A. (1990) *J. Magn. Reson.*, **89**, 496–514.

Laskowski, R.A., MacArthur, M.W., Moss, D.S. and Thornton, J.M. (1993) *J. Appl. Crystallogr.*, **26**, 283–290.

Layton, M.J., Owczarek, C.M., Metcalf, D., Clark, R.L., Smith, D.K., Treutlein, H.R. and Nicola, N.A. (1994) *J. Biol. Chem.*, **269**, 29891–29896.

Levitt, M.H., Freeman, R. and Frenkiel, T. (1982) *J. Magn. Reson.*, **47**, 328–330.

Live, D.H., Davis, D.G., Agosta, W.C. and Cowburn, D. (1984) *J. Am. Chem. Soc.*, **106**, 1939–1941.

Marion, D. and Wüthrich, K. (1983) *Biochem. Biophys. Res. Commun.*, **113**, 967–974.

Marion, D., Driscoll, P.C., Kay, L.E., Wingfield, P.T., Bax, A., Gronenborn, A.M. and Clore, G.M. (1989a) *Biochemistry*, **28**, 6150–6156.

Marion, D., Ikura, M., Tschudin, R. and Bax, A. (1989b) *J. Magn. Reson.*, **85**, 393–399.

Markley, J.L. and Kainosho, M. (1993) In *NMR of Macromolecules. A Practical Approach* (Ed., Roberts, G.C.K.), IRL Press, Oxford, U.K., pp. 101–152.

Maurer, T., Smith, D.K., Owczarek, C.M., Layton, M.J., Zhang, J.-G., Nicola, N.A. and Norton, R.S. (1994) *Growth Factors*, **11**, 271–276.

McDonald, N.Q., Panayotatos, N. and Hendrickson, W.A. (1995) *EMBO J.*, **14**, 2689–2699.

McKay, D.B. (1992) *Science*, **257**, 412–413.

Messerle, B.A., Wider, G., Otting, G., Weber, C. and Wüthrich, K. (1989) *J. Magn. Reson.*, **85**, 608–613.

Metcalf, D. (1992) *Growth Factors*, **7**, 169–173.

Milburn, M.V., Hassell, A.M., Lambert, M.H., Jordan, S.R., Proudfoot, A.E.I., Graber, P. and Wells, T.N.C. (1993) *Nature*, **363**, 172–176.

Miyajima, A., Hara, T. and Kitamura, T. (1992a) *Trends Biochem. Sci.*, **17**, 378–382.

Miyajima, A., Kitamura, T., Harada, N., Yokota, T. and Arai, K. (1992b) *Annu. Rev. Immunol.*, **10**, 295–331.

Morton, C.J., Bai, H., Zhang, J.-G., Hammacher, A., Norton, R.S., Simpson, R.J. and Mabbutt, B.C. (1995) *Biochim. Biophys. Acta*, **1249**, 189–203.

Mott, H.R., Baines, B.S., Hall, R.M., Cooke, R.M., Driscoll, P.C., Weir, M.P. and Campbell, I.D. (1995) *J. Mol. Biol.*, **247**, 979–994.

Mott, H.R. and Campbell, I.D. (1995) *Curr. Opin. Struct. Biol.*, **5**, 114–121.

Muhandiram, D.R., Farrow, N.A., Xu, G.-Y., Smallcombe, S.H. and Kay, L.E. (1993) *J. Magn. Reson.*, **B102**, 317–321.

Neidig, K.-P., Geyer, M., Görler, A., Antz, C., Saffrich, R., Beneike, W. and Kalbitzer, R. (1995) *J. Biomol. NMR*, **6**, 255–270.

Nicola, N.A., Cross, B. and Simpson, R.J. (1993) *Biochem. Biophys. Res. Commun.*, **190**, 20–26.

Nicola, N.A. (Ed.) (1994) In *Guidebook to Cytokines and Their Receptors*, Sambrook and Tooze, New York, NY, U.S.A., pp. 1–7.

Nishimura, C., Watanabe, A., Gouda, H., Shimada, I. and Arata, Y. (1996) *Biochemistry*, **35**, 273–281.

Olejniczak, E.T. and Eaton, H. (1990) *J. Magn. Reson.*, **87**, 628–632.

Otting, G. and Wüthrich, K. (1988) *J. Magn. Reson.*, **76**, 569–574.

Owczarek, C.M., Layton, M.J., Metcalf, D., Lock, P., Willson, T.A., Gough, N.M. and Nicola, N.A. (1993) *EMBO J.*, **12**, 3487–3495.

Pandit, J., Bohm, A., Jancarik, J., Halenbeck, R., Koths, K. and Kim, S.-H. (1992) *Science*, **258**, 1358–1362.

Pennica, D., King, K.L., Shaw, K.J., Luis, E., Rullamas, J., Luoh, S.-M., Darbonne, W.C., Knutzon, D.S., Yen, R., Chien, K.R., Baker, J.B. and Wood, W.I. (1995a) *Proc. Natl. Acad. Sci. USA*, **92**, 1142–1146.

Pennica, D., Shaw, K.J., Swanson, T.A., Moore, M.W., Shelton, D.L., Zioncheck, K.A., Rosenthal, A., Taga, T., Paoni, N.F. and Wood, W.I. (1995b) *J. Biol. Chem.*, **270**, 10915–10922.

Peppard, J.V., Loo, P., Sills, M.A., Munster, D., Pomponi, S.A. and Wright, A.E. (1996) *J. Biol. Chem.*, **271**, 7281–7284.

Piotto, M., Saudek, V. and Sklenář, V. (1992) *J. Biomol. NMR*, **2**, 661–665.

Powers, R., Garrett, D., March, C.J., Frieden, E.A., Gronenborn, A.M. and Clore, G.M. (1992) *Science*, **256**, 1673–1677.

Powers, R., Garrett, D., March, C.J., Frieden, E.A., Gronenborn, A.M. and Clore, G.M. (1993) *Biochemistry*, **32**, 6744–6762.

Presta, L.G. and Rose, G.D. (1988) *Science*, **240**, 1632–1641.

Redfield, C., Smith, L.J., Boyd, J., Lawrence, M.P., Edwards, R.G., Smith, R.A.G. and Dobson, C.M. (1991) *Biochemistry*, **30**, 11029–11035.

Redfield, C., Boyd, J., Smith, L.J., Smith, R.A.G. and Dobson, C.M. (1992) *Biochemistry*, **31**, 10431–10437.

Redfield, C., Smith, L.J., Boyd, J., Lawrence, M.P., Edwards, R.G., Gershater, C.J., Smith, R.A.G. and Dobson, C.M. (1994) *J. Mol. Biol.*, **238**, 23–41.

Robinson, R.C., Grey, L.M., Staunton, D., Vankelecom, H., Vernallis, A.B., Moreau, J.F., Stuart, D.I., Heath, J.K. and Jones, E.Y. (1994) *Cell*, **77**, 1101–1116.

Rohl, C.A. and Baldwin, R.L. (1994) *Biochemistry*, **33**, 7760–7767.

Rucker, S.P. and Shaka, A.J. (1989) *Mol. Phys.*, **68**, 509–517.

Senda, T., Shimazu, T., Matsuda, S., Kawano, G., Shimizu, H., Nakamura, K.T. and Mitsui, Y. (1992) *EMBO J.*, **11**, 3193–3201.

Shaka, A.J., Barker, P.B. and Freeman, R. (1985) *J. Magn. Reson.*, **64**, 547–552.

Shaka, A.J., Lee, C.J. and Pines, A. (1988) *J. Magn. Reson.*, **77**, 274–293.

Smith, L.J., Redfield, C., Boyd, J., Lawrence, G.M.P., Edwards, R.G., Smith, R.A.G. and Dobson, C.M. (1992) *J. Mol. Biol.*, **224**, 899–904.

Smith, D.K., Treutlein, H.R., Maurer, T., Owczarek, C.M., Layton, M.J., Nicola, N.A. and Norton, R.S. (1994) *FEBS Lett.*, **350**, 275–280.

Smith, D.K. (1996) Ph.D. Thesis, The University of Melbourne, Melbourne, Australia.

Somers, W., Ultsch, M., De Vos, A.M. and Kossiakoff, A.A. (1994) *Nature*, **372**, 478–481.

Spera, S. and Bax, A. (1991) *J. Am. Chem. Soc.*, **113**, 5490–5492.

Sprang, S.R. and Bazan, J.F. (1993) *Curr. Opin. Struct. Biol.*, **3**, 815–827.

Stewart, C.L., Kasoar, P., Brunet, L.J., Bhatt, H., Gadi, I., Kontgen, F. and Abbondanzo, S.J. (1992) *Nature*, **359**, 76–79.

Vuister, G.W. and Bax, A. (1992) *J. Magn. Reson.*, **98**, 428–435.

Vuister, G.W. and Bax, A. (1993) *J. Am. Chem. Soc.*, **115**, 7772–7777.

Walter, M.R., Cook, W.J., Zhao, B.G., Cameron, R.P., Ealick, S.E., Walter, R.L., Reichert, P., Nagabhushan, T.L., Trotta, P.P. and Bugg, C.E. (1992a) *J. Biol. Chem.*, **267**, 20371–20376.

Walter, M.R., Cook, W.J., Ealick, S.E., Nagabhushan, T.L., Trotta, P.P. and Bugg, C.E. (1992b) *J. Mol. Biol.*, **224**, 1075–1085.

Walter, M.R. and Nagabhushan, T.L. (1995) *Biochemistry*, **34**, 12118–12125.

Walter, M.R., Windsor, W.T., Nagabhushan, T.L., Lundell, D.J., Lunn, D.J., Lunn, C.A., Zauodny, P.J. and Narula, S.K. (1995) *Nature*, **376**, 230–235.

Ware, C.B., Horowitz, M.C., Renshaw, B.R., Hunt, J.S., Liggitt, D., Koblar, S.A., Gliniak, B.C., McKenna, H.J., Papayannopoulou, T., Thoma, B., Cheng, L., Donovan, P.J., Peschon, J.J., Bartlett, P.F., Willis, C.R., Wright, B.D., Carpenter, M.K., Davidson, B.L. and Gearing, D.P. (1995) *Development*, **121**, 1283–1299.

Werner, J.M., Breeze, A.L., Kara, B., Rosenbrock, G., Boyd, J., Soffe, N. and Campbell, I.D. (1994) *Biochemistry*, **33**, 7184–7192.

Wishart, D.S., Sykes, B.D. and Richards, F.M. (1991) *J. Mol. Biol.*, **222**, 311–333.

Wishart, D.S. and Sykes, B.D. (1994) *Methods Enzymol.*, **239**, 363–392.

Wishart, D.S., Bigam, C.G., Yao, J., Abildgaard, F., Dyson, H.J., Oldfield, E., Markley, J.L. and Sykes, B.D. (1995) *J. Biomol. NMR*, **6**, 135–140.

Wlodawer, A., Pavlovsky, A. and Gustchina, A. (1992) *FEBS Lett.*, **309**, 59–64.

Wüthrich, K. (1986) *NMR of Proteins and Nucleic Acids*, Wiley, New York, NY, U.S.A.

Zdanov, A., Schalk-Hihi, C., Gustchina, A., Tsang, M., Weatherbee, J. and Wlodawer, A. (1995) *Structure*, **3**, 591–601.

Zink, T., Ross, A., Lüers, K., Cieslar, C., Rudolph, R. and Holak, T. (1994) *Biochemistry*, **33**, 8453–8463.