The High-Resolution, Three-Dimensional Solution Structure of Human Interleukin-4 Determined by Multidimensional Heteronuclear Magnetic Resonance Spectroscopy

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ABSTRACT: The high-resolution three-dimensional solution structure of recombinant human interleukin-4 (IL-4), a protein of ~15 kDa which plays a key role in the regulation of B and T lymphocytes, has been determined using three- and four-dimensional heteronuclear NMR spectroscopy. The structure is based on a total of 2973 experimental NMR restraints, comprising 2515 approximate internuclear distance restraints, 102 distance restraints for 51 backbone hydrogen bonds, and 356 torsion angle restraints. A total of 30 structures was calculated by means of hybrid distance geometry–simulated annealing, and the atomic rms distribution about the mean coordinate positions for residues 8–129 is 0.44 ± 0.03 Å for the backbone atoms, 0.83 ± 0.03 Å for all atoms, and 0.51 ± 0.04 Å for all atoms excluding disordered side chains. The N- and C-terminal residues (1–7 and 130–133, respectively) appear to be disordered. The structure of IL-4 is dominated by a left-handed four-helix bundle with an unusual topology comprising two overhand connections. The linker elements between the helices are formed by either long loops, small helical turns, or short strands. The latter include a mini anti-parallel β-sheet. A best fit superposition of the NMR structure of IL-4 with the 2.25 Å resolution crystal structure [Wlodawer, A., Pavlovsky, A., & Gutschina, A. (1992) FEBS Lett. 309, 59–64] yields a backbone atomic rms difference of 1.37 Å which can be mainly attributed to tighter packing of the helices in the crystal structure. This is indicated by an approximately 20% reduction in the axial separation of three pairs of helices (αA–αC, αA–αD, and αC–αD) in the crystal structure relative to the NMR structure and may reflect the greater flexibility of the molecule in solution which is reduced in the crystal due to intermolecular contacts. Comparison of the NMR structure of IL-4 with the X-ray structures of two other related proteins, granulocyte–macrophage colony stimulating factor [Diedrichs, K., Boone, T., & Karplus, P. A. (1992) Science 254, 1779–1782] and human growth hormone [de Vos, A. M., Ultsch, M., & Kossiakoff, A. A. (1992) Science 255, 306–312], that bind to the same hematopoietic superfamily of cell surface receptors reveals a remarkably similar topological fold, despite the absence of any significant overall sequence identity, and substantial differences in the relative lengths of the helices, the lengths and the nature of the various connecting elements, and the pattern and number of disulfide bridges. Indeed, the Cα atom coordinates of 72 and 79 residues of IL-4 can be superimposed on the Cα coordinates of granulocyte–macrophage colony stimulating factor and human growth hormone with rms differences of ~1.7 and 2.0 Å, respectively.

Interleukin-4 (IL-4)† is a member of the cytokine family of proteins and plays a key role in the immune and inflammatory systems [see Paul and Ohara (1987), Yokota et al. (1988), Finkel et al. (1990), Paul (1991), and Boulay and Paul (1992) for reviews]. IL-4 regulates B-cell proliferation and differentiation, modulates the survival, proliferation, and differentiation of T-cells, acts as a growth factor on mast cells, regulates macrophage activation, and induces the expression of VCAM-1 on endothelial cells. IL-4 is a potent inducer of human cytotoxic CD8+ T-cells, an activity which has been shown to be responsible for establishing tumor-specific systemic immunity to an established renal cancer by injection of renal tumor cells genetically engineered to secrete large doses of IL-4 locally (Golumbek et al., 1991). The very potent antitumor activity of IL-4 at the primary tumor site is also associated with the elicitation of a localized inflammatory infiltrate, dominated by eosinophils (Tepper et al., 1989, 1992). Additionally, IL-4 induces the expression of class I and II MHC molecules and the IgE low-affinity receptor

† Abbreviations: IL-4, human recombinant interleukin-4; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser enhancement spectroscopy; ROE, rotating frame Overhauser effect; ROESY, rotating frame Overhauser enhancement spectroscopy; HNHB, homonuclear Hartmann-Hahn spectroscopy; HMQC, heteronuclear multiple quantum coherence; CT, constant time; HCACO, CαH proton to α-carbon to carbonyl carbon correlation; HNNB, amide proton to nitrogen to Cα proton correlation; HN(CO)HB, amide proton to nitrogen to Cα proton (via carbonyl carbon); 3D, three-dimensional; 4D, four-dimensional; TPPI, time-proportional phase incrementation; SA, simulated annealing; rms, root mean square.

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† The coordinates of the 30 final simulated annealing structures of IL-4, together with the coordinates of the restrained minimized mean structure, (SA)r, and the complete list of experimental NMR restraints have been deposited in the Brookhaven Protein Data Bank. The accession numbers are 1ITI for the coordinates and 1ITI-MR for the complete set of experimental NMR restraints.
‡ Immunex Corporation.
on resting B-cells. IL-4 causes immunoglobulin class switching of activated B-cells to IgE and IgG1, thereby generating and sustaining in vivo IgE responses and ensuring the dominance of IgG1 in a T-cell-dependent immune response (Kuhn et al., 1991).

To further understand the structure-function relationship of IL-4 and its mode of interaction with its receptor, we have set out to elucidate the high-resolution, three-dimensional structure of IL-4 in solution by NMR spectroscopy. Recently, we presented the complete 1H, 15N, and 13C resonance assignments of IL-4 (Powers et al., 1992a), the elucidation of its secondary structure (Garrett et al., 1992), and the determination of a low-resolution (second generation) tertiary structure (Powers et al., 1992b). At the same time, another low-resolution NMR structure was published by Smith et al. (1992). In this article, the focus is extended to the determination of a high-resolution three-dimensional structure of IL-4 using three- and four-dimensional heteronuclear NMR spectroscopy. The resulting fourth generation (Clore & Gronenborn, 1991a,b) structure is based on 2973 experimental NMR restraints, and the atomic rms distribution about the mean coordinate positions (excluding residues 1-7 and 130-133 at the N- and C-termini, which are disordered) is 0.44 ± 0.03 Å for the backbone atoms, 0.83 ± 0.03 Å for all atoms, and 0.51 ± 0.04 Å for all atoms excluding disordered side chains. This presents an approximate doubling in the number of experimental NMR restraints and an increase by a factor of more than 2 in the precision and accuracy of the coordinates relative to the previous NMR structure determinations of IL-4 (Powers et al., 1992b; Smith et al., 1992).

**EXPERIMENTAL PROCEDURES**

**Sample Preparation.** Uniformly (>95%) 15N- and 15N/13C-labeled human recombinant IL-4 was expressed in yeast and purified as described previously (Powers et al., 1992a). The version of recombinant IL-4 used comprises the tetrapeptide sequence Glu-Ala-Glu-Ala added to the N-terminus of the natural sequence. In the present article, residues are numbered from the N-terminal Glu as residue 1, so that the natural IL-4 sequence starts at residue 5. In addition, the two potential N-linked glycosylation sites at Asn 42 and Asn 109 in the natural sequence were changed to Asp by site-directed mutagenesis to prevent hyperglycosylation in the yeast host (Curtis et al., 1991). Samples for NMR contained 2 mM 12N- or 13C/15N-labeled IL-4, pH 5.7, dissolved in either 90% H2O/10% D2O or 99.996% D2O.

**NMR Spectroscopy.** All spectra were recorded at 36 °C on Bruker AMX600 and AM600 spectrometers equipped with a triple-resonance 1H/13C/15N probe. Quadrature detection in the indirectly detected dimensions was obtained using the TPPi-States method (Marion et al., 1989a). For spectra recorded in H2O, water suppression was achieved by the use of spin-lock pulses to randomize magnetization of protons not attached to 15N (Messerle et al., 1989). In the case of the 4D 13C/15N-edited NOESY spectrum, suppression of undesired coherence transfer pathways was achieved by means of pulse field gradients (Bax & Pochapsky, 1992), enabling a minimal two-step phase cycle to be employed (Vuister et al., 1993).

The present structure is based on the following series of spectra: 2D HMQC-J (Kay & Bax, 1990; Forman-Kay et al., 1990), 3D 15N- (Marion et al., 1989b; Zuiderweg & Fesik, 1989) and 13C/15N-edited HSQC (Vuister et al., 1991), 3D 13C-15N- and 13C/15N-edited HCACO (Vuister et al., 1992), and 4D 15N/13C-edited ROESY (Clore et al., 1990b), and 13C/15N/13C-edited NOESY (Ikura et al., 1990), 3D 15N- (Clore et al., 1990a) and 13C-edited ROESY (Clore et al., 1991a), 3D 15N-edited HHAHO (Marion et al., 1989b; Clore et al., 1991a), 3D HN(CO)HB (Grzesiek et al., 1992), 3D HNHB (Archer et al., 1991), 3D long-range 13C-15N correlation (Bax et al., 1992), 3D coupled CT-HCACO (Powers et al., 1991; Vuister et al., 1992), and 4D 15N/13C- (Kay et al., 1990) and 13C/15N/13C-edited NOESY (Clore et al., 1991b; Zuiderweg et al., 1991; Vuister et al., 1993). Examples of the quality of spectra not shown here are found in previous publications, together with details of their experimental implementation: see Garrett et al. (1992) and Powers et al. (1992a,b). Experimental details regarding spectra not reported in these publications are provided below.

The 4D 13C/15N-edited NOESY spectrum was recorded with 2 scans per increment and a total measuring time of 84 h using sine bell shaped pulse field gradients (of strength 7 G/cm at their maximum) applied along the z axis. The pulse scheme and experimental setup employed were exactly as described by Vuister et al. (1993). The 4D 12N/13C-edited NOESY spectrum was recorded with 4 scans per increment and a total measuring time of 6 days using the pulse scheme described by Kay et al. (1990). Because this experiment includes a double 15N/13C heteronuclear filter which is highly efficient at eliminating any potential artifacts arising from undesired coherence transfer pathways, no pulse field gradients were employed.

The spectral widths for the 3D long-range 13C-15N correlation experiment in the indirectly detected 13C dimensions were 87.196 ppm for F1 and 3.657 ppm for F2 with the carrier at 30 ppm, and the spectral width in the 1H acquisition dimension was 8.011 ppm with the carrier at 2.67 ppm. The spectral widths for the coupled 3D CT-HCACO experiment in the indirectly detected 13C dimensions were 12.05 ppm for F1(13CO) and 33.13 ppm for F2(13CO) with the carrier at 176.68 ppm for F1, and 56 ppm for F2, and the spectral width in the 1H acquisition dimension was 4.17 ppm with the carrier at 4.67 ppm. The spectral widths for the 4D 13C/13C-edited NOESY, 3D 13C-edited ROESY, 4D 12N/13C-edited NOESY, and the 3D 15N/13C-edited ROESY NMR experiments in the indirectly detected 15N and/or 13C dimensions were 30.01 and 20.71 ppm, respectively, with the carrier at 115 and 63.71 ppm, respectively. For the 3D 13C-edited ROESY experiment, the spectral width in the indirectly detected 1H dimension was 9.26 ppm with the carrier at 3 ppm, and the spectral width in the acquisition dimension was 19.84 ppm with the carrier at 8.21 ppm. For the 3D 12N-edited ROESY experiment, the spectral width in the indirectly detected 1H dimension was 11.41 ppm with the carrier at 4.67 ppm, and the spectral width in the 1H acquisition dimension was 13.44 ppm with the carrier at 4.67 ppm. For the 4D 13C/15N-edited NOESY experiment, the spectral width in the indirectly detected 1H dimension was 11.41 ppm with the carrier at 4.67 ppm, and the spectral width in the 1H acquisition dimension was 10.04 ppm with the carrier at 4.67 ppm. For the 3D 13C-13C-edited NOESY experiment, the spectral width in the indirectly detected 1H dimension was 8.96 ppm with the carrier at 3.98 ppm, and the spectral width in the 1H acquisition dimension was 10.04 ppm with the carrier at 4.67 ppm.

For the 3D long-range 13C-13C correlation experiment, the number of points acquired in the various dimensions was 128 complex points in F1(13C), 16 complex points in F2(13C), and 256 complex points in F3(1H). For the coupled 3D CT-HCACO experiment, the number of points acquired in the various dimensions was 64 complex points in F1(13CO), 32 complex points in F2(13CO), and 256 complex points in F3(1H). For the 3D 13C- and 12N-edited ROESY experiments, the number of points acquired in the various dimensions was...
128 complex points in $F_1(\text{H})$, 32 complex points in $F_2(\text{13C}$ or $\text{15N}$), and 512 complex points in $F_2(\text{H})$. For the 4D $\text{13C}$/
$\text{15N}$-edited NOE experiment, the number of points acquired in the various dimensions was 16 complex points in $F_1(\text{15N})$,
64 complex points in $F_2(\text{H})$, 16 complex points in $F_3(\text{13C})$, and 256 complex points in $F_4(\text{H})$. For the 4D $\text{13C}$/
$\text{15C}$-edited NOE experiment, the number of points acquired in the various dimensions was 18 complex points in $F_1(\text{13C})$,
64 complex points in $F_2(\text{H})$, 16 complex points in $F_3(\text{13C})$, and 300 complex points in $F_4(\text{H})$. One zero-filling was used in all
dimensions, and in the case of the 4D $\text{13C}$/
$\text{13C}$-edited NOE and the 4D $\text{13C}$/
$\text{15N}$-edited NOE experiment, linear prediction by means of the mirror image technique (Zhu & Bax, 1990) was used with zero-filling to further extend the data in the $F_1(\text{13C})$ and $F_2(\text{15N}$ or $\text{13C}$) dimensions.

All spectra were processed on a Sun Sparc Workstation using in-house routines for Fourier transformation (Kay et al., 1989; G. Vuister, unpublished) and linear prediction (Zhu & Bax, 1990), together with the commercially available software package NMRZ (New Methods Research, Inc., Syracuse, NY) for the processing of 2D planes. Analysis of the 3D and 4D spectra was carried out using the in-house programs PIPP and STAPP (Garrett et al., 1991; D.S. Garrett, unpublished).

The mixing times employed for the heteronuclear-edited NOE and ROE experiments were as follows: 120 ms for the 3D $\text{13C}$- and $\text{15N}$-edited NOE experiments; 150 ms for the 3D $\text{13H}$-$\text{15N}$ HMBC-NOE- HMBC experiment; 110 and 107 ms for the 4D $\text{13N}$/
$\text{13C}$- and $\text{13C}$/
$\text{13C}$-edited NOE experiments, respectively; and 35 and 40 ms for the $\text{15N}$- and $\text{13C}$-edited ROE experiments, respectively.

Interproton Distance Restraints. NOEs were assigned from 3D and 4D heteronuclear-edited NOE spectra recorded with mixing times ranging from 100 to 150 ms. The NOEs were classified into strong, medium, and weak corresponding to interproton distance restraints of 1.8-2.7 Å (1.8-2.9 for NOEs involving NH protons), 1.8-3.3 Å (1.8-3.5 for NOEs involving NH protons), and 1.8-5.0 Å, respectively (Williamson et al., 1985; Clore et al., 1986). Thus, the lower bounds for the interproton distance restraints were set to the sum of the van der Waals radii of two protons. Upper distance limits for distances involving methylic protons and non-stereospecifically assigned methylene protons were corrected appropriately for center averaging (Wüthrich et al., 1983), and an additional 0.5 Å was added to the upper distance limits for NOEs involving methylic protons (Clore et al., 1987; Wagner et al., 1987).

Torsion Angle Restraints and Stereospecific Assignments. $\phi$, $\psi$, and $\chi_1$ torsion angle restraints and stereospecific assignments of $\beta$-methylene protons were derived from the following data. Approximate intraresidue and sequential interproton distance restraints involving the NH, C=O, and C=H protons were obtained from ROE data to avoid complications arising from spin diffusion (Bothner-By et al., 1984; Bax & Davis, 1985; Bax et al., 1986) using 3D $\text{13C}$-edited ROE (40 ms mixing time; Clore et al., 1991a) and 3D $\text{15N}$-edited ROE (35 ms mixing time; Clore et al., 1990a) experiments. 3D $\text{15N}$-edited HOAHA (44.6 ms mixing time), HNHB, and HN(CO)HB experiments were used to obtain a qualitative estimate of the magnitude of the $J_{\phi\beta}$, $J_{\text{NN-HB}}$, and $J_{\text{CC-HB}}$ coupling constants, respectively (Clore et al., 1991a; Archer et al., 1991; Grzesiek et al., 1992). Finally, $J_{\text{HNHA}}$ coupling constants were obtained from an HMQC-J spectrum (Kay & Bax, 1989), as described by Forman-Kay et al. (1990).

![Figure 1: Scheme for obtaining stereospecific assignments of $\beta$-methylene protons and $\chi_1$ rotamers on the basis of a qualitative analysis of the relative intensities of cross peaks observed in the 3D $\text{15N}$-edited ROESY, $\text{13C}$-edited ROESY, $\text{15N}$-edited HOAHA, HNHB, and HN(CO)HB experiments (Clore et al., 1991a; Archer et al., 1991; Grzesiek et al., 1992). The relative intensities of the NH–COH cross peaks in the 3D $\text{15N}$-edited HOAHA, HNHB, and HN(CO)HB spectra are dependent on the size of the $J_{\text{CN}}$, $J_{\text{HH}}$, and $J_{\text{CO}}$ coupling constants, respectively, where a large coupling constant (i.e., trans conformation) results in a strong cross peak. The expected pattern of cross-peak intensities is indicated for the three preferred $\chi_1$ rotamers ($-60^\circ$, $180^\circ$, and $+60^\circ$). A strong cross peak is indicated by the letter W, and a weak or missing peak is indicated by the letter S. Analysis of high-resolution well-refined X-ray structures indicates that $<20^\circ$ of $\chi_1$ angles deviate by more than 20° from the staggered rotamer positions (Ponder & Richards 1987; McGregor et al., 1987; Nilges et al., 1990).

The majority of the $\beta$-methylene stereospecific assignments and the $\phi$, $\psi$, and $\chi_1$ torsion angle restraints were obtained from $J_{\text{HH}}$, coupling constants, and approximate distance restraints for intraresidue and sequential NOEs involving NH, C=O, and C=H protons were obtained by means of the conformational grid search program STEROSEARCH (Nilges et al., 1990), as described previously (Kraulis et al., 1989). These were verified and in some cases extended on the basis of the relative intensities of cross peaks observed in the 3D $\text{15N}$- and $\text{13C}$-edited ROESY, $\text{15N}$-edited HOAHA, HNHB, and HN(CO)HB experiments, as depicted in Figure 1. Stereospecific assignments for the methyl groups of the valine residues were obtained on the basis of the relative intensity of the intraresidue NH–C=H and C=H–C=H NOEs as described by Zuiderweg et al. (1985).

The Ile and Leu $\chi_2$ torsion angle restraints, as well as the stereospecific assignment of the methyl groups of the Leu residues, were determined from $J_{\text{COC}}$ coupling constants obtained from the relative intensity of the C=O and C=O cross peaks in a 3D long-range $\text{13C}-\text{13C}$ NMR correlation spectrum (Bax et al., 1992), in conjunction with the relative intensities of intraresidue NOEs involving NH, C=O, C=H, and C=H protons expected for the three $\chi_1$ rotamers (Table I). The latter are necessary to distinguish $\chi_2$ angles of $60^\circ$ and $180^\circ$ for Leu and $-60^\circ$ and $+60^\circ$ for Ile. These restraints were verified by examining the resulting structures for consistency with the long-range NOEs involving the methyl groups of Ile and Leu. An example of a typical $^1H(F_2)-^13C(F_1)$ plane of the 3D long-range $\text{13C}-\text{13C}$ correlation spectrum is illustrated in Figure 2.

Cysteine disulfide bridge $\chi_2$ and $\chi_3$ torsion angle restraints were based on the following: (a) a comparison of the C=O–C=O
method of Nilges et al. (1988a) with minor modifications calculations.

distances observed in the simulated annealing (SA) structures calculated during the course of the iterative refinement, with distances observed in the simulated annealing (SA) structures only introduced subsequent to the initial set of structure calculations. The hybrid distance geometry4ynamical simulated annealing (Clore et al., 1990b), making use of the program XPLOR protons and the pattern of sequential and interstrand NOES

Hydrogen-Bonding Restraints. The hydrogen bond restraints were deduced on the basis of slowly exchanging NH protons and the pattern of sequential and interstrand NOEs involving the NH and C=O protons (Wüthrich, 1986). Slowly exchanging NH protons were identified from a series of 2D 15N-1'H Overbodenhausen correlation spectra (Bodenhausen & Ruben, 1980; Baz et al., 1990; Norwood et al., 1990) acquired over a period of ~24 h starting within 5 min of dissolving an unexchanged sample of lyophilized protein in D2O [see Garrett et al. (1992)]. Two distance restraints were used for each hydrogen bond: one between the hydrogen and the acceptor atom of 1.5-2.3 Å and one between the donor and the acceptor atom of 2.4-3.3 Å. These were only introduced subsequent to the initial set of structure calculations.

Structure Calculations. Structures were calculated using the hybrid distance geometry–dynamical simulated annealing method of Nilges et al. (1988a) with minor modifications (Clore et al., 1990b), making use of the program XPLOR

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* The pattern NOEs, classified as strong (s), medium (m), and weak (w), for the various pairs of x1 and x2 torsion angles was obtained by examining the corresponding interproton distances using the molecular graphics program FRODO (Jones, 1978).

FIGURE 2: Example of a 13C(F1)–H(F3) plane taken at 180° ppm of the 3D 13C–13C long-range correlation spectrum of IL-4. Because of the large disparity in the intensity of cross peaks in the Cα region (equivalent to cross peaks arising from long-range 1H–15C–13C–13C bond couplings) and the Cγ/Cε region (equivalent to diagonal peaks), these two regions are plotted at different threshold levels and superimposed onto one plot for clarity. (The starting contour level is a factor of 2 lower for the diagonal peaks.) The relative intensities of the Cα and Cγ cross peaks for Ile 36, Leu 83, and Leu 120 were used directly to determine the 1J(Cα,Cγ) coupling constants (1J(Cα,Cγ) = tan−1(l_{CαCγ}/l_{CαCγ})/2π, Cr was determined from the intensities of the Cα(F1)–Cγ(H1) and Cγ(F1)–Cγ(H1) cross peaks, respectively, and 2r = 1/J(Cα) = 29.4 ms, from which information on the corresponding x2 torsion angles was derived. Negative cross peaks are indicated by dashed contour lines.

(Brünger et al., 1986; Brünger, 1992; Kuszewski et al., 1992).

The protocol involves calculating an initial set of substructures incorporating only one-third of the atoms by projection from n-dimensional distance space into Cartesian coordinate space, followed by simulated annealing with all atoms. The target function that is minimized during simulated annealing (as well as during conventional Powell minimization) comprises only quadratic harmonic terms for covalent geometry (that is, bonds, angles, planes, and chirality), square-well quadratic potentials for the experimental distance and torsion angle restraints (Clore et al., 1986), and a quartic van der Waals repulsion term for the nonbonded contacts (Nilges et al., 1988–c). All peptide bonds were constrained to be planar and trans. There were no hydrogen-bonding, electrostatic, or 6–12 Lennard-Jones empirical potential energy terms in the target function.

Iterative Structure Refinement. As in previous structure determinations form this laboratory (Kraulis et al., 1989; Clore et al., 1990b, 1991c; Omichinski et al., 1990; Forman-Kay et al., 1991; Gronenborn et al., 1991), an iterative refinement strategy was employed, incorporating more experimental restraints at each successive stage as the quality of the structure improves. The starting point for the current refinement was the initial second generation structure of IL-4 (Powers et al., 1992b). This structure was based on a total of 921 NOE restraints without stereospecific assignments and 183 backbone (φ, ψ) torsion angle restraints, and it had a backbone atomic
rms distribution about the mean coordinate positions of 0.95 ± 0.16 Å for residues 8–129.

Using an iterative approach, we were able to resolve all ambiguities in the assignment of NOEs from the 3D 15N- and 13C-edited and 4D 15N/13C- and 13C/13C-edited NOESY experiments. Thus, it was required that the assignment of any given ambiguous NOE must be consistent with the current structure, thereby eliminating unreasonable assignments with interproton distances greater than 6 Å. More importantly, the current refined structure also aided in the identification of misassigned NOE pairs, which arose as a result of the very limited 1H chemical shift dispersion exhibited by the IL-4 spectrum (Powers et al., 1992a). The simulated annealing calculation was then repeated after each update of the restraint list, and a new set of structures was generated for further analysis of the NMR data.

Since the 3D and 4D heteronuclear-edited NOESY spectra were recorded with mixing times ranging from 100 to 150 ms, it was apparent that the classification of NOEs into strong, medium, and weak categories could be influenced by spin-diffusion effects. Consequently, NOEs in the restraint list were evaluated for spin-diffusion effects at each iteration on the basis of observed distances and spatial relationships in the current ensemble of structures. If the resulting distance was significantly longer than that predicted from an isolated two-spin interpretation of the experimentally observed NOE intensity, and a spin-diffusion pathway was highly probable on the basis of the spatial geometry of the relevant protons, as well as a qualitative interpretation of NOE intensities calculated by a full-relaxation matrix procedure (Brünger, 1992), the corresponding upper bound distance limit for the NOE was either adjusted upward (i.e., into the next distance range class if the NOE was originally classified as strong or medium) or the NOE was removed from the restraint list (if the NOE was originally classified as weak). This procedure was employed specifically for two types of situations: first, in cases where two methylene protons (attached to the same carbon) exhibit an NOE to the same spin, but one of the two methylene protons clearly points away from this spin at a distance greater than 5 Å; second, in cases where the complete ring system of a Phe shows NOEs to a particular spin, but where it is clear from the spatial configuration that only the C'H proton is actually close in space to this spin (i.e., so that the NOEs observed to the C'H and C'H protons arise from an indirect cross-relaxation pathway via the C'H proton).

As the approximate interproton distance restraints are classified into three ranges, the resulting distinct cutoffs can result in systematic errors for interproton distances whose values lie at the boundary of two distance ranges. This can be corrected by examining the distribution of violations in the ensemble of calculated structures (Clore et al., 1993). Thus, if a particular interproton distance restraint was systematically violated in all of the structures, even by as little as 0.1 Å, we reclassified it into the next class (i.e., from strong to medium and from medium to weak), as discussed by Clore et al. (1993).

From our experience, a conservative approach to the assignment of NOE restraints results in more accurate structures, even if this is achieved at the expense of a slight decrease in precision. Thus, as the lower bound is always set to the sum of the van der Waals radii of two protons (i.e., 1.8 Å), an increase in the upper bound limit of an NOE restraint simply reduces its information content but does not introduce any errors. Conversely, an erroneous NOE or an NOE that is incorrectly assigned to a stronger class (i.e., a smaller upper bound) will introduce errors in the restraint list and consequently have a detrimental effect on the accuracy of the structure. A typical example would be the observation of a skewed χ1 rotamer conformation in a case where the coupling constants clearly indicate that the χ1 angle lies in one of the three preferred conformations (i.e., 60°, −60°, or 180°); another example would be the observation of a single, tightly clustered, skewed χ1 rotamer conformation in a case where the coupling constants are clearly indicative of multiple rotamer populations.

The large number of fully processed 2D planes in the 4D 15N/13C- and 13C/13C-edited NOESY spectra (4096 and 16 384, respectively) required novel processing tools for analysis. The new tools incorporate the current set of refined structures, the XPLOR interproton distance restraint list, and the chemical shift (1H, 15N, and 13C) assignment table with the 4D data into the programs PIPP and STAPP (Garrett et al., 1991; D. S. Garrett, unpublished) to identify potentially unassigned cross peaks in the 4D NMR spectra. The program STAPP identifies potential new interproton distance restraints from the 4D NOE spectra that are consistent with the chemical shift assignments, have interproton distances of less than 6 Å in the current set of structures, and are not in the XPLOR interproton distance restraint list. The cross peaks associated with the new interproton distance restraints can then be examined visually using PIPP to verify the presence of a real peak or to eliminate artifacts. This approach greatly facilitated the analysis of the 4D NOE spectra and permitted the incorporation of a substantial number of new NOEs into the restraint list. Indeed, the 4D 13C/13C-edited NOESY spectrum was particularly valuable in the identification of NOEs between methyl protons with very similar or identical 1H chemical shifts.

Examples of the quality of the 4D 13C/13C-edited NOESY and 4D 15N/13C-edited NOESY spectra are provided in Figures 3 and 4, respectively. Figure 3 shows two 2D cross-sections taken through the 4D 13C/13C-edited NOESY spectrum, perpendicular to the F(1) (13C) and F(2) (1H) coordinates of the C methyl group of Ile 36 and the C' methylene group of Lys 88. Figure 4 shows a 2D cross-section taken through the 4D 15N/13C-edited NOESY spectrum, perpendicular to the F(1) (15N) and F(2) (1H) coordinates for the NH groups of Ile 9, Phe 77, Val 105, Glu 107, and Phe 116.

At the end of the iterative refinement cycle, stereospecific assignments were obtained for 52 of the 96 residues with β-methylene protons, for the methyl groups of two of the three Val residues, and for the methyl groups of 13 of the 16 Leu residues. The majority of the residues without stereospecific assignments corresponded to surface residues with disordered side chains or residues with degenerate β-methylene protons. The validity of the stereospecific assignments was confirmed by being properly accounted for in further refined structures. A table of the complete 1H, 13C, and 15N assignments of IL-4, including all stereospecific assignments obtained, is provided in the supplementary material.

For well-defined Phe and Tyr residues, the NOE distance restraints to the C'H and C'H protons can often be assigned to only one side of the ring, thereby discriminating between the C'H and C'H or the C' and C'H protons (Froman-Kay et al., 1991a). In the case of IL-4, all six Phe and both Tyr residues were well defined and had low surface accessibilities, making it possible to assign NOE restraints to only one of the pair of C'H and C'H protons when it was clear that the NOE could only be to one of them. 1Hans coupling constants obtained from a coupled 3D CT-HCACO spectrum were used to ascertain the presence of
High-Resolution Solution Structure of Interleukin-4

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**FIGURE 3:** Examples of typical $^{13}$C($F_2$)–$^1$H($F_3$) planes taken at two different $^1$H($F_2$)–$^{13}$C($F_3$) cross-sections through the 4D $^{13}$C/$^{13}$C-edited NOESY spectrum of IL-4. The cross-sections shown correspond to the $^1$H and $^{13}$C chemical shifts of (A) Ile 36 and (B) Lys 88, with partial overlap of Asp 109$\beta_2$. These cross-sections display $^1$H–$^{13}$C correlations of protons that have an NOE interaction with (A) Ile C$^\beta$H and (B) Lys 88 C$^\beta$H or Asp 109 C$^\alpha$H. Since the spectral width in the $^{13}$C dimension was set to 20.71 ppm, resonances were extensively folded in $F_1$ and $F_3$ resulting in both negative and positive cross peaks. Negative cross peaks are indicated by dashed contour lines.

Hence, the purpose of incorporating these restraints was not to increase the precision of the local structure but to assist in the convergence of the simulated annealing calculations.

The incorporation of $\psi$ restraints in the simulated annealing protocol can be problematic in regions which do not correspond to secondary structure elements. Therefore, most $\psi$ restraints were added near the end of the iterative refinement procedure when the structures were fairly well defined and most, if not all, erroneous interproton distance restraints were corrected. It was our empirical observation that the quality and precision of each ensemble of structures were primarily dependent on the number and correctness of the interproton distance restraints employed in the simulated annealing calculations. The presence of torsion restraints mainly assists in the convergence rate of the calculations, particularly since the torsion angle restraint boundaries were always much larger than the resulting standard deviations for the corresponding torsion angles in the calculated ensemble of structures.

**FIGURE 4:** Example of a typical $^1$H($F_2$)–$^{13}$C($F_3$) plane taken at the $^{15}$N($F_2$) = 123.3 ppm and $^1$H($F_3$) = 8.43 ppm cross-section through the 4D $^{15}$N/$^{13}$C-separated NOESY spectrum of IL-4. The cross-sections shown correspond to the $^{15}$N and NH chemical shifts of residues Ile 9, Phe 77, Val 105, Glu 107, and Phe 116 and displays $^1$H–$^{13}$C correlations of protons that have an NOE interaction with the backbone amide protons of these residues. Since the spectral width in the $^{13}$C dimension was set to 20.71 ppm, resonances were extensively folded in $F_1$ resulting in both negative and positive cross peaks. Negative cross peaks are indicated by dashed contour lines.

non-glycine residues with positive $\phi$ backbone torsion angles, since a value of less than 134 Hz is indicative of a positive $\phi$ torsion angle (Vuister et al., 1992). No values less than 134 Hz were observed. Hence, all non-glycine $\phi$ torsion angles were, as a minimum, restrained to be negative ($-2^\circ$ to $-178^\circ$), provided that the corresponding $^1$H$\text{C}^{\alpha}$ coupling constant could be accurately measured from the 3D CT-TCACO spectrum. In addition, some $\phi$ and $\psi$ torsion angle restraints were added for defined backbone regions where the $\phi$ and $\psi$ torsion angles in the current set of structures had observed ranges of less than $\pm 20^\circ$. The boundaries for these restraints, however, were significantly larger ($\pm 50^\circ$) than the observed $\pm 20^\circ$ range.

Tightly Bound Water. Upon completion of the simulated annealing calculations, we identified the presence of two tightly bound water molecules (lifetime $> 1$ ns) in the IL-4 structure from the 3D $^{15}$N-edited ROESY spectrum by the observation of ROEs from the water frequency to NH protons (Otting & Wüthrich, 1989; Clore et al., 1990a; Forman-Kay et al., 1991b; Clore & Gronenborn, 1992). The 3D $^{15}$N-edited ROESY experiment for the $F_1$($^1$H) plane corresponding to the chemical shift of water (4.67 ppm) is illustrated in Figure 5. The cross peaks corresponding to ROEs between bound water (W) and the side-chain NH$_2$ groups of Gln 24, Gln 76, and Gln 82 are indicated. The two bound water molecules were not included in the refinement process because of the difficulty of correctly placing the water molecules in the structure. A number of other cross peaks to bound water are also observed in this
RESULTS AND DISCUSSION

The Converged Structures. The structural statistics for the final 30 simulated annealing (SA) structures of human IL-4 are summarized in Table II, and best fit superpositions of the backbone atoms and selected side chains are shown in Figures 6 and 7, respectively. The atomic rms distributions about the mean coordinate positions and the angular rms deviations for the backbone atoms and selected side chains are shown in Figure 8 as a function of residue number. As is evident from the data in Figures 6–8, the final structures are of high precision and accuracy. In addition, they display very small deviations from idealized covalent geometry and satisfy the experimental NMR restraints within experimental errors (Table II). Thus, there are no interproton distance and torsion angle violations greater than 0.1 Å and 1°, respectively. There are no bad nonbonded contacts, as indicated by the small value of the quartic van der Waals repulsion term, $F_{\text{rep}}$ (3.4 kcal mol$^{-1}$). This is further supported by the calculated value of two other energy parameters which were not included in the target function for simulated annealing, namely, the large negative values for the Lennard-Jones–van der Waals energy ($-466 \pm 10$ kcal mol$^{-1}$) and the solvation free energy of unfolding ($-133 \pm 3$ kcal mol$^{-1}$). The latter is consistent with a protein the size of IL-4 (Eisenberg & McLachlan, 1986; Chiche et al., 1990) and is indicative of an appropriate distribution of hydrophobic and hydrophilic residues within the interior and exterior of the protein.

Excluding residues 1–7 and 130–133 at the N- and C-termini, which are poorly defined by the data (cf. Figure 8), the atomic rms distribution of the 30 SA structures about the mean coordinate positions is 0.44 ± 0.33 Å for the backbone atoms, 0.83 ± 0.03 Å for all atoms, and 0.51 ± 0.04 Å for all atoms excluding disordered side chains (Table II). In addition, the mean standard deviations for the $\phi$ and $\psi$ backbone torsion angles of residues 8–129 are 8.4 ± 7.0° and 8.4 ± 6.8°, respectively. The backbone of the core of the protein comprising the elements of regular secondary structure is defined to an even greater degree of precision and accuracy, with an atomic rms distribution about the mean coordinate positions of 0.34 ± 0.04 Å and mean standard deviations of 6.1 ± 4.2° and 6.3 ± 3.9° for the $\phi$ and $\psi$ backbone torsion angles, respectively. Not surprisingly, surface side chains, and in particular residues with longer side chains such as Lys and Arg, tend to be partially disordered, especially beyond the C$\alpha$ position. This is clearly evident in Figure 8, where the standard deviations of the $\chi_1$ and $\chi_2$ torsion angles are plotted together with surface accessibility as a function of residue. In addition to the N- and C-termini, the largest backbone atomic and angular ($\phi, \psi$) rms deviations occur in the surface-exposed loop regions comprising residues 25–31, 35–43, 67–72, and 99–109 (Figures 6 and 8), which correlates with their higher degree of mobility as seen in $^{15}$N relaxation measurements (Redfield et al., 1992).

Most of the backbone torsion angles for non-glycine residues lie within the allowed and expected $\alpha$-helix and $\beta$-sheet regions of the Ramachandran plot (Figure 9). The few non-glycine residues that fall outside these regions correspond to residues located at the structural interfaces of helices and connecting loops (Ala 72, Asp 109), in loop regions (Ile 36, Lys 41, and Lys 106), or at the C-terminus (Lys 130). Gly 99 and Gly 71 are the only residues which exhibit a positive $\phi$ angle.

The backbone atomic rms shift from the average coordinates of our previous second generation structure (Powers et al., 1992b) to the average coordinates of the present high-resolution fourth generation structure is ~1.7 Å (for residues 8–129). This difference is ~0.3 Å larger than the sum of the backbone atomic rms deviations of the two ensembles of simulated annealing structures about their respective means. Hence, the previous average coordinates lie just outside the current ensemble of highly refined simulated annealing structures. This finding is completely in line with previous observations on the determination of the solution structure of interleukin-18 at various stages of refinement (Clore et al., 1990c, 1991c), as well as with a series of extensive theoretical calculations aimed at probing the effects of the number of experimental NMR restraints on the precision and accuracy of the resulting coordinates (Clore et al., 1993). In particular, an increase in the number of restraints not only reduces the atomic rms spread of the ensemble of simulated annealing structures but also results in a concomitant and significant increase in the accuracy of both the ensemble and mean coordinates. Further, the precision of an ensemble of structures is always greater than its accuracy (Clore et al., 1993). Indeed, in model calculations the backbone rms difference between an ensemble of NMR structures and the true structure (i.e., the accuracy) is 50–70% larger than the corresponding rms difference between the same ensemble of structures and their mean coordinate.
A. Structural Statistics

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<th>(SA)r</th>
<th>X-ray</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.0016 ± 0.0009</td>
<td>0.0043</td>
<td>0.609</td>
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<tr>
<td></td>
<td>0.0022 ± 0.0019</td>
<td>0.0061</td>
<td>0.457</td>
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<tr>
<td>interresidue sequential (</td>
<td>i</td>
<td>-</td>
<td>j</td>
</tr>
<tr>
<td></td>
<td>0.0009 ± 0.0003</td>
<td>0.0021</td>
<td>0.792</td>
</tr>
<tr>
<td>H-bonds (102)*</td>
<td>0.0027 ± 0.0008</td>
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</tr>
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<td>H-bonds (Å)</td>
<td>0.068 ± 0.020</td>
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<td>FNOE kcal mol⁻¹/°</td>
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<tr>
<td>Fsum kcal mol⁻¹</td>
<td>0.11 ± 0.07</td>
<td>0.15</td>
<td>23.10</td>
</tr>
<tr>
<td>Fiso kcal mol⁻¹</td>
<td>0.39 ± 0.82</td>
<td>0.17</td>
<td>108.13</td>
</tr>
<tr>
<td>FTJ kcal mol⁻¹*</td>
<td>-466 ± 10</td>
<td>-477</td>
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</tr>
<tr>
<td>EBB E kcal mol⁻¹*</td>
<td>-133 ± 3</td>
<td>-149</td>
<td>-141</td>
</tr>
</tbody>
</table>

deviations from idealized covalent geometry

B. Atomic rms Differences (Å)

<table>
<thead>
<tr>
<th></th>
<th>backbone atoms</th>
<th>all atoms</th>
<th>backbone atoms</th>
<th>all atoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>residues 8–129 (SA) vs SA</td>
<td>0.44 ± 0.03</td>
<td>0.83 ± 0.03</td>
<td>0.34 ± 0.04</td>
<td>0.78 ± 0.04</td>
</tr>
<tr>
<td>secondary structure</td>
<td>0.46 ± 0.03</td>
<td>0.91 ± 0.04</td>
<td>0.36 ± 0.04</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>(SA) vs (SA)r</td>
<td>0.13</td>
<td>0.37</td>
<td>0.11</td>
<td>0.37</td>
</tr>
<tr>
<td>SA vs X-ray</td>
<td>1.35</td>
<td>2.16</td>
<td>1.04</td>
<td>1.88</td>
</tr>
<tr>
<td>(SA) vs X-ray</td>
<td>1.37</td>
<td>2.24</td>
<td>1.04</td>
<td>1.94</td>
</tr>
<tr>
<td>(SA) vs X-ray</td>
<td>1.42 ± 0.04</td>
<td>2.31 ± 0.05</td>
<td>1.09 ± 0.07</td>
<td>2.03 ± 0.06</td>
</tr>
</tbody>
</table>

ordered side chains

positions (i.e., the precision), independent of the number of experimental NMR restraints, provided these are sufficient to define the polypeptide fold (Clore et al., 1993).

There are a number of reasons for the above observations. First, as the nonbonded contacts are represented by a simple quartic van der Waals repulsion term, the only attractive forces present in the target function are those associated with the interproton distance restraints. Hence, the lower the number of interproton distance restraints, the smaller the contribution of the attractive forces to the target function, which will tend to result in a systematic expansion of the calculated structures (Clore et al., 1993). Second, the exact backbone conformation is critically dependent on the detailed packing of internal side chains, which in the case of IL-4 involves an unusually large number of Leu and Ile residues. If the side-chain conformations of these internal Leu and Ile residues, as well as those of the other hydrophobic residues within the protein core, are poorly defined due to either a small number of interproton distance restraints or the absence of stereospecific assignments and associated χ₁ and χ₂ torsion angle restraints, the correct packing of their side chains will not be attained. As a result, the probability of generating, under these circumstances, either an individual structure or an average structure which is very close to the true structure is likely to be infinitesimally small (Clore et al., 1990c). In this respect, it should be noted that the experimental restraints used to compute the earlier low-resolution structures did not include any stereospecific assignments or side-chain torsion angle restraints (Powars et al., 1992b; Smith et al., 1992). In addition to these two factors, which are intrinsic to NMR protein structure determination, it should be kept in mind that the experimental NMR restraints used to calculate the low-resolution structures are likely to contain errors (e.g., misassignment of NOEs, incorrect torsion angle restraints). That this is clearly the case can be ascertained by examining the overall rms of the violations of all upper interproton distance bounds, and all upper and lower
torsion angle bounds. These have mean values of 0.076 Å
and 0.389°, respectively, in the low-resolution structures of
Smith et al. (1992), 0.033 Å and 0.293°, respectively, in the
low-resolution structures of Powers et al. (1992b), and 0.0016
Å and 0.068°, respectively, in the current set of high-resolution
structures (Table II). Thus, the high-resolution structures
display 20–50- and 4–5-fold improvements in agreement with
the interproton distance and torsion angle restraints, respec-
tively, relative to the previous low-resolution structures,
consistent with the large increase in the number and correctness
of the experimental restraints.

Description of the Structure. The secondary structure
elements present in IL-4 are summarized in Table III. The
overall structure of IL-4 consists of four α-helices arranged
in a left-handed helical bundle. A unique characteristic of
the IL-4 structure is the orientation of the helices in which
adjacent helices are anti-parallel while nonadjacent opposing
helices are parallel. This results in two overhand connections
in the form of two long loops with some irregular β-strand
characteristics. Indeed, part of these two long loops actually
forms a short antiparallel β-sheet between residues 32–34
and 110–112. Helix αA (residues 8–24) leads into a five-
residue helical turn (htA, residues 27–31), followed by a strand
(βA, residues 32–34) that runs in an anti-parallel direction
to helix αA and a loop (residues 35–43) that leads into helix αB
(residue 44–66). Helices αB and αC (residues 73–98) are
connected by a short loop (residues 67–72) that contains a
global helical turn (htB, residues 67–72) that runs anti-parallel to αC and leads directly into
the fourth helix, αD (residues 113–128).

The structure of IL-4 contains three disulfide linkages. The
N- and C-termini of the protein are connected by the disulfide
bond between Cys 7 and Cys 131, which is ill-defined. The
disulfide bridge between Cys 28 and Cys 69 connects the two
helical turns, htA and htB. The average Cα-Cα distance
between Cys 28 and Cys 69 was only 5.1 ± 0.1 Å. This very
short Cα-Cα distance resulted in x1(Cys 28), x2(Cys 28), x3,
x4(Cys 69), and x5(Cys 69) angles of -133 ± 4°, 58 ± 2°,
75 ± 7°, -167 ± 6°, and -44 ± 12°, respectively. The disulfide
bridge between Cys 50 and Cys 103 connects helix αB to the
loop from residues 99–109. The average Cα-Cα distance
between Cys 50 and Cys 103 was 6.9 ± 0.02 Å, indicative of
an extended conformation typically observed in disulfides that
span the β-barrels in immunoglobulins or short loops, with
x1(Cys 50), x2(Cys 50), x3, x4(Cys 103), and x5(Cys 103)
angles of 160 ± 7°, -160 ± 6°, -117 ± 4°, -145 ± 1°, and
-176 ± 11°, respectively.

The IL-4 molecule is oblong in shape with approximate
dimensions of ~41 x ~28 x ~19 Å. This is reflective of
the four relatively long α-helices of approximately equal lengths
which are packed into the left-handed bundle (Tables III
and IV). The angles and axial separations between the long axes
of the four anti-parallel helical pairs, αA-αC, αC-αB, αB-αD,
and αD-αA, are -157° and 11.1 Å, -154° and 10.2 Å, -152°
and 7.7 Å, and -148° and 11.8 Å, respectively. The

![Figure 6: Two stereoviews showing best fit superpositions of the backbone (N, Cα, C) atoms of the 30 final SA structures. The view in B is related to that in A by an approximately 90° rotation about the x axis. The three disulfide bridges are also shown in A. Residues 7–131 and 9–129 are shown in A and B, respectively.](image-url)
respectively. Thus, the angle $\Omega$ (Chothia, 1984) between the long axes of adjacent anti-parallel helices, when projected onto their plane of contact, ranges from $+23^\circ$ to $+32^\circ$. In terms of the "ridges into grooves" model of helix packing, this corresponds to class 3-4 packing in which one helix uses $\pm 4\pi$ ridges and the other uses $\pm 3\pi$ ridges (Chothia et al., 1977, 1981) (i.e., the ridges on the helix surface are formed by side chains separated by four and three residues in the sequence, respectively).

The nature of the helix packing is dependent on side-chain–side-chain interactions. The core of the IL-4 protein is entirely hydrophobic. The residues involved in the helix packing are Leu 11, Ile 14, Leu 18, and Leu 21 of helix $\alpha_A$, Phe 49, Ala 52, Ala 53, Leu 56, Phe 59, and Tyr 60 of helix $\alpha_B$, Leu 83, Ile 84, Leu 87, Leu 90, Leu 94, and Leu 97 of helix $\alpha_C$, and Leu 113, Phe 116, Leu 117, Leu 120, Met 124, and Tyr 128 of helix $\alpha_D$. The interactions of these side chains at the interface of helices $\alpha_A$, $\alpha_B$, and $\alpha_D$, helices $\alpha_A$, $\alpha_C$, and $\alpha_D$, and helices $\alpha_B$, $\alpha_C$, and $\alpha_D$ are illustrated in Figure 7A–C, respectively. In addition, there are a number of hydrophobic interactions between strand $\beta_A$ and helices $\alpha_B$ and $\alpha_D$ that are illustrated in Figure 7A. Specifically, Val 33 of strand $\beta_A$ is in contact with Val 55 and Phe 116, Ile 36 is in contact with Ala 52, and Phe 37 is in contact with Ile 123.

It appears from Figure 6A that residues 73–80 in helix $\alpha_C$ extend beyond the helix bundle and are solvent-exposed. The orientation of this segment of helix $\alpha_C$ with respect to the core of the protein, however, is well-defined and is stabilized by numerous hydrophobic side-chain–side-chain interactions involving the helical turns $\tau_A$ and $\tau_B$. In particular, Gln 76 is in close proximity to Thr 26 and Leu 27, Phe 77 is in close proximity to Leu 70, Arg 79 is in close proximity to Gln 24 and Thr 26, and His 80 is in close proximity to His 63, Glu 64, Cys 69, and Leu 70 (Figure 7C).

The presence of potential hydrogen bonds or salt bridges involving side chains and bound water in the IL-4 structure was ascertained after the final 30 SA structures were calculated. The restrained minimized average structure,
Figure 8: Atomic rms distribution of the 30 individual SA structures about the mean structure \( \overline{\text{SA}} \), for the backbone (N, C\( \alpha \), C, O) atoms, all atoms, and side-chain atoms as a function of residue number, together with the variation in surface accessibility of each residue (left). Standard deviation of the backbone \( \phi \) and \( \psi \) and side-chain \( \chi_1 \) and \( \chi_2 \) torsion angles for the 30 SA structures as a function of residue number (right). The black circles represent the average values at each residue, and the error bars indicate the standard deviations in these values. The bottom of the figure presents a schematic diagram of the secondary structure of IL-4, with helices shown as helical coils and \( \beta \)-strands shown as arrows.

Figure 9: Ramachandran \( \phi, \psi \) plot for the restrained minimized mean structure, \( (\text{SA})_\text{r} \), of IL-4. Non-glycine residues that lie outside the allowed regions of the Ramachandran plot (indicated by the solid lines) and glycine residues lying in the positive \( \phi \) region of the plot are labeled.

\( (\text{SA})_\text{r} \), of IL-4 with side chains involved in potential hydrogen bonds, salt bridges, and interactions with tightly bound water is illustrated in Figure 10. The approximate positions of the two tightly bound water molecules (lifetime > 1 ns) in the average IL-4 structure are indicated by solid spheres, since the exact positioning of the water molecules could not be determined.

The presence of tightly bound water was determined from ROEs between water and side-chain amide groups observed in the 3D \( ^{15}\text{N} \)-edited ROESY spectrum (Figure 5). Three

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<th>HGH*</th>
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<td>98-102</td>
<td></td>
</tr>
</tbody>
</table>

* From the X-ray structures of GM-CSF (Diederichs et al., 1991) and HGH (De Vos et al., 1992). These residues comprise two small helices located in the connecting loop between helices A and B (i.e., they are significantly longer than the helical turns present in IL-4 and GM-CSF).
Table IV: Interhelical Angles and Axial Separations between Pairs of Helices Comprising the Left-Handed Four-Helix Bundle Observed in the X-ray Structures of IL-4, GM-CSF, and HGH

<table>
<thead>
<tr>
<th>helical pairs</th>
<th>IL-4 (NMR)</th>
<th>IL-4 (X-ray)</th>
<th>GM-CSF#</th>
<th>HGH#</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-C</td>
<td>-157° (11.1)</td>
<td>-153° (8.8)</td>
<td>-151° (8.6)</td>
<td>-145° (10.7)</td>
</tr>
<tr>
<td>A-D</td>
<td>-158° (10.2)</td>
<td>-152° (8.5)</td>
<td>-149° (10.4)</td>
<td>-155° (6.0)</td>
</tr>
<tr>
<td>B-C</td>
<td>-152° (7.7)</td>
<td>-151° (7.9)</td>
<td>-167° (7.4)</td>
<td>-157° (8.9)</td>
</tr>
<tr>
<td>B-D</td>
<td>-148° (11.8)</td>
<td>-149° (11.9)</td>
<td>-141° (10.0)</td>
<td>-155° (10.1)</td>
</tr>
<tr>
<td>A-B</td>
<td>40° (14.9)</td>
<td>41° (14.8)</td>
<td>39° (14.5)</td>
<td>43° (13.9)</td>
</tr>
<tr>
<td>C-D</td>
<td>36° (15.1)</td>
<td>41° (12.3)</td>
<td>40° (11.0)</td>
<td>34° (12.5)</td>
</tr>
</tbody>
</table>

# The interhelical angles (degrees) between each helical pair is followed by the axial separation (Å). The first four helical pairs are anti-parallel, while the last two are parallel.  
# IL-4 (NMR) is the restrained minimized mean NMR structure, (SA) to Table II.  
# IL-4 (X-ray) is the 2.25 Å resolution crystal structure of IL-4 (Wlodawer et al., 1992).  
# From the X-ray structure of GM-CSF (Diederichs et al., 1991).  
# From the X-ray structure of HGH (De Vos et al., 1992).

such ROEs were observed involving the side-chain amide of Gln 24, Gln 76, and Gln 82. The close proximity of Gln 24 and Gln 82 in the structure suggests that a single bound water (W1) bridges these two side chains. W1 probably adds additional stability to the C-terminus of helix αA since Gln 24 is the C-terminal residue of this helix. Further, W1 also increases the packing stability of helix αA to helix αB by removing a potentially destabilizing side-chain interaction.

The second bound water (W2) is in close proximity to the side-chain amide group of Gln 58 in helix CYB. The close proximity of Gln 24 and Gln 82 in the structure suggests that a single bound water (W2) is involved in a network of bridging hydrogen bonds comprising the side-chain amide group of Gln 58 and the backbone carbonyl of Ala 39 in the loop connecting helix αB and strand βA. Finally, there are two interactions which stabilize the positioning of strand βA with respect to helices αB and αD, namely, the carboxylate side chain of Asp 35 at the end of strand βA is hydrogen-bonded to the guanidinium group of Arg 119 in helix αD, and the backbone carbonyl of Ala 39 in the loop connecting helix αB and strand βA is hydrogen-bonded to the guanidinium group of Arg 51 in helix αB. The hydrogen bond/salt bridge involving Asp 35 is of particular interest as it serves to neutralize the negative charge on the completely buried side chain of Asp 35, which has a surface accessibility of only ~6 Å.

Comparison of the Solution Structure of IL-4 with the X-ray Structure

Shortly after the publication of our initial low-resolution (second generation) NMR structure of IL-4 (Powers et al., 1992b), two crystal structures of IL-4 were determined: one at 2.25-Å resolution (Wlodawer et al., 1992) and the other at 2.35-Å resolution (Walter et al., 1992a). The superposition of the backbone atoms of the restrained minimized mean, (SA), high-resolution NMR structure and the 2.25-Å resolution X-ray structure of Wlodawer et al. (1992) is shown in Figure 11, together with a plot of the backbone atomic rms differences as a function of residue. Clearly the two structures are similar, both in terms of overall topology and alignment of the four-helix bundle, as evidenced by the very good agreement in the calculated interhelical angles (Table III). Nevertheless, there are some significant differences, as evidenced by the relatively large rms differences between the two structures. Thus, excluding residues 1–7 and 130–133 at the N- and C-termini, respectively, the atomic rms difference between the restrained minimized mean NMR structure, (SA), and the X-ray structure is 1.37 Å for the backbone atoms and 2.24 Å for all atoms (Table II). When only residues involved in secondary structure are considered, these values drop to 1.04 and 1.94 Å, respectively.

These relatively large rms differences can be attributed to rigid-body displacements of the helices such that the four-helix bundle is more tightly packed in the crystal structure than the NMR structure. In particular, the axial separation for three pairs of helices, αA–αC, αA–αD, and αC–αD, is reduced by approximately 20% from 11.1, 10.2, and 15.1 Å, respectively, in the NMR structure to 8.8, 8.5, and 12.3 Å, respectively, in the crystal structure. This is also accompanied by a decrease in the radius of gyration from 14.7 Å in the NMR structure to 14.2 Å in the crystal structure. The higher degree of compactness of the crystal structure can probably be attributed to the mobility and flexibility of the protein in solution and to crystal packing forces arising from interactions with neighboring protein molecules in the crystal lattice. In this regard it is interesting to note that in the crystal lattice residues 66–76 in the loop connecting helices αB and αC are in contact with the N-terminus of helix αSA and the C-terminus of helix αC of a second molecule and with the C-terminus of helix αD of a third molecule.

The conformation of the Cys 28–Cys 69 disulfide bridge, which is associated with a very short Cε-Cε' distance, is the same in the NMR and X-ray structures, the χ angles differing...
on average by no more than 20–25°. The conformation of the Cys 50–Cys 103 disulfide bridge, however, differs significantly between the two structures. Whereas the χ angles in the NMR structure are typical of an extended disulfide bridge conformation (see above), those in the X-ray structure are highly unusual and skewed (Richardson, 1981) with χ1(Cys 50), χ2(Cys 50), χ3, χ3'(Cys 103), and χ1'(Cys 103) angles of 169°, -94°, 154°, 114°, and -93°, respectively. Indeed, there do not appear to be any examples of χ3 angles which does not lie within a ±30° range of either +90° or -90° (Richardson, 1981).

The other major differences between the NMR and crystal structures lie in three loop regions comprising residues 35–43, 67–72, and 99–109, which connect helices αA and αB, helices αC and αD, and helices αC and αD, respectively. Again, mobility in solution and crystal packing are likely to be major contributors to these differences. Thus, for example, residues 40–45 of one molecule lie anti-parallel to residues 40–45 of a second molecule in the crystal lattice with four hydrogen bonds between the two molecules at this location [Ser 40-(CO)–Thr 48(OH), Thr 43(NH)–Asn 42(O), Thr 44-(NH)–Lys 41(CO), and Thr 44(NH)–Ser 40(CO)]. Similarly, the orientation with respect to the core of the protein of the loop comprising residues 67–72 is clearly influenced by crystal packing, as described above. In solution, on the other hand, 15N heteronuclear relaxation measurements indicate that loops comprising residues 35–43 and 99–109 exhibit a significant degree of motion on the picosecond time scale and are characterized by low (0.2–0.6) values for the overall order parameters, S2 (Redfield et al., 1992).

The significance of the structural differences between the NMR and crystal structures is indicated by the very high values of the NOE and torsion angle restraint energies (Table II) and the large number of interproton distance and torsion angle violations greater than 2 Å and 60°, respectively, exhibited by the X-ray structure (Table V). Many of the larger interproton distance violations are a result of different χ rotamers between the X-ray and NMR structures. A particular example is Trp 95, which has χ1 and χ2 angles of 176 ± 5° and -91 ± 4°, respectively, in the NMR structures and -63° and 108°, respectively, in the crystal structure. This difference is real and is verified by a significant number of NOEs which could only be present in the χ1,χ2 = 180°, -90° conformer and by the absence of NOEs which could only be present in the χ1,χ2 = -60°,90° conformation.

The χ1 torsion angles of a number of internal residues in the X-ray structure are significantly skewed from the normal rotamer positions. The distortion of these torsion angles, which may be a reflection of the tighter helix packing in the crystal structure, also results in significant NOE and torsion angle violations, but does not affect the general character of the side-chain–side-chain interactions.

The backbone φ and ψ restraint violations seen in the crystal structure are located in the two mobile loop regions (residues 35–43 and 99–109). These violations are probably of little significance since the NMR structure corresponds to an average structure and the crystal structure corresponds to a particular "frozen-out" conformation that is also influenced by crystal packing forces.

It is also interesting to note that, from an energetic viewpoint, the value of the Lennard–Jones–van der Waals energy is less negative for the crystal structure (-368 kcal mol-1) than for the NMR structure (-477 kcal mol-1). This may be a reflection of the tighter packing of the helices, resulting in some poor nonbonded contacts. There is no significant difference, however, in the values of the solvation free energy of folding for the solution and crystal structures (Table II).

Comparison of the Solution Structure of IL-4 with the Crystal Structures of Human Growth Hormone and Granulocyte–Macrophage Colony Stimulating Factor. The lefthanded four-helix bundle motif with two overhand connections observed in the structure of IL-4 is relatively unique. To our knowledge, it has only been observed in four other proteins, all of which bind to the same superfAMILY of hematopoietic receptors (Boulay & Paul, 1992): growth hormone (Abdel-Meguid et al., 1987; de Vos et al., 1992), granulocyte–macrophage colony stimulating factor (GM-CSF; Diedrichs et al., 1991; Walter et al., 1992b), interleukin-2 (Bazan, 1992; McKay, 1992), and macrophage colony stimulating factor (Pandit et al., 1992). The coordinates of human growth hormone (HGH) and GM-CSF were kindly provided to us by Drs. A. M. de Vos and A. Kossiakoff and Dr. P. A. Karplus.
High-Resolution Solution Structure of Interleukin-4

Biochemistry, Vol. 32, No. 26, 1993 6757

A

<SA> vs X-ray: Backbone atoms

B

Residue

RMS difference [Å]

3.0
2.0
1.0
0.0
10 20 30 40 50 60 70 80 90 100 110 120 130

FIGURE 11: Best fit superposition of the backbone (N, Cα, C) atoms of the restrained minimized mean NMR (thick line) structure and the X-ray (thin line) structure of IL-4 for residues 7-131 (A). Backbone (N, Cα, C, O) atomic rms difference between the 30 SA structures and the X-ray structure as a function of residue number (B). The black circles represent the average values at each residue and the bars the standard deviations of these values. The X-ray structure is that of Wlodawer et al. (1992).

respectively, and the results of a comparison with the NMR structure of IL-4 are discussed below.

Even though there is no significant overall sequence identity between IL-4, HGH, and GM-CSF, their overall topology is very similar, as illustrated both by the ribbon diagrams shown in Figure 12 and by the very similar values for the interhelical angles and axial separation within the four-helix bundle, listed in Table III. There is one significant topological difference, however, between IL-4 and GM-CSF on the one hand and HGH on the other. Namely, in the case of IL-4 and GM-CSF, the N-terminal end of the overhand loop connecting helices αA and αB passes behind helix αD in the view shown in Figure 12, while in the case of HGH it passes in front of helix αD.

A least-squares match of the Cα coordinates using the program O (Jones & Kjeldgaard, 1990) reveals that 72 residues of IL-4 and GM-CSF and 79 residues of IL-4 and HGH can be superimposed with atomic rms differences of 1.7 and 2 Å, respectively (Table VI). The resulting best fit superpositions are also shown in Figure 12, and the corresponding sequence alignments are given in Figure 13. The extent of sequence identity within these matched regions of structure is small, namely, 25% for IL-4 and GM-CSF and only 10% for IL-4 and HGH.

The principal differences between IL-4, GM-CSF, and HGH reside in the relative lengths of the helices, the nature and lengths of connecting elements, and the number and pattern of disulfide bonds. A comparison of the secondary structure elements present in the three structures is listed in Table III. Inspection of the ribbon drawings shown in Figure 12 indicates that GM-CSF is smaller than IL-4, whereas HGH is larger than IL-4. This is reflected in the lengths of all helices in the three structures. The helices in GM-CSF, IL-4, and HGH range from 10 to 20 residues, 17 to 26 residues, and 21 to 33 residues, respectively, in length. In addition, the relative lengths of the helices in each structure differ. In the case of HGH, helices αA and αD are longer than helices αB and αC, whereas the reverse is true for IL-4. For GM-CSF, helices αA and αC are longer than αB and αD. These differences result in a distinct alteration in the surface at the top of the front face in the view shown in Figure 12.

The connecting elements between the various helices also differ in the three structures. IL-4 has two helical turns, one connecting helices αA and αB and the other connecting helices αB and αC, and a small anti-parallel β-sheet connecting the two loops between helices αA and αB and helices αC and αD. In GM-CSF, the helical turn between helices αB and αC is missing. This is probably due to the fact that this connecting
element is significantly shorter in GM-CSF (three residues) than in IL-4 (six residues). Conversely, the anti-parallel \( \beta \)-sheet is two residues longer in GM-CSF than in IL-4. The differences in the connecting elements between IL-4 and HGH are more distinctive. In HGH, the anti-parallel \( \beta \)-sheet is absent and an additional short (seven residue) helix is present between helices \( \alpha_A \) and \( \alpha_B \). This additional helix can be formed because the linker between helices \( \alpha_A \) and \( \alpha_B \) is approximately twice as long as the linker in IL-4 (19 residues compared to 37 residues) and, hence, does not need to adopt an entirely extended conformation to complete the link between the two helices.

In addition, the pattern and number of disulfide linkages differ in the three structures. IL-4 has three disulfide bridges, whereas both HGH and GM-CSF have only two disulfide linkages. The disulfide linkages in GM-CSF connect helix \( \alpha_B \) to the loop region between helices \( \alpha_C \) and \( \alpha_D \), and the C-terminus to helix \( \alpha_C \). The disulfide linkages in HGH connect helix \( \alpha_B \) to the loop region between helices \( \alpha_A \) and \( \alpha_B \), and the C-terminus to helix \( \alpha_D \).

**Biological Implications.** The striking qualitative and quantitative resemblance of the topologies of IL-4, GM-CSF, and HGH illustrated in Figure 12 was unexpected given the very limited amount of overall sequence similarity. One rationale for this observation may be provided by the fact that the receptors for these three proteins belong to the same hematopoietic superfamily. If this interpretation is accurate, then one would expect all three proteins to interact with their cell surface receptor in a similar manner. The crystal structure of the HGH–receptor complex has been determined (de Vos et al., 1992), and it reveals that a single molecule of HGH is bound to two molecules of receptor. This lends support to the hypothesis that dimerization is responsible for signal transduction by the hematopoietic superfamily of receptors (Cunningham et al., 1991; Fuh et al., 1992; Boulay & Paul, 1992). It may therefore be possible to infer details of the interaction of IL-4 with its cognate receptor on the basis of the structural alignment of IL-4 and HGH presented above (Figure 12).

By analogy with the HGH–receptor complex, we therefore propose that there are two distinct sites of interaction on IL-4.
The interaction site for the first receptor molecule would involve the surface formed mainly by helix $\alpha_A$, strand $\beta_A$, and the loop connecting strand $\beta_A$ to helix $\alpha_B$ (bottom left-hand side of Figure 6A and bottom of Figure 6B), while the interaction site for the second receptor molecule would involve the surface formed by helices $\alpha_A$ and $\alpha_C$ (bottom right-hand side of Figure 6A and top right of Figure 6B). The structure-based sequence alignment in Figure 13, which is principally restricted to the helical regions, then permits one to infer potential residues of IL-4 involved in contacting the two receptor molecules by direct analogy with the HGH system. Residues comprising the first site in HGH are His 18 in helix $\alpha_A$ and Arg 167, Lys 168, Asp 171, Thr 175, and Arg 178 in helix $\alpha_B$, which correspond to Asn 19, Glu 114, Asn 115, Glu 118, Thr 122, and Arg 125, respectively, in IL-4. In addition to these residues, we would also predict the potential involvement in IL-4 of Lys 121, Glu 126, and Tyr 128. Residues constituting the second site in HGH are Asn 12, Leu 15, Arg 16, and Arg 19 in helix $\alpha_A$ and Asp 116 and Glu 119 in helix $\alpha_C$, which correspond to Glu 13, Lys 16, Thr 17, Ser 20, Arg 89, and Arg 92, respectively, in IL-4. Other likely residues in this site that could potentially make contact with the IL-4 receptor are Thr 10 in helix $\alpha_A$ and Asn 93 in helix $\alpha_C$.

Three mutational studies are of interest in the light of the above prediction. First, analysis of a series of human–mouse GM-CSF chimera indicated that residues 21–31 and 78–94, which correspond to the second interaction site described above, are important for recognition (Kauhsansky et al., 1989). Second, site-specific mutagenesis data on GM-CSF have implicated a critical role for Glu 21 (equivalent to Glu 114 in IL-4; cf. Figure 13) in high-affinity binding to the $\beta$-receptor chain (Lopez et al., 1992). Third, a mutation in the first interaction site of Tyr 128 to Asp in IL-4 results in the generation of a competitive IL-4 antagonist which still binds to the receptor with high affinity (~60% of wild type), but no longer possesses any proliferative activity for T-cells (Kruse et al., 1992).

It could be argued that the effect of the Tyr 128 → Asp mutation in IL-4 arises from the removal of an important hydrophobic interaction with the receptor molecule. This is unlikely since the aromatic ring of Tyr 128 has only limited surface accessibility (~50 Å²) and only the hydroxyl group is directly solvent-exposed (cf. Figures 7C and 8). If the interaction of the hydroxyl group with the receptor molecule were crucial for IL-4 activity, one would speculate that adding the negatively charged Asp residue might actually increase the stability of the interaction or, at the least, have no effect. In fact, replacement of Tyr 128 by a positively charged Lys resulted in a mutant with a higher level of activity than the Tyr 128 → Asp mutant (Kruse et al., 1992). In addition, complete removal of the hydroxyl group in the Tyr 128 → Phe mutant resulted in a protein for which complete activity and binding affinity were preserved (Kruse et al., 1992). This indicates that the effect of the Tyr 128 → Asp mutation is probably not the result of a direct loss of a specific interaction with the receptor molecule, but in all likelihood arises from a local loss of the structural integrity of IL-4, a very likely occurrence given the fact that the side chain at position 128 resides on the interior face of the helix.

Tyr 128 is the C-terminal residue of helix $\alpha_D$, which is part of the first proposed binding site. Any local unwinding or disruption of helix $\alpha_D$ could be severe enough to eliminate this binding site. There is a significant amount of evidence that suggests that electrostatic interactions are important in $\alpha$-helix formation (Shoemaker et al., 1987). These results suggest that appropriately charged groups at the N- and C-termini of a helix can interact favorably with the positive and negative poles, respectively, of the $\alpha$-helix macrodipole, thereby stabilizing the helix. Therefore, by analogy, the mutation of Tyr 128 to the negatively charged Asp residue probably destabilizes the $\alpha_D$ helix because of its unfavorable interaction with the negative pole of the $\alpha$-helix macrodipole. Indeed, the probability of finding an Asp residue at the C-terminus of an $\alpha$-helix in native proteins is very low (Shoemaker et al., 1987). This also explains why the Tyr 128 → Lys mutation has a higher relative activity than the Tyr 128 → Asp mutation, since the positively charged Lys would interact favorably with the negative pole of the helix macrodipole. Even though the Tyr 128 → Lys mutation probably stabilizes the formation of helix $\alpha_D$, the mutant is significantly less active than native IL-4, presumably because of a conformational effect on receptor binding due to the introduction of a positive charge at this location. The Tyr 128 → Asp mutation is obviously only severe enough to eliminate one receptor binding site, consistent with the 60% decrease in receptor binding affinity. [Note that the stoichiometry of receptor binding for the wild-type and mutant proteins was not established (Kruse et al., 1992)]. The other mutations at position 128 (Phe, His, Asn, Lys, Gly) all have similar receptor binding affinity to wild-type IL-4, but exhibit varying degrees of reduced biological efficacy. This suggests that the effects of these various Tyr 128 mutations only result in local structural perturbations affecting a single receptor binding site. Hence, the observation that the Tyr 128 → Asp mutation eliminates IL-4 efficacy and acts as an antagonist is consistent with the view that signal transduction in the IL-4 system involves multiple binding sites on IL-4, possibly causing receptor dimerization. Thus, the antagonist properties of the Tyr 128 → Asp mutant appear to arise from the loss of one binding site and the concomitant prevention of receptor dimerization.

The availability of the high-resolution solution structure of IL-4 presented in this article will permit the design and interpretation of future mutational studies aimed at probing structure–function relationships and delineating the receptor binding sites in more detail. In addition, this structure and
the associated NMR spectroscopic data will provide the basis for future studies aimed at determining the three-dimensional structure of the IL-4-receptor complex in solution.

ACKNOWLEDGMENT

We thank Drs. Ad Bax and Geerjen Vuister for many useful discussions, Drs. Andrew Karplus, Alex Wlodawer, and Abraham de Vos and Anthony Kossiakoff for providing us with the X-ray coordinates of GM-CSF, IL-4, and HGH, respectively, and Rolf Tschudin for technical assistance.

SUPPLEMENTARY MATERIAL AVAILABLE

One table giving a complete list of $^1$H, $^{13}$C, and $^{15}$N resonance assignments for IL-4, including stereospecific assignments of $\beta$-methylene protons and methyl groups of Leu and Val (6 pages). Ordering information is given on any current masthead page. In addition, the coordinates of the 30 final simulated annealing structures of IL-4, together with the coordinates of the restrained minimized mean structure, (SA)r, and the complete list of experimental NMR restraints, have been deposited in the Brookhaven Protein Data Bank.

REFERENCES


