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Assignment of $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$, ^{13}CO and $^{13}C^{\beta}$ resonances in a 67 kDa p53 dimer using 4D-TROSY NMR spectroscopy

Frans A.A. Mulder[a], Ayeda Ayed[b], Daiwen Yang[a], Cheryl H. Arrowsmith[b] & Lewis E. Kay[a]
[a]*Protein Engineering Centers of Excellence and Departments of Medical Genetics, Biochemistry and Chemistry, The University of Toronto, Toronto, ON, Canada M5S 1A8 and* [b]*Division of Molecular and Structural Biology, Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, 610 University Avenue, Toronto, ON, Canada M5G 2M9*

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Abstract

The p53 tumor suppressor is a transcription factor that plays a crucial role in the activation of genes in response to DNA damage. As a first step towards detailed structural studies of the molecule aimed at understanding its regulation, we have used 4D-TROSY triple resonance NMR spectroscopy to obtain nearly complete $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$, ^{13}CO and $^{13}C^{\beta}$ resonance assignments of a dimeric form of the protein comprising DNA-binding and oligomerization domains (67 kDa). A simple comparison of 4D spectra recorded on p53 molecules consisting of DNA-binding and oligomerization domains with and without the regulatory domain establishes that both constructs have essentially identical chemical shifts. Although the affinity of p53 for target DNA is decreased in constructs containing the regulatory domain, the chemical shift results reported here suggest that this decrease is not due to specific domain interactions involving the regulatory portion of the molecule, or alternatively, that such interactions require the presence of DNA.

The p53 protein is a 393-residue tumor suppressor that activates genes involved in the control of the cell cycle and cell death in response to DNA damage (Levine, 1997). Because over half of all human cancers involve mutations in the molecule there has been a great deal of interest in structural studies of wildtype and mutant forms of the protein (Arrowsmith and Morin, 1996). Full length p53 is a tetramer comprising an acidic transactivation domain at the N-terminus (residues 1–70), followed by a DNA-binding domain (90–300), a tetramerization domain (324–355) and finally a basic regulatory domain (355–393) at the C-terminal end of the molecule. Recently, both NMR (Clore et al., 1994, 1995; Lee et al., 1994) and X-ray (Jeffrey et al., 1995) studies of the tetramerization domain have been reported as well as an X-ray study of a monomer of the DNA-binding domain in complex with target DNA (Cho et al., 1994). While these structures have provided important insights, including an understanding of the clustering of cancer-causing mutations in specific regions of the molecule (Cho et al., 1994), there are many remaining questions that can only be addressed through a study of larger constructs containing several of the domains. For example, knowledge of interdomain interactions, quaternary structure and potential allosteric modulation of the protein are particularly relevant to the regulation of p53.

A number of significant developments in NMR spectroscopy over the past few years, including triple resonance spectroscopy of ^{15}N, ^{13}C, ^{2}H-labeled proteins (Farmer and Venters, 1998; Gardner and Kay, 1998) and TROSY spectroscopy (Pervushin et al., 1997), now allow NMR studies of proteins in the 50–100 kDa molecular weight range in some cases. These advances facilitate the study of large proteins, such as p53. We report here the nearly complete backbone $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$, ^{13}CO and sidechain $^{13}C^{\beta}$ chemical shift assignments of a 67 kDa dimeric construct of p53 consisting of both DNA-binding and tetramerization domains (residues 82–360) using new

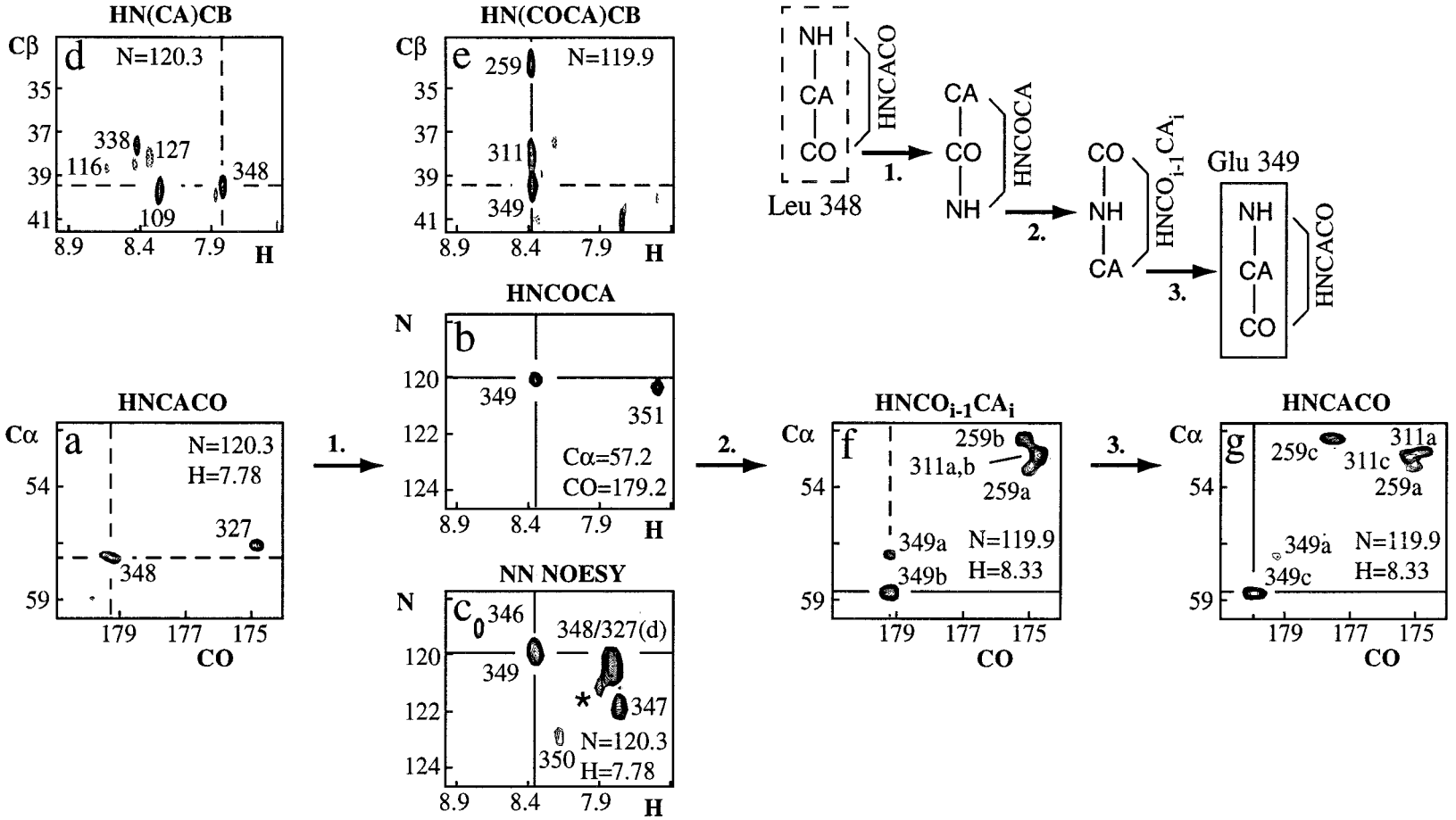

Figure 1. Slices from 3D- and 4D-TROSY based triple resonance experiments recorded on a uniformly ^{15}N, ^{13}C, ^{2}H-labeled sample of dimeric $p53^{82-360}_{MQ,LR}$ (0.4 mM in dimer, 25 mM phosphate, 250 mM NaCl, 10 mM DTT, 5 mM $MgCl_2$, pH 6.5, 10% D_2O, 600 MHz field strength, 25 °C). Pulse schemes (including delays) and processing parameters are as described previously (Konrat et al., 1999; Yang and Kay, 1999). All of the data was processed using NMRPipe (Delaglio et al., 1995) and analyzed with NMRView (Johnson and Blevins, 1994) software. In each spectrum cross peaks are identified by the residue number of the $^{15}N,^{1}H^{N}$ pair of the correlation. In the HNCO$_{i-1}$CA$_i$ cross peaks are denoted 'a' or 'b', depending on whether the correlation connects ($^{13}C^{\alpha}_{i-1}$,$^{13}CO_{i-1}$,$^{15}N_i$,$^{1}H^{N}_i$) or ($^{13}C^{\alpha}_{i}$,$^{13}CO_{i-1}$,$^{15}N_i$,$^{1}H^{N}_i$) respectively. In cases where correlations linking both ($^{13}C^{\alpha}_{i}$,$^{13}CO_i$,$^{15}N_i$,$^{1}H^{N}_i$) and ($^{13}C^{\alpha}_{i-1}$,$^{13}CO_{i-1}$,$^{15}N_i$,$^{1}H^{N}_i$) are observed in the 4D-HNCACO they are distinguished by 'c' and 'a', respectively, while no identifier is used in cases where only the ($^{13}C^{\alpha}_{i}$,$^{13}CO_i$,$^{15}N_i$,$^{1}H^{N}_i$) correlation is observed (see spectrum a). In the 4D $^{15}N,^{15}N$-edited NOESY diagonal peaks are denoted by '(d)', while * is an exchange peak between Leu 348 in the $p53^{82-360}_{MQ,LR}$ dimer and the monomer. Spins from Leu 348 are indicated by crosshairs with dashed lines, while those from Glu 349 are denoted with continuous lines. Negative peaks (due to spectral aliasing) are drawn with dashed contours.

4D TROSY-based triple resonance experiments (Konrat et al., 1999; Yang and Kay, 1999) recorded on a ^{15}N, ^{13}C, ^{2}H-labeled sample. In order to facilitate NMR studies of this large system we have prepared a construct spanning residues 82–360 of p53 which contains two mutations in the tetramerization domain, Met340Gln and Leu344Arg (referred to as $p53^{82-360}_{MQ,LR}$ in what follows). These mutations result in formation of a dimer (two molecules, each comprising DNA-binding and oligomerization domains), as opposed to a tetramer (Davidson and Arrowsmith, unpublished data). Biochemical data suggest that the dimer behaves in a similar manner to the intact tetramer in terms of its regulation by the R-domain (A. Ayed and C.H. Arrowsmith, unpublished data).

The strategy used for assignment is illustrated schematically in the upper right hand corner of Figure 1. Three 4D triple resonance data sets are recorded, including the 4D TROSY-HNCACO ($^{13}C^{\alpha}_{i}$,$^{13}CO_{i}$,$^{15}N_{i}$,$^{1}H^{N}_{i}$) (Yang and Kay, 1999), the 4D TROSY-HNCOCA ($^{13}C^{\alpha}_{i-1}$,$^{13}CO_{i-1}$,$^{15}N_{i}$,$^{1}H^{N}_{i}$) (Yang and Kay, 1999) and the 4D TROSY-HNCO$_{i-1}$CA$_{i}$ ($^{13}C^{\alpha}_{i}$,$^{13}CO_{i-1}$,$^{15}N_{i}$,$^{1}H^{N}_{i}$) (Konrat et al., 1999). The spins that are correlated in each of the experiments are indicated in parentheses with the subscript *i* denoting the residue number. In some cases correlations of the form ($^{13}C^{\alpha}_{i-1}$, $^{13}CO_{i-1}$,$^{15}N_{i}$,$^{1}H^{N}_{i}$) are observed in the 4D-HNCACO and 4D-HNCO$_{i-1}$CA$_{i}$ along with the more intense connectivities listed above (Yang and Kay, 1999). In cases where ($^{13}C^{\alpha}$,^{13}CO) and (^{15}N,$^{1}H^{N}$) spin pairs are unique it is possible to use the 4D-HNCACO and the 4D-HNCOCA exclusively for backbone assignment. For example, the ($^{13}C^{\alpha}$,^{13}CO) chemical shifts of residue *i* obtained from a 4D-HNCACO slice can be linked with the corresponding carbon shifts in the 4D-HNCOCA, thereby identifying the ^{15}N,$^{1}H^{N}$ shifts of the subsequent residue, $i + 1$ (step 1). These ^{15}N,$^{1}H^{N}$ shifts in turn allow assignment of the $i + 1$ $^{13}C^{\alpha}$,^{13}CO shifts using the 4D-HNCACO. In cases where there are degeneracies in ($^{13}C^{\alpha}$,^{13}CO) or (^{15}N,$^{1}H^{N}$) pairs of chemical shifts, additional experiments must be analyzed, as illustrated in Figure 1.

Figure 1 shows spectra obtained from 4D and 3D data sets recorded on a 0.4 mM (dimer concentration) sample of $p53^{82-360}_{MQ,LR}$. In (a) the $^{13}C^{\alpha}$ and ^{13}CO shifts of Leu 348 of the tetramerization domain are obtained from the 4D-HNCACO slice at $^{15}N = 120.3$ ppm, $^{1}H^{N} = 7.78$ ppm. Using these carbon shifts in the 4D-HNCOCA (b) two possible candidate ^{15}N,$^{1}H^{N}$ spin pairs for Glu 349 are obtained (step 1); this ambiguity is resolved by inspection of a slice from the 4D ^{15}N,^{15}N-edited NOESY (Grzesiek et al., 1995; Venters et al., 1995) (c, $^{15}N = 120.3$ ppm, $^{1}H^{N} = 7.78$ ppm) where the $^{15}N/^{1}H^{N}$ shifts of one of the NOE cross peaks and one of the candidate peaks in the 4D-HNCOCA slice (b) are identical ($^{15}N = 119.9$ ppm, $^{1}H^{N} = 8.33$ ppm). Further confirmation of this assignment is obtained from the 3D TROSY-HN(CA)CB ($^{13}C^{\beta}_{i}$,$^{15}N_{i}$,$^{1}H^{N}_{i}$) (Salzmann et al., 1999; Yang and Kay, 1999, see supplementary material) and the 3D TROSY-HN(COCA)CB ($^{13}C^{\beta}_{i}$,$^{15}N_{i+1}$,$^{1}H^{N}_{i+1}$) (Salzmann et al., 1999; Yang and Kay, 1999, see supplementary material) data sets (d and e). The $^{15}N/^{1}H^{N}$ shifts of Glu 349, Asp 259 and Asn 311 are degenerate, illustrated in panel g, and it is therefore not possible to assign the intra-residue $^{13}C^{\alpha}/^{13}CO$ shifts of residue 349 from a combination of the 4D-HNCOCA and 4D-HNCACO. However, since the assignments of Leu 348 ^{13}CO and Glu 349 $^{15}N/^{1}H^{N}$ are known it is possible to obtain the $^{13}C^{\alpha}$ shift of Glu 349 from the HNCO$_{i-1}$CA$_{i}$ (f, step 2), allowing unambiguous assignment of the Glu 349 ^{13}CO from the HNCACO (g, step 3).

Using the assignment procedure described above, over 95% of the $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$, ^{13}CO and $^{13}C^{\beta}$ shifts have been obtained for $p53^{82-360}_{MQ,LR}$. Interestingly, we also observe an additional set of resonances for the residues in the tetramerization domain that arise from a minor (<25%) monomer species in solution that is unfolded. A complete list of assignments of the dimer and the monomer shifts is provided in the supplementary material.

The chemical shifts reported here provided a foundation upon which to pursue further NMR studies of p53 and to establish whether there are interactions between the domains. Because the linkers between the domains in p53 are flexible (average ^{15}N T_2s of 36±5, 57±12 and 223±70 ms for the DNA-binding domain, the tetramerization domain and the linker region between these domains, respectively) it may prove difficult to crystallize multi-domain constructs of the molecule and hence obtain information about domain-domain interactions from X-ray diffraction studies.

A comparison of 4D-HNCO$_{i-1}$CA$_{i}$ data sets recorded on dimers with (residues 82–393) and without (residues 82–360) the regulatory domain establishes that there are no chemical shift differences of any significance (the root-mean-squared difference in

$^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$ and ^{13}CO chemical shifts between the two constructs is less than half of the spectral resolution in each of the appropriate dimensions). This suggests that the presence of the regulatory domain has little influence on the conformation of either the DNA-binding domain, the tetramerization domain or the intervening linker region of p53. This result is significant in that the C-terminal regulatory residues have been proposed to allosterically regulate the conformation of the p53 protein (Hupp and Lane, 1994).

In summary, assignments of backbone resonances in a dimer of p53 comprising DNA-binding and oligomerization domains have been obtained using a series of new 4D triple resonance experiments. These assignments are extremely valuable in providing a framework by which to compare structural properties of a variety of p53 molecules.

Supplementary information available

One table of $^{1}H^{N}$, ^{15}N, $^{13}C^{\alpha}$, ^{13}CO and $^{13}C^{\beta}$ chemical shifts of dimeric $p53^{82-360}_{MQ.LR}$ and the oligomerization domain from the unfolded monomer and three figures consisting of slices from the 3D and 4D data sets used for assignment of residues 204–207. The material is available by e-mail from L.E.K. (kay@pound.med.utoronto.ca)

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