Tandem Dimerization of the Human p53 Tetramerization Domain Stabilizes a Primary Dimer Intermediate and Dramatically Enhances its Oligomeric Stability

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Tetramerization of the human p53 tumor suppressor protein is required for its biological functions. However, cellular levels of p53 indicate that it exists predominantly in a monomeric state. Since the oligomerization of p53 involves the rate-limiting formation of a primary dimer intermediate, we engineered a covalently linked pair of human p53 tetramerization (p53tet) domains to generate a tandem dimer (p53tetTD) that minimizes the energetic requirements for forming the primary dimer. We demonstrate that p53tetTD self-assembles into an oligomeric structure equivalent to the wild-type p53tet tetramer and exhibits dramatically enhanced oligomeric stability. Specifically, the p53tetTD dimer exhibits an unfolding/dissociation equilibrium constant of 26 fM at 37 °C, or a million-fold increase in stability relative to the wild-type p53tet tetramer, and resists subunit exchange with monomeric p53tet. In addition, whereas the wild-type p53tet tetramer undergoes coupled (i.e. two-state) dissociation/unfolding to unfolded monomers, the p53tetTD dimer denatures via an intermediate that is detectable by differential scanning calorimetry but not CD spectroscopy, consistent with a folded p53tetTD monomer that is equivalent to the p53tet primary dimer. Given its oligomeric stability and resistance against hetero-oligomerization, dimerization of p53 constructs incorporating the tetramerization domain may yield functional constructs that may resist exchange with wild-type or mutant forms of p53.

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Introduction

The human tumor suppressor p53 is a 393 amino acid residue transcription factor involved in cell cycle control, programmed cell death and cellular differentiation.1 Tetramerization of the human p53 protein occurs through its p53tet domain (residues 325 to 355) and it is in the context of this oligomeric arrangement that it serves a major role in directing cells with genetic lesions to undergo DNA repair, cell-cycle arrest, or apoptosis.1,2 Mutations in p53 leading to a loss of its tumor-suppressive function are known to occur in more than 50% of known human cancers.3 The majority of these mutations occur in the DNA-binding domain (residues 110 to 286) and result in p53 mutants that self-associate or form heterotetramers with wild-type p53 pools.3,4 These p53 heterotetramers exhibit transdominant effects including prolonged cellular half-lives and altered p53 functions relative to their wild-type counterpart.5 Most significantly, they fail to suppress tumor growth in vivo.6 Since tetramerization is a central feature in the maintenance of normal p53 function as well as its loss of function in tumorigenesis, any strategy aimed at restoring the tumor suppressive action of wild-type p53 in cancer cells necessitates an understanding of both the structural and thermodynamic parameters guiding
the oligomerization and stability of the tetramerization domain. The structure of the p53 tetramerization domain (p53tet) has been characterized extensively by X-ray crystallography and NMR spectroscopy. Within the compact homotetrameric complex, each subunit consists of a β-sheet and two antiparallel α-helices. Two primary dimers in turn pair through complementary contacts involving their α-helical interfaces. Hydrophobic interactions drive the formation of both the primary dimer and the tetramer, although ionic interactions have been shown to direct the specificity of the interactions in the homotetramer. Inter-subunit symmetry suggests a "dimer of dimers" architecture in which "primary dimers" are formed by an antiparallel β-sheet and two antiparallel α-helices. Two primary dimers in turn pair through complementary contacts involving their α-helical interfaces. Hydrophobic interactions drive the formation of both the primary dimer and the tetramer, although ionic interactions have been shown to direct the specificity of the interactions in the homotetramer. Interestingly, thermodynamic experiments have supported a strictly two-state dissociation event (from folded tetramers to unfolded monomers) at equilibrium under a wide range of pH and salt conditions. Specifically, under physiological conditions, the primary dimer is not significantly populated, although it has been shown that a dimeric species represents an obligatory kinetic intermediate in the assembly of tetramers.

Thermodynamic analyses have highlighted in the past that the population of tetrameric p53 depends on the p53 concentration in solution, with the greatest change in oligomer population occurring in the micromolar range. At 20 °C and pH 7, for example, a 10 μM solution of p53(303-393) peptides would display 83% of the peptides as tetramers, a value that falls rapidly to 28% at 1 μM, and to only 0.1% at 0.1 μM. Since typical intracellular concentrations of p53 are in the nanomolar range (or lower) and its tumor-suppressive functions are directly linked to its quaternary structure, this concentration dependence has three biologically relevant consequences: (i) functionally inactive monomers dominate the cellular pool of p53, (ii) active, tetrameric p53 complexes are transiently assembled from monomer pools and are rapidly dissociated, and finally (iii) both mutant and wild-type p53 subunits have an equal probability of exchanging rapidly and being present within tetramer pools. While these features serve as part of the regulatory mechanism for p53 function, they are also responsible for transdominant inhibition. Specifically, in cancer cells harboring heterozygous p53 mutations, the level of wild-type p53 homotetramers is severely perturbed resulting ultimately in a reduction to homozygosity and enhanced cell survival. This hypothesis is supported by the general observation that induction of wild-type p53 function can restore apoptotic mechanisms in tumor cells. Towards this end, efforts have been made to engineer more stable and exchange-resistant p53 tetramers by screening site-directed mutants and p53 orthologs both in vitro and in silico. We propose a different approach wherein a thermodynamically favored p53tet dimer is generated to drive the formation of a wild-type p53-like tetramer. More specifically, a covalently linked dimer of the p53tet domain would stabilize the primary dimer intermediate, pushing the oligomerization equilibrium towards oligomer formation. We report the design of such a dimer, termed p53tetTD, which consists of two copies of the p53tet domain (residues 310 to 360) separated by a dipeptide linker (Figure 1). Structural and thermodynamic analyses of this construct in relation to wild-type p53tet indicate that, in solution, p53tetTD self-assembles into a four-domain, dimeric complex equivalent to the wild-type tetrameric p53tet but exhibits substantially higher stability against thermal and chemical denaturation. The p53tetTD dimer is also resistant to subunit exchange with monomeric p53tet domains. Moreover, we found that tethering two p53tet is sufficient to stabilize a folded primary dimer at equilibrium, p53tetTD is the first example of a stabilized p53tet primary dimer using the native p53tet sequence and represents a potentially useful scaffold for building highly stable and exchange-resistant p53 constructs for overcoming transdominant inhibition in cancer cells harboring p53 mutations.

**Results**

We have designed a novel construct, termed p53tetTD, based on the human p53 tetramerization domain (p53tet) in which two consecutive copies of human p53(310-360) have been fused in tandem (Figure 1). The minimum tetramerization domain encompasses residues 325 to 355 of human p53. A linker consisting of a Gly-Thr dipeptide was introduced following residue 360 of the leading p53tet tandem, resulting in a spacer spanning a total of 23 residues. The calculated molecular masses of p53tetTD (Mₜ, 12,257.6) and of the wild-type p53tet (Mₜ, 6265.0) peptides were confirmed by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry. Size-exclusion chromatography in 25 mM Na₂HPO₄ (pH 7.2) at 25 °C indicated that both peptides eluted with identical retention times (Figure 2(a)), between the 17 and 44 kDa protein markers. These observations are consistent with the wild-type p53tet tetramer and p53tetTD dimer having comparable oligomeric sizes. Equilibrium sedimentation experiments further confirmed similar apparent molecular masses for p53tet (Mₜ, 23,968±541; 3.8× monomers) and p53tetTD (Mₜ, 26,235±1065; 2.1× monomers), corresponding to a four-domain structure for both peptides. Finally, glutaraldehyde crosslinking (0.125% v/v) of p53tetTD led to the formation of a dimeric species that co-migrated with tetrameric p53tet in SDS-PAGE (Figure 2(b)). Taken together, these three experiments demonstrate that p53tetTD self-assembles to the same stoichiometry, i.e. a four-domain, dimeric structure, as expected for the tetrameric form of the wild-type p53tet domain. The conformation of the p53tetTD dimer was further analyzed by NMR and far-UV CD spectroscopy. The 1H-15N heteronuclear single quantum
**Figure 1.** Recombinant self-assembling p53-based oligomeric constructs used in this study. The control wild-type p53tet peptide encompasses residues 310–360 of the human p53 protein, which includes the minimum p53 tetramerization domain (residues 325 to 355; PDB ID: 1PES). The p53tetTD construct is a covalently linked tandem dimer of p53tet. Ribbon models of the predicted structure are shown and were generated by homology modeling of the 1PES structure using SWISS-MODEL. The first and second domains of the tandem are colored in red and green, respectively. Unstructured residues are displayed in grey.
coherence (HSQC) spectrum of p53tetTD yielded peaks at the chemical shifts reported for the wild-type p53tet domain (as determined by Lee et al.9; Figure 3(a), Table 1). The average differences in the chemical shifts for the two oligomers were 0.07 ppm and 0.48 ppm, respectively. The far-UV CD spectra of the wild-type p53tet domain and of the p53tetTD construct were also substantially overlapping (Figure 3(b)), with a secondary structure content of 18% α-helix, 27% β-sheet, 22% turns, and 33% disordered for p53tetTD,26,27 similar to previous estimates for p53tet(303-366).12 Overall, these data strongly support the view that the dimeric, four-domain structure found in the p53tet dimer

![Figure 2](image2.png)

**Figure 2.** The p53tetTD peptide dimerizes to an equivalent quaternary structure as the wild-type p53tet tetramer at equilibrium. (a) High performance size-exclusion chromatography indicates that both peptides elute with equivalent retention times. Samples of wild-type p53tet domain (0.2 mg) and p53tetTD (0.2 mg) dissolved in 10 mM Na2HPO4 (pH 7.4) were separated on a Superdex 75HR. The column was equilibrated at 25 °C in the same buffer and the flow rate was 0.8 ml/min. Protein peaks were detected at 254 nm. The column was calibrated with thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and cyanocobalamin (1.35 kDa). (b) Time-course of glutaraldehyde-crosslinking of p53tet (400 μM) and p53tetTD (200 μM) at 25 °C in 25 mM Na2HPO4, 100 mM NaCl (pH 7.4) followed by separation by SDS-PAGE. Protein markers (in kDa) are shown in the center lane.

![Figure 3](image3.png)

**Figure 3.** The wild-type p53tet and p53tetTD constructs exhibit identical secondary and tertiary structures. (a) 1H-15N HSQC NMR spectrum of p53tet at 40 °C in 25 mM Na2HPO4, 100 mM NaCl (pH 7.4). Chemical shift values of assigned peaks are listed in Table 1. Unassigned peaks are marked with asterisks. (b) Far-UV CD spectra of wild-type p53tet (filled squares) and p53tetTD (open squares) at 25 °C in 10 mM Na2HPO4 (pH 7.4). Lines represent best-fits of the data to secondary structure deconvolution by CDSSTR from 185 to 240 nm.26,27
is essentially identical to the wild-type p53tet tetramer.

**Comparative thermodynamic analysis of the oligomerization of p53tet and p53tetTD**

To what extent is the oligomeric structure of p53tetTD stabilized by tandem dimerization of the wild-type p53tet domain in the linear peptide sequence? Previous investigations have shown that dissociation of the wild-type p53tet domain is consistent with a two-state transition, i.e. from a folded tetramer to four unfolded monomers, over a broad range of pH and salt concentrations. The so-called primary dimer suggested by published crystal and NMR structures is not detected at equilibrium. Freire and co-workers found that at concentrations above 30 μM near physiological pH, the wild-type p53tet tetramer was stable \((T_m>70^\circ C)\) irrespective of salt concentrations down to 25 mM NaCl. With decreasing pH, however, the tetramer is not only significantly destabilized but also becomes increasingly sensitive to low salt concentrations. Our CD spectroscopic and calorimetric data for the control wild-type p53tet domain also confirmed a two-state transition, as neither denaturation by guanidinium (GdnH\(^+\); Figure 4) nor temperature (Figures 5 insets and 6(a)) revealed a detectable equilibrium intermediate. The derived thermodynamic parameters for this peptide (Table 2) were also in agreement with those reported previously, indicating an enthalpically driven folding/association to the p53tet tetramer.

For the p53tetTD peptide, at equivalent concentrations in terms of the p53tet domains (and therefore half the concentration of the control p53tet peptide), the self-assembled dimer exhibited significantly greater resistance to GdnH\(^+\) denaturation at pH 7.4. Thus, at 25 °C the concentration of Gdn\(^+\) at which 19 μM p53tetTD peptides are half-dissociated is 1.3 M greater than the corresponding value for wild-type p53tet peptides at 38 μM (Figure 4). Calorimetric measurements, however, indicate that while the wild-type p53tet tetramer undergoes coupled (i.e. two-state) dissociation-unfolding (Figure 5 insets), the differential scanning calorimetry (DSC) melting profile for p53tetTD exhibited a bimodal profile particularly as peptide concentration dropped below about 100 μM. Specifically, above this concentration, the DSC profile was manifestly two-state; below this concentration, a shoulder could be observed with a slightly lower transition temperature (Figure 5(a) and (b)). This qualitative difference from p53tet was not artifactual as only a single species of peptide, namely the two-domain p53tetTD, was present in the sample (as verified by SDS-PAGE and mass spectrometry) and that this peptide was quantitatively dimerized at room temperature (as confirmed by size-exclusion chromatography and equilibrium sedimentation). The simplest explanation would be a three-state transition wherein the p53tetTD dimer \((N_2)\) dissociates to

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**Table 1.** NMR HSQC resonance assignments for p53tetTD (corresponding to human p53 (323–358)) at 40 °C, pH 7.0

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<th>Residue</th>
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<th>N</th>
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</tr>
<tr>
<td>D324</td>
<td>8.19 (8.22)</td>
<td>120.9 (121.3)</td>
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<tr>
<td>G325</td>
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<tr>
<td>E326</td>
<td>7.88 (8.02)</td>
<td>120.3 (121.0)</td>
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<td>Y327</td>
<td>7.80 (7.85)</td>
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<td>F328</td>
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<td>T329</td>
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<td>116.9 (117.4)</td>
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<td>A255</td>
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<td>122.5 (123.0)</td>
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<tr>
<td>G356</td>
<td>8.08 (8.20)</td>
<td>108.5 (107.6)</td>
</tr>
<tr>
<td>K357</td>
<td>7.92 (7.92)</td>
<td>120.3 (120.7)</td>
</tr>
<tr>
<td>E358</td>
<td>8.38 (8.36)</td>
<td>123.6 (123.4)</td>
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</table>

Chemical shifts are referenced to internal \(^1\)H\(_2\)O (4.58 ppm) and external CH\(_3\)\(^15\)N\(_2\)O (78.98 ppm).

* Values in parentheses are reference values for the wild-type p53tet domain determined at 45 °C, pH 7.0.

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**Figure 4.** Isothermal guanidinium titration of the wild-type p53tet domain and p53tetTD. Denaturation curves for wild-type p53tet domain (38 μM; filled squares) and p53tetTD (19 μM; open squares) in 10 mM Tris (pH 7.4). Changes in ellipticity values at 226 nm were monitored as a function of increasing concentrations of GdnHCl at 25 °C. Lines represent the best-fit of the data to the linear extrapolation model.
folded monomeric intermediates (I, corresponding to a covalently tethered version of the primary dimer envisaged from structural data), which subsequently denature to unfolded monomers (U) within the covalent dimer:

\[ \text{N}_2 \rightleftharpoons 2\text{I} \rightleftharpoons 2\text{U} \]  

To test this mechanistic model, we used a statistical thermodynamic formalism as described by Thompson et al. 28 Specifically, fitting the data (to the low-concentration data where the two transitions are discernable) to this model directly provides an estimate of the thermodynamic parameters for (i) the dissociation of the p53tetTD (four-domain) dimer to folded (two-domain) monomers (N2 \rightleftharpoons 2I; \Delta H_{N2/2I}, \Delta C_{P,N2/2I}, and \text{T}_{N2/2I}°) and (ii) the combined dissociation of the p53tetTD dimer and unfolding of the peptide (N2 \rightleftharpoons 2U; \Delta H_{N2/2U}, \Delta C_{P,N2/2U}, and \text{T}_{N2/2U}°). (For both sets of parameters, N2 is the reference state; see Materials and Methods.) We found that the three-state model fitted the low as well as the higher concentration data (Figure 5(a) and (b)). Quantitatively, the overall dissociation-unfolding thermodynamics (\Delta C_{P,N2/2U} and \Delta H_{N2/2U}) for p53tetTD were essentially double that of the wild-type p53tet domain, thus providing additional, thermodynamic evidence that the p53tetTD peptides assemble to an equivalent oligomeric structure as the wild-type p53tet domain. (\Delta H values are temperature-dependent and were extrapolated to a common temperature for comparison.)

To further test the physical validity of the three-state model for p53tetTD, we determined the structurally derived thermodynamics for the dissociation step, using the p53tet tetramer (PDB ID: 1PES) and its primary dimer as models of the initial and intermediate states, respectively (Table 3). Specifically, since the primary dimer interface is extensively hydrophobic, 12 the change in heat capacity for the dissociation of the p53tetTD dimer should reflect...
hydration of this exposed, primarily hydrophobic interface of the dissociated dimer. Indeed, the structural ΔC_p value of 360 cal/(K mol peptide) is in excellent agreement with the values of ΔC_p,N2/2I obtained independently from data fitting (from 332 to 380 cal/(K mol peptide); Table 2) (1 cal=4.184 J).

These data reinforce the view that the folded p53tetTD monomer assumes the conformation of the primary dimer for the wild-type p53tet domain.

When probed by far-UV CD spectroscopy, the p53tet tetramer yielded expected melting profiles for coupled dissociation/unfolding that were sensitive to peptide concentrations in the low micromolar range, demonstrating the sensitivity of tetrameric stability to these levels of concentrations (Figure 6 (a)). The corresponding behavior of p53tetTD, however, was altogether different. Not only were the transition temperatures considerably higher than for the wild-type p53tet domain at comparable peptide concentrations, there was also no detectable shift in melting profiles at peptide concentrations from 86 to 14 μM; in contrast, a comparable change in equivalent domain concentrations of p53tet resulted in a shift of T_m of greater than 7 °C. It was therefore mechanistically appropriate to treat the far-UV CD melting of p53tetTD as a monomeric transition, which gave a good fit of the data (Figure 6 (b)). The observation that far-UV CD spectroscopy does not detect any intermediate in the course of either thermal or GdnH+ denaturation (Figure 4) is a self-consistent result for a native-like p53tetTD monomeric intermediate.

### Table 2. Thermodynamic parameters associated with the thermal dissociation-unfolding of wild-type p53tet or p53tetTD peptides

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<tr>
<th>Peptide</th>
<th>Cosolute</th>
<th>Modelb (Probe)</th>
<th>[Peptide]c (μM)</th>
<th>T°d (°C)</th>
<th>Tm°e (°C)</th>
<th>ΔH(T°)e (kcal/mol)</th>
<th>ΔS(T°)e (cal/(K mol)</th>
<th>ΔC_p,e (cal/(K mol)</th>
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<td>Wild-type p53tet domain (n=4)</td>
<td>A (DSC)</td>
<td>174.1</td>
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<td>814.0±0.1</td>
<td>849.0±0.1</td>
<td>54.1±0.2</td>
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<td>446±9</td>
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<tr>
<td></td>
<td>A (CD)</td>
<td>70.3</td>
<td>126.9±0.3</td>
<td>77.8±0.3</td>
<td>73.7±0.3</td>
<td>53.1±0.2</td>
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<td>(446)</td>
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<td></td>
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When probed by far-UV CD spectroscopy, the p53tet tetramer yielded expected melting profiles for coupled dissociation/unfolding that were sensitive to peptide concentrations in the low micromolar range, demonstrating the sensitivity of tetrameric stability to these levels of concentrations (Figure 6 (a)). The corresponding behavior of p53tetTD, however, was altogether different. Not only were the transition temperatures considerably higher than for the wild-type p53tet domain at comparable peptide concentrations, there was also no detectable shift in melting profiles at peptide concentrations from 86 to 14 μM; in contrast, a comparable change in equivalent domain concentrations of p53tet resulted in a shift of T_m of greater than 7 °C. It was therefore mechanistically appropriate to treat the far-UV CD melting of p53tetTD as a monomeric transition, which gave a good fit of the data (Figure 6 (b)). The observation that far-UV CD spectroscopy does not detect any intermediate in the course of either thermal or GdnH⁺ denaturation (Figure 4) is a self-consistent result for a native-like p53tetTD monomeric intermediate.
since unpaired p53tet primary dimers in the
absence of unfolding would yield a comparable
secondary structure.

Equilibrium dissociation/unfolding constants
\( (K_D, \text{the monomer concentration at which unfolded}) \) were
calculated using thermodynamic data from Table 2
for the p53tetTD dimer and wild-type p53tet
tetramer under physiological conditions (37 °C, pH
7.4, 150 mM Na\(^+\)). The \( K_D \) value of 71.8 nM (\( K_D=K_{411/3} \); see
Materials and Methods) for the wild-type p53tet
domain is in general agreement with previous
measurements by analytical equilibrium ultracentrifugation.\(^ {14} \) For the p53tetTD peptide, the calculated
\( K_D \) was 26.4 fM (normalized with respect to
the oligomer), an impressive 6.4-log unit gain in
stability. Thus tandem dimerization of the p53tet
domain represents a highly effective method of
increasing the stability of the p53 oligomeric
structure, much more so than would be expected
from site-directed mutants.

To study the stability of the wild-type p53tet
domain and p53tetTD under mildly denaturing
conditions, we also analyzed their dissociation/
unfolding in the presence of 1.0 M GdnH\(^+\). This
concentration was chosen using data from GdnH\(^+\)
titrations at 25 °C (Figure 4) showing that both p53
constructs exist as oligomers in solution at the
outset of a DSC upscan. For the wild-type p53tet
domain, the presence of 1.0 M GdnH\(^+\) substan-
tially reduced the thermal stability of the tetramer:
the \( T_m \) for the wild-type p53tet domain at 143 μM
is 13 °C higher under physiological conditions
(150 mM Na\(^+\)) than in the presence of 1.0 M at
twice the concentration (287 μM; Table 2). More
significantly, p53tetTD at dimer concentrations as
low as 17 μM exhibited no bimodal melting in
contrast with native conditions at comparable
concentrations (Figure 5(c)), and remained more
stable (by 6 °C) than the wild-type p53tet tetramer
at over four times the oligomer concentration
(72 μM). Thermodynamically, the presence of
1.0 M GdnH\(^+\) caused a 70% rise in \( \Delta H \) (extrap-
olated to 25 °C) and 30% reduction in \( \Delta C_p \)
relative to native conditions, although the values
(per mol peptide) for p53tetTD were again almost

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**Figure 6.** Far-UV CD detects a single monomolecular transition in p53tetTD thermal unfolding. Thermal melting of the wild-type p53tet domain and p53tetTD dimer was monitored spectroscopically by recording changes in ellipticity values at 222 nm. All peptides were dissolved in 25 mM Na\(_2\)HPO\(_4\) (pH 7.4). Profiles shown have been normalized and baseline-subtracted. Melting profiles for the wild-type p53tet domain were obtained at peptide concentrations of 8.8, 17.6, 35.1, and 70.3 μM. Lines represent the best-fit of the data to a coupled dissociation-unfolding model, using the calorimetrically determined value of \( \Delta C_p = 446 \text{ cal}/(\text{K mol peptide}) \) for p53tet unfolding. Melting profiles for p53tetTD at peptide concentrations of 14.3, 28.5, and 85.5 μM. The line represents the best-fit to a monomolecular transition using the calorimetric \( \Delta C_p = 956 \text{ cal}/(\text{K mol peptide}) \) for p53tetTD unfolding. Inset: original melting profiles (path length was 1 mm). Parameter estimates are reported in Table 2.
exactly double that for the wild-type p53tet domain.

Dimeric p53tetTD resists exchange with single-domain subunits

A biologically relevant aspect of characterizing the p53tetTD construct is its resistance against the formation of hetero-oligomers, since this exchange mechanism may be responsible for the inhibition of wild-type p53 by mutated forms of the protein. To this end, we co-incubated 6×His-tagged wild-type p53tet domain or 6×His-tagged p53tetTD peptide with equimolar domain concentrations of untagged p53tet domain in PBS at 37 °C over a two-day period. The 6×His-tagged hetero-oligomers were purified at various time intervals with Ni-NTA resin and the resulting oligomer compositions were analyzed by SDS-PAGE and densitometry (Figure 7). The time-dependent appearance of untagged p53tet domain therefore provided an estimate of the extent to which subunit exchange was occurring among the 6×His-tagged oligomers with the untagged pool of wild-type p53tet domain. For 6×His-tagged p53tet, the fraction of untagged p53tet recovered followed the statistically expected value of \( \frac{7}{8} \) (0.875; see legend to Figure 7). For 6×His-tagged p53tetTD, the fractional recovery of untagged p53tet approached \( 0.13\pm0.01 \), significantly below the statistically expected value of \( \frac{1}{3} \) (assuming all peptide interactions were equally likely). Clearly, the four-domain p53tetTD dimer was relatively stable against exchange with single-domain subunits such as wild-type p53tet domain, and by extension the majority of p53 mutants, which carry mutations outside the p53tet domain.

Discussion

The oligomeric instability at physiological concentrations and the rapid exchange of wild-type p53 monomers with mutant p53 proteins are two important properties responsible for the loss of p53 functions in cancer cells. An understanding of the biophysical basis of p53 tetramerization is therefore essential to devising effective strategies for overcoming this phenomenon. To this end, we have engineered and characterized a p53tet peptide construct (p53tetTD, tandem dimer) that harbors two consecutive copies of the human p53 tetramerization domains (residues 310–360) in an effort to analyze and bypass a critical thermodynamic barrier affecting the oligomerization of human p53, namely the formation of a primary dimer. We have shown that the p53tetTD peptide self-assembles into a dimeric structure that is topologically equivalent to the p53tet homotetramer (Figures 1, 2, 3; Table 1) but exhibited substantially greater resistance to thermally or chemically induced dissociation (Figures 4–6), and also against subunit exchange with monomeric p53tet domains (Figure 7). In summary, the thermodynamic and structural data on p53tetTD relative to the wild-type p53tet domain indicate that linear linkage of two p53 tetramerization domains in the p53tetTD peptide stabilizes the inter-domain interactions to such an extent that the two-domain primary dimer (in the context of a folded p53tetTD monomer) exists at equilibrium and dimerizes to the final four-domain complex in an essentially rigid-body association.
Covalent linkage of subunits as a strategy for driving oligomerization of the p53tet domain

The formation of quaternary structure is an entropically costly process as it requires the reduction in translational and rotational degrees of freedom for each subunit. Clearly, this entropic cost must be recovered by favorable processes (e.g. hydrophobic effect, formation of hydrogen bonds and salt-bridges) if oligomerization is to be energetically favored. Assuming that solvent–protein and protein–protein interactions are not significantly different in the final complex, the thermodynamic value of covalent linkage of subunits lies in reducing the sampling space of destabilized unfolded states (by severely limiting intrasubunit motion) and thus favoring the free energy advantage of oligomerization. This benefit has been demonstrated for a number of dimeric proteins, for which fusion of the two subunits renders the transition independent of overall concentration.30–35 In some cases, the enhanced stability is used to leverage another coupled molecular event such as DNA binding.31–33

In the case of the p53 tetramerization domain, the rate-limiting step in forming a tetramer from four unfolded monomeric p53tet peptides has been spectroscopically shown to be the dimerization of unfolded monomers into transient, native-like dimeric intermediates, two of which concomitantly assemble into tetramers to produce a dimers of dimers.15 Previously, the equilibrium properties of the p53tet primary dimer were studied using site-specific mutants that trap these peptides into native-like dimers (e.g. M340E/L344K, M340Q/L344R, L348A, K351E) rather than a tetramer.31,36–38 In our experiments, tandem dimerization represents an alternative route for stabilizing this rate-limiting primary dimer intermediate using the native p53tet sequence. As with other tethered dimers, the p53tetTD peptide unfolds independently of concentration when monitored by far-CD spectroscopy, which does not detect the rigid-body dissociation of the p53tetTD dimer (Figure 6(b)). By forming a

![Figure 8](image_url)

**Figure 8.** Unfolding/dissociation equilibria for p53tetTD under physiological conditions. Values of equilibrium constants at 37 °C were calculated using thermodynamic data obtained at pH 7.4 in 150 mM Na+ (Table 2), normalized with respect to the oligomer. The p53tetTD dimer unfolds via an equilibrium folded monomer (equivalent to a covalently linked primary dimer) that is intermediate in energy between the folded dimer and unfolded monomers. $K_D$ represents the overall dissociation/unfolding equilibrium constant, equal to the monomer concentration at which the concentrations of folded oligomers and unfolded monomers are equimolar; $K_U$ is the unfolding constant for the p53tetTD monomer. Note that for this thermodynamic cycle, $K_D = [\frac{[U]^2}{[N]^2}] = K_{N+1}/K_{U}$. For comparison, $K_D = 72$ nM for the wild-type p53tet tetramer.
tethered primary dimer that is intermediate in energy between the folded dimer and unfolded monomers (Figure 8), the p53tetTD peptide decouples the formation of the primary dimer and that of the ultimate four-domain complex, giving rise to a three-state dissociation/unfolding transition for the p53tetTD dimer (Figure 5(a) and (b)). Interestingly, despite the apparent loss of the tethered primary dimer intermediate at equilibrium in the presence of 1.0 M Gdn+, the p53tetTD dimer maintains some oligomeric stability over the wild-type p53tet tetramer. This indicates a direct role for a reduction in the transition valency, in addition to stabilization of the rate-limiting primary dimer intermediate, in promoting oligomeric stability of p53tet by tandem dimerization. The magnitude of this stability enhancement is remarkable: from our thermodynamic data, under physiological conditions (pH 7.4, 150 mM Na+), the overall unfolding/dissociation constant (i.e. the concentration of monomer at which folded oligomers and unfolded monomers are equimolar) for the p53tetTD dimer is 26.4 fM, more than six orders of magnitude lower than the corresponding value for the wild-type p53tet domain (71.8 nM). To further illustrate the stability of the p53tetTD dimer over the wild-type p53tet tetramer, we calculated their respective Tm as a function of decreasing peptide concentration (Figure 9). We use Tm, the temperature at which the oligomer is 50% dissociated and/or unfolded, rather than the more customary transition temperature Tn per sé because Tm is ambiguous for transitions involving multiple steps. Indeed, at concentrations below 10 nM, wild-type p53tet peptides do not achieve 50% tetramers at any temperature, whereas the p53tetTD construct at a concentration of 0.1 nM would exist with half of its molecules paired as dimers at 37 °C. Thus, our results demonstrate a potentially effective strategy for maintaining the essential quaternary structure of p53 at nanomolar concentrations without the need to introduce site-specific mutations. Dawson and co-workers have also engineered a chemically crosslinked p53tet dimer although the use of unnatural residues and loss of all termini limit its usefulness in biological applications.

**Potential applications of p53tetTD as a design scaffold**

One strategy aimed at alleviating transdominant inhibition is to replace non-functional p53 mutants with exogenous, functional p53 constructs. However, simply re-introducing wild-type p53 (without correcting the existent mutations) into cells may not significantly restore tumor suppressor activity, since they remain highly sensitive to concentration and mutant p53 would continue to perturb the cellular pool of all p53 species. Thus, an effective rescue p53 oligomer must be stable at nanomolar (and lower) concentrations and resist exchange with mutant p53 monomers. To this end, it has been shown that stabilization of the tetramerization domain per sé is sufficient to produce functional, exchange-resistant p53-based constructs: specifically, p53-based constructs in which the native tetramerization domain has been substituted with various engineered leucine zippers (and assemble into tetrameric or even dimeric coiled coils) resist subunit exchange and restore tumor suppression in cancer cells harboring p53 mutations.

**Materials and Methods**

**Cloning of p53tetTD**

The plasmid pET15b-p53wt, in which a N-terminally 6×His-tagged construct encoding residues 310 to 360 of human p53 and a thrombin cleavage site were inserted into the Ndel/BamHI sites of the vector (Novagen, Madison, WI), was a gift from Dr Cheryl H. Arrowsmith. Using this plasmid as template, the insert was amplified by PCR in two separate reactions: reaction 1 employed a common forward primer and p53lnkR (GTCAAGTTACCCTGGCTTCCCTGCCAGCTGGCTCTGG), and reaction 2 employed a common reverse primer and p53lnkF (GTCAAGTTACCACAAACACCCAGCTCCCTC-
Pelleted cells from 2 l cultures were sonicated into 30 ml of Buffer A, followed by a second 4 l of Buffer D (10 mM Tris-HCl (pH 8.0), 2 M NaCl, 1 mM CaCl2). After adding five units of thrombin (GE Healthcare, Piscataway, NJ), the renatured protein was diazylated (MWCO 12,000–14,000) overnight against 4 l of fresh Buffer D at room temperature. Thrombin was removed by incubating the protein solution with 2 ml of benzamidine-agarose resin (Qiagen, Hilden, Germany) at 4 °C for 2 h, and poured into polypropylene columns (Bio-Rad, Hercules, CA). The resin was gravity-washed with an additional 50 ml of Buffer A, followed by 50 ml of Buffer B (6 M GdnHCl, 0.1 M Na2HPO4, pH 6.3). The peptides were finally eluted with 25 ml of Buffer C (6 M GdnHCl, 0.1 M sodium acetate, pH 4.8). Fractions (25 ml) were collected and 100 μl aliquots of each fraction were precipitated in ice-cold ethanol and analyzed by SDS-PAGE. Fractions containing the pure construct were pooled and dialyzed (MWCO 3500, Spectrum Labs) against 4 l of 2 M NaCl containing 100 mM NaCl.

Analytical size-exclusion chromatography

A Superdex 75 HR column (GE Healthcare) attached to a Waters HPLC system (Milford, MA) was equilibrated in 25 mM Na2HPO4 (pH 7.2). Peptides (0.2 mg dissolved in up to 400 μl of buffer) were loaded onto the column and eluted at a flow rate of 0.8 ml/min. Protein peaks were detected at 254 nm and the Superdex 75HR column was calibrated with molecular mass standards (M, 1350 to 670,000; Bio-Rad).

Analytical ultracentrifugation

Sedimentation equilibrium ultracentrifugation experiments were performed on a Beckman Optima XL-I analytical ultracentrifuge (Fullerton, CA) using an AN50-Ti rotor with six-channel charcoal Epon cells. Peptides at three different concentrations (0.125, 0.25, and 0.5 mg/ml) were centrifuged at 20 °C at 3, 20, 32, and 44 × 1000 rpm. Radial absorbance data at 230 nm was fitted using a single species model for estimation of molecular masses.

Circular dichroism (CD) spectroscopy

Thermal melting and isothermal guanidinium titrations were performed with an Aviv 62A DS spectrometer equipped with built-in temperature control (Lakewood, NJ). For simple scans and thermal denaturation experiments, a 1 mm cuvette was used and heated at 1 deg.C increments. After equilibration for 30 s, the ellipticity at 222 nm was recorded with an integration time of 30 s. Control renaturation experiments conducted at the same rate of cooling revealed no significant hysteresis between the heating and cooling curves. For isothermal guanidinium titrations, 2 ml of peptide (in 10 mM Tris, pH 7.4) was prepared in a 10 mm cuvette, and guanidinium concentration was increased by successive replacements of a predetermined aliquot with an equal volume of peptide dissolved at the same concentration in the same buffer containing 8.0 M GdnHCl. After mixing, the solution was allowed to equilibrate at the set temperature for at least 5 min before recording the spectrum from 240 nm.

Differential scanning calorimetry (DSC)

Peptides at concentrations between 33 and 287 μM were scanned at 0.75 deg.C/min in a N-DSC II instrument (Calorimetry Sciences, Lindon, UT). The final dialyze was used as a buffer reference. An overpressure of 3.00 atm was applied to retard bubbling at elevated temperatures up to 120 °C. Subtraction of the buffer-buffer baseline and conversion from power to molar heat capacity were performed with CpCalc software (version 2.1; Applied Thermodynamics) before model-dependent thermodynamic analysis as described below.

NMR spectroscopy

Uniformly 15N-labeled p53tetTD was produced in M9 minimal medium supplemented with 0.1 mM CaCl2, 1 mM MgCl2, 0.01 mM FeCl3, 2 mg/l thiamine, 2 mg/l biotin, 5 g/l glucose as the carbon source, and 1 g/l 15NH4Cl (15N, 98%+; Cambridge Isotope Laboratories, Cambridge, MA) acting as the nitrogen source. 1H–15N TROSY-HSQC spectra were recorded on a Varian Inova 500-MHz spectrometer at 40 °C in 25 mM Na2HPO4 (pH 7.0) containing 100 mM NaCl.
Kinetics of subunit exchange

The rate and extent of exchange in p53tetTD dimers for the wild-type p53tet domain to form hetero-oligomers were studied as described elsewhere.\textsuperscript{11} Briefly, 6× His-tagged p53tetTD (100 μM) was co-incubated with 200 μM untagged p53tet in PBS at 37 °C over a period of 48 h. A control mixture of 200 μM 6×His-tagged and untagged p53tet domain was also prepared. At various time intervals, 300 μl aliquots were removed and mixed with 50 μl of a Ni-NTA resin slurry (Qiagen) at room temperature for 10 min. The resin was pelleted by centrifugation at 10,000 g for 30 s and subsequently washed five times with 200 μl of PBS. Bound proteins were eluted in the presence of 100 μl of PBS containing 0.5 M imidazole and aliquots were analyzed by SDS-PAGE. Untagged p53tet band intensities were quantified in the absence of 6×His-tagged constructs. All experiments were performed in triplicate.

Data analysis

The content of secondary structure in the p53tet peptides was estimated by deconvoluting their buffer-subtracted CD spectra using the CDSSTR algorithm from the online server DICHROWEB.\textsuperscript{26,27} CD spectra and subtracted CD spectra using the CDSSTR algorithm from peptides was estimated by deconvoluting their buffer-

\begin{equation}
\Delta G(T) = \Delta H^0 \left(1 - \frac{T}{T^0}\right) + \Delta C_p \left(T - T^0 + T \ln \frac{T}{T^0}\right)
\end{equation}

where \(T^0\) is the reference temperature at which \(\Delta G = 0\), and \(\Delta C_p\) is taken to be temperature-independent. The thermodynamic parameters are given in terms of per mol of monomer and \(K_{n1}\) (expressed in terms of dissociation of the oligomer) is therefore related to \(\Delta G\) by:

\begin{equation}
K_{n1} = \exp \left(-\frac{n \Delta G}{RT}\right)
\end{equation}

where \(n\) is the stoichiometry of the oligomer, and \(R\) is the gas constant. For two-state oligomeric transitions, the equilibrium constant \(K\) and fractional dissociation \(f\) are related by the following equation:\textsuperscript{45,46}

\begin{equation}
K_{n1} = np_{t}^{n-1} \frac{p_{t}^{n}}{1-f}
\end{equation}

where \(p_{t}\) is the total peptide concentration (i.e. monomers). Analytical solutions of equation (4) for \(f\) in terms of \(K\) are algebraically cumbersome\textsuperscript{47} (or impossible when \(n>4\)) and are more readily solved numerically by Newton’s method. For DSC data, the molar heat capacity \(C_p\) is given by:

\begin{equation}
C_p = \frac{n f (1-f)}{n-f [n-1]} \frac{\Delta H}{RT^2} + \Delta C_p f
\end{equation}

where \(\Delta H = \Delta H^0 (T-T^0)^2\) For thermal denaturation monitored by CD spectroscopy, the observed CD signal \(\Theta\) is related to \(f\) by:

\begin{equation}
\Theta = f [(\Theta_f + b_f T) - (\Theta_n + b_n T)] + (\Theta_f + b_f T)
\end{equation}

Equation (6) thus incorporates the pre- and post-transition baselines \((b_n\) and \(b_f\) respectively) directly in the analysis.

For p53tetTD, an alternative mechanistic model is a three-state transition, i.e. equation (1), in which the folded dimer (N2) dissociates to folded monomeric intermediates (I), which then denature to unfolded monomers (U). In this case, we use a statistical mechanical approach that was used to analyze the dissociation/unfolding of the dimeric basic leucine zipper GCN4.\textsuperscript{28} Taking N2 as the reference state, we define one set of thermodynamic parameters (equations (2) and (3)) for dimer dissociation to folded monomers (\(\Delta H_{N2/2I}, \Delta C_p_{N2/2I}, T_{N2/2I}, K_{N2/2I}, \text{etc.}\)) and another for the overall dissociation/unfolding transition (\(\Delta H_{N2/2U}, \Delta C_p_{N2/2U}, T_{N2/2U}, K_{N2/2U}, \text{etc.}\)). These transitions are related by the partition function \(Q\):

\begin{equation}
Q = 1 + K_{N2/2I} \frac{N2}{2\sqrt{N2}} + K_{N2/2U} \frac{N2}{2\sqrt{N2}}
\end{equation}

In the statistical thermodynamic model, \(K\) is normalized with respect to the monomeric species (I and U) to remain consistent with equation (7), i.e. \(K_{N2/2I} = \frac{[I]}{[N2]}\) and \(K_{N2/2U} = \frac{[U]}{[N2]}\). \([N2]\) is calculated from the known total peptide concentration \(p_t\) by \([N2] = A - \sqrt{T^2 - 4p_t^2}\) where \(A = 4p_t + (K_{N2/2I} + K_{N2/2U})^2\). The fractional populations \(f\) for the three conformers are:

\begin{align*}
& f_n = \frac{1}{Q} \\
& f_i = \frac{K_{N2/2I}}{2Q\sqrt{[N2]}} \\
& f_u = \frac{K_{N2/2U}}{2Q\sqrt{[N2]}}
\end{align*}

Since the temperature derivatives of equation (8) are algebraically unwieldy, DSC profiles are numerically integrated and it is the average excess enthalpy function \(<\Delta H>\) that is fitted:

\begin{equation}
<\Delta H> = \int_{T_i}^{T_f} (C_p) dT = f_n \Delta H_{N2/2I}(T) + f_u \Delta H_{N2/2U}(T)
\end{equation}

For isothermal guanidinium titrations, \(\Delta G\) is taken as linearly dependent on the molar denaturant concentration \(c\):\textsuperscript{47}

\begin{equation}
\Delta G = \Delta G(H_2O) - nc
\end{equation}

where \(\Delta G(H_2O)\) is the extrapolated value to zero denaturant concentration and \(n\) is the molar dependence of \(\Delta G\) on guanidinium concentration. In this case, the observed CD signal \(\Theta\) is also described by equation (6). (with \(c\) instead of \(T\) as the independent variable), although sloping baselines were generally not observed in these experiments.

Non-linear regression of thermodynamic data to the models was performed with the software Origin (version 7.5; Northampton, MA). Where appropriate, multiple experiments differing in peptide concentration were fitted
Acknowledgements

We thank the following colleagues: Dr Cheryl H. Arrowsmith for providing the original pET15b-p53wt plasmid; Dr Ayeda Ayed for assistance with the NMR TROSY-HSQC experiments; Drs Tigran V. Chalikian, Robert B. Macgregor, Jr, and Avi Chakrabarty for access to spectroscopic instruments and laboratory facilities; Dr James W. Wells and the reviewers for helpful comments. We also thank Miss Allison D. Hutton for kind editorial assistance. This investigation was financially supported by Canadian Breast Cancer Research Alliance in association with the Canadian Cancer Society.

References


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