Hydrophobic side-chain size is a determinant of the three-dimensional structure of the p53 oligomerization domain

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The p53 tumor suppressor oligomerization domain, a dimer of two primary dimers, is an independently folding domain whose subunits consist of a β -strand, a tight turn and an α -helix. To evaluate the effect of hydrophobic side-chains on three-dimensional structure, we substituted residues Phe341 and Leu344 in the α -helix with other hydrophobic amino acids. Substitutions that resulted in residue 341 having a smaller side-chain than residue 344 switched the stoichiometry of the domain from tetrameric to dimeric. The three-dimensional structure of one such dimer was determined by multidimensional NMR spectroscopy. When compared with the primary dimer of the wild-type p53 oligomerization domain, the mutant dimer showed a switch in α -helical packing from anti-parallel to parallel and rotation of the α -helices relative to the β -strands. Hydrophobic side-chain size is therefore an important determinant of a protein fold.

Keywords: hydrophobic effect/NMR spectroscopy/p53 tumor suppressor/protein design/protein structure

Introduction

The three-dimensional structure of a protein is determined by its amino acid sequence (Anfinsen, 1973), but predicting structure from sequence is not yet feasible. Since not all residues of a protein determine its structure (Hecht *et al.*, 1983; Matthews, 1987; Reidhaar-Olson and Sauer, 1988; Bowie *et al.*, 1990; Heinz *et al.*, 1992), the first step in solving the sequence–structure problem might be to identify which residues contain the informational content that specifies the protein fold.

The genetic approach to identifying residues that are important determinants of protein folds is to search for amino acid substitutions that change the fold without leading to denaturation. No such substitutions have been described for monomeric globular proteins (Lim and Sauer, 1989, 1991; Eriksson *et al.*, 1992; Baldwin *et al.*, 1993) and it has been argued that changing a native fold would require substituting at least half the amino acids of a protein (Rose and Creamer, 1994). Interestingly, the packing and stoichiometry of coiled coils is altered by conservative

substitutions targeting the hydrophobic residues at the subunit interface (Harbury *et al.*, 1993; Lovejoy *et al.*, 1993).

The p53 tumor suppressor protein is a sequence-specific transcription factor of fundamental significance to the pathogenesis and therapy of human cancer (Harris, 1993). The tumor suppressor activity of p53 requires homo-oligomerization (Pietenpol *et al.*, 1994), which is mediated by an oligomerization domain at the C-terminus of the protein (Sakamoto *et al.*, 1994; Wang *et al.*, 1994). This domain is well suited for structural studies, because it is only 30 residues long and its function can be easily assayed, yet it has features of larger globular proteins in that it has a well-defined globular hydrophobic core, contains both β -sheet and α -helical secondary structure elements and does not require metal ions or other prosthetic groups for folding (Clare *et al.*, 1994, 1995; Lee *et al.*, 1994a; Jeffrey *et al.*, 1995). Inspired by the effects of conservative hydrophobic amino acid substitutions on packing of coiled coils, we explored whether similar substitutions affect the fold of the oligomerization domain of the p53 tumor suppressor protein.

Results

Oligomerization of p53 mutants

The four identical subunits of the p53 oligomerization domain are arranged as a dimer of dimers; in the primary dimer the β -strands pack antiparallel, as do the α -helices. Across primary dimers, the α -helices pack parallel at an 81° angle (Figure 1). Residues Phe341 and Leu344 in the hydrophobic core of the domain are critical for oligomerization (Waterman *et al.*, 1995). Phe341 is positioned at the interface of the subunits that form the primary dimers, whereas Leu344 forms the interface between the primary dimers (Figure 1). We substituted Phe341 with Val, Ile or Leu, and Leu344 with Ile, Phe or Trp, preserving the hydrophobic/polar profile of the domain. Full-length p53 proteins with these substitutions and all combinations thereof, retained their ability to bind DNA, although some, such as p53IF, migrated faster in native gel electrophoresis than wild-type p53 (p53wt; Figure 2A). This behavior correlated with the relative side-chain sizes of residues 341 and 344 and was not dependent on a specific side-chain geometry, such as β - or γ -branching. We reasoned that the substitutions were affecting oligomerization stoichiometry, because p53IF comigrated with p53Ala344 (Figure 2B), a known dimeric mutant (Waterman *et al.*, 1995). Furthermore, DNA binding of p53IF was unaffected by replacing Met340 by Lys and/or replacing Phe344 by Tyr (Figure 2B), residues which map to the dimer–dimer interface in p53wt (Figure 1B). Tolerance to these substitutions suggests that p53IF is a dimer with no dimer–dimer interface. In contrast, p53wt was partially

Fig. 1. Structure of the p53wt oligomerization domain. (A) Primary amino acid sequence and secondary structure elements. (B) Three-dimensional structure (PDB code: 1SAK). Color saturation distinguishes the two subunits within a primary dimer; color hue distinguishes the two primary dimers. Residue types are indicated in the single-letter code.

dissociated into dimers when Met340 was replaced by Lys (Figure 2B).

Migration of p53–DNA complexes on native gels is an indirect measure of the stoichiometry of the p53 oligomerization domain. The oligomerization domains of p53Ile341Tyr344 (p53IY) and p53Lys340Ile341Tyr344 (p53KIY) were therefore expressed in *Escherichia coli*, purified to homogeneity and assayed directly for subunit stoichiometry by glutaraldehyde crosslinking and gel filtration chromatography. In the glutaraldehyde crosslinking assay, p53IY and p53KIY crosslinked only up to dimers, whereas p53wt crosslinked up to tetramers (Figure 3A). The p53IY and p53KIY dimers were quite stable, since they did not dissociate into monomers when crosslinking was performed at temperatures as high as 55°C (data not shown). On the gel filtration column, p53KIY eluted more slowly than p53wt, confirming that p53KIY has a smaller molecular size and lower subunit stoichiometry than p53wt (Figure 3B). We note that both p53wt and p53KIY eluted faster than expected based on their molecular weights of 27.3 and 13.7 kDa, respectively; this behavior has been previously described for p53wt and probably reflects the non-spherical shape of these domains (Lee *et al.*, 1994a).

NMR structural analysis

The structural basis for the switch in p53 oligomerization stoichiometry was probed by multidimensional solution NMR spectroscopy. Of the two dimeric domains purified to homogeneity, p53IY and p53KIY, only the latter could be maintained at 40°C for prolonged periods of time without aggregation, a prerequisite for structural analysis, whereas p53IY aggregated in less than an hour.

Comparison of the p53KIY and p53wt domains showed that the backbone ^1H and ^{15}N amide resonance frequencies

Fig. 2. Native gel electrophoretic mobility shift analysis of full-length p53 proteins with hydrophobic amino acid substitutions targeting the oligomerization domain. (A) Amino acid substitutions targeting residues 341 and 344. The names of the mutants indicate the hydrophobic residues at positions 341 and 344, respectively; p53FL is wild-type p53. (B) Comigration of the DNA complexes of p53IF (Ile341 and Phe344), p53IY (Ile341 and Tyr344) and p53KIY (Lys340, Ile341 and Tyr344) with the previously characterized dimeric mutant p53A344 (Ala344).

Fig. 3. Subunit stoichiometry of purified recombinant p53 oligomerization domains. (A) Glutaraldehyde (Gl.) crosslinking of p53wt, p53IY and p53KIY oligomerization domains at 0.1 mM protein concentration. Crosslinking was incomplete, as indicated by the presence of monomers. The molecular weight markers (MWM) are, from left to right, 97.4, 66.2, 45, 31, 21.5 and 14.4 kDa. (B) Gel filtration chromatography of p53wt and p53KIY oligomerization domains at 0.1 mM protein concentration. The MWM are, from left to right, 670, 158 (overlapping peaks), 44, 17 and 1.3 kDa.

Fig. 4. Intersubunit short-range NOEs in the p53KIY oligomerization domain identified with a ^{12}C -filtered, ^{13}C -edited HMQC-NOESY-HMQC experiment. Greek letters refer to amino acid protons and the peaks are labeled by the last two digits of the residue codon.

Fig. 5. Superimposition of 30 simulated annealing structures corresponding to residues 327–353 of human p53. Color hue distinguishes the two subunits.

of residues Gly325, Leu330, Gln331, Arg333, Arg337, Phe338, Glu339, Glu343, Asn345, Glu346, Ala347 and Lys351 differed by more than 0.4 p.p.m. in the proton frequency and/or by more than 1.2 p.p.m. in the nitrogen frequency. Such large chemical shift differences are suggestive of changes in three-dimensional structure (Wuthrich, 1986; Cavanagh *et al.*, 1996), especially since some of the above residues, such as Gln331 and Arg333 are 10 and 7 Å, respectively, away from the nearest substituted residue in the established p53wt structure. Intersubunit NOEs can be very informative regarding changes in the three-dimensional structure of oligomers. To monitor such NOEs an equilibrated 1:1 mixture of ^{15}N - and ^{15}N , ^{13}C -labeled p53KIY polypeptides was subjected to a ^{12}C -filtered, ^{13}C -edited HMQC-NOESY-HMQC experiment (Figure 4). The intersubunit contacts of residues Thr329 and Leu330 were consistent with an antiparallel β -sheet, as in p53wt. However, the short-range contacts involving Ile341, Ala347, Leu348 and Leu350 were inconsistent with antiparallel packing of the α -helices as observed in the primary dimers of p53wt. Using 678 experimentally derived NMR restraints, 30 simulated annealing structures for residues 327–353 of p53KIY were calculated. These structures overlap well (Figure 5) and none of them have distance restraint violations greater than 0.3 Å or dihedral angle violations greater than 3°. The refined average structure has atomic root mean

Table I. Structure statistics^a

	$\langle \text{SA} \rangle$	$(\text{SA})_r$
R.m.s.d. from experimental distance restraints (Å) ^b		
intrasubunit (384)		
intraresidue (122)	0.001 ± 0.0003	0.000
sequential (120)	0.053 ± 0.012	0.097
short range (124)	0.031 ± 0.013	0.035
long range (18)	0.064 ± 0.020	0.061
intersubunit (200)	0.044 ± 0.007	0.077
hydrogen bonds (38)	0.073 ± 0.011	0.100
R.m.s.d. from experimental angle restraints (°)		
dihedral angles (56)	0.62 ± 0.21	0.87
R.m.s.d. from idealized geometry		
bonds (Å)	0.0028 ± 0.0001	0.0062
angles (°)	0.55 ± 0.026	0.69
impropers (°)	0.47 ± 0.078	0.66
Lennard–Jones energy (kcal/mol) ^c	-407 ± 23	-437
Atomic r.m.s.d. from average (Å)		
backbone	0.51	
all atoms	1.41	
core sidechains	1.05	

^a $\langle \text{SA} \rangle$ is the ensemble of the 30 final simulated annealing structures and $(\text{SA})_r$ is the refined average structure. The number of restraints is given in parentheses and applies to the entire dimer.

^bNone of the simulated annealing structures exhibit distance violations greater than 0.3 Å or dihedral angle violations greater than 3°.

^cThe Lennard–Jones energy was calculated using the CHARMM22 forcefield (Brooks *et al.*, 1983).

deviations from the 30 simulated annealing structures of 1.41 Å for all atoms, 1.05 Å for all atoms in the hydrophobic core and 0.51 Å for all backbone atoms (Table I).

The p53KIY oligomerization domain is a symmetrical dimer (Figure 6). As in p53wt, each subunit consists of three secondary structure elements: a β -strand encompassing residues 328–332 (as compared with residues 326–333 in p53wt), a turn and an α -helix encompassing residues 335–353 (as compared with residues 335–355 in p53wt). In both p53KIY and p53wt, the β -strands form an antiparallel β -sheet. In contrast, the α -helices pack parallel at an 83° angle in p53KIY. This differs significantly from the 155° interhelical angle within the primary dimer of p53wt, but is similar to the 81° interhelical angle with which the primary dimers of p53wt pack against each other. Packing of the α -helices in p53KIY appears to be stabilized by hydrophobic interactions substantially involving Tyr344, which interacts with Ile341 of the same subunit and with Tyr344 and Leu348 of the other subunit (Figure 6).

To evaluate the switch between p53KIY and p53wt, the structures of the two protein domains were superimposed. Superimposition of p53KIY and p53wt via residues 328–332 shows that in both domains the β -sheet presents a similar hydrophobic surface for packing of the N-termini of the α -helices (Figure 7A). Nevertheless, the position of the α -helices relative to the β -strands differs significantly in the two structures as revealed by the angle of the α -helices relative to the β -strands, the position of the N-termini of the α -helices and the orientation of the side-

Fig. 6. Orthogonal views of the p53KIY oligomerization domain corresponding to residues 327–353 of human p53. Color saturation and the presence or absence of a prime following the residue number distinguish the two subunits. Residue numbers followed by an asterisk refer to both subunits.

Fig. 7. Superimposition of the p53wt (residues 325–355) and p53KIY (residues 327–353) oligomerization domains via residues 328–332 of their β -strands (A) or via residues 347–350 of their α -helices (B). (C) The orientations of p53wt in panels A and B are labeled a and b, respectively, and are related to each other by the indicated rotations (in degrees). Color-coding is as in Figures 1B and 6. Residue numbers with no appended symbols refer to p53KIY; numbers followed by a prime refer to p53wt; asterisked numbers refer to both p53wt and p53KIY. In panel A one of the p53wt α -helices is not shown in its entirety.

chains of residues 347, 348 and 350. Superimposition via residues 347–350 reveals that the way in which the C-termini of the α -helices in p53KIY are packed is very similar to the way they pack across primary dimers in p53wt (Figure 7B). Thus, the major difference in the

p53KIY and p53wt structures relates to the packing of the central regions of the α -helices.

Three amino acid substitutions (Met340 to Lys, Phe341 to Ile and Leu344 to Tyr) distinguish p53KIY from p53wt and account for the observed structural switch. To

Fig. 8. Backbone amide resonance frequencies of Gln331 and Arg333 in p53wt and selected mutant oligomerization domains. The amino acid substitutions in the mutants are: Ile341 and Phe344 (p53IF); Lys340, Ile341 and Phe344 (p53KIF); Lys340, Ile341 and Tyr344 (p53KIY); Lys340 (p53K340); and Phe344 (p53FF). The peaks are labeled by the last two digits of the residue number; boxes mark the resonance frequencies of Gln331 and Arg333 in p53wt.

determine which of the three substitutions are most critical, we obtained two-dimensional $^1\text{H}/^{15}\text{N}$ HSQC spectra of p53Ile341Phe344 (p53IF), p53Lys340Ile341Phe344 (p53KIF), p53Lys340 (p53K340) and p53Phe344 (p53FF) proteins. The first three mutant proteins were examined as crude bacterial lysates (Gronenborn and Clore, 1996), whereas p53FF was purified. A number of well-resolved resonances were monitored, including the resonances of residues Gln331 and Arg333 in the β -strand, which are more than 7 Å away from the nearest substituted amino acid in both p53wt and p53KIY, and which shift as a result of the global structural change in p53KIY. The shifts characteristic of p53KIY occurred in p53KIF and p53IF, but not in p53K340 or p53FF (Figure 8). We therefore attribute the structural switch in p53KIY predominantly to the change in sizes of the hydrophobic side-chains at positions 341 and 344.

Discussion

One possible interpretation for the structural switch of the p53 oligomerization domain invokes the hydrophobic effect, which favors large hydrophobic side-chains being shielded in the protein interior by the side-chains of other residues (Kauzmann, 1959; Dill, 1985; Livingstone *et al.*, 1991; Sharp *et al.*, 1991; Pace, 1992). In p53wt, the largest hydrophobic side-chain of the α -helix maps to Phe341 and is shielded from solvent by intersubunit interactions with Leu344, Asn345 and Leu348 (Figure 9A). These interactions favor antiparallel packing of the α -helices. In p53KIY, the largest hydrophobic side-chain of the α -helix maps to Tyr344 and is shielded by intersubunit interactions with Tyr344 (equivalent to Leu344 in p53wt), Asn345 and Leu348, which stabilize parallel packing of the α -helices (Figure 9B). Thus, hydrophobic intersubunit interactions involving residues 344, 345 and 348 are redistributed from the side-chain of residue 341 in p53wt to the side-chain of residue 344 in p53KIY, reversing the orientation of α -helical packing. According to this model, the switch in subunit stoichiometry is secondary to the change in α -helical packing, since in p53KIY parallel packing of

Fig. 9. Hydrophobic packing of Phe341 in p53wt (A) and of Tyr344 in p53KIY (B). Color-coding is as in Figures 1B and 6. The primes following the residue numbers distinguish the two subunits. Asterisked numbers refer to both subunits. p53wt is oriented as in Figure 5C(a) and one of its α -helices is not shown. Figures 1B, 6, 7 and 9 were prepared with the programs MOLSCRIPT and RENDER (Bacon and Anderson, 1988; Kraulis, 1991; Merritt and Murphy, 1994).

the α -helices does not allow residues 340 and 344 to form a hydrophobic surface for assembly of primary dimers into tetramers (compare Figures 1 and 6). An alternative interpretation of the structural switch is that the primary consequence of the substitutions is to disrupt tetramerization, leading to primary dimers with altered structure. However, this would not explain why the structural switch requires substitution of residue Phe341, which is not at the dimer–dimer interface (Figure 1). Furthermore, all known examples of protein domains that can change between monomers and higher order oligomers, show no change in secondary structure element packing as a result of the switch in stoichiometry (Bennett *et al.*, 1995).

In leucine zippers, α -helices that assemble as parallel dimeric coiled coils and consist of heptad repeats $(a.b.c.d.e.f.g)_n$ with mostly Val at position *a* and Leu at position *d* (Hodges *et al.*, 1981; Landschulz *et al.*, 1988), substitutions of the hydrophobic residues at positions *a* and *d* with other hydrophobic residues changes their subunit stoichiometry from dimeric to trimeric or tetrameric (Harbury *et al.*, 1993; Lovejoy *et al.*, 1993). This switch can either maintain the parallel packing of the subunits or can lead to antiparallel packing, but is apparently different from the switch in p53 oligomerization domain structure. First, the three-dimensional structure switch of coiled coils affects subunit packing and stoichiometry, but not the structure of the individual subunits. In contrast, the switch of the p53 oligomerization domain changes the tertiary structure of the subunits. Second, the

critical determining factor for the coiled coil switch is the geometry of the hydrophobic side-chains, which affects the 'knobs in holes' packing, whereas the determining factor for the p53 oligomerization domain switch is hydrophobic side-chain size. Third, we note that the only structural features shared by coiled coils and the p53 oligomerization domain are their oligomeric nature and small size. Coiled coils have a single secondary structure element and a cylindrical hydrophobic core. The p53 oligomerization domain has a globular hydrophobic core and contains β -strand, turn and α -helical secondary structure and the arrangement of the α -helices in p53 is atypical of leucine zipper motifs, where the helices have smaller interhelical angles (Jeffrey *et al.*, 1995).

Most native protein structures are stabilized by the hydrophobic effect (Kauzmann, 1959; Dill, 1985; Livingstone *et al.*, 1991; Sharp *et al.*, 1991; Pace, 1992). Our results suggest that hydrophobic side-chain size through the hydrophobic effect may not only stabilize protein structure, but also specify it. The inability to observe global changes in the folds of monomeric globular proteins in response to hydrophobic amino acid substitutions (Lim and Sauer, 1991; Eriksson *et al.*, 1992; Lim *et al.*, 1992) could indicate that hydrophobic side-chain size is a structural determinant for only a few protein domains. Alternatively, hydrophobic side-chain size may be a general determinant, but its influence may be apparent only in a few domains, such as the p53 oligomerization domain, whose oligomeric nature and small size amplify the effects of a limited set of amino acid substitutions.

Materials and methods

DNA binding

Plasmids encoding mutant full-length p53 proteins were generated by PCR-directed mutagenesis of pGEMhump53wtB (Waterman *et al.*, 1995). DNA binding was examined by native gel electrophoretic mobility shift assays using 32 P-labeled oligonucleotide BC.S10 and *in vitro* translated p53 (Waterman *et al.*, 1995).

Protein expression and purification

A PCR fragment with 5' *Bam*HI and 3' *Hind*III restriction sites encoding a methionine and amino acids 304–363 of human wild-type p53 was cloned in the vector pT5T (Eisenberg *et al.*, 1990). Equivalent plasmids were constructed for the mutants. Proteins were expressed in *E. coli* (BL21) grown at 30°C in unlabeled or isotopically labeled 15 N- or 13 C-labeled minimal medium (Isotec, Miamisburg, OH). After overnight induction with IPTG, the cells were pelleted, stirred on ice for 20 min in glycerol, 0.7% (v/v) Triton-X and 0.4% (v/v) β -mercaptoethanol, and then for another 15 min in lysis buffer (10 mM Tris pH 8.0, 500 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.6 mg/ml lysozyme) containing Pefabloc SC (Pentapharm, Basel, Switzerland) and pepstatin (Sigma, St Louis, MO, USA). 120 U/ml DNase I (Sigma) was added and stirring was continued for another 30 min. The lysate was cleared by centrifugation at 300 000 g for 70 min, adjusted to 1.5 M ammonium sulfate and cleared again by centrifugation at 300 000 g for 15 min. p53 was purified in three steps: (i) on a 50 ml phenyl-Sepharose column (Pharmacia, Piscataway, NJ, USA) equilibrated with 1.5 M ammonium sulfate, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA and eluted by decreasing salt concentration on a GradiFrac system (Pharmacia); (ii) on an 8 ml phenyl-Superose column (Pharmacia) equilibrated with 1.7 M ammonium sulfate, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA and eluted by decreasing salt on a SMART system (Pharmacia); and (iii) on a 1 ml Resource S column (Pharmacia) equilibrated with 50 mM sodium phosphate (pH 7.0), 50 mM NaCl, 0.01 mM EDTA and eluted by increasing NaCl concentration on the SMART system. Purified p53 was adjusted to 50 mM sodium phosphate (pH 7.0), 50 mM NaCl, 0.1 mM deuterated EDTA (Isotec), 0.1 mM sodium azide by gel filtration on a Sephadex G-50 column (NAP,

Pharmacia) and concentrated to 0.25 ml by ultrafiltration (Amicon, Beverly, MA, USA) to a final concentration of 1–2 mM.

Glutaraldehyde crosslinking

The purified p53 oligomerization domains at a 0.1 mM concentration in 200 mM sodium phosphate, 50 mM Tris-HCl (pH 7.5) buffer were incubated with 0.1% (v/v) glutaraldehyde (Sigma) for 15 min at 37°C and subjected to SDS-PAGE.

Gel filtration chromatography

The purified p53 oligomerization domains at a 0.1–1 mM concentration in 50 mM sodium phosphate (pH 7), 200 mM NaCl, 5 mM EDTA buffer were loaded on a Superdex 75 gel filtration column (Pharmacia). The elution profile was established by plotting absorbance (280 nm) against elution volume.

NMR structure determination

All experiments were performed at 40°C on 750 and 600 MHz Bruker DMX instruments. Assignments were made from HNCA, HNCOC, CBCACONH and HNCACB 3D triple (^1H , ^{13}C , ^{15}N) resonance, TOCSY-HMQC and NOESY-HMQC 3D double (^1H , ^{15}N) resonance and 2D COSY, 2D TOCSY, 3D TOCSY-HMQC and 3D HCCH-TOCSY experiments (Wuthrich, 1986; Marion *et al.*, 1989; Bax *et al.*, 1990; Grzesiek and Bax, 1992a,b; Wittekind and Mueller, 1993; Lohr and Ruterjans, 1995; Cavanagh *et al.*, 1996). NOE restraints were obtained from 2D NOESY, 3D ^{15}N -edited NOESY-HMQC and ^{13}C -edited HSQC-NOESY experiments (Majumdar and Zuiderweg, 1993). Inter- and intra-subunit NOEs were differentiated with ^{12}C -filtered/ ^{13}C -edited and ^{13}C -filtered/ ^{13}C -edited HMQC-NOESY-HMQC experiments performed on equilibrated 1:1 mixtures of ^{12}C - and ^{13}C -labeled samples (Lee *et al.*, 1994b). Coupling constants were obtained from HMQC-J, HNHA and HNCA-J measurements and hydrogen bonds from proton-deuterium exchange (Wuthrich, 1986; Vuister and Bax, 1993; Cavanagh *et al.*, 1996). The structures were folded from random and distance geometry embedded starting points by simulated annealing using experimental restraints and a non-crystallographic symmetry term (Brünger, 1992). NOE intensities were classified as strong, medium or weak, corresponding to distance restraints of 1.8–3.2, 1.8–4.0 and 1.8–5.0 Å, respectively. Dihedral angles were restrained to $-140 \pm 60^\circ$ or to $60 \pm 55^\circ$ for $^3J_{\text{HNH}\alpha}$ measurements of >8.5 or <5 Hz, respectively. Hydrogen bond restraints were incorporated as two NOEs restraining O–NH to 1.7–2.3 Å and N–O to 2.8–3.3 Å. Pseudo-atom restraints were used whenever stereospecific assignments could not be made. The average structure was calculated from 30 simulated annealing structures and was refined using restrained minimization and a repulsive term to simulate the van der Waals potential (Brünger, 1992). Protein geometry was evaluated with PROCHECK and PROMOTIF (Laskowski *et al.*, 1993). Coordinates will be deposited with the Brookhaven Protein Database and can be obtained either from that database or directly from the authors.

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