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Refolding and structural characterization of the human p53 tumor suppressor protein

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Abstract

The human tumor suppressor p53 is a conformationally flexible and functionally complex protein that is only partially understood on a structural level. We expressed full-length p53 in the cytosol of *Escherichia coli* as inclusion bodies. To obtain active, recombinant p53, we varied renaturation conditions using DNA binding activity and oligomeric state as criteria for successful refolding. The optimized renaturation protocol allows the refolding of active, DNA binding p53 with correct quaternary structure and domain contact interfaces. The purified protein could be allosterically activated for DNA binding by addition of a C-terminally binding antibody. Analytical gelfiltration and chemical cross-linking confirmed the tetrameric quaternary structure and the spectroscopic analysis of renatured p53 by fluorescence and circular dichroism, suggested that native p53 is partially unstructured.

Keywords: p53; Cancer; Renaturation; Protein folding; Loosely folded protein

1. Introduction

The p53 tumor suppressor protein is a sequence-specific DNA binding protein that functions to maintain genome integrity. Loss of its normal function by mutation is a key molecular event in the development of tumors, which can be detected in >50% of all human cancers. The protein is activated upon various stresses, i.e. DNA damage and enhances the transcription of a network of genes involved in the regulation of cell cycle arrest and apoptosis [1–3].

Regulation occurs mainly via phosphorylation, dephosphorylation and acetylation in the N- and C-terminal regions of the p53 protein [4–8] and the binding of the oncoprotein Mdm2 to the N-terminal transactivation domain of p53. Mdm2 binding targets p53 for degradation by the ubiquitin–proteasome pathway and ensures the apparent low cellular stability of p53 [9–11].

The p53 tumor suppressor protein is a flexible protein that seems to exist in two conformations in the cell. Conformation-sensitive monoclonal

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antibodies have led to the identification of two mutually exclusive conformations of p53 (wild-type and mutant p53) which differ in activity, association with heat shock proteins and stability [12–15,75]. The wild-type conformation is active for DNA binding whereas the mutant conformation is inactive for DNA binding [75].

The p53 protein can be divided into at least four functional domains. The N terminal region (residues 1–94) is an acidic transcriptional activation domain, which seems to be unfolded prior to binding to the transcription activation complex [16,17]. The DNA binding domain of p53 has been localized to the center of the protein (residues 102–292) [15,18], whose crystal structure in complex with DNA has been determined [19]. Most of the oncogenic mutations can be mapped to the core domain and lead to a thermodynamic destabilization of this domain [20]. Six so-called hot-spot mutations in the core domain do not influence the overall tertiary fold compared to that of the wild-type, but lead to characteristic local structural changes as detected by NMR spectroscopy [21]. The conformation of this domain is essential for p53 activity.

Human and murine p53 exist predominantly as tetramers of unusual shape and to a smaller extent as higher oligomers in solution [30,48,62,63]. It has been shown that p53 binds to its specific DNA consensus sequence (PuPuPu-C(A/T)(T/A)G-PyPyPy)$_2$ as a tetramer, each monomer interacting with five base pairs of the consensus sequence [22–24]. The structure of the p53 tetramerization domain (residues 326–353) has been determined by both NMR spectroscopy and X-ray crystallography [25,26]. The tetramerization domain is a dimer of dimers and was found to be very resistant to thermal unfolding at physiological pH and salt concentration [27]. Folding and unfolding of the tetramerization domain is a reversible process, without the formation of stable intermediates [28]. An analysis of its folding pathway established, however, the existence of a transient, highly structured dimeric intermediate [29]. The last 30 C-terminal amino acids comprise a basic negatively regulatory domain of p53, which is a target of various acetylation and phosphorylation processes [2,7,30]. These modifications regulate p53 activity. Bacterially expressed, post-translationally unmodified p53 is latent for specific DNA-binding. The conversion of latent p53 into active p53 can be achieved by several means, all targeting the regulatory C-terminal domain: (i) by phosphorylation, (ii) by acetylation, (iii) by DnaK binding, (iv) by antibody binding at the basic C-terminus (PAb 421 or PAb 122), (v) by deletion of the last 30 amino acids or (vi) by a C-terminal peptide [30–32].

In comparison to the core and the tetramerization domain of p53, there is only a limited amount of structural data available for the full-length p53 protein. Comprehensive studies of the structure–function relationship clearly demand sufficient quantities of protein in a homogeneous and native form. The absence of eucaryotic post-translational modifications in *Escherichia coli* make bacterial expression systems an excellent source of unmodified p53. However, soluble expression and purification of p53 in bacteria have proven difficult and only low yields have been obtained so far [30,33]. High-level expression of recombinant proteins in *E. coli* often results in the accumulation of insoluble inclusion bodies (IBs). The formation of these inclusion bodies can be attributed to the insufficient capacity of the folding machinery of the cell during the overexpression of the recombinant protein. Usually, IBs are homogenous and the protein of interest can be extracted and isolated easily. In order to obtain native protein, several refolding conditions (temperature, pH, buffer mixture) have to be optimized to achieve biologically active protein [34–36].

In this study, we report the solubilization and renaturation of full-length, wild-type human tumor suppressor protein p53. The renaturation strategy was optimized by variation of physicochemical parameters, in order to achieve a maximum yield of active tetrameric p53 protein. Renatured p53 protein was purified to homogeneity and was characterized in comparison to wild-type protein regarding structure, DNA-binding activity and oligomeric status.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany) or

ICN (Meckenheim, Germany). Ampicillin and 3-(1-Pyridinio)-1-propanesulfonate (PPS) were obtained from Sigma (St. Louis, USA). Chromatography materials were from Amersham Pharmacia (Uppsala, Sweden).

2.2. Expression of human p53

The *E. coli* strain BL21(DE3) containing the plasmid pET3a-p53 [33] was used for the expression of human wild-type p53. Fermentation was carried out in Luria Broth (LB) medium with ampicillin (100 μg/ml) at 37 °C and at a constant pH of 7.2. Recombinant protein expression was induced at an $OD_{578\ nm}$ of 0.6 by addition of 1 mM isopropyl-β-D-thiogalactopyranosid (IPTG). During fermentation, samples were collected at regular intervals to check for protein expression by SDS-PAGE. Three and a half hours after induction, cells were harvested by centrifugation and stored at −70 °C.

2.3. Isolation and solubilization of inclusion bodies

The inclusion body preparation of the cell pellet was performed according to Rudolph et al. [37]. Briefly, the cell pellet was resuspended in 0.1 M Tris–HCl, 1 mM EDTA, pH 7 at 4 °C. Disruption of the cells was carried out by incubation with lysozyme (1.5 mg/g cells, 30 min, 4 °C) and high-pressure homogenization. In order to digest DNA, 10 μg/ml DNase and 3 mM $MgCl_2$ were added and the lysate was incubated for 30 min at 25 °C. Then, 0.5 vol. of 60 mM EDTA, 6% Triton X-100, 1.5 M NaCl, pH 7 was added and the mixture was incubated for 30 min at 4 °C. The inclusion bodies (IBs) were harvested by centrifugation at 31 000×*g* for 10 min. To further remove cell debris and other proteins, the IB pellet was washed again with 0.1 M Tris–HCl pH 7, 30 mM EDTA, 3% Triton X-100, 0.8 M NaCl and after centrifugation, a washing step with 0.1 M Tris–HCl pH 7, 20 mM EDTA was performed. The pellet was stored at −70 °C.

For solubilzation, the IB pellet was resuspended in a small volume of 100 mM Tris–HCl, 6 M GuanidiniumCl (GdmCl), 50 mM DTT, pH 8. After stirring at room temperature for 2 h, the pH was shifted to pH 2 by the addition of 1 M HCl. Insoluble material was removed by centrifugation (30 000×*g*, 30 min). After estimating the protein concentration by the Bradford assay [38], the solubilized inclusion bodies were stored in aliquots at −70 °C.

2.4. Refolding of p53 inclusion bodies

Renaturation was initiated by a rapid 100- or 150-fold dilution of the solubilized protein into refolding buffer. If not indicated otherwise, the buffer contained 50 mM sodium diphosphate (NaPPhos), 1 M L-arginine, 2 mM DTT, 0.2 mM $ZnCl_2$, pH 8. In the analytical experiments the protein concentration was 75–100 μg/ml. The samples were incubated for 10 h at 15 °C or as indicated. Afterwards, the solution was dialyzed overnight against 50 mM NaPPhos, 4 mM DTT, 5% (v/v) glycerol, pH 8 at 4 °C. To remove aggregates formed during refolding and/or dialysis, the dialyzed samples were centrifuged (at 30 000×*g*) for 30 min at 4 °C. Prior to analysis or purification, the renatured proteins were precipitated in the presence of 50% ammonium sulfate and resuspended in 1/5 vol. of dialysis buffer.

For a large-scale production, p53 was renatured in 200 ml 50 mM NaPPhos, 1 M L-arginine, 2 mM DTT, 0.2 mM $ZnCl_2$, pH 8 at 15 °C. To increase the volume yield, the pulse renaturation method (solubilisate was added to the renaturation buffer six times with 150 μg/ml per step and an interval of 90 min between each addition [39]) was applied and the final protein concentration was 900 μg/ml. After dialysis against 50 mM NaPPhos, 5% (v/v) glycerol, 4 mM DTT, pH 8, the protein solution was centrifuged for 1 h at 4 °C with 30 000×*g*.

2.5. Purification of renatured p53

After dialysis and centrifugation, the renaturation solution was loaded onto a Heparin Sepharose CL-6B column (Amersham Pharmacia), equilibrated in 30 mM NaPPhos, 100 mM KCl, 3% (v/v) glycerol, 4 mM DTT, pH 7.5. The protein was eluted by a linear KCl gradient. Fractions active for DNA binding were pooled, concentrated by

50% ammonium sulfate precipitation and loaded onto a Superose 6 gel filtration column (Amersham Pharmacia), equilibrated in 30 mM NaPPhos, 300 mM KCl, 3% (v/v) glycerol, 4 mM DTT, pH 7.5. Fractions active for DNA binding were pooled, concentrated by 50% ammonium sulfate precipitation and re-loaded onto the same column in order to remove all oligomeric p53 species. Fractions with pure and tetrameric protein were collected, concentrated, dialyzed against 30 mM NaPPhos, 50 mM KCl, 5% (v/v) glycerol, 2 mM DTT, pH 7.5 at 4 °C and stored in aliquots at −70 °C. The concentration was determined by UV spectroscopy using the extinction coefficient of 0.785 for a 1-mg/ml solution in a 1-cm cuvette at 280 nm, calculated according to Gill and von Hippel [40].

2.6. DNA binding assay (*EMSA*)

The specific oligonucleotides containing the 20-mer p53 consensus DNA binding site (PG; [23]) with HindIII-compatible ends were: 5′-AGCTTA-GACATGCCTAGACATGCCTA-3′ and 3′-ATCTGTACGGATCTGTACGGATTCGA-5′. The complementary oligonucleotides were end-labeled with [γ-^{32}P]-ATP, as described by Sambrook et al. [41] and annealed. Unincorporated nucleotides were removed by MicroSpin G25 columns (Amersham Pharmacia). The DNA binding buffer contained 15% (v/v) glycerol, 40 mM Hepes, 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mg/ml BSA, 5 mM DTT, 50 mM KCl, pH 8. Renatured protein (50–400 ng) was incubated in DNA binding buffer with 5–10 ng of labeled, specific, double-stranded p53 consensus-site oligonucleotide and 100 ng of supercoiled, unlabelled competitor DNA (p-Bluescript II SK+, Stratagene). Activating antibody PAb 421 (Calbiochem) was added to a final concentration of 200 ng per reaction. The reaction mix was loaded on a 4% native polyacrylamide gel and separated at 175 V for 2 h at 4 °C. The running buffer consisted of 0.33×TBE (30 mM Tris–HCl, 30 mM boric acid, 0.5 mM EDTA, pH 8.4) with 0.1% Triton X-100. DNA was detected and quantified using a PhosphorImager (Molecular Dynamics).

2.7. Cross-linking by Glutaraldehyde

Purified renatured p53 (0.3 mg/ml in 25 mM Hepes, 50 mM KCl, 2 mM DTT, pH 8) was incubated with increasing amounts of glutaraldehyde/water (final concentrations from 0 to 0.1%) for 3 min at 37 °C (reaction volume: 25 μl). The cross-linking reaction was stopped by addition of excess Tris–HCl. Samples were analyzed by 7.5% SDS-PAGE according to Laemmli [42].

2.8. Size exclusion chromatography by HPLC

Analytical gelfitration was performed using a TosoHaas TSK3000SW column or a Phenomenex BioSep SEC-3000 column. Gelfiltration runs were carried out at 18 °C in 25 mM Hepes, 200 mM KCl, 2 mM DTT, pH 8 with a flow rate of 0.75 ml/min. Protein was detected by fluorescence emission at 330 nm (excitation at 280 nm) using a JASCO FP-920 fluorescence detector.

2.9. Spectroscopic techniques

Far-UV-circular dichroism (CD) measurements were performed in a J-715 spectropolarimeter with a PTC343 peltier unit (Jasco, Tokyo, Japan). After dialyzing the protein overnight against 20 mM NaPPhos, 1.5 mM TCEP, 1 mM $ZnCl_2$, pH 7.5, the sample was centrifuged and the concentration was determined by UV spectroscopy. CD spectra were recorded from 250 to 196 nm for the native protein and from 250 to 210 nm for the denatured protein, at a constant temperature of 15 °C in a 0.1-cm quartz cuvette. The spectra were accumulated 12 times with an integration time of 0.5 s. All spectra were corrected for buffer contributions and converted to mean residue weight ellipticity, according to Schmid [43]. For secondary structure analysis, the program JFIT was applied which uses poly-L-lysine model data [44].

The fluorescence of p53 was determined using a Spex FluoroMax-2 fluorimeter and 1-cm quartz cuvette at 15 °C. The excitation wavelength was set to 280 nm. Spectra were recorded from 295 to 400 nm. The spectral bandwidth was 5 nm for both excitation and emission. All spectra were corrected for buffer contributions. Prior to the

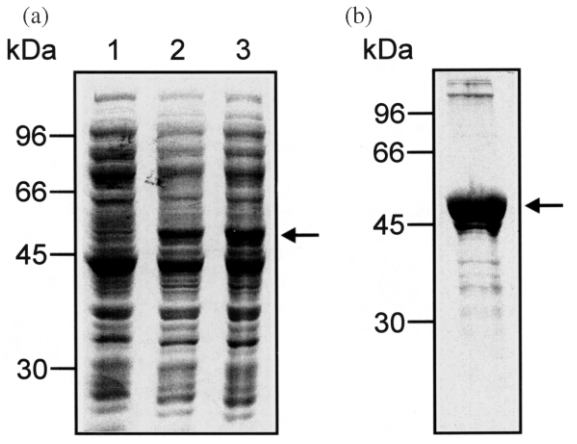

Fig. 1. SDS-PAGE of *E. coli* bacterial extracts and human wild-type p53. SDS-PAGE was carried out according to Laemmli [42] with a 10% polyacrylamide gel. (a) lane 1, total cell lysate obtained before induction; lane 2, cell lysate 1 h after induction; lane 3, cell lysate 5 h after induction. The arrow indicates the p53 protein. (b) solubilized inclusion body proteins.

experiment, p53 was dialyzed overnight at 4 °C, centrifuged and protein concentration was determined by UV spectroscopy.

The UV spectrum of renatured p53 was determined from 240 to 400 nm in a JASCO V-550 UV/Vis spectrophotometer at 18 °C.

3. Results

3.1. Expression and refolding of human p53

p53 was expressed in *E. coli* with a yield of approximately 5% of the total protein (Fig. 1a). SDS-PAGE analysis of soluble and insoluble protein fractions showed that p53 was deposited to a large extent in inclusion bodies (IBs). At a temperature of 37 °C, the protein was produced almost exclusively in an insoluble form. Lowering the temperature to 22 °C led to an increase in the production of soluble protein to approximately 10–20% of recombinant p53. Since the amount of soluble protein was low and prone to proteolytic digestion during expression, expression as IBs was preferred. IBs have a relatively high density and can therefore be separated from the soluble protein fraction and cell debris by centrifugation after cell disruption. The IBs were solubilized in the presence of the strong denaturing reagent GdmCl and the reducing agent DTT. Approximately 80% of total protein content of the IBs was shown to be p53 (Fig. 1b).

To obtain native protein, a refolding strategy was developed; p53 is a multi-functional protein, made up of several domains with different functions. To follow the folding and association of p53 during renaturation we chose two functions of the protein to assay the refolding yields quantitatively: quaternary structure and specific DNA binding activity. The quaternary structure of the refolded protein was analyzed by HPLC size exclusion chromatography. DNA binding and activation of the refolded protein was tested by an electrophoretic mobility shift assay (EMSA), using a C-terminally binding and activating antibody.

Refolding of p53 was initiated by the dilution of solubilized protein into renaturation buffer. All buffers contained Zn^{2+}-ions, because each p53 monomer binds one Zn^{2+}-ion in its core domain [19]. The Zn^{2+}-ion is coordinated by one histidine and three cysteine residues. To keep the cysteines reduced during refolding a small amount of reducing agent was added to all buffers.

Firstly, we analyzed aggregation during renaturation of p53 by turbidity (Fig. 2). Depending on the renaturation buffer there was almost complete suppression of aggregation, (e.g. 1 M-arginine), limited amount of aggregation (0.1 M Tris–HCl) or strong aggregation (50 mM Hepes). Interestingly, the aggregation process is very fast and happens in the minute time range, implying the existence of early intermediates sensitive for aggregation.

As can be seen in Table 1, we analyzed a large variety of renaturation buffers containing different components or additives for p53 DNA binding activity and tetrameric protein. Only such buffers that suppressed aggregation reactions effectively (-arginine, PPS, urea) led to the formation of p53 species active in DNA binding (Fig. 3). A first step to optimize refolding was the variation of the concentration of these additives. The best results were achieved either by 1 M L-arginine, 2 M urea or 1.3 M PPS. These substances are known to labilize misfolded or improperly associated molecules and thereby suppress their aggregation [45].

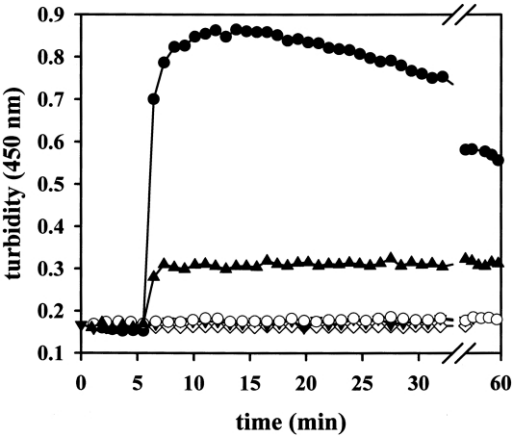

Fig. 2. Aggregation of the p53 protein during refolding. Turbidity of the refolding solution at 450 nm is shown indicating the aggregation of proteins at the initial stage of the renaturation. Solubilized inclusion bodies were diluted 1:200 at 15 °C to a final concentration of 100 μg/ml in 1 M L-arginine pH 8 (◇), 50 mM NaPPhos pH 8 (▼), 50 mM Hepes pH 8 (●), 1 M Tris–HCl pH 8 (○), 0.1 M Tris–HCl pH 8 (▲). The turbidity was measured immediately after the refolding reaction was initiated and followed for one hour.

Both the DNA binding activity and the oligomeric state of refolded p53 correlated well in this screen, indicating that active and tetrameric p53 protein was obtained.

3.2. Optimization of refolding conditions

The pH is an important parameter influencing the stability of folding intermediates and the state of thiol groups of cysteines. In p53, all cysteines are in the reduced state. This is favored by acidic to neutral pH values. However, the stability and association of the protein might demand a more alkaline pH. To determine the optimum pH range for p53 renaturation, we varied the pH between 6.0 and 9.0 (Fig. 4a). No reactivation was observed under acidic conditions, as measured by DNA binding. Renaturation yields increased with increasing pH reaching an optimum at approximately pH 8.0. At more alkaline pH the yield decreased slightly.

Aggregation reactions are one of the main obstacles during refolding of proteins. They depend on the concentration of the folding protein and the temperature [46]. As aggregation seemed to be a major side reaction in the refolding of p53, we analyzed the renaturation reaction under two buffer conditions at various temperatures. Maximum renaturation yields were achieved between 15 and 20 °C, according to DNA binding activity analysis (Fig. 4b). At temperatures below 15 °C there was a slight decrease in yield, whereas at higher temperatures DNA binding activity decreased strongly and aggregation predominated.

Like temperature, the protein concentration is an important factor in the partitioning between active and aggregated protein during refolding. Aggregation processes are of a reaction order >2 and therefore the concentration of non-native polypeptide is of critical importance [47]. In order to determine the concentration with the highest yield of active p53, we analyzed the renaturation reaction at different protein concentrations. Maximum yields were obtained between 25 and 200 μg/ml (Fig. 4c). Interestingly, the amount of aggregated protein increased steadily in this concentration range, correlating well with a higher order reaction. Concentrations above 200 μg/ml led to a sharp decrease in active protein and to a high level of aggregation.

3.3. Kinetics and pulse renaturation

After dilution into renaturation buffer, the aggregation of p53 occurs rapidly (within minutes), implying the existence of an early aggregation-sensitive intermediate. However, reactivation of p53 is completed in approximately 2 h, as measured by the appearance of DNA binding activity (Fig. 4d). The kinetic of reactivation is concentration-dependent, with a doubling of the protein concentration leading to almost a doubling in the apparent reaction rate. This indicates that the association to a tetramer might be a critical step in the refolding of p53. To increase the volume yield, it should be possible to add denatured protein in a stepwise fashion, allowing each aliquot to refold to active protein before the next one is added [39,46]. Based on our experiments, the time between pulses was set to 90 min at a protein concentration of 150 μg/ml. Surprisingly, denatured p53 could be added six times with an overall

Table 1
Screen of different renaturation buffers for DNA binding activity

Renaturation buffer	DNA binding activity (%)
50 mM MOPS pH 8	1
50 mM Hepes pH 8	0
50 mM sodium phosphate pH 7.5	4
10 mM Tris–HCl pH 8	0
100 mM Tris–HCl pH 8	4
1 M Tris–HCl pH 8	47
30 mM NaPPhos pH 8	22
50 mM NaPPhos pH 8	51
1 M L-arginine/HCl pH 8	95
50 mM NaPPhos pH 8, 1 M L-arginine	**100**
40 mM NaPPhos pH 8, 0.5 M PPS	82
40 mM NaPPhos pH 8, 1 M PPS	85
40 mM NaPPhos pH 8, 1.3 M PPS	**100**
50 mM NaPPhos pH 8, 1 M Urea	59
50 mM NaPPhos pH 8, 1.5 M Urea	65
50 mM NaPPhos pH 8, 2 M Urea	82
50 mM NaPPhos pH 8, 2.5 M Urea	59
50 mM NaPPhos pH 8, 3 M Urea	58
50 mM NaPPhos pH 8, 3.5 M Urea	11
50 mM NaPPhos pH 8, 9 mM β-CD	45
50 mM NaPPhos pH 8, 15 mM M-β-CD	30
30 mM NaPPhos pH 8, 6 mM CTAB/18 mM M-β-CD	5

NaPPhos: sodium diphosphate; β-CD: β-Cyclodextrin; M-β-CD: Methyl-β-Cyclodextrin; PPS: 3-(1-Pyridinio)-1-propanesulfonate.

yield corresponding to that of six separately performed renaturations (Fig. 4e). Increasing the number of additions further had only a small effect on the yield of active protein, probably due to aggregation processes and the high content of denaturing agent.

3.4. Activity of renatured p53

After a large-scale renaturation, p53 was purified to homogeneity by affinity and size-exclusion chromatography (see Section 2). Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry and automated Edman degradation confirmed the molecular mass of 43.7 kDa (monomer) and the identity of the refolded p53 protein, indicating that no modifications had occurred during the production process (data not shown).

The DNA binding activity of renatured and purified p53 protein was tested by EMSA with the p53 consensus sequence. The renatured protein was latent and could be activated for DNA binding by addition of a C-terminal specific antibody [30,31] (Fig. 5). After addition of a large access of unspecific competitor DNA, the DNA binding diminished only slightly, whereas addition of unlabelled consensus sequence abolished the signal to a large degree (data not shown). Taken together, we could show that refolded p53 binds its consensus sequence specifically and is regulated by allosteric activation.

3.5. Quaternary structure of renatured p53

The tetrameric state of p53 is of critical importance for its function in the living cell. Especially, high affinity DNA binding depends on the formation of tetrameric protein. Therefore, the quaternary structure of the purified protein was analyzed by HPLC size exclusion chromatography (Fig.

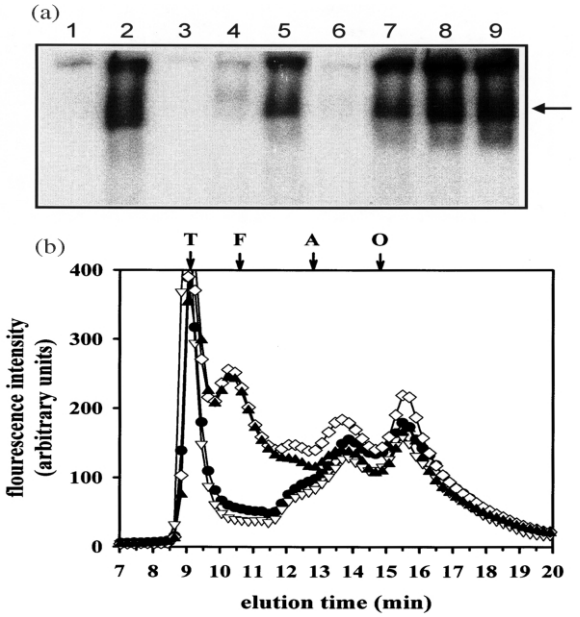

Fig. 3. Analysis of renatured p53 protein from different renaturation buffers for DNA binding and quaternary structure. Refolding was initiated by rapid 1:150 dilution of solubilized p53 into various renaturation buffers, containing 2 mM DTT and 0.2 mM $ZnCl_2$. The final protein concentration was 75 μg/ml. The samples were incubated for 10 h at 10 °C and dialyzed against 50 mM NaPPhos, 5% (v/v) glycerol, 4 mM DTT, pH 8 at 4 °C overnight. After a centrifugation step to remove aggregated protein, the solution was concentrated by 50% ammonium sulfate precipitation by the factor of 5. (a) Approximately 1/50 vol. of the concentrated renaturation solution was subjected to analysis by an electrophoretic mobility shift assay. The upper half of an exemplary electrophoretic mobility shift assay gel using the p53 consensus sequence is shown. Lane 1, control without protein; lane 2, control with wild-type protein; lane 3, 10 mM Tris–HCl pH 8; lane 4, 100 mM Tris–HCl pH 8; lane 5, 1 M Tris–HCl pH 8; lane 6, 50 mM Hepes pH 8; lane 7, 50 mM NaPPhos pH 8; lane 8, 1 M L-arginine pH 8; lane 9, 50 mM NaPPhos, 1 M L-arginine, pH 8. The arrow indicates the p53-DNA complex. (b) Approximately 1/10 vol. of the concentrated renaturation solution was loaded onto a TSK3000SW SEC-column (TosoHaas). The column was run in 25 mM Hepes, 200 mM KCl, 2 mM DTT, pH 8 at 18 °C. Protein was detected by fluorescence emission at 330 nm. Elution profile of renaturation assays in 0.1 M Tris–HCl pH 8 (●), 50 mM Hepes pH 8 (▽), 1 M L-arginine pH 8 (◇) and 50 mM NaPPhos, 1 M L-arginine, pH 8 (▲) as renaturation buffer are shown exemplary. All renaturation buffers contained 2 mM DTT and 0.2 mM $ZnCl_2$. T: thyroglobulin (669 kDa); F: ferritin (440 kDa); A: aldolase (158 kDa); O: ovalbumin (43 kDa).

6a). The apparent molecular mass of 550 kDa is approximately a factor of three larger than the calculated molecular mass of 178 kDa, but this value is in good agreement with published data [48]. Friedman et al. found a similar elution behavior for p53, expressed in baculovirus-infected insect cells. They suggested that this may be caused by the ellipsoid conformation of the p53 tetramer. In order to confirm the tetrameric state of the renatured protein, we performed cross-linking experiments with glutaraldehyde as cross-linking agent. With a saturating amount of glutaraldehyde, only one band with a molecular mass of 190 kDa corresponding to the tetrameric species was found on SDS-polyacrylamide gels (Fig. 6b).

3.6. Characterization of renatured p53

The UV spectrum of purified p53 showed an absorbance maximum at 278 nm and a shoulder at 283 nm, which is typical for a protein containing tyrosine and tryptophane residues (Fig. 7a). The protein preparation is essentially nucleotide-free ($A_{280\ nm}$:$A_{260\ nm}$ = 1.7). At 320 nm, a small absorption was detected, which could not be removed by dialysis.

To gain insight into the secondary and tertiary structure of p53, we recorded far UV-CD and fluorescence spectra of native and chemically denatured protein (Fig. 7b). The fluorescence spectrum of refolded p53 gave a peak at 347 nm. Denaturation with the chaotropic agent GdmCl led to a red shift of the emission maximum to 353 nm, corresponding to the tryptophane signal and a smaller maximum at 306 nm, corresponding to the tyrosine signal.

The far UV-CD spectrum displayed a minimum at 203 nm, with a mean residue ellipticity of approximately $-9000°$ cm^2 $dmol^{-1}$ (Fig. 7c). Minima in the range between 210 and 220 nm, normally indicating α-helices or β-sheets were absent. However, the spectrum of refolded p53 is clearly different from that of the chemically denatured protein. The mean residue ellipticity is in the range of a β sheet protein. Using the JFIT CD analysis program [44], we calculated the secondary structure to consist of 6% α-helices, 34% β-strands

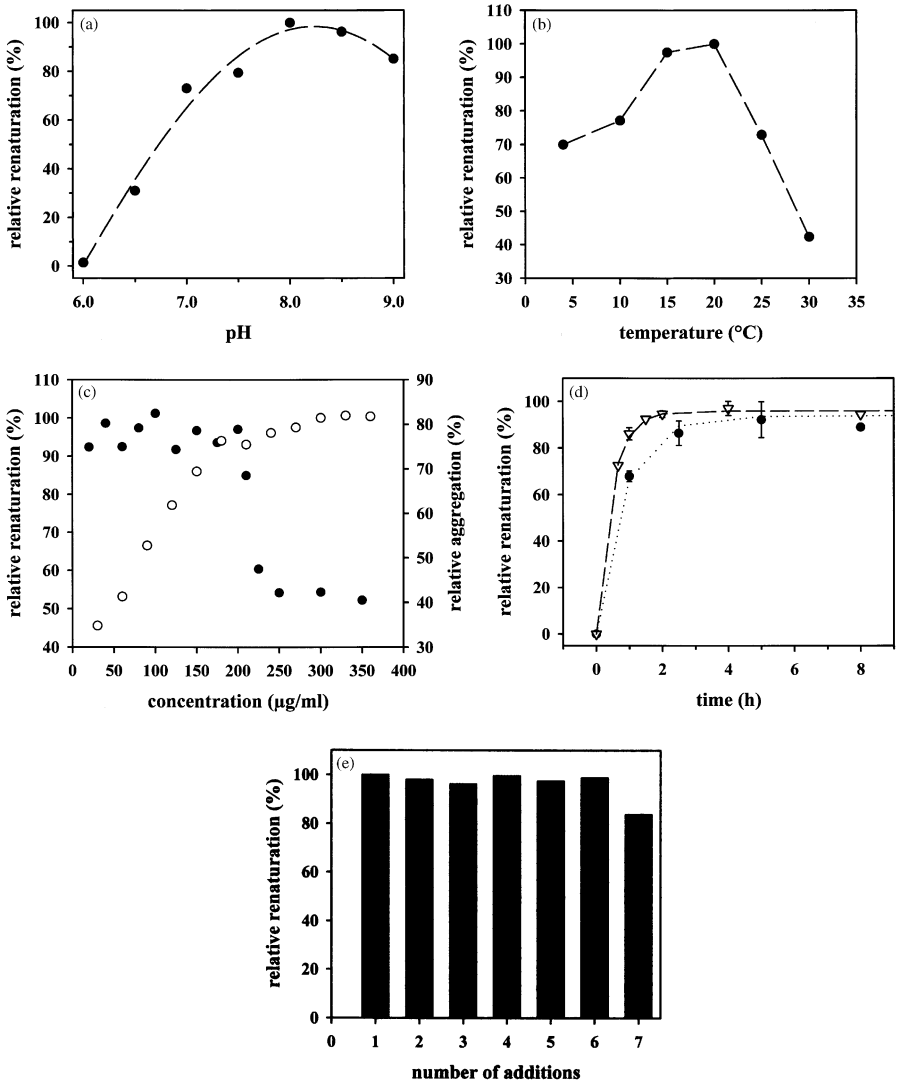

Fig. 4. Optimization of renaturation conditions: refolding was initiated by rapid 1:150 dilution of solubilized p53 into 50 mM NaPPhos, 1 M L-arginine, 2 mM DTT, 0.2 mM $ZnCl_2$, pH 8, to a final protein concentration of 75 μg/ml. After renaturation, the refolding samples were dialyzed, centrifuged, precipitated and the renaturation yield was determined by DNA binding assays (EMSA). The highest activity was set to 100% and the other values are expressed relative to that. (a) pH dependence: refolding was performed as described, with the renaturation buffer adjusted to various pH. (b) Temperature dependence: the renaturation samples were incubated at different temperatures for refolding and then the experiment was continued as described. (c) Concentration dependence: refolding was performed with various protein concentrations at 15 °C. DNA binding activity (●) was measured as described. Aggregation (○) was analyzed by SDS-PAGE to determine the amount of soluble protein after refolding and dialysis. (d) Time dependence: p53 was renatured at 15 °C, with a final protein concentration of 75 μg/ml (●) and 150 μg/ml (▽). After various times, samples were taken, dialyzed overnight and analyzed for DNA binding activity. The data was fitted with a single exponential fit. (e) Pulse renaturation: refolding was started by dilution of the solubilized protein at 15 °C, to a starting protein concentration of 150 μg/ml. This process was repeated six times, yielding a total concentration of 950 μg/ml, with a time interval of 1.5 h between each addition. The refolding yield was quantified for each process by analysis of the DNA binding activity of renatured protein. 100% relative renaturation corresponds to a 3% overall renaturation.

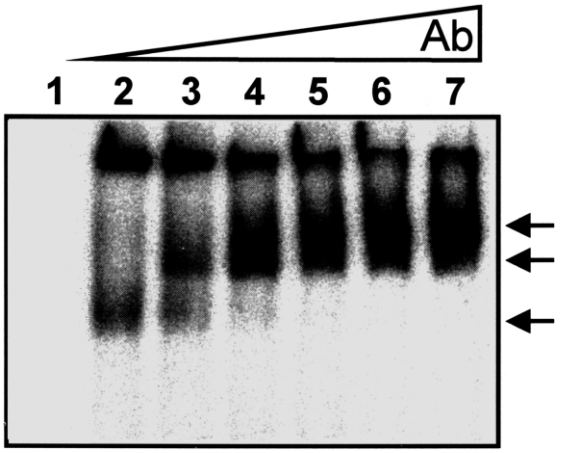

Fig. 5. DNA binding activity of renatured p53 protein. An electrophoretic mobility shift assay (upper half of the gel is shown) using the p53 consensus sequence of purified renatured p53 protein, in the presence of activating antibody PAb 421 was performed. Binding reaction mixtures contained no protein (lane 1), 100 ng p53 with 0 ng PAb 421 (lane 2), 100 ng PAb 421 (lane 3), 200 ng PAb 421 (lane 4), 300 ng PAb 421 (lane 5), 400 ng PAb 421 (lane 6), 500 ng PAb 421 (lane 7). The arrows indicate (from bottom to top) latent p53, p53-tetramer in complex with one antibody molecule and p53-tetramer in complex with two antibody molecules.

and 60% coil structure. So far, only the secondary structure of the p53 core domain, the N-terminal domain and the oligomerization domain is known. The core domain consists mainly of β-sheets, the N-terminal domain (aa 1–73) is devoid of secondary structure and the oligomerization domain (326–353) consists of an α-helix and a β-strand. As the α-helical content is small and β-sheets do not contribute significantly to the ellipticity in comparison to coil structures and α-helices, one can expect that the far UV-CD spectra are strongly influenced by the coil elements.

4. Discussion

4.1. Expression and renaturation of the p53 tumor suppressor protein

p53 plays a central role in orchestrating the decision between life or death of a cell by integrating a number of signals under stress conditions resulting in growth arrest or apoptosis [49]. However, so far little is known on the structure/function relationship of the full-length human protein. We decided to express full-length human p53 in *Escherichia coli* to avoid inhomogeneity due to different levels of phosphorylation and thus

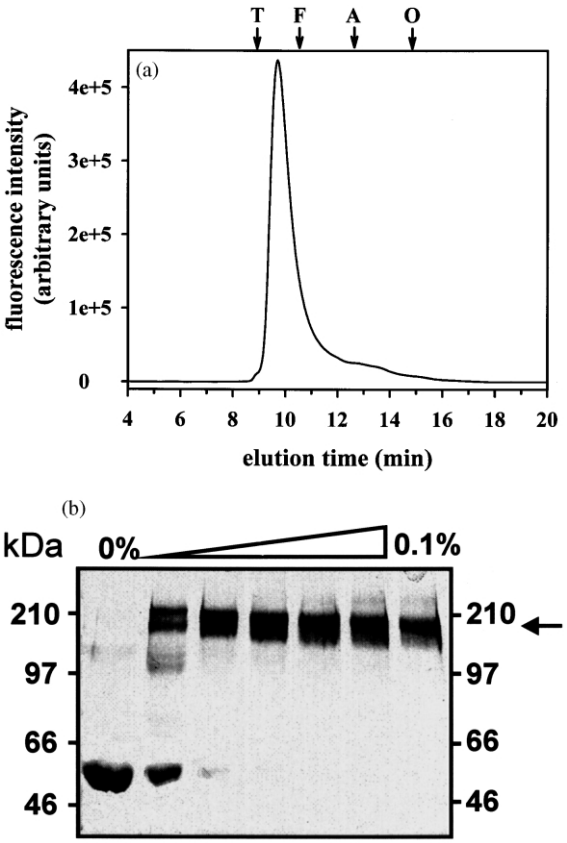

Fig. 6. Quaternary structure of renatured p53: (a) analytical gelfiltration, 100 ng of purified renatured p53 (0.3 mg/ml) were applied to a BioSep SEC-3000 column (Phenomenex). The column was calibrated using LMW and HMW gel filtration calibration kits (Amersham Pharmacia). T: thyroglobulin (669 kDa); F: ferritin (440 kDa); A: aldolase (158 kDa); O: ovalbumin (43 kDa). p53 peaks at 550 kDa. (b) p53 protein (0.3 mg/ml) was cross-linked with increasing amounts of glutaraldehyde (concentrations ranging from 0 to 0.1%) under binding conditions (25 mM Hepes, 50 mM KCl, 2 mM DTT, 0.5 mM EDTA, pH 8) at 37 °C and complexes were analyzed by 7.5% SDS-PAGE. Plotting the log of the molecular masses of the markers against the gel migration of each protein standard gave a standard curve, allowing the determination of the apparent molecular masses of the cross-linked p53 species. The arrow indicates the p53 tetramer.

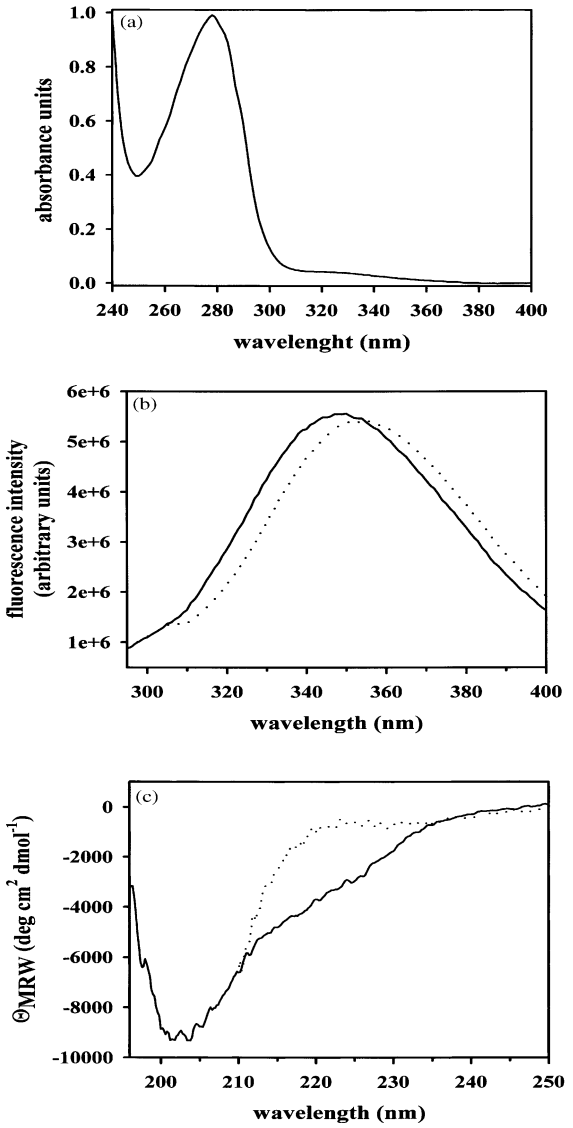

Fig. 7. Spectroscopic characterization of renatured p53: (a) UV spectrum, purified renatured p53 protein in 40 mM Hepes, 50 mM KCl, 2 mM DTT, 0.5 mM EDTA, 5% glycerol, pH 8. (b) Fluorescence spectra of native and denatured p53, fluorescence emission spectra of renatured p53 (25 μg/ml) in 20 mM NaPPhos, 1.5 mM TCEP, 1 mM $ZnCl_2$, pH 7.5 at 15 °C (—), measured with excitation at 280 nm. The protein was denatured in the same buffer for 4 h at room temperature (····). (c) Secondary structure of native and denatured p53: far-UV-CD spectra of renatured p53 (150 μg/ml) were recorded in 20 mM NaPPhos, 1.5 mM TCEP, 1 mM $ZnCl_2$, pH 7.5at 15 °C (—), or in the same buffer with 6 M GdmCl after 4 h incubation at room temperature (····).

conformationally different p53 populations present in eucaryotic expression systems. In *E.coli*, the p53 protein is almost exclusively expressed as insoluble IBs. The deposition of heterologously expressed protein in *E. coli* in IBs is a commonly observed phenomenon, especially for oligomeric, multi-domain proteins [50]. In the last years, several protocols have been developed to recover functional, native protein from inclusion body material [35,46].

To obtain a maximum yield of active recombinant p53 for structural analysis, we varied renaturation conditions using DNA binding activity and oligomeric state as criteria for successful refolding. The renaturation levels were observed to be low due to non-specific protein aggregation. It had been shown previously that aggregation is influenced by several biophysical parameters, especially temperature and protein concentration, and can be suppressed by the addition of low molecular weight molecules. Here, we used L-Arginine and the sulfobetaine PPS [51] to increase the refolding yield. L-Arginine was shown to be very effective as a folding enhancer for several multi-domain proteins [45,46,52–54]. L-arginine reduces unproductive aggregation of unfolded polypeptides or folding intermediates by increasing their solubility during folding [54,55]. Similar effects were proposed for the action of sulfobetaines [51,56].

Interestingly, the renaturation process of p53 displays features observed previously in the refolding of other tetrameric proteins [57–59]. Usually, folding follows a sequential folding-association mechanism with side-reactions of misfolding and misassembly, leading to a kinetic partitioning between correct reconstitution and aggregation [58]. Aggregation is a reaction of higher order and can therefore be minimized by lowering the protein concentration. Interestingly, p53 renaturation yields were identical up to a concentration of 200 μg/ml, despite increasing amounts of aggregated protein. At higher concentrations, aggregation dominated the refolding process and lead to a sharp decline in yield. This reflects the balance between unwanted irreversible aggregation and desired association of p53 monomers to dimers and tetramers. Both reactions are favored by higher concentrations, but only correct association leads

to native DNA binding protein. Similar to the refolding of LDH [57], the renaturation of p53 is concentration-dependent, indicating that tetramerization is a critical and presumably rate-limiting step in the folding process. In the gelfiltration analysis of the refolded protein (Fig. 3b), oligomers consisting of more than four p53 monomers could be detected. They are formed in the renaturation process and had to be removed during the purification of renatured p53 (data not shown). Consistent with this finding, it was shown that different p53 batches or populations from baculovirus expression systems could form higher oligomers [48,60]. Formation of higher oligomers of p53 might be a frequent but unproductive event even in the living cell.

Folding of the tetramerization domain of p53 is fully reversible and proceeds via a highly structured dimeric intermediate that dimerizes to form the native tetramer as a dimer of dimers [29]. The initial association between highly unstructured molecules follows a nucleation–condensation mechanism, whereas the second dimerization step follows the framework mechanism of preformed stable elements [29]. It is very likely that the full-length protein tetramerizes also as a dimer of dimers, but the presence of the other p53 domains complicates the folding process and offers the possibility of non-productive aggregation and misassembly.

Renaturation at low protein concentrations causes severe problems for industrial-scale production because large renaturation volumes are necessary. Compared to the overall refolding kinetics (hours), aggregation of p53 is very rapid (minutes), implying that non-native species of molecules prone to aggregate are formed immediately after dilution of the denatured protein into refolding buffer. Later in the refolding process, folding intermediates seem to be protected from non-specific aggregation, probably because most of the interacting surfaces become buried in the interior of the protein [46]. In the case of p53, it is therefore possible to apply the method of pulse renaturation successfully by adding denatured protein up to six times into the refolding buffer. After purification, a yield of approximately 3% of the total p53 protein was reached corresponding to a maximal volume yield of approximately 20 μg/ml.

4.2. Biochemical characterization of renatured p53 tumor suppressor protein

The specific DNA binding activity of p53 is essential for its function as a tumor suppressor. Therefore, we investigated the DNA binding activity of the purified, renatured p53 protein by an electrophoretic mobility shift assay; p53 tetramers, expressed in *E. coli*, are latent for DNA binding and have to be activated by a concerted, allosteric mechanism [30,31]. In our assay, we used the C-terminally binding antibody PAb 421 to switch between latent and active forms. One prerequisite for the allosteric mechanism is the correct conformation of the p53 domains involved in the switching and their proper interaction with each other in order to communicate the binding of the antibody at the C-terminus to the core domain of p53. Our results therefore confirm that the different domains of p53 and their contact interfaces are correctly folded.

An additional requirement for efficient DNA binding and transcriptional activation is the formation of tetramers; p53 tetramers show the highest affinity for DNA, whereas dimers bind only weakly [61]. Tetrameric binding correlates well with the four pentanucleotide binding motifs in the DNA consensus sequence [23]. The dominant form of renatured p53 is a tetramer, as shown by cross-linking studies with glutaraldehyde. Analysis by gel filtration established an apparent molecular weight of approximately 550 kDa. Friedman et al. [48] showed that tetrameric p53 has an unusual, ellipsoid shape, which is responsible for an apparent molecular mass of approximately 500 kDa in gelfiltration experiments. Taken together, our data is in good agreement with data published for human and murine p53 [48,63].

The spectroscopic analysis of renatured p53 revealed that the protein is partially unstructured. To date, structural data exist only for the core domain (aa 102–292 [19]) and the oligomerization domain (aa 326–353 [25,26,64,65]). The N-terminal region of p53 (aa 1–73) is an acidic transcription activation domain. As shown by

NMR, this domain of p53 is mostly unstructured and lacks secondary structure elements, besides the existence of short and unstable structural elements [17,69]. This seems to be a typical feature of acidic transcription activation domains [66–68]. Several studies have suggested that the linker region between the core domain and the tetramerization domain is also essentially unstructured and confers the structural flexibility required for p53 function [27,28]. A peptide derived from the C-terminal region of p53 (aa 367–388) was found to have no regular structure in its native form by NMR spectroscopy [70].

In agreement with the notion that p53 is a protein containing unstructured domains, the fluorescence signal of native p53 displayed a maximum at 347 nm. The fluorescence signal of the core domain is dominated by the eight tyrosine residues, whereas the contribution of the single tryptophane, which is 25% solvent exposed, is low [20]. Because of the high solvent accessibility of the tyrosine 327, there is essentially no difference between folded or unfolded protein in the fluorescence signal of the tetramerization domain [28]. The remaining three tryptophanes (W23, W53, W91) are part of the mostly unstructured N-terminal region and are therefore very likely solvent exposed, explaining the large red shift of the emission maximum of full-length p53 and the small difference between the spectra of folded and unfolded protein.

In summary, our results and previous data [12,13,15,18,74–77] suggest that p53 is a flexible, partially unstructured native protein [71–73]. p53 has to integrate many different signals by interacting with numerous cellular and viral binding partners as an inducer of various downstream events. Flexibility and a modular architecture may be the prerequisites to fulfill these diverse functions at the checkpoint of proliferation and apoptosis. However, conclusive structure–function studies have been complicated by difficulties in obtaining sufficient amounts of homogeneous tetrameric full-length p53. Based on our experiments it is now possible to produce tetrameric full-length p53 in order to accomplish a detailed biochemical and biophysical structure–function analysis.

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