FOR THE RECORD

Validation of helical tilt angles in the solution NMR structure of the Z domain of Staphylococcal protein A by combined analysis of residual dipolar coupling and NOE data

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Abstract

Staphylococcal protein A (SpA) is a virulence factor from *Staphylococcus aureus* that is able to bind to immunoglobulins. The 3D structures of its immunoglobulin (Ig) binding domains have been extensively studied by NMR and X-ray crystallography, and are often used as model structures in developing de novo or ab initio strategies for predicting protein structure. These small three-helix-bundle structures, reported in free proteins or Ig-bound complexes, have been determined previously using medium- to high-resolution data. Although the location and relative orientation of the three helices in most of these published 3D domain structures are consistent, there are significant differences among the reported structures regarding the tilt angle of the first helix (helix 1). We have applied residual dipolar coupling data, together with nuclear Overhauser enhancement and scalar coupling data, in refining the NMR solution structure of an engineered IgG-binding domain (Z domain) of SpA. Our results demonstrate that the three helices are almost perfectly antiparallel in orientation, with the first helix tilting slightly away from the other two helices. We propose that this high-accuracy structure of the Z domain of SpA is a more suitable target for theoretical predictions of the free domain structure than previously published lower-accuracy structures of protein A domains.

Keywords: Residual dipolar coupling; structure refinement; Z domain

Staphylococcal protein A (SpA) is a 42-kD cell-wall-bound virulence factor of *Staphylococcus aureus*. Its extracellular portion consists of a tandem repeat of five immunoglobulin (Ig)-binding domains that share high sequence similarities with one another. The size of these domains is relatively small; each contains ~58 amino acid residues. The solution structures of two of these domains, the B and E domains, as well as the very similar Z domain, have been determined by NMR spectroscopy (Gouda et al. 1992; Jendeberg et al. 1996; Starovasnik et al. 1996; Tashiro et al. 1997). These structural analyses revealed that these IgG-binding domains adopted a classical "up–down" three-helical bundle fold. The structure of the B domain in complex with the F_c domain of IgG has also been determined by X-ray crystallography (Deisenhofer 1981). The structure of the third helix of the B domain in this complex was not determined because of poor electron density (Deisenhofer 1981), but was subsequently shown to exist in the complex by solution NMR (Tashiro et al. 1997; Gouda et al. 1998). The Ig-binding domains of SpA also bind to the antigen recognition (F_{ab}) fragment of Ig antibodies. For example, a study of the complexes formed between the D domain of protein A and

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Abbreviations: Ig, immunoglobulin; IgG, immunoglobulin G; RDC, residual dipolar coupling

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fragments of IgM illustrates the structural basis of such dual binding activity (Graille et al. 2000): Helix 1 and helix 2 interact with F_c, while helix 2 and helix 3 bind to the F_{ab} domain of IgM.

The Z domain is an engineered mutant of the B domain generated by substituting Ala 1 with Val and Gly 29 with Ala (Nilsson et al. 1987). These substitutions have no effect on its affinities for F_c fragments of IgG antibodies (Jendeberg et al. 1995, 1996). Although the overall folds of the B and Z domains are the same, there are subtle differences in the reported structures; in particular, the tilt angle of helix α1 is ~30° with respect to helices α2 and α3 in the B domain (Gouda et al. 1992), but these angles are ~10°–15° in the Z domain (Tashiro et al. 1997). The reported Z-domain structure (Tashiro et al. 1997) is more similar to the structures of the E domain (Starovasnik et al. 1996) and the F_c-bound B domain (Deisenhofer 1981) than the reported B-domain structure (Gouda et al. 1992). The same helical orientations (Tashiro et al. 1997) were observed in the bound conformation of the Z domain in a complex with an affibody (a variant of the Z domain binding wild-type Z domain; Högbom et al. 2003; Wahlberg et al. 2003). The structural differences between the free B- and Z-domain structures could perhaps be attributed to the different protein constructs used in those studies, or to the different energy functions used during structure generation. On the other hand, this difference may also reflect the flexible nature of the first helix and/or the limited number of NOE constraints connecting the first helix to the rest of the B- or Z-domain structure. In any case, it is important to establish definitively the helix orientations in the free Z domain, and if indeed there are small but energetically significant rearrangements of the helix packing upon IgG (or affibody) complex formation.

Residual dipolar coupling (RDC) measurements, measuring angular orientations of internuclear vectors relative to the principal axis system of the molecular alignment tensor, provide valuable NMR data for defining relative orientations of individual segments of a macromolecular structure (Tjandra and Bax 1997a; Bax et al. 2001). The application of RDC constraints of global nature, together with locally based NOE and torsion angle constraints, in structure generation calculations provides structures of significantly better precision and accuracy (Tjandra et al. 1997; Clore et al. 1999). Protein structures refined with RDC data are higher quality with considerably better stereochemical geometry than those originally determined without RDC data (Schwalbe et al. 2001; Stauffer et al. 2002). More importantly, the relative orientations of domains and/or subdomains in these RDC-refined structures are more precisely defined (Fischer et al. 1999; Markus et al. 1999; Stauffer et al. 2002).

The IgG-binding domains of SpA have been extensively used in developing computational methods of protein folding and in algorithm development for de novo structure prediction (Boczko and Brooks 1995; Olszewski et al. 1996; Lee et al. 1999). Although the locations of the three helices is identified correctly in these theoretical studies, as the B domain has been used as the reference structure, there remains some uncertainty regarding the accuracy of these predictions with respect to helical packing and orientation. For these reasons, it is particularly valuable to provide a refined structure of the Z domain with accurate and unambiguous helical orientations. A refined Z-domain structure is also valuable for characterizing the amplitude of structural rearrangements resulting from interaction of antibodies with the IgG-binding domains of SpA. Here, we present a refined solution NMR structure of the Z domain, applying RDC data together with published NOE and scalar coupling constraints. Our results provide a small refinement of the relative helical tilt angles, and confirm with RDC data our previous conclusion that the Z domain is composed of three nearly perfectly antiparallel α-helices.

Results and Discussion

$^1D_{NH}$, $^1D_{C\alpha H\alpha}$, and $^1D_{C\alpha C'}$ residual dipolar couplings were measured on a ^{13}C, ^{15}N-enriched Z-domain sample partially aligned in pf1 phage media. A total of 126 residual dipolar couplings (34 $^1D_{NH}$, 43 $^1D_{C\alpha H\alpha}$, 49 $^1D_{C\alpha C'}$) were obtained, excluding degenerate and ambiguous data. Figure 1A summarizes the distribution of these RDC data along the Z-domain primary sequence. Overall, these RDC data are spread evenly among the three helices, although there are only a few $^1D_{NH}$ RDCs in the first helix. The initial axial ($D_a = -15.8$ Hz) and rhombic ($R = 0.58$) components of the alignment tensor were estimated from the normalized distribution of these 126 RDC values (Fig. 1B; Clore et al. 1998a). Following the grid search strategy described by Clore and colleagues (Clore et al. 1998b), we found the optimum values of D_a (−17.3 Hz) and R (0.47) used for subsequent structure generation. In these refinement calculations, the force constant for the RDC constraint term was increased from 0.001 to 1.0 kcal mole^{-1} Hz^{-2}, where the final value reflected our average experimental error of ~1.5 Hz in the $^1D_{NH}$ measurement (Clore et al. 1998b).

The previously described NMR structure of the Z domain (Tashiro et al. 1997) was calculated using the program CONGEN with 536 conformational distance constraints (488 NOE constraints and 48 hydrogen-bond constraints) and 107 dihedral angle constraints, corresponding to 11.1 constraints per restrained residue. These constraints were used in these refinement calculations together with 126 newly acquired RDC data as input to CNS 1.0 (Brünger et al. 1998). To compare Z-domain structures with and without RDC data using the same force field, we also recalculated Z-domain structures with CNS using only the distance and dihedral angle constraints, without RDC constraints. The same CNS routines and parameters were used for both sets

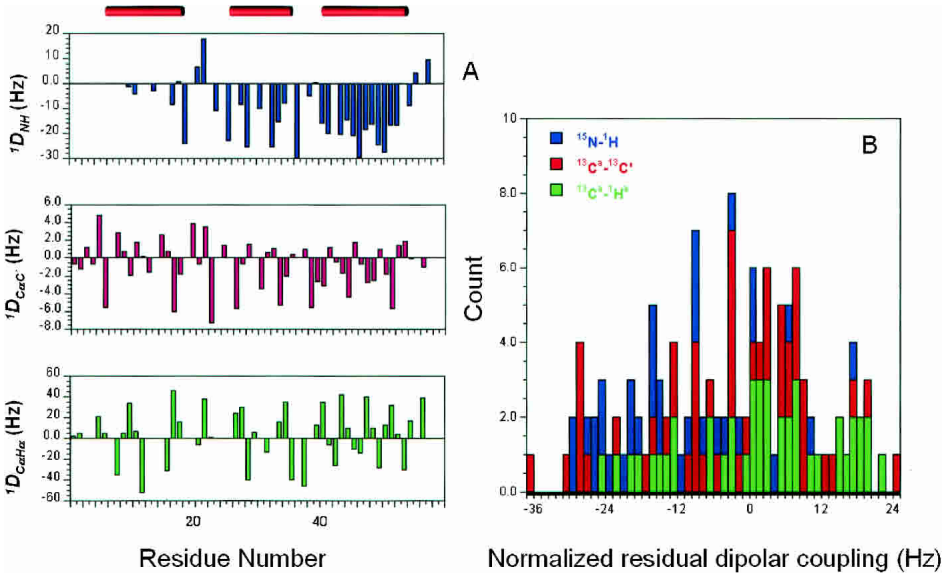

Figure 1. (*A*) Plot of $^1D_{NH}$, $^1D_{C\alpha H\alpha}$, and $^1D_{C\alpha C'}$ residual dipolar couplings versus residue number of the Z domain. The locations in the sequence of three helices are indicated by red horizontal bars at the *top*. (*B*) Histogram plot of distributions of $^1D_{NH}$, $^1D_{C\alpha H\alpha}$, and $^1D_{C\alpha C'}$ data. The data are normalized to the $^1D_{NH}$ data based on bond length and gyromagnetic ratios (Bax et al. 2001).

of structure generation calculations. Figure 2 shows the representative Z-domain structure ensembles generated with or without RDC data. The resulting structures exhibit no significant violations of experimental constraints. For the ensemble of structures generated without RDC data, 87.6% and 11.8% of defined backbone ϕ, ψ values are mapped to the most favored regions and additionally allowed regions of the Ramachandran plot (Laskowski et al. 1993). The corresponding values are improved to 91.4% and 8.4%, respectively, for the ensembles generated with RDC constraints. Three α-helices were identified; K7 to L17 (α1), E25 to D36 (α2), and S41 to A54 (α3). Backbone and all heavy atoms root mean square deviations (RMSD) were calculated over mean coordinates by MOLMOL (Koradi et al. 1996) using residues within these three helices. For the structure ensemble generated without RDC data (Fig. 2A), the RMSD values based on backbone and all heavy atoms are 0.7 ± 0.3 Å and 1.3 ± 0.3 Å, respectively. The structures generated with RDC constraints (Fig. 2B) are somewhat better converged; the backbone and heavy atom RMSD values are 0.6 ± 0.1 Å and 1.5 ± 0.2 Å, respectively. The observed improvements result from the better defined relative orientations of the three helices, rather than refinement of the conformations of the individual helices (data not shown). The two ensembles generated with or without RDC data are very similar (Fig. 2C). Pairwise RMSD values for the ensembles computed with and without the RDC data are 0.7 ± 0.2 Å and 1.5 ± 0.2 Å for backbone and all heavy atoms, respectively. In addition, the RDC values predicted by the program PALES (Zweckstetter and Bax 2000) using the coordinates generated without RDC constraints fit quite well to the experimental RDC data (Fig. 3A). Their correlation is high, with a correlation coefficient of 0.794 between the calculated and observed RDC data. However, the RDC-refined structures agree significantly better with the observed RDC values, with a correlation coefficient of 0.997 (Fig. 3B). These results indicate that our previously reported Z-domain structure (Tashiro et al. 1997; PDB 2SPZ) is consistent with the newly acquired RDC data and thus highly accurate, although the refinement described here results in a slightly more accurate structure.

We calculated the helical tilt angles of the refined Z-domain structures using subroutines in the molecular graphics program MOLMOL (Koradi et al. 1996). For comparison, we also calculated helical tilt angles of other Ig-binding domains of SpA. Table 1 summarizes these statistics. Compared with our previously described NMR structure of the Z domain generated without RDC constraints, the first helix (α1) in the RDC-refined Z-domain structures exhibits a slightly larger tilt with respect to helices α2 and α3 (Fig. 2C; Table 1). Nevertheless, this result shows that the relative orientations of the three helices in the Z domain are almost perfectly antiparallel. In particular, the tilt angles between helices α1 and α2 are essentially identical in these structures of the Z domain and the X-ray crystal structure of the F_c-bound B domain (Deisenhofer 1981). In contrast, the helical tilt angles of the free B domain (PDB id: 1BDC) are significantly different from other domains (Table 1). Furthermore, the tilt angles of our refined Z domain agree remarkably well with the 15° tilt angle reported for the first helix of the free E domain of SpA (Starovasnik et al. 1996) and the D domain of SpA in complex with the F_{ab} fragment

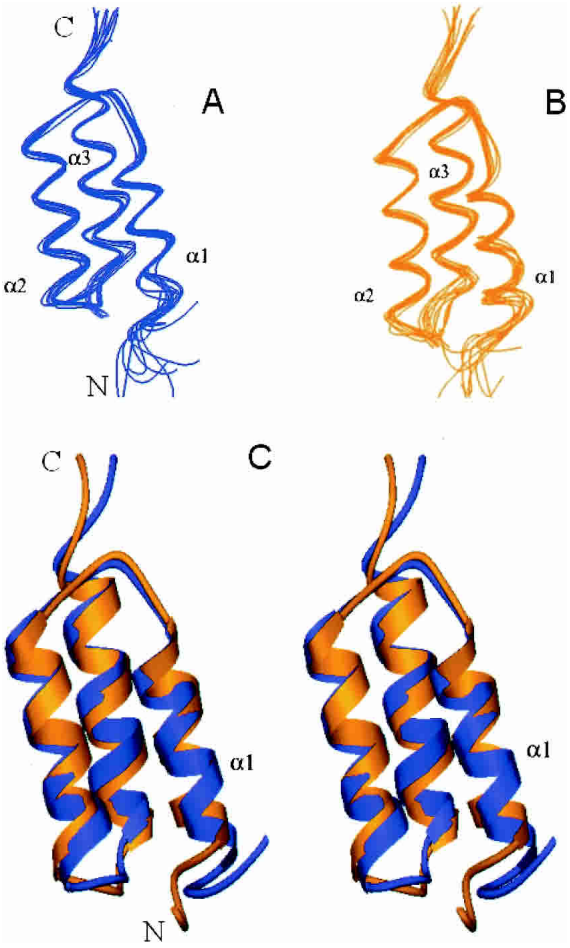

Figure 2. Representations of refined Z-domain structure. (*A*) Structures generated with CNS without using RDC data. (*B*) Structures generated with CNS using RDC constraints. (*C*) Stereo view of superimposition of representative structures generated with (yellow-orange) and without RDC (blue) data.

of IgM antibody (Graille et al. 2000). Recently, the Z-domain structure has also been solved in complex with an affibody (a variant of the Z domain selected to bind wild-type Z domain) both by X-ray crystallography (Högbom et al. 2003) and NMR (Wahlberg et al. 2003). In these bimolecular complexes, the three helices of the Z domain have essentially the same tilt angles as we reported here for the free Z domain (Table 1). Therefore, we conclude that the three helices of IgG-binding domains of SpA are indeed antiparallel, with very small interhelical tilting in the free Z domain. These refined helical orientations should be considered in evaluating the algorithms and approaches of theoretical structure predictions.

Two additional lines of evidence also support the helical tilt angles of the Z domain reported in Table 1. First, we acquired a second set of $^1D_{NH}$ RDC data by establishing partial alignment of the Z domain in a second alignment medium using DMPC/DHPC bicelles (Ottiger and Bax 1998). When these $^1D_{NH}$ RDC data were included in our structure generation, we obtained virtually the same structures as those using only the RDCs from pf1 media (Table 1). However, this set of $^1D_{NH}$ RDC data was rather small (37 RDCs), and the derived alignment tensor may not be estimated reliably. However, as shown in Table 1, the resulting tilt angles in structures generated with RDCs from two media are similar to those obtained using the single alignment media data. Second, we evaluated the degree to which the published B-domain structure is consistent with our RDC data for the Z domain. The overall RDC data measured for the Z domain fit reasonably well with overall B-domain coordinates, with a correlation coefficient of 0.82. However, this good fit is attributed to the high similarity between the Z and B domains in relative orientations of helices α2 and α3. The structures of the Z domain and the B domain can indeed be superimposed and aligned very well, except for the orientation of helix α1. To evaluate how this orientation is defined from our RDC data, all the long-range (i.e., interhelical) NOE constraints connecting the first helix α1 (up to residue 16) to the rest of the structure were deleted from our Z-domain constraint lists. Using the remaining NMR constraints (with RDC data), we generated well-converged structures that have orientation of helix α1 similar to those obtained using a full data set (data not shown). Thus, the orientation of helix α1 is indeed determined by these RDC data. Interestingly, but not surprisingly, if we replaced the experimental Z-domain RDC constraints with RDC data simulated from the NMR structure of the B domain, we obtained a structure ensemble whose first helix adopted the orientation of the B domain (data not shown). We could not do the reverse test because the experimental B domain constraints are not available. These results demonstrate that these residual dipolar coupling data accurately and precisely define helical orientations in the Z domain that are different from those reported for the B domain.

As discussed above, the helical orientations of the Z domain obtained using NOE and scalar coupling data with or without RDC data are indistinguishable from each other, but clearly distinguishable from the structure reported for the free B domain (Gouda et al. 1992). Although the Z-domain construct has 14 extra residues at the N terminus and two amino acid substitutions (i.e., Ala 1 → Val, Gly 29 → Ala; Jansson et al. 1996) that make it different from the B domain, these differences do not appear to be the basis of the reported 3D structural differences, considering that the structures of several other homologous Ig-binding domains have the same helical orientations as the Z domain (Table 1). Our results, in combination with other studies in the literature, indicate that all of these protein A Ig-binding domains adopt similar three-helical antiparallel structures. These results also indicate that there is not a large conformational rearrangement of the three-helix Ig-binding do-

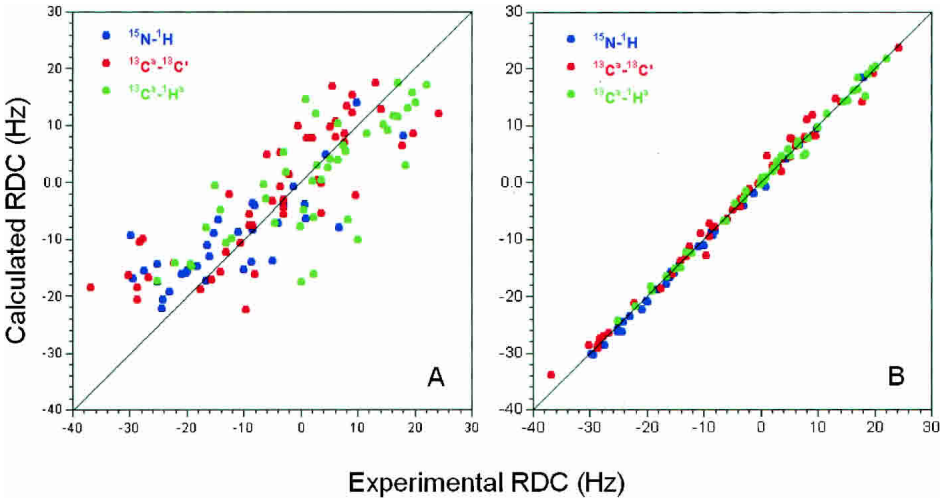

Figure 3. Correlation between RDC data measured in pf1 phage media and RDC data calculated from final Z-domain coordinates generated (*A*) without RDC and(*B*) with RDC constraints. All data are normalized to the $^{1}D_{NH}$ data.

mains upon F_c binding, and finally resolve the controversy regarding the proposed conformational changes in these domains during complex formation. The relative orientations of helices in our refined structure of the Z domain are accurate and precise, and therefore serve as a better template than the available B-domain structure in developing algorithms for protein structure prediction. The atomic coordinates and complete NMR constraints lists for this refined Z-domain structure have been deposited in the Protein DataBank (PDB id: 1Q2N).

Materials and methods

Uniformly ^{13}C, ^{15}N-enriched Z domain of *Staphylococcus aureus* protein A was prepared as described previously (Jansson et al. 1996; Tashiro et al. 1997). An isotropic NMR sample was prepared at 1.1 mM protein concentration in 20 mM NH_4OAc buffer with 5% D_2O at pH 6.5 ± 0.05. The sample used for RDC measurements was prepared with filamentous phage as described (Hansen et al. 1998). The ^{13}C, ^{15}N-enriched sample was first concentrated using a 0.5-mL Ultrafree concentrator (Millipore) and then diluted with appropriate amounts of pf1 phage stock solution (ASLA) and buffer to a final concentration of 18 mg/mL pf1 phage, 0.9 mM Z-domain protein in 20 mM NH_4OAc buffer containing 100 mM NaCl, and 7% D_2O at pH 6.6 ± 0.05. NMR samples were transferred into a 5-mm susceptibility-matched Shigemi tube for data collection.

All NMR spectra were acquired at 20°C on a four-channel Varian INOVA 500 NMR spectrometer, equipped with a 5-mm triple-resonance probe. After a brief (~30 min) equilibration in the magnetic field, alignment of pf1 media was confirmed by ^{2}H quadrupole splitting, which remained constant throughout the data collection (Q = 18.2 ± 0.1 Hz). ^{15}N–H^{N}, ^{13}C′–$^{13}C_{\alpha}$, and $^{13}C_{\alpha}$–H_{α} splittings were measured on the isotropic and partially aligned samples using 2D IPAP (in-phase/antiphase) ^{15}N–^{1}H HSQC (Ottiger et al. 1998), 3D C_{α} (F1) coupled HNCO (Bax et al. 2001), and 3D C_{α} (F1) coupled HAcacoNH experiments (Tjandra and Bax 1997b), using sweep widths of 5500 Hz in the ^{1}H, 1500 Hz in the ^{15}N, 2000 Hz in the C′, and 2250 Hz in the H_{α} dimensions, respectively. 2D IPAP ^{15}N–^{1}H HSQC was acquired with data matrices of 256 × 2K complex points, processed with Gaussian multiplication and zero filling to 4K × 4K. 3D C_{α} (F1) coupled HNCO and 3D C_{α} (F1) coupled HAcacoNH were collected with 128 × 40 × 1K and 96 × 40 × 1K complex points. These 3D spectra were processed with linear prediction in F1 and F2 dimensions,

Table 1. *Helical tilt angles of Ig-binding domains of* Staphylococcal *protein A*

	α1/α2	α1/3α	α2/α3
Z domain refined using CNS with RDC constraints	169.5 ± 2.9	17.0 ± 3.4	172.2 ± 1.8
Z domain refined using CNS without RDC constraints	172.5 ± 4.1	10.2 ± 4.4	171.9 ± 2.2
Z domain refined with RDC constraints from 2 alignment media	171.4 ± 3.4	15.2 ± 3.9	172.3 ± 1.8
Z domain reported previously (PDB ID: 2SPZ; Tashiro et al. 1997)	170.2 ± 4.3	13.1 ± 2.9	173.4 ± 2.1
B domain (PDB ID: 1BDC; Gouda et al. 1992)	150.0 ± 2.4	39.7 ± 3.7	168.8 ± 2.8
D domain in complex (PDB ID: 1DEE; Graille et al. 2000)	174.2	14.6	168.2
E domain (PDB ID: 1EDL: Starovasnik et al. 1996)	166.0 ± 2.9	15.0 ± 4.3	168.1 ± 1.3
Fc-bound B domain (PDB ID: 1EC2; Deisenhofer 1981) (helix α3 not observed)	173.3	—	—
Z domain in complex with affibody (PDB ID: 1LP1; Högbom et al. 2003)	177.8	10.8	167.7
Z domain in complex with affibody (PDB ID: 1H0T; Wahlberg et al. 2003)	171.4 ± 1.7	18.2 ± 2.1	169.2 ± 1.9

Helical tilt angles were calculated from the X-ray structures (D domain and B domain in complex) or ensemble of NMR conformers using the program MOLMOL (Koradi et al. 1996). The helix locations for previously reported structures were taken from the papers describing these structures.

and Gaussian multiplication, and zero filling to 2K × 256 × 1K. The individual RDC data were determined by subtracting the ^1J splittings measured in the isotropic sample from the ^1J (now with dipolar coupling contribution) values obtained in the weakly aligned sample. All spectra were analyzed in SPARKY (Goddard and Kneller 1991).

The program CNS 1.0 (Brünger et al. 1998) was used for structure generation with the SANI module for RDC analysis (Clore et al. 1998b). The 536 distance constraints and 107 dihedral angle constraints were identical to those used previously (Tashiro et al. 1997), but reformatted for CNS. All structures were generated from an extended strand with random initial velocities using the default simulated annealing protocol of the CNS package. The averaging method for analyzing NOE constraints is summation. We calculated 100 conformers, and the 10 structures with lowest values of the CNS target function were selected to represent the solution structure. MOLMOL 2K.1 (Koradi et al. 1996), ProCheck (Laskowski et al. 1993), and PDBStat (R. Tejero and G. Montelione, unpubl. software) were used for analyzing the final structures. Figures of protein structures were generated using the program Ribbons 2.0 (Carson 1991).

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