Neutrophil-activating peptide 2 (NAP-2), which demonstrates a range of proinflammatory activities, is a 72-residue protein belonging to the α-chemokine family. Although NAP-2, like other α-chemokines, is known to self-associate into dimers and tetramers, it has been shown that the monomeric form is physiologically active. Here we investigate the solution structure of monomeric NAP-2 by multi-dimensional 1H-NMR and 15N-NMR spectroscopy and computational modelling. The NAP-2 monomer consists of an amphipathic, triple-stranded, antiparallel β-sheet on which is folded a C-terminal α-helix and an aperiodic N-terminal segment. The backbone fold is essentially the same as that found in other α-chemokines. 15N T1, T2 and nuclear Overhauser effects (NOEs) have been measured for backbone NH groups and used in a model free approach to calculate order parameters and conformational exchange terms that map out motions of the backbone. N-terminal residues 1 to 17 and the C-terminus are relatively highly flexible, whereas the β-sheet domain forms the most motionally restricted part of the fold. Conformational exchange occurring on the millisecond time scale is noted at the top of the C-terminal helix and at proximal residues from β-strands 1 and 2 and the connecting loop. Dissociation to the monomeric state is apparently responsible for increased internal mobility in NAP-2 compared with dimeric and tetrameric states in other α-chemokines.

Key words: chemokine, internal motions, protein.

INTRODUCTION

Neutrophil-activating peptide 2 (NAP-2) [1], low-affinity platelet factor 4 (LA-PF4) [2] [also named connective-tissue-activating protein III (CTAP-III) [3]] and β-thromboglobulin (βTG) [4] are platelet-specific, naturally occurring N-terminal cleavage products of platelet basic protein (PBP) [2]. All are homologues of platelet factor 4 (PF4) [5] and are members of the growing family of C-X-C proteins or α-chemokines [6], which include interleukin 8 (IL-8) [1,7] and Gro-α [8]. Collectively, this protein family is involved in inflammation, blood clotting and wound healing.

NAP-2 is exceptionally potent at neutrophil activation and acts like homologous IL-8 in neutrophil chemotaxis [9]; it competes with IL-8 for binding to neutrophil cell-surface IL-8 receptors and subsequent G-protein activation [10,11]. Because NAP-2 devoid of its C-terminal segment is at least partly biologically active, the NAP-2 N-terminal domain, which includes the N-terminus and topologically related residues, apparently possesses the neutrophil activation properties [12]. Even though NAP-2 at millimolar concentrations exists in solution as an equilibrium between monomeric, dimeric and tetrameric states [13], like PF4 [14], it is primarily monomeric under physiological conditions, in which its concentration is considerably lower [13,15]. However, at nanomolar concentrations, at which NAP-2 induces changes in cytosolic free [Ca2+] and neutrophil chemotaxis and exocytosis, it certainly must be monomeric. In support of our original proposal that α-chemokines such as NAP-2 are probably active as monomers [15], Rajarathnam et al. [16,17] synthesized monomeric forms of IL-8, NAP-2 and melanoma growth stimulatory activity (MGSA) and showed that these α-chemokines are indeed active as monomers. In this respect, the study of the NAP-2 monomer state is most relevant to developing structure–function relationships and understanding how this chemokine functions biologically. Although the X-ray crystal structure of tetrameric NAP-2 is known [18], the present NMR study provides an added dimension by addressing the solution structure and motional dynamics of monomeric NAP-2 in the absence of conformationally stabilizing/modifying subunit associations.

In previous NMR studies of NAP-2 [13,19], the self-association-induced exchange broadening of resonances and the presence of multiple aggregate states were overcome by using various agents (salts and organic compounds) to disaggregate NAP-2. The most effective of these is 2-chloroethanol [20], which at low concentration [3–4% (v/v)] essentially dissociates NAP-2 to the monomeric state [19]. This allowed most 1H sequence-specific assignments to be made for monomeric NAP-2 and secondary structural elements to be deduced from a qualitative interpretation of 1H nuclear Overhauser effects (NOEs), 1H chemical shifts and coupling constants and long-lived backbone NH groups [19]. As with other α-chemokines whose structures are known (e.g. PF4 [21–23], IL-8 [24] and SDF-1 [25]), monomeric NAP-2 contains a triple-stranded anti-parallel β-sheet with strand 2 (residues 34–41) hydrogen-bonded to strands 1 (residues 22–28) and 3 (residues 46–49). Strands 1 and 2 are connected by a loop that is folded proximally to the neutrophil-activating N-terminal tripeptide sequence Glu-Leu-Arg. An α-helix extends from residue 55 to the C-terminus, with the last five C-terminal residues being undefined by the data. In that 1H-NMR study [19], structural modelling could not be completed owing to a lack of key long-range NOEs that were required to position the C-terminal α-
helix with respect to the β-sheet. In the present study, recombinant NAP-2 was uniformly enriched in 15N and 13C-N/1H, 2-dimensional (2D) and three-dimensional (3D) NMR experiments permitted a detailed structural analysis. In addition, 15N, 1H, 19F, T1, T2, and NOE relaxation terms were acquired and used to derive backbone motional parameters. The motional study, in particular, provides a unique understanding of the physical state of monomeric NAP-2.

**MATERIALS AND METHODS**

**Isolation of recombinant NAP-2**

The synthetic gene for human NAP-2 was expressed as a non-fusion protein in *Escherichia coli* (BL21) cells and grown on a 10-litre scale. NAP-2 was purified, cleaved and refolded essentially as described previously [26]. Purity was assessed by Coomassie staining of SDS/PAGE gels, analytical C18 reverse-phase HPLC and amino acid analysis. Typically, several hundred milligrams of material that was more than 95% pure were isolated from 100 g of starting material. Protein concentration was determined by using the bicinchoninic acid assay [27].

**NMR spectroscopy**

Samples for 1H-NMR measurements were freeze-dried and redissolved in D2O or H2O immediately before the experiment. For work in D2O solutions, 10% (v/v) H2O was routinely used. The solution also contained 4% (v/v) perdeuterated 2-chloroethanol to dissociate dimer-tetramer aggregate states [19, 20]. The final protein concentration was 18 mg/ml. The pH was adjusted to pH 3.6 by adding microlitre increments of NaOH or HCl to a 0.6 ml sample. The pH was not adjusted for isotope effects. Most experiments were acquired at 313 K. NMR spectra were recorded in the Fourier mode on a Bruker AMX-500 or on Varian Inova Unity Plus-500 and/or Varian Inova Unity Plus-600 spectrometers. The D2O deuterium signal was used as a field-frequency lock. All chemical shifts are quoted in parts per million (p.p.m.) downfield of sodium 2,2-dimethyl-2-silapentane sulphonate.

Sequence-specific assignments for most 1H resonances of NAP-2 have been reported by Mayo et al. [19] from analysis of COSY, double-quantum-filtered COSY, homonuclear Hartman–Hahn spectroscopy (HOHAHA) and NOE/JOE experiments. Heteronuclear 1H–15N NMR experiments have been done in this paper to provide 15N and complete 1H resonance assignments as well as to acquire additional NOE constraints for computational modelling and backbone dynamics studies.

2D-NMR and 3D-NMR 1H–15N heteronuclear multiple quantum coherence (HMQC)-HOHAHA and NOE/JOE [28–31] experiments were acquired to assign 15N resonances. The water signal was suppressed by using direct irradiation or square pulses (100 ms for 0.6 s) of 1024 points especially for the 1H–15N spectra. The mixing time on 1H–15N HMQC-HOHAHA experiments was 60 ms. HMQC-NOE experiments were acquired by using mixing times of 50, 75, 100 and 200 ms. The 1H carrier was placed at the water frequency or in the centre of the 1H–15N region, with 15N carrier at 125.0 p.p.m. The acquired data matrices for each 2D experiment were 512 (t1) × 1024 (t2) complex data points, with spectral widths of 7200 Hz in F1 (1H) and 3600 Hz in the F2 dimension of 15N. Spectra were recorded in the pure absorption mode by using the time-proportional phase incrementation (TPPI) and TPPI-states methods for quadrature detection in t1 and t2 respectively. Zero-filling and linear prediction were employed to yield spectra of 1024 (F1) × 1024 (F2) points. The acquired data matrices for each 3D experiment were 128 (t1) × 32 (t2) × 1024 (t3) complex data points, with spectral widths of 7200, 3600 and 7200 Hz in the F1 (1H), F2 (15N) and F3 (1H) dimensions respectively. The spectra were recorded in pure absorption mode by using the TPPI and TPPI-states methods for quadrature detection in t1 and t2 respectively. Zero-filling was employed to yield spectra of 512 (F1) × 64 (F2) × 1024 (F3) points. Additional suppression of the water signal was achieved by convolution of the time-domain data [28, 29]. Data were processed either directly on the spectrometer computer or off-line on an SGI Indigo workstation computer (using the program FELIX (Molecular Simulations).

A high-resolution 2D 1H–15N HSQC experiment [32] was acquired (4096 × 1024 points) to measure the JHH,N coupling constants. For this experiment, protons were not decoupled during the t2 evolution, thereby allowing 1H–1H couplings to evolve during acquisition. The 1H and 15N carriers were placed at the water frequency and 125.0 p.p.m. respectively, with spectral widths of 3600 Hz (1H) and 3648 Hz (15N). The O1 for the 1H dimension was centred within the NH resonance region. The water resonance was folded over in the downfield region of the spectral window. Water was saturated by using phase-shifted square pulses (100 ms for 0.6 s) of 1024 points. The TPPI-states method was used to obtain quadrature detection in both the t1 and t2 dimensions. Zero-filling yielded a final spectrum of 4096 × 2048 points.

**Structural modelling**

Interproton distance constraints were derived from NOEs assigned in 2D 1H homonuclear and 2D and 3D 1H–15N heteronuclear-edited NOE/JOE spectra acquired with mixing times of 50, 75, 100 and 200 ms. An analysis of NOE growth curves indicated that, whereas backbone to backbone inter-proton NOEs were normally maximal at 100 ms, side-chain to side-chain and side-chain to backbone NOEs were maximal at 100–200 ms. Generally, by using 75–100 ms NOE data sets, NOEs were classified as strong, medium, weak or very weak corresponding to upper bound distance constraints of 2.8, 3.3, 4.0 and 4.5 Å respectively. The lower bound restraint between non-bonded protons was set at 1.8 Å. Pseudo-atom corrections were added to the upper-bound distance constraints where appropriate [33] and a 0.5 Å correction was added to the upper bound for NOEs involving methyl protons [34, 35]. Averaging was applied to NOEs involving non-stereospecifically assigned methylene protons [36]. Averaging was applied to NOEs involving non-stereospecifically assigned methylene protons [36, 37]. For JHH,N > 8 Hz and JHH,N < 5 Hz, the angle ϕ was constrained to be −120 ± 40° and −65 ± 25° respectively. The minimum range employed for dihedral angle constraints in ϕ was ±25°. Stereospecific assignments for β-methylene protons of His16, Asn19, Gln21, Glu24, Lys28, His31, Asn33, Gln34, Asp34, Cys50 and Asp50 and for the γ-CH groups of Val60 were derived from interproton distances obtained from 2D NOE/JOEs and 3D 15N-edited NOE/JOEs experiments and from qualitative estimates of the magnitude of some JHH,N coupling constants obtained from 2D HOHAHA, double-quantum-filtered COSY and 3D 15N-edited HOHAHA experiments. Hydrogen-bond constraints were identified from the pattern of sequential and interstrand NOEs involving NH and C=H protons, together with evidence of slow exchange of protons between amides and solvent. Each hydrogen bond identified was defined by using two distance constraints: rNH = 1.7–2.3 Å and rC=O = 2.5 to 3.3 Å, except for those hydrogen bonds that occur at the ends of secondary structure units, in which rNH = 1.7 to 2.4 Å and rC=O = 2.5 to 3.4 Å.

Derived internuclear distance and angular constraints were used in calculating structures for NAP-2 by using X-PLOR [38].
NAP-2 was created by using parallhgd.pro force fields. For NOE constraints, a soft potential function was used. The first step in our X-PLOR protocol created a template coordinate set by using the Template routine. Then random structures were generated and refined by using the Random and Refine routines respectively. This dynamical simulated annealing procedure ran high-temperature dynamics (2000 K for 50 ps), cooled down (to 100 K in 50 K steps with 1.3 ps molecular dynamics at each temperature step) and energy-minimized the structures. The distance symmetry force constant was kept at 4.2 kJ/mol Å² throughout the calculation. The NOE force constant was doubled at the beginning of each cycle, from an initial value of 2 to a maximum value of 210 kJ/mol Å² while the van der Waals force constant was increased slowly from 0.42 to 16.8 kJ/mol Å². Powell minimization was then performed at 100 K for 250 steps. The resulting structures were subjected to the same Refine routine four times. Significant decreases in $E_{\text{total}}$ and $E_{\text{NOE}}$ were noted with each Refine cycle. Structures were superimposed by using the BIOSYM INSIGHT viewer and analysed by using X-PLOR analysis routines. Final structures were subjected to the X-PLOR Accept routine with a violation threshold for NOEs of 0.5 Å and dihedral angles of 5°.

**15N–H motional dynamics**

1H/O-water. For by using phase-shifted square pulses or a Messerle pulse on the middle of the NH region and the water resonance was saturated performed for each measurement. The transmitter was set in the routine four times. Significant decreases in high-power 120 s relaxation delay. Performed with and without proton saturation during the $T^E$INEPT transfer experiments [39]. The time delays for $T^E$ were used between pulses. In the absence of $I^E$ chemistry. In this case, relaxation equations for $I^E$--edited spectrum and $I^E$-edited NOE were used to calculate the NOE relaxation time $\tau_{\text{NOE}}$. In that study, outside the hydrophobic faces of the amphipathic $\beta$-sheet and $\alpha$-helix were observed for use in modelling studies.

To calculate $T^H$, $T^E$, and NOE values, peak heights from one-dimensional slices through cross-peaks were measured for the different time delays. Peak intensities decreased exponentially [$I = I_0e^{-t/\tau}$] for both $T^H$ and $T^E$ data. Regression line analysis was performed, and $T^H$ or $T^E$ values were obtained from the line of best fit. NOEs are reported as the ratio $I_{\text{NOE}}/I_{\text{ref}}$, where $I$ is the intensity of the resonance. Analysis of these relaxation data is based on the assumption that 15N relaxation is dominated by dipole–dipole interactions with the bonded protons and by chemical-shift anisotropy. In this case, relaxation equations for $R_s^E = 1/T^E_s$, $R_s^H = 1/T^H_s$, and NOE can be written as described by Daragan and Mayo [43]. Contributions from chemical exchange processes, $R_s^E$, $R_s^H$, to $R_s^E$ have been included in this analysis.

Relaxation data were analysed by using the Lipari and Szabo [44,45] model free approach, in which it is assumed that the shape of the protein is spherical with an overall correlation time of $\tau_s$, and that the internal motions of each NH bond can be described by a correlation time $\tau_B$ and an order parameter $S$. For this model, the motional spectral density function can be written [44,45]:

$$J(\omega) = S^2\tau_s^2/(1 + \omega^2\tau_s^2) + (1 - S^2)/\tau_s^2$$

(1)

where $1/\tau_s = 1/\tau_B + 1/\tau_s$. The overall correlation time $\tau_s$ was initially estimated by minimizing the following target function:

$$F_{\text{error}} = [(R^E_{\text{obs}} - R^E_{\text{calc}})/R^E_{\text{obs}}]^2 + [(R^H_{\text{obs}} - R^H_{\text{calc}})/R^H_{\text{obs}}]^2 + [(I_{\text{NOE}} - I_{\text{calc}})/I_{\text{obs}}]^2$$

(2)

for each residue separately. $R^E_{\text{obs}}$, $R^E_{\text{calc}}$ and NOE$_{\text{obs}}$ are experimental values; $R^E_{\text{calc}}$, $R^H_{\text{calc}}$ and NOE$_{\text{calc}}$ are calculated values. In a subsequent minimization, an average value of $\tau_s$ was used with only $R_s^H$ and NOE, and $\tau_s, \tau^E, \text{and } S^E$ were varied to minimize the target function. $R_s^E$ values were then essentially calculated from the difference of observed and calculated values for $R_s^H$. Inclusion of these values in the analysis generally improved overall fits.

**RESULTS AND DISCUSSION**

**Resonance assignments**

Monomeric NAP-2 was generated by adding to the solution 4% (v/v) 2-chloroethanold, which is known to dissociate the dimeric and tetrameric aggregation states of the protein [19,20]. Because 1H-NMR sequence-specific assignments for monomeric NAP-2 are known [19], heteronuclear 1H–15N 2D-NMR and 3D-NMR experiments were performed to acquire 15N resonance assignments and additional NOE distance constraints and motional dynamics information. For uniformly 15N-enriched NAP-2, a 1H–15N HSQC 2D-NMR contour plot is shown in Figure 1. 1H–15N sequence-specific resonance assignments labelled in Figure 1 have been derived by starting with known 1H resonance assignments [19] and following connectivities through 15N-edited HOHAHA and NOESY 3D-NMR spectra (results not shown).

**Solution structure**

Elements of secondary structure have been deduced from a qualitative interpretation of 1H NOEs, $J_{\phi,\psi}$ coupling constants and long-lived backbone NH groups [19]. In that study, outside the $\beta$-sheet domain, few long-range side-chain to side-chain NOEs could be identified given the fact that NAP-2 contains only one tyrosine residue and two histidine residues and a preponderance of aliphatic hydrophobic residues. It is these NOEs that best define overall folding. For NAP-2 the derivation of secondary structural elements was therefore limited to folding within the $\beta$-sheet and $\alpha$-helix domains [19]. In 1H–15N NOESY 3D-NMR experiments, significant numbers of side-chain to backbone NOEs could be identified. In particular, several NOEs between the hydrophobic faces of the amphiphilic $\beta$-sheet and C-terminal $\alpha$-helix were observed for use in modelling studies.

A total of 858 NOE distance constraints were derived from an analysis of both homonuclear and heteronuclear NOESY spectra. These included 420 intra-residue, 148 sequential, 123 medium-range ($|i-j| < 5$) and 167 long-range ($|i-j| > 5$) constraints. In addition, torsion angle constraints were obtained for 42 values of $\phi$. A total of 16 hydrogen bonds were identified from long-lived backbone NH groups and by inspection of initial NAP-2 structures, giving rise to 32 hydrogen-bond distance constraints. These hydrogen-bond constraints account for the slowly exchanging amide protons identified. The total number of experimentally derived constraints was therefore 932, giving a total of approx. 13 constraints per residue.

Initially, 100 structures for NAP-2 were calculated as described in the Materials and methods section. The best-fit superpositions
Figure 1  $^{15}$N-$^1$H HSQC of NAP-2

A $^{15}$N-$^1$H HSQC contour plot for NAP-2 in the 2-chloroethanol-induced monomer state is shown. This 2D-NMR data set was acquired as discussed in the Materials and methods section. Cross-peaks are labelled with sequence-specific resonance assignments.

Figure 2 Structure of monomeric NAP-2

Structures for monomeric NAP-2, based primarily on NOEs, were calculated as discussed in the Materials and methods section. The $\beta$-sheet domains of 20 structures were superimposed. Of backbone C$\alpha$ atoms for the final 20 structures, along with an average structure, are shown in Figure 2. These structures showed no NOE violations greater than 0.5 Å [46]. Structural statistics are summarized in Table 1. The less structurally defined N-terminus and the end of the C-terminal helix are apparent. The structures satisfy experimental constraints quite well. Excluding N-terminal residues 1–5 and C-terminal residues 66–72, which are least well defined by the data, atomic root-mean-square (RMS) differences with respect to the mean coordinate positions are $0.63 \pm 0.07$ Å for backbone (N, C$\alpha$, C) atoms and $1.5 \pm 0.3$ Å for all heavy atoms. For the three $\beta$-sheet strands (Gln-21–Lys-28, Gln-34–Thr-40 and Lys-46–Leu-49) as well as for the loop connecting strands 2 and 3 (Leu-41–Arg-45), the average backbone RMS deviation is 0.38 Å. In addition, $\phi$ and $\psi$ angular order parameters are all more than 0.8. The angular order parameter is defined such that a value of 1 indicates an exactly defined angle and a value of 0 results from complete dihedral heterogeneity [47]. Backbone torsion angles for all non-glycine residues lie within the allowed region of a Ramachandran $\phi$–$\psi$ plot. Taken together, the above data indicate that these structures used to represent the solution conformation of NAP-2 are well converged.

Even though the C-terminal helix in itself is structurally well defined, the position of the C-terminal helix on top of the $\beta$-sheet domain could not be established initially [19] owing to a lack of inter-domain NOEs resulting from the presence of only hydrophobic aliphatic residues lying between the helix and the sheet. Use of $^{15}$N-edited 2D-NMR and 3D-NMR experiments, however, has allowed a number of these constraints to be identified. Residues Ile-56, Ile-59, Val-60, Leu-64 and Ala-65 at the top of the helix interact with $\beta$-sheet residues Leu-23, Val-25, Val-35, Ala-39 and Leu-41. In the crystal, NAP-2 forms tetramers; the X-ray structure shows that the C-terminal helix is uniquely positioned, with the same inter-domain contact residues [19].

The overall structural folding of monomeric NAP-2 is generally conserved relative to other $\alpha$-chemokines such as IL-8 [24,48,49], Gro-$\alpha$ [50], bovine PF4 [21] and human PF4 [22,23]. Significant
Table 1 Structural statistics

None of the 20 final structures exhibited distance restraint violations greater than 0.5 Å or dihedral angle violations greater than 5°. RMS deviations are means ± S.D. for the 20 structures. The final values of the NOE ($E_{NOE}$), torsion angle ($E_{CDIH}$) and NCS ($E_{NCS}$) potentials were calculated with force constants of 210 kJ/mol per Å$^2$, 840 kJ/mol per rad$^2$ and 126 kJ/mol per Å$^2$ respectively.

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<td>Angles (degrees)</td>
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<td>Improperss (degrees)</td>
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<table>
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<tr>
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Figure 3 15N–1H HSQC relaxation spectra of NAP-2

A series of three 15N–1H HSQC relaxation spectra for NAP-2 are shown. The delay times, $T_2$, for these plots were 7.7 ms (left panel), 96 ms (middle panel) and 173.5 ms (right panel). The acquisition of these 2D-NMR data sets was as discussed in the Materials and methods section.

Figure 4 15N relaxation decay curves for NAP-2

Four representative 15N $T_1$ decay curves are shown for monomeric NAP-2: □, Val-25; ○, Leu-4; △, Gly-66; ▽, Ile-55. Peak intensities were derived from slices through cross-peaks as discussed in the text.
Using NMR relaxation [43]. The average internal correlation $T_1$ from differential Figure 3 to exemplify changes in cross-peak intensities resulting three time-modulated as described in the Materials and methods section. A series of $15N–H$ backbone dynamics internal flexibility in general in NAP-2. This in turn suggests that subunit association might also dampen terminal helix conformation with respect to the $\alpha$-sheet domain. Order parameters generally decrease at the termini, as expected for more mobile segments. A value of 0.3 is close to unrestricted motion. To check that $R_ex$ does indeed result from conformational exchange and not from anisotropy, relaxation data were acquired at two spectrometer frequencies (500 and 600 MHz). In all cases, the ratio between the relaxation terms at 600 and 500 MHz fell between 1.4 and 1.5, which is around the expected value of 1.44, indicating that $R_ex$ results from conformational exchange.

**15N–H backbone dynamics**

$15N$ $T_1$ and $T_2$ relaxation times and $15N;1^H$ NOEs were measured as described in the Materials and methods section. A series of three time-modulated $^1H–15N$ 2D-NMR spectra are shown in Figure 3 to exemplify changes in cross-peak intensities resulting from differential $^1H$ relaxation rates. $T_1$, $T_2$ and NOE values were calculated by measuring peak heights from one-dimensional slices through cross-peaks for the different time delays. Peak intensities decreased exponentially $[I = I_0e^{-t/T}]$ for both $T_1$ and $T_2$ data as exemplified by $T_1$ data shown in Figure 4. The solid line through data points represents the line of best fit from regression analysis. Experimental $15N$ $R_1$, $R_2$ and NOE values are plotted against the NAP-2 residue number in Figure 5.

Using the Lipari and Szabo model free approach [44,45] and the minimization procedure described in the Materials and methods section, the best value for the overall correlation time, $\tau_\sigma$, was 3.7 ns. In terms of the dependence of $\tau_\sigma$ on the number of residues in a protein, this value for monomeric NAP-2 compares reasonably well with $\tau_\sigma$ values of other proteins obtained by using NMR relaxation [43]. The average internal correlation time was 170 ps. $15N$ motional order parameters, $S_{NH}^2$, along with $R_ex$ chemical exchange terms, $R_{ex}$, are plotted against the NAP-2 residue number in Figure 6. Inclusion of the $R_ex$ exchange term generally improves the fit in the model free analysis of the data. $S_{NH}^2$ values range from approx. 0.3 at the termini to 0.3–0.7 with some loops/turns to 0.8–0.93 within the $\beta$-sheet and helix domains. Order parameters generally decrease at the termini, as expected for more mobile segments. A value of 0.3 is close to unrestricted motion. To check that $R_ex$ does indeed result from conformational exchange and not from anisotropy, relaxation data were acquired at two spectrometer frequencies (500 and 600 MHz). In all cases, the ratio between the relaxation terms at 600 and 500 MHz fell between 1.4 and 1.5, which is around the expected value of 1.44, indicating that $R_ex$ results from conformational exchange.

**Figure 5 15N $R_1$, $R_2$ and NOE values for NAP-2**

$15N$ $R_1$, $R_2$ and NOE values for monomeric NAP-2 are plotted in bar graph format against the NAP-2 sequence. Values were derived as discussed in the text. Results shown are averages of two separate measurements; standard deviations (results not shown) were on average ±15% for $R_1$, ±25% for $R_2$ and ±20% for NOE.

**Figure 6 Motional order parameters and exchange terms for NAP-2**

Motional order parameters, $S_{NH}^2$, and conformational exchange terms, $R_ex$, are plotted against the NAP-2 sequence. Values were derived as discussed in the text.
scale and conformational exchange on the chemical-shift time scale is not apparent. Segments Gly-27-Thr-30 and Leu-41-Asp-50 fall into this category. This latter stretch of the sequence includes the α-chemokine folding initiation site (Leu-41-Arg-45) [51] and contains many of the longest lived NH groups consistent with its position at the core of the protein. Structurally, this is one of the best defined regions in NAP-2. Finally, case 3 defines the situation in which the structure is well defined but internal motions occur on the millisecond chemical-shift time scale. Sequences that fall within this motional time range include Met-7-Thr-11, Glu-24-Ile-26, His-31-Asn-33, Glu-36-Thr-40 and Asp-52-Gln-61. Structurally, the respective sequences are at the N-terminus, the end of β-strand 1, the loop between strands 1 and 2, and the N-terminus and the end of β-strand 3, and the C-terminal α-helix, and all are proximal to each other in the structure (see Figure 2).

The overall structural dynamics picture of NAP-2 is a that of an aperiodic, highly mobile N-terminal segment that leads into a well-defined β-sheet scaffold on which is folded a C-terminal helix. Fast N-terminal motions are apparently slowed by Cys-6 and Cys-8, which form disulphide bridges to Cys-32 and Cys-48 respectively. Even these bridged sequences, however, are flexible on the millisecond chemical-shift time scale; note that Cys-32 displays the largest R<sub>cex</sub> value. These slower motions continue from Ile-9 to Thr-12, probably as the result of this sequence’s being close to disulphide bond-constrained Cys-6 and Cys-8 and the fact that Thr-11 and Thr-12 form a very short β-sheet with Lys-46-Leu-49. The next four residues, Ser-13-His-16, show increased mobility. In general the aperiodic N-terminus in NAP-2 is inherently flexible and is constrained only in specific places when stabilized by interactions with the β-sheet. On the C-terminal side of Pro-17 leading into the first β-sheet strand, motions are more attenuated. Near the middle of this first strand and into the large inter-strand loop (residues Ile-26, Gly-27, Lys-32, and Cys-8, which form disulphide bridges to Cys-32 and Cys-48) and into the large inter-strand loop (residues Ile-26, Gly-27, Lys-32, and Cys-8), with its position at the core of the protein. Structurally, this is the largest internal motions to have a more complete picture of the physical state of a structure.

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REFERENCES


Conclusions

The NMR solution structure of monomeric NAP-2 is essentially the same as that found in other α-chemokines with a three-stranded anti-parallel β-sheet on which is folded a C-terminal α-helix and an aperiodic N-terminal sequence. Dissociation to the monomeric state, however, increases internal mobility owing to a loss of stabilizing inter-subunit interactions. The present study emphasizes the fact that when elucidating protein structures in solution (or in the crystal), it is important to take into account internal motions to have a more complete picture of the physical state of a structure.

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