Rapid Validation of the Overall Structure of an Internal Domain-Swapped Mutant of the Anti-HIV Protein Cyanovirin-N Using Residual Dipolar Couplings

Carole A. Bewley

Laboratory of Bioorganic Chemistry
National Institute of Diabetes and Digestive
and Kidney Diseases, National Institutes of Health
Bethesda, Maryland 20892

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The value of residual dipolar couplings in NMR structure determination has now been well established.[1−4] In addition to providing long-range orientational information for structure refinement,[5,6] dipolar couplings can be used for protein structure validation and protein fold recognition,[7] by comparison of the best fit of the experimentally determined dipolar couplings to those predicted for a known structure either by direct minimization with the set of atomic coordinates, using procedures such as singular value decomposition (SVD),[8] or by back-calculation on the basis of molecular shape.[9] Recently we have shown the power of combining these methods by using them to determine the relative orientation of the monomeric subunits of a symmetrical domain-swapped dimer of the anti-HIV protein cyanovirin-N (CV-N), for which 18 residual dipolar couplings could be measured.[10] In this communication we make use of these methods to rapidly assess the overall structure of an internal domain-swapped monomeric mutant of CV-N and briefly describe its design and biological activity.

CV-N is a cyanobacterial protein that potently (1−10 nM) inhibits all strains of HIV through high-affinity interactions with the HIV surface envelope glycoprotein gp120.[11] CV-N is under joint investigation by NCI and NIAID as a topical anti-HIV agent,[11] thus further structural studies that will shed light on its precise mechanism of action are of significant interest. At physiological pH CV-N exists predominantly as an 11 kD monomer (>90%), and a 22 kD symmetrical domain-swapped dimeric structure can be formed at low pH in the presence of low percentages of organic solvents.[12] High-resolution structures have been solved for the monomer by NMR,[6] and for the domain-swapped dimer by X-ray crystallography at low pH[12] and by NMR at neutral pH.[10] CV-N has dual elements of internal 2-fold symmetry: its sequence comprises two homologous 50 amino acid repeats, D1 and D2, which display 70% similarity (Figure 1a), and its structure displays C_2 pseudosymmetry (Figure 1b).

Prior to the NMR and X-ray structures, Mori et al. constructed many CVN mutants, the more interesting of which included tandem domain repeats D1D1 and D2D2, and a circularly permuted version comprising D2D1.[13] The tandem mutants were inactive, and the activity of D2D1 was reduced over 200-fold. While it is not readily apparent why D2D1 in particular exhibits such attenuated antiviral activity, two structural features come to mind. First, relative to CV-N, the first and last three amino acids of D2D1, which are critical for activity,[13] have been altered. Second, the linker comprising residues 50−53 which connects domains 1 and 2 by crossing over the first β-hairpin[6] (Figure 1a,b) has been interrupted. Based on these observations we designed an internal domain-swapped mutant where the cores of D1 and D2 have been swapped, but the first and last three amino acids and the linker remain unaltered (Figure 1a), the rationale being that these residues might be important for structural integrity. We refer to this mutant as domain-swapped CV-N (dsCV-N).

A synthetic gene encoding the sequence of dsCV-N shown in Figure 1b was constructed and expressed in *E. coli* with standard protocols for uniform ^{15}N-labeling. Fifty-one resonances for dsCV-N were effortlessly assigned by a direct overlay of the ^{1}H−^{15}N correlation spectra of CV-N and dsCV-N (Figure 2a), and 3D ^{15}N-separated NOE experiments confirmed all of these assignments (spectra provided as Supporting Information). The NOE spectra also confirmed equivalent positions for the pair of disulfide bonds in CV-N and dsCV-N, and therefore the preservation of the pair of triple-stranded β-sheets.[6] Resonances that could not be readily assigned from the ^{1}H−^{15}N correlation spectrum alone largely correspond to residues in or proximal to the N- and C-termini and the linker region, as would be expected if the mutant shares the same fold.

^{15}N relaxation measurements at 35 °C show that dsCV-N exists predominantly as a monomer with average ^1H_N and ^{15}N T_2 values of ∼50 and ∼145 ms, respectively, and a rotational correlation time of ∼4.9 ns.[14] Thirty-one residual dipolar couplings for dsCV-N measured in a neutral liquid crystalline bicelle medium were readily obtained from F_1-coupled ^{1}H−^{15}N correlation spectra, allowing analysis by SVD[8] and the steric obstruction model.[9] The latter requires that the modest alignment of solute occurs through steric interactions between the liquid crystalline medium and solute as opposed to attractive or long-range repulsive interactions. We note here that although residues of D1 and D2 have been exchanged in dsCV-N, they are predicted to reside in near-identical structural elements (a triple-stranded antiparallel β-sheet and a β-hairpin) as in CV-N providing the mutant is folded. Thus CV-N numbering has been used in the calculations so that D1 of dsCV-N is being compared directly to D2 of wild-type CV-N, as indicated in Figures 1a and 2a. As shown in Figure 2b, very good agreement is observed between dipolar couplings for dsCV-N and those predicted for the X-ray monomer (which comprises the AB′ subunit of the dimer[10,12]) with SVD (Figure 2b), which yields values of 8.5 Hz for the magnitude of the axial component of the alignment tensor (D_a^{NH}), 0.156 for the rhombicity (η), an rmsd of 1.46 Hz, a dipolar coupling R-factor[15] (R_{dip}) of 15.4%, and a correlation coefficient of 0.96. Similarly, dipolar couplings back-calculated on the basis of molecular shape predict values for D_a^{NH} and η of 10.0 Hz and 0.05, respectively; and the predicted

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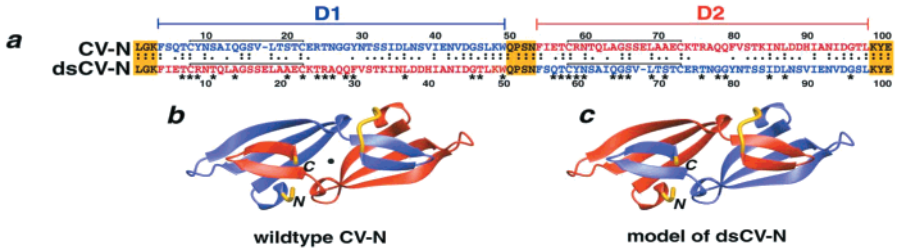

Figure 1. Comparison of sequence and structure of CV-N and an internal domain-swapped mutant dsCV-N. (a) Alignment of wildtype and domain-swapped mutant CV-N sequences. Residues corresponding to the natural sequence of domains 1 (D1) and 2 (D2) are colored blue and red, respectively. The preserved N- and C-termini (three amino acids each) and four-amino acid linker (residues 50−53) are highlighted with a yellow box. Residues for which dipolar couplings were measured are marked with an asterisk. (b) Ribbon diagram of the three-dimensional structure of CV-N with D1 colored blue, D2 colored red, N- and C-termini and linker colored yellow, and the C_2 axis of pseudosymmetry indicated by a black circle. (c) Ribbon diagram of the model of dsCV-N designed to have the same fold as wild-type CV-N; residues corresponding to D1 and D2 of CV-N are colored blue and red, respectively, and the preserved N- and C-termini and linker are colored yellow. Note the exchange in positions of D1 and D2.

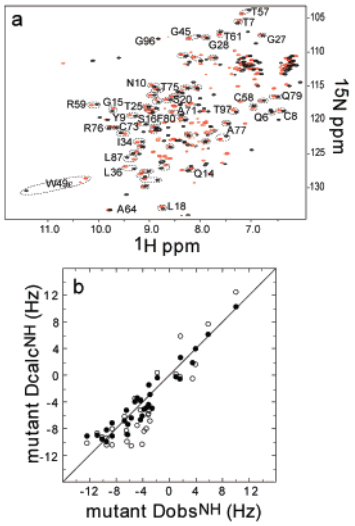

Figure 2. Spectral and structural similarity between CV-N and dsCV-N. (a) Overlay of ^{1}H-^{15}N correlation spectra of CV-N (black) and dsCV-N (red) recorded at 27 °C on a Bruker DMX600 spectrometer. Labels denote CV-N assignments, and dotted circles enclose equivalent residues in CV-N and dsCV-N as shown in Figure 1a. Both samples contained ~0.5 mM protein in 10 mM sodium phosphate, pH 6.5. (b) Comparison of the 31 experimentally measured dipolar couplings (D_{obs}^{NH}) of dsCV-N with values calculated (D_{calc}^{NH}) by best-fitting, using SVD (solid circles), or predicted from the molecular shape (open circles) on the basis of the X-ray coordinates of the monomer which correspond to the AB′ half of the X-ray dimer.[12] The dipolar couplings plotted are limited to the 31 couplings that were measured for dsCV-N in F_1-coupled ^{1}H−^{15}N correlation spectra under isotropic and weakly aligned (3% 3:1 DMPC:DHPC at 35 °C) conditions. SVD and predictions made on the basis of molecular shape were carried out with the program SSIA as described in ref 9.

couplings agree well with the observed couplings as indicated by values of 22.9% and 0.89 for R_{dip} and the correlation coefficient, respectively. Thus, the 31 residues in dsCV-N for which dipolar couplings could be rapidly measured reside in near-identical structural elements as their counterparts in wild-type CV-N, and the results from the steric obstruction model indicate that dsCV-N is monomeric and its overall shape (and therefore the position of residues for which dipolar couplings were not measured) must be the same as in CV-N. We can therefore conclude that the internal domain-swapped CV-N mutant shares an essentially identical structure to that of wild-type CV-N.

We have tested dsCV-N in a quantitative HIV-1 envelope-mediated cell fusion assay and found it to exhibit an ~500-fold diminution in activity. While we have demonstrated that wild-type CV-N and dsCV-N have the same overall shape and should therefore share near identical overall structures, substantial changes in chemical shifts are observed for a number of resonances in the spectra recorded at 27 °C versus those recorded in the presence of bicelles at 35 °C. These differences suggest that dsCV-N is not as stable as CV-N[16] (the spectra of which change minimally with increasing temperature), and that loss of activity might be attributed to subtle changes in intradomain structure. The details leading to such differences will have to await determination of a high-resolution structure of dsCVN.

As we enter the era of genomics, significant efforts are underway to obtain high-resolution three-dimensional structures for as many gene products as possible. As these goals continue to be met, the need to rapidly assess three-dimensional structures for proteins sharing sequence or structural homology with those already reported will grow. To this end several groups have demonstrated the utility of analyzing dipolar couplings with respect to existing structures for structure validation and domain orientation.[7−10] The application presented here provides a practical example of using these methods for structure validation, and underscores how rapidly one can accurately assess the three-dimensional structures of homologous proteins by measuring relatively few dipolar couplings,[8,10] in this case less than one-third of the total. We expect that such methods will continue to find great utility in rapidly obtaining good structural models for other homologous and chimeric proteins for which structures are already available, and for structural analysis of mutations.

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Supporting Information Available: Strips from 3D ^{15}N-separated NOE spectra corresponding to ^{15}N resonances residing in an equivalent β-strand of CV-N and dsCV-N (PDF); coordinates for dsCV-N model are available from the author upon request. This material is available free of charge via the Internet at http://pubs.acs.org.

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(16) These results are in agreement with findings by L. Barrientos and coworkers, submitted.