FOR THE RECORD

Overcoming the problems associated with poor spectra quality of the protein kinase Byr2 using residual dipolar couplings

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Abstract

For the Ras-binding domain of the protein kinase Byr2, only a limited number of NOE contacts could be initially assigned unambiguously, as the quality of the NOESY spectra was too poor. However, the use of residual ¹H–¹⁵N dipolar couplings in the beginning of the structure determination process allows to overcome this problem. We used a three-step recipe for this procedure. A previously unknown structure could be calculated reasonably well with only a limited number of unambiguously assigned NOE contacts.

Keywords: Byr2; residual dipolar couplings; structure calculation; NMR

Small GTPases like Ras exhibit a central role in cellular signal transduction. A high percentage of human tumors are linked to mutations in the Ras gene (Barbacid 1987). In the GTP-bound form, Ras can interact with various effector molecules. It is, therefore, important to learn more about Ras/effector interactions. Known effectors are, for example, the serine/threonine kinase c-Raf1 and the Ral guanine-nucleotide exchange factors RalGDS, Rgl, and Rlf. An important step toward the understanding of the Ras/effector interactions is detailed knowledge about the structures of these molecules. Solution structures are known for the Ras-binding domains (RBDs) of c-Raf1 (Terada et al. 1999), Rgl (Kigawa et al. 1998), Rlf (Esser et al. 1998), and RalGDS (Geyer et al. 1997). The structure of the Ras-binding domain of the protein kinase Byr2 (Byr2-RBD) from *Schizosaccharomyces pombe*, a homolog to the Raf kinase of

higher eukaryotes, is still unknown. Byr2-RBD was identified as a fragment of 116 residues in the amino-terminal part of the molecule and is only weakly related in primary sequence to other known effectors. A multiple sequence alignment of the Ras-binding domains of Byr2, Rgl, RalGDS, c-Raf1, and Rlf was performed using the program XALIGN (Wishart et al. 1994). For Byr2, the sequence identity with, for example, RalGDS and c-Raf1, amounts to only 15% and 14%, respectively.

One major obstacle in structure determination of the protein was the low stability, as well as the poor spectral quality caused by protein aggregation. To keep the protein in solution and stabilize the monomeric state, 200 mM of deuterated glycine was added to the protein solution. This, in turn, considerably increased the line width of the nuclear magnetic resonance (NMR) spectra to ≥ 17 Hz by increasing the viscosity of the solution. Further problems are related to the fact that $\sim 30\%$ of the molecule is disordered. Despite the resulting signal overlap, almost complete resonance assignments could be obtained by heteronuclear methods (Huber et al. 2000). The quality of the NOESY spectra, however, did not allow to extract a sufficient number of unambiguous distance constraints to calculate a three-dimensional structure. On the other hand, Clore et al. (1999) have shown for

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proteins of known structure that the three-dimensional structure can still be determined by using residual dipolar couplings even if the majority of NOE contacts has been omitted. Here, we describe the use of residual dipolar couplings at a very early stage of structure determination of Byr2 when only a limited set of NOE contacts was available.

Results and Discussion

The preparation of Byr2 and sequential assignment of the NMR signals of Byr2 have been described elsewhere (Huber et al. 2000). Residual dipolar couplings are induced by the addition of phospholipid bicelles to the protein solution (Tjandra and Bax 1997). Solutions containing a bicelle-forming phospholipid mixture of dimyristoylphosphatidylcholine (DMPC) and dihexanoylphosphatidylcholine (DHPC) were prepared following the procedure described by Losonczi and Prestegard (1998) in 50 mM phosphate buffer at pH 6.2. The measurements were carried out at a total phospholipid concentration of 5 wt%. The presence and long-term stability of magnetically oriented bicelles

were detected by ^2H NMR spectroscopy (i.e., by the observation of the characteristic doublet splitting of the signal of $^2\text{H}_2\text{O}$) (see, e.g., Ottiger and Bax 1998). One-bond ^1H - ^{15}N residual dipolar couplings could be measured for 28 of the 116 amino acid residues by a heteronuclear single quantum coherence (HSQC) experiment without ^1H decoupling during the ^{15}N evolution period (t_1). The above described overlap problems are responsible for the relatively small number of measured residual dipolar couplings. From the distribution function of the observed residual dipolar couplings one can estimate (Clore et al. 1998) the anisotropy of 15 Hz and the rhombicity of 0.3 of the molecular alignment tensor of Byr2. The following three-step "recipe" was used to overcome these problems (Fig. 1). In the first step 61, 339, and 160 NOE contacts were identified from the homonuclear two-dimensional, heteronuclear three-dimensional ^{15}N and ^{13}C separated NOESY spectra, respectively. However, only 103 of them were long-range contacts and a considerable number of these were ambiguous. Because of the relatively low number of identified long-range contacts, 19 ambiguous contacts had to be included where one assignment seemed to be more likely than the other possibilities.

Fig. 1. Application of residual dipolar couplings for protein structure determination with a limited number of unambiguously assignable NOEs. Strategy for residual dipolar coupling aided NOE cross-peak assignment and structure calculation (a). The structure and relative orientation of the two α -helices H1 and H2 are displayed (b). The complete backbone of Byr2 is shown (c) in green, the helices H1 and H2 are colored in blue. One structure was selected from the ensemble obtained in step 3. For comparison, the backbone of human ubiquitin is shown in red (Cornilescu et al. 1998). Structures for Byr2 were obtained using the CNS 1.0 package (Brünger et al. 1998) and the standard dynamical annealing protocol delivered with CNS.

Twelve hydrogen bond restraints were identified from an analysis of the slowly exchanging amide protons. Structure calculations were started with this relatively small number of distance restraints without using residual dipolar couplings. Because of the relatively small number of restraints, the resulting structures only showed the rough general fold of the molecule in which the orientation of the various secondary structure elements relative to each other was poorly defined. Therefore, on the basis of these structures it was impossible to unambiguously identify more NOE contacts or to verify the ambiguous NOE contacts. It is demonstrated in Figure 1 how the orientation of the secondary structure elements relative to each other changes during the three steps of structure calculation. As an example, the two α -helices of Byr2 (H1: residues 22–32, H2: 59–63) are shown. In step 1 the two helices are almost parallel (Fig. 1a,b, step 1). In the second step, 28 residual dipolar couplings were included in addition to the distance restraints of step 1. The residual dipolar couplings were particularly helpful for defining the general arrangement of the various secondary structure elements (i.e., to determine their relative orientation). The axes of the two α -helices in Figure 1b are now almost perpendicular relative to each other. However, α -helix H1 predicted by these calculations exhibits an unrealistic deformation (Fig. 1a,b, step 2). The second turn is twisted by $\sim 180^\circ$. For each step of the structure determination process, the 10 structures of lowest total energy were superimposed and average rmsd values were calculated. To show the influence of the inclusion of residual dipolar couplings, Table 1 summarizes the results of steps 1 and 2 obtained with and without residual dipolar couplings, respectively.

Identical sets of NOE contacts were used in steps 1 and 2. Note that the inclusion of residual dipolar couplings (step 2) only results in a small decrease of the rmsd value calculated for the C α atoms, whereas the other rmsd values remain constant or increase significantly. This behavior indicates the presence of misassigned NOE contacts. From the 19 ambiguous long-range contacts so far included into the structure calculations, 8 could be confirmed or unambiguously reassigned using the improved backbone structure (Fig. 1b). The above-mentioned deformation of H1 was caused by the erroneous assignment of two of these ambiguous NOE contacts. Eleven contacts remained ambiguous and were excluded from the following structure calculations. In addition, using back-calculated NOESY spectra (Görler and Kalbitzer 1997) it was possible to identify 43 new unambiguous long-range contacts and two additional hydrogen bonds. In the third stage, new structures were calculated using the residual dipolar couplings and the improved set of distance restraints (Fig. 1a,b, step 3). The rmsd value calculated for the C α atoms of the 10 best structures obtained in step 3 amounts to 3.52 Å. A generally accepted measure for the quality of a structure is the rmsd value. Comparing the rmsd values for the C α atoms of the selected structures for step 1 (5.47 Å), step 2 (5.17 Å), and step 3 (3.52 Å), it becomes clear that the quality of the obtained structures improves after the addition of the residual dipolar couplings and the correction of the ambiguous NOEs incorrectly assigned at the beginning. From the backbone structures obtained in step 3 (Fig. 1c) it can be seen that Byr2 exhibits an ubiquitin-like fold similar to other Ras-binding domains (e.g., RalGDS).

Table 1. Comparison of rmsd values obtained for Byr2 structures calculated in step 1 and step 2 of the protocol described in Fig. 1

	rmsd (C α)/Å	rmsd (NOE)/Å	rmsd (bonds)/Å	rmsd (angles)/degree	rmsd (impropers)/degree
Step 1	5.47	0.105 $\pm$ 0.011	0.0067 $\pm$ 0.0002	0.719 $\pm$ 0.037	0.557 $\pm$ 0.066
Step 2	5.17	0.111 $\pm$ 0.010	0.0081 $\pm$ 0.0005	0.932 $\pm$ 0.050	0.795 $\pm$ 0.090

10 structures of lowest total energy were superimposed and average rmsd values of C α atoms to the mean structure were calculated. The other rmsd values are calculated with respect to ideality as defined in HC^a. All structure calculations were started from extended strand conformations after the standard-simulated annealing protocol suggested by CNS. It should be noted that the force constant for residual dipolar couplings was set to a small value during the high temperature molecular dynamics stage and reached its maximum value at the second slow cooling stage, when the structures were already mainly folded (see below). Quadratic harmonic potential terms (force constants of 1000 kcal mol⁻¹ Å⁻², 500 kcal mol⁻¹ rad⁻², and 500 kcal mol⁻¹ rad⁻² for bonds, angular, and improper terms, respectively; 1 cal = 4.184 J) are used to maintain the covalent geometry very close to ideality. The following parameters were used: for high temperature annealing: torsion angle dynamics, temperature = 50,000 K, 1000 steps, force constant for NOE energy = 150 kcal mol⁻¹ Å⁻², for residual dipolar coupling energy = 0.01 kcal mol⁻¹ s², and for dihedral energy = 100 kcal mol⁻¹ rad⁻², time step = 0.015 ps. For first slow cooling: torsion angle dynamics, temperature = 50,000 K, 1000 steps, force constant for NOE energy = 150 kcal mol⁻¹ Å⁻², for residual dipolar coupling energy = 0.001–1.0 kcal mol⁻¹ s² and for dihedral energy = 200 kcal mol⁻¹ rad⁻², time step 0.015 ps, slow cooling temperature step 250 K. For second slow cooling: Cartesian dynamics, temperature = 3000 K, 3000 steps, force constant for NOE energy = 150 kcal mol⁻¹ Å⁻², for residual dipolar coupling energy = 1.0–50.0 kcal mol⁻¹ s² and for dihedral energy = 200 kcal mol⁻¹ rad⁻², time step 0.005 ps, slow cooling temperature step 25 K. Final energy minimization: Powell method, 2000 steps, force constant for NOE energy = 75 kcal mol⁻¹ Å⁻² and for dihedral energy = 400 kcal mol⁻¹ rad⁻².

Work is in progress to identify unambiguously more NOE contacts based on the structures of step 3 and to measure other residual dipolar couplings (^1H - ^{13}C , ^{13}C - ^{15}N , $^{13}\text{C}^\alpha$ - $^{13}\text{C}'$) to obtain a highly resolved three-dimensional structure of Byr2.

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