Local Structural Elements in the Mostly Unstructured Transcriptional Activation Domain of Human p53*

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*p53 is a tumor suppressor protein involved in the negative feedback of cell proliferation (1) and is composed of a few discrete functional domains, such as a transactivation domain (TAD) (2), a DNA-binding domain (3), and an oligomerization domain (4, 5). The full TAD of p53 consists of the N-terminal 73-residues and has a net charge of –17 because it is rich in acidic amino acid residues, such as Asp and Glu (2). Hence, the p53 TAD is a member of “acidic” (6) activation domains (AADs), which interact with a variety of target proteins that bind to specific sites on DNA (2, 7–11). The DNA-binding domain and the oligomerization domain of p53 have been subjected to extensive structural investigations, and their structures are well determined (3–5). On the contrary, little is known about the structure of the TAD of p53 except the fact that it is inherently devoid of tertiary structure, as are most AADs (12–14), and that short p53 TAD fragments are able to form “induced” helices upon binding to target proteins (7, 15, 16). The structural models proposed for AADs, such as “acidic blobs and negative noodles” (12) or a “polypeptide lasso” (13), are useful to some extent to describe the overall structural state of the intact p53 TAD in a target-free environment.

However, these models do not suggest the possibility that some specific structural determinants may exist in the unbound state of AADs and mediate recognition of AADs by target proteins. Despite the speculation (11, 12, 17) that transcriptional activity or TAD-target binding would be governed by some specific structural determinants, such as an amphipathic helix (18), acquiring direct physical evidence for the putative helical structure in the unbound form of TADs has been mostly unsuccessful (13, 19–25). For example, two full-length TADs, the VP16 TAD from herpes simplex virus (21, 22) and the TAD in yeast heat shock transcription factor (13), were shown by CD spectropolarimetry and NMR spectroscopy to have no secondary structure in aqueous solution. For the former, no evidence of any secondary structure could be found, even in a hydrophobic solvent such as 80% methanol. Even though the minimal activation domain from the human glucocorticoid receptor was shown to contain multiple helices, this observation was possible only in the presence of a strong helix-promoting solvent (23). The presence of some secondary structures was noted by CD measurements in the activation domains of GCN4 and GAL4 in a hydrophobic solvent, but the dominant form was controversially found to be antiparallel β-sheet rather than the putative α-helix (24, 25). The results of NMR studies on short TAD fragments are also controversial. In the case of the VP16 TAD, an unbound 17-residue fragment was reported to have no detectable secondary structure, which becomes helical upon target binding (26). In contrast, a 16-residue p53 TAD fragment was shown to form, even in the absence of a target protein, an ordered structure at 2 °C with two contiguous β-turns, which is very similar to a helix (27). For short TAD fragments (<20 residues), helices have been observed only in their target-bound state (7, 16, 26). However, helices were detected if a longer fragment, such as the 46-residue CAMP-response element-binding protein kinase-inducible transcription domain (pKID) (28) or the 88-residue long full-length TAD from the activating transcription factor-2 (29), was examined. In particular, the latter has, unlike typical AADs, extremely well defined secondary structures, such as a zinc finger-like motif and short β-strands in aqueous solution. An interesting exception is the full-length TAD of yeast heat shock transcriptional activation domain.
transcription factor, which, even as an intact domain, is completely devoid of secondary structure (13). As the structural features of TADs vary widely, ranging from being totally unstructured to having well defined secondary structures, it is not clear whether one would be able to find a general structural description for unbound TADs.

Functionally different from but structurally similar to TADs are several proteins that are also known to be inherently unstructured or loosely folded (30–36). The observation that these proteins may function without necessarily relying on tertiary structure has in fact suggested establishment of a new view on protein structure (30, 37). The unbound full-length p53 TAD, largely unstructured in aqueous solution, contains a small fraction of secondary structure (9), as do many other TADs (28, 38, 39). In order to fully understand the potential ramification of such minimal secondary structures for transcriptional activity, we have carried out a detailed multidimensional NMR study on a uniformly 15N-labeled full-length p53 TAD. Thorough characterization of the overall structural state of the p53 TAD not only should help in establishing a better structure-function relationship for TADs but also may provide additional insight into the new view on protein structure.

EXPERIMENTAL PROCEDURES

Protein Preparation—The 75-residue human p53 TAD was prepared as described previously (9). The DNA fragment encoding a GS linker plus residues 1–73 of p53 was amplified by polymerase chain reaction using two primers (5′-GGTCGGATCCCATGAGCCCGCTCA-3′ and 3′-GGTGAACTTACACGGGGAGCAGCCTC-5′) and subcloned into BamHI and HindIII sites of pSK(-) (Stratagene) to construct pSK-p53-TAD (9). The DNA fragment of the p53 TAD in pSK(-) was subcloned into BamHI and HindIII sites of the Escherichia coli expression vector pGEX-4B. The recombinant DNA containing the p53-TAD DNA fragment was introduced into the E. coli DH5α and subjected to expression as glutathione-S-transferase fusion p53-TAD peptides. Cells were grown in 2× YTA medium (16 g/liter tryptone, 10 g/liter yeast extract, 5 g/liter NaCl) with 0.05 mg/ml ampicillin (Sigma) at 37 °C, pH 7.0, for 4 h. 0.3 ml isopyropyl-1-thio-β-D-galactopyranoside was added when the A600 reached 0.7, and cells were further grown at 37 °C for 4 h. The final A600 was approximately 1.2. Cells were centrifuged for 30 min at 8000 rpm. Pellets were resuspended in 50 mM sodium phosphate, pH 7.8, and then reacted with hen egg white lysozyme (0.25 mg/ml, Sigma) for 1 h at 4 °C. The suspension was then sonicated and centrifuged at 4 °C, 12,000 rpm for 20 min. The fusion protein was bound to glutathione-Sepharose affinity resin (Amersham Pharmacia Biotech) and cleaved with thrombin (Roche Molecular Biochemicals) to release the p53-TAD peptide. The peptide was further chromatographically purified using SOURCE 15Q (PE 4.6 mm inner diameter × 100 mm long, Amersham Pharmacia Biotech) ion exchange and C18 218TP1010 (10 mm inner diameter × 100 mm long, Vydac) preparative reverse-phase high pressure liquid chromatography to homogeneity.

To obtain the uniformly 15N-labeled p53 TAD, the E. coli was initially grown without labeling for 10 h at 37 °C, pH 7.4, in M9 medium supplemented with 0.4% glucose, 2.0 mg/liter biotin, 2.0 mg/liter thiamine, 1 mM MgSO4, 0.1 mM CaCl2, and 0.05 mg/ml ampicillin. This was followed by a period of 5–10 h during which the cells were resuspended in 50 mM sodium phosphate, pH 7.8, and then reacted with isopyropyl-1-thio-β-D-galactopyranoside and was performed as described above. The purified peptide was subjected to amino acid composition analysis and mass spectrometry to confirm its identity.

NMR Experiments—Protein samples in a concentration of ∼0.3 mM were prepared in 90% H2O/10% D2O or 100% H2O containing 50 mM acetate buffer with a final pH of 6.3. All NMR experiments were done at 5 and 25 °C using a Varian Unity 500 or Unity INOVA 600 spectrometer equipped with a triple-resonance probe in order to avoid spectral overlap as much as possible, to monitor temperature dependent structural changes and to calculate temperature coefficients of backbone amide protons. Pulse sequences used were as described previously (40) except that current 15N-1H HSQC spectra consisted of 150 complex t1 increments with spectral widths of 920 Hz in the F1 (15N) dimension and 6600 Hz in the F2 dimension (1H). For the three-dimensional 15N-edited TOCSY-HSQC and NOESY-HSQC spectra (41, 128 complex t1 and 32 complex t2 increments were acquired with spectral widths of 6600 Hz in the F1 (1H), 920 Hz in the F2 (15N), and 6600 Hz in the F3 (1H) dimension. Hydrogen-deuterium exchange was monitored by one-dimensional 1H methods at 5 °C. Pulsed field gradients were used in all 1H-detected heteronuclear experiments in order to minimize spectral artifacts as well as to select desired coherences using an enhanced sensitivity approach (42). Also, water-selective pulses were employed to achieve minimal solvent saturation (43, 44). Mixing times for TOCSY-HSQC spectra were 55–70 ms, whereas a mixing time of 190 or 250 ms was used for NOESY-HSQC experiments. The three-bond 1JHNH coupling constants were measured by 3D HNHA technique (45). Temperature coefficients for the backbone amide protons (3JHNH) were calculated from the 1H resonance assignments obtained at two temperatures (5 and 25 °C). For measurements of 15N relaxation times a series of 1H-15N HSQC spectra were recorded with seven different relaxation delays. The 15N-H1 heteronuclear steady-state NOEs were measured from a pair of spectra recorded with and without a proton presaturation; in the former, a net recycle delay of 5 s was used, whereas for the latter, a recycle delay of 2 s was followed by a 2 s proton presaturation period.

Data were processed and analyzed on a Sun SPARCstation using Varian Vnmr, nmrPipe/nmrDraw (46), and PIPP software (47).

RESULTS

Resonance assignment for the full-length 73-residue human p53 TAD was achieved using three-dimensional 15N-edited TOCSY-HSQC, NOESY-HSQC techniques (41, 48), and 1H homonuclear two-dimensional COSY, TOCSY, and NOESY experiments according to the sequential resonance assignment procedure (49). The first two N-terminal residues of the recombinant protein, Gly and Ser, were excluded from the amino acid numbering of the peptide as they originated from the N-terminal glutathione-S-transferase fusion linker. Unambiguous assignment of the backbone 15N, amide and aliphatic protons was possible (Table I) for all residues except for 14 prolines and Glu52. The level of achieved resonance assignment, however, was sufficient for subsequent structural characterization of the p53 TAD. Fig. 4 shows an 15N-1H HSQC spectrum of the full-length 73-residue human p53 TAD with resonance assignment.

Summarized in Fig. 1 are T1 and T2 relaxation times of the backbone 15N and 15N-H steady-state heteronuclear NOEs for the unbound 73-residue p53 TAD. Two regions in the full-length p53 TAD have noticeably different local backbone dynamics from the rest of the molecule; a pronounced region near residues 18–30 and another encompassing residues 47–55. The former, interestingly, contains the functionally important conserved hydrophobic residues in the p53 TAD such as Phe18, Leu26, Trp23 and Leu55 (7–9). Then, these local structural orders were further analyzed using NOE and chemical shift indices (50) as shown in Fig. 2. The continuity of sequential δ15N NOEs indicates that three regions, near residues 18–30, 39–45, and 48–55 of the p53 TAD, have helical propensity. The AGADIR (51), a robust algorithm predicting helical content of a protein in the absence of tertiary structure, predicts that the unbound p53 TAD possess three helices, which correspond to the three regions that we found. Among the three predicted regions, the first, encompassing residues Gin14–Leu25, was predicted to be the most helical.

Fig. 3 shows details on the chemical shift index analysis for the Hα chemical shift (50), deviation of the three-bond coupling constants 1JHNH, from random coil values (52), and the temperature coefficients of the backbone amide protons (3JHNH). In consistent with the AGADIR prediction, an amphipathic helix seems to be formed by residues Thr18–Leu26, whereas other two regions showing helical propensity form only narrow turns. The INHCA technique has a tendency of underestimating the 1JHNH values by −0.5 Hz when compared with other methods (45). As shown in Fig. 3, even if such a tendency is taken into consideration, one finds that the observed 1JHNH values associated with the helix are not only sufficiently small
| Residue | ¹⁵N | NH | H₂ | H₂O | Others |
|---------|-----|----|----|-----|--------|
| Met¹    | 122.01 | 8.71 | 4.50 | 2.04/2.10 | 2.47 |
| Glu²    | 121.56  | 8.45 | 4.28 | 2.05/1.91 | 2.25 |
| Gln³    | 123.39  | 8.42 | 4.57 | 2.02/1.88 | 2.28 |
| Pro⁴    | 121.25  | 8.24 | 4.27 | 2.03/1.88 | 2.23 |
| Ser⁵    | 118.05  | 8.52 | 4.42 | 3.82   |       |
| Asp⁶    | 123.84  | 8.59 | 4.89 | 2.78/2.53 |       |
| Pro⁷    | 116.07  | 8.61 | 4.40 | 3.86   |       |
| Val⁸    | 121.56  | 7.99 | 4.17 | 2.10   | 0.90  |
| Glu⁹    | 126.58  | 8.49 | 4.56 | 2.01/1.86 | 2.29 |
| Pro¹⁰   |       |     |     |       |       |
| Pro¹¹   |       |     |     |       |       |
| Leu¹²   | 122.78  | 8.49 | 4.32 | 1.66   | 1.60  |
| Ser¹³   | 116.99  | 8.48 | 4.42 | 3.86   |       |
| Gln¹⁴   | 122.78  | 8.63 | 4.33 | 2.12/1.94 | 2.33 |
| Glu¹⁵   | 122.32  | 8.56 | 4.25 | 1.89   | 2.17  |
| Thr¹⁶   | 115.31  | 8.24 | 4.21 | 4.09   | 1.11  |
| Phe¹⁷   | 122.47  | 8.36 | 4.58 | 3.13/3.02 |       |
| Ser¹⁸   | 119.99  | 8.21 | 4.33 | 3.86/3.72 |       |
| Asp¹⁹   | 122.32  | 8.39 | 4.54 | 2.58   |       |
| Leu²⁰   | 121.25  | 8.03 | 4.08 | 1.53   | 1.41  |
| Trp²¹   | 115.73  | 7.34 | 4.49 | 3.30   |       |
| Lys²²   | 120.49  | 7.61 | 3.99 | 1.85   | 1.66  |
| Ser²³   | 119.73  | 8.89 | 4.18 | 1.97   | 2.18  |
| Asn²⁴   | 118.97  | 8.34 | 4.74 | 2.78/2.65 |       |
| Asn²⁵   | 121.88  | 8.33 | 4.72 | 2.81/2.70 |       |
| Val²⁶   | 120.49  | 8.16 | 4.07 | 2.07   | 0.93  |
| Ser²⁷   | 125.98  | 8.44 | 4.39 | 1.62   | 1.62  |
| Asp²⁸   | 118.51  | 8.39 | 4.72 | 3.85/3.77 |       |
| Leu²⁹   | 124.15  | 8.47 | 4.57 | 1.58   | 1.58  |
| Ser³⁰   | 116.07  | 8.49 | 4.37 | 3.86   |       |
| Glu³¹   | 121.56  | 8.65 | 4.21 | 2.04/1.93 | 2.30 |
| Ala³²   | 125.98  | 8.47 | 4.29 | 1.36   |       |
| Met³³   | 128.87  | 8.48 | 4.46 | 2.08/2.01 | 2.60 |
| Asp³⁴   | 120.03  | 8.57 | 4.54 | 2.71/2.57 |       |
| Leu³⁵   | 122.01  | 8.20 | 4.23 | 1.67   | 1.58  |
| Trp³⁶   | 121.49  | 7.99 | 4.03 | 1.83   | 1.37/1.12 |
| Ser³⁷   | 118.66  | 8.64 | 4.77 | 3.87   |       |
| Asp³⁸   | 119.27  | 8.33 | 4.54 | 2.69/2.53 |       |
| Asp³⁹   | 120.34  | 8.23 | 4.59 | 2.66   |       |
| Leu⁴⁰   | 120.49  | 8.40 | 4.32 | 1.88   | 2.18  |
| Thr⁴¹   | 116.99  | 8.09 | 4.24 | 4.07   | 1.15  |
| Glu⁴²   | 123.69  | 8.43 | 4.24 | 2.01/1.89 | 2.24 |
| Asp⁴³   | 123.84  | 8.60 | 4.84 | 2.73/2.53 |       |
| Gly⁴⁴   | 128.87  | 8.49 | 4.07 |       |       |
| Pro⁴⁵   | 121.25  | 8.36 | 4.54 | 2.63   |       |
| Asp⁴⁶   | 127.04  | 8.42 | 4.54 | 1.36   |       |
| Asp⁴⁷   | 122.32  | 8.61 | 4.31 | 1.78/1.72 | 1.62 |
| Met⁴⁸   | 123.84  | 8.66 | 4.81 | 2.04/1.96 | 2.65/2.57 |
| Pro⁴⁹   | 121.25  | 8.70 | 4.35 | 2.13/1.96 | 2.40 |
| Ala⁵⁰   | 125.67  | 8.45 | 4.27 | 1.37   |       |
| Ala⁵¹   | 125.37  | 8.45 | 4.57 | 1.35   |       |
| Pro⁵²   | 124.30  | 7.86 | 4.00 | 2.05   | 0.91  |
helix and the turns (TAD spectrum shown in Fig. 4). Existence of a similar preexisting helix has been noted in the pKID (28). The relative intensities of the sequential d_{NN} and d_{NN} NOEs show that the preexisting helices found in the full-length p53 TAD and in the pKID (28) are as helical as the bound-state or induced amphipathic helix formed by the VP16 TAD fragment upon binding to TAF_{p}31 (26) or that in the mdm2-bound state of a short p53 TAD fragment (16). The preexisting helix in the p53 TAD is as highly amphipathic as the mdm2-induced helix (7, 16) and thus has a hydrophobic surface formed by Phe^{28}, Leu^{22}, Trp^{23}, Leu^{25}, and Leu^{26}. A potential helix-stabilizing salt bridge exists between the side chains of Asp^{22} and Lys^{24}. In addition to the preexisting amphipathic helix, the unbound full-length p53 TAD seems to contain two nascent turns, one formed by the residues Met^{40}–Met^{44} (turn I) and the other by Asp^{18}–Trp^{23} (turn II), even though evidence for the presence of these turns is somewhat weaker than for the helix. Interestingly, these turns are weakly amphipathic; their hydrophobic surfaces are formed by Met^{40}, Leu^{43}, and Met^{44} and by Ile^{50}, Trp^{53}, and Phe^{54}, respectively. Because the TAD-binding pocket in mdm2 is hydrophobic (7), these turns may have a potential to bind to mdm2.

**DISCUSSION**

Clearly, AADs in the unbound state do not have any discernible tertiary structure (9, 11–14). Accordingly, the aim of the current investigation was not to determine the three-dimensional structure of the unbound full-length p53 TAD but to delineate the details of its local structural elements using heteronuclear multidimensional NMR spectroscopy. We have found a preexisting amphipathic helix and two nascent turns in the unbound full-length p53 TAD. Because the preexisting helix is almost identical to the induced helix, the structural transition associated with this region of the p53 TAD upon target binding would not be coil-to-helix type but would just involve tightening of the preexisting helix into a stable helix. A short fragment of the p53 TAD encompassing residues Leu^{14}–Glu^{28} was able to form, even in the absence of a target protein, a structure with two contiguous β-turns at low temperature, at which backbone dynamics of the peptide is reduced (27). Similarly, reduction in backbone flexibility of the residues Leu^{14}–Glu^{28} due to neighboring residues in the full-length p53 TAD seems to be sufficient to cause conversion of the two β-turn structure into a helix even at ambient temperature. Despite the belief that an amphipathic helix is necessary for the transcriptional activity of TADs (18), obtaining unequivocal evidence for the presence of such a structure in the unbound forms of TADs turned out to be difficult. The induced helices were found to form for short fragments of TADs as a result of target binding (7, 16, 26), but preexisting helices are found in the absence of target proteins for a longer fragment of a TAD (28) or in full-length TADs (29, this work). Because the specificity determinants for TAD-target interactions are features that probably exist a priori and facilitate TAD-target recognition, the secondary structures found in this study and by others (28, 29) may constitute such determinants that act as “hydrophobic antennae,” which may become more structured in the course of target binding. From the structural viewpoint, the unbound full-length p53 TAD is closer to a “molten globule” (55, 56) or a “compact intermediate” (57) than to acid blobs.

As expected, addition of a helix-promoting solvent makes the p53 TAD more helical (9). Here, we have studied the structure of the p53 TAD in aqueous solution. Because the TAD-binding pocket in target proteins is known to be hydrophobic (7, 16, 26), state of the p53 TAD in order to distinguish it from the induced helix that forms upon target binding.

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2 We use the term “preexisting” for the helix found in the unbound form of a TAD.
the observed helices in the nonphysiological solvent condition could well be relevant to the transcriptional activity (23, 28) despite the fact that such an extrapolation may not be valid in general. We show in Fig. 2 that some NOEs are associated with the secondary structures delineated. This result suggests that conformational changes of a TAD upon target binding may be global rather than being locally restricted to the helical region, so that the target-bound full-length p53 TAD is much more structured than the unbound counterpart. One possible structural change is conversion of the two nascent turns found in the unbound state into an amphiphatic helix due to structural restraints imposed by a target protein. The fact that overall helical content of several AADs increases upon addition of hydrophobic solvents (9, 19, 20, 38, 39) that might mimic the hydrophobic environment a target protein provides supports this possibility. The other possibility is formation of two long helices formed by residues Ser15–Leu32 and Gln38–Asp57 with a symmetric Ser33-Pro34-Leu35-Pro36-Ser37 linker between the two helices. Such a speculation is supported by the fact that mutation of two prolines, which are neither acidic nor conserved hydrophobic residues, to hydrophilic residues results in significant (∼25%) activity loss (9).
Amide protons are assigned except for 14H2O. Near 109.5 ppm instead of 128.87 ppm.

The full-length p53 TAD can be divided into two synergizing subdomains, an N-terminal subdomain consisting of residues 1–42 and a C-terminal subdomain containing residues 43–73; the transcriptional activity of an intact p53 TAD is higher than the sum of the individual activities of the two subdomains (9). Because the TAD-target binding almost entirely relies on hydrophobic steric fit (7–9, 16, 26), not only the N-terminal subdomain containing the strongly amphipathic helix but also the C-terminal subdomain containing weakly amphipathic turns is expected to be able to bind to a same target protein. The fact that binding affinity of a p53 TAD fragment (residues 1–57) to mdm2 is higher by ~50% than that of a shorter fragment (residues 15–29) is consistent with such a prediction (7). The N-terminal subdomain, having an amphipathic helix, should bind more efficiently and produce stronger activity than the C-terminal subdomain that contains a nascent turn (note that neither subdomain contains the turn I formed by residues Met40–Met44 due to the particular way the full-length p53 TAD is split). Remarkably, this is precisely what was observed (9). The N-terminal subdomain of the p53 TAD (~35% of the full activation domain) is ~6 times more potent than the C-terminal subdomain (~6% of the full activation domain). The transcriptional potency of a fragment containing only the turn I is unknown, but it would be similar to that of the C-terminal subdomain as both turn I and turn II are weakly amphipathic. Therefore, the synergistic transcriptional activity of the p53 TAD appears to be “intramolecular,” meaning that multiple hydrophobic surfaces touch a single target protein, as opposed to “intermolecular” synergism, in which more than one TAD is involved (10, 11, 14). Conceivably, the fraction of “ligand-bound” target protein would be higher when a target protein interacts with the full-length p53 having multiple hydrophobic surfaces than when it interacts with a subdomain. The interesting fact that the local amphipathic secondary structures in the unbound p53 TAD are found at positionally conserved locations strongly suggests that presence of multiple clusters of hydrophobic residues (hydrophobic antennae) observed in several AADs (9, 17, 19, 22, 23, 58, 59) may not be a mere coincidence. The VP16 TAD (19, 59) and the Rta TAD in Epstein-Barr virus (58) also have multiple clusters of hydrophobic residues, which can be divided into subdomains, and exhibit synergism similar to that of the p53 TAD. Thus, the preexisting minimal secondary structures found in the p53 TAD may not be unique for the p53 TAD but may also exist in other AADs.

As was suggested earlier (12), a less traditional perspective on protein structure may be needed to understand the relationship between transcriptional activity and the “unstructured” structure of TADs. Our study has shown that one aspect of such a new perspective might be associated with careful characterization of minimal secondary structures in proteins from which tertiary structure is absent. In other intrinsically unstructured proteins the loosely folded structures are indeed necessary or even optimal for their function (30, 31). Similarly, one may speculate that being loosely folded is perhaps advantageous for TADs to carry out their promiscuous function, i.e. binding to diverse, not one specific, target proteins (10, 11). Undoubtedly, hydrophobic interactions are most critical for TAD-target interactions (7–9, 16, 17, 26, 58–60). Yet other interactions, such as electrostatic interactions, are utilized as well for TAD-target binding (60). Furthermore, the structural details of the amphipathic helices found in the p53 TAD fragments (7, 16) and in the VP16 TAD fragment (26) are not exactly same, suggesting the possibility that such subtle structural differences may confer some specificity to different TADs despite the common hydrophobic principle.

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