Kinetic Instability of p53 Core Domain Mutants

IMPLICATIONS FOR RESCUE BY SMALL MOLECULES

Oncogenic mutations in the tumor suppressor protein p53 are found mainly in its DNA-binding core domain. Many of these mutants are thermodynamically unstable at body temperature. Here we show that these mutants also denature within minutes at 37 °C. The half-life ($t_{1/2}$) of the unfolding of wild-type p53 core domain was 9 min. Hot spot mutants denatured more rapidly with increasing thermodynamic instability. The highly destabilized mutant I195T had a $t_{1/2}$ of less than 1 min. The wild-type p53-(94–360) construct, containing the core and tetramerization domains, was more stable, with $t_{1/2}$ at 37 °C, similar to full-length p53. After unfolding, the denatured proteins aggregated, the rate increasing with higher concentrations of protein. A derivative of the p53-stabilizing peptide CDB3 significantly slowed down the unfolding rate of the p53 core domain. Drugs such as CDB3, which rescue the conformation of unstable mutants of p53, have to act during or immediately after biosynthesis. They should maintain the mutant protein in a folded conformation and prevent its aggregation, allowing it enough time to reach the nucleus and bind its sequence-specific target DNA or the p53 binding proteins that will stabilize it.

The tumor suppressor protein p53 is at the center of a network of many proteins whose role is to guard the cell from potential cancer (1, 2). Missense point mutations in p53 (see the mutation data base in www.iacr.fr/p53) lead to its inactivation and are involved in many human cancers (3, 4). These are mapped mainly to the DNA-binding core domain (5). Classification of the core domain mutants according to their DNA binding and thermodynamic stability properties results in the following three major phenotypes (6): 1) DNA contact mutants, which are unable to bind DNA and are as stable and almost as folded as the wild-type; 2) locally distorted and/or weakly destabilized mutants, partly unfolded, which are not fully able to bind DNA and are usually destabilized by less than −2 kcal/mol (6); and 3) globally destabilized mutants, which possess a high degree of unfolding and are destabilized by more than −3 kcal/mol. Accordingly, one of the important targets in cancer therapy is the rescue of p53 mutants (4, 7–10). Small molecules that bind the native but not the denatured state can restore the activity of unfolded p53 mutants by shifting the equilibrium toward the native state (7–10). We have recently demonstrated the feasibility of this approach and developed a peptide, FL-CDB3, which is able to stabilize p53 core domain and rescue its sequence-specific DNA binding activity (8).

Here, we have studied the kinetics of unfolding of p53 core domain mutants to gain a deeper understanding of the basis for their inactivation and the requirements for their rescue. We find that p53 core domain mutants are kinetically unstable, with an in vitro half-life of only a few minutes at 37 °C. We find a clear correlation between the thermodynamic and kinetic instability of p53 core domain mutants, the more unstable the mutant, the shorter the half-life. We also show that peptides can slow down the unfolding rate of the p53 core domain. We conclude that peptides like FL-CDB3, which rescue the conformation of destabilized p53 mutants, must act during or immediately after biosynthesis and maintain the mutant protein in its native state until it reaches the nucleus and binds its sequence-specific DNA.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—Human p53 core domain wild-type and mutants (residues 94–320) and human tetrameric p53 (residues 94–360) were cloned, expressed, and purified as described (11). Details of full-length p53 purification will be published elsewhere.2

**Peptide Studies**—The peptide CDB38 (Lys (Mca)-REDEDEIE-NH$_2$) was synthesized on a Pioneer peptide synthesizer (Applied Biosystems) using standard Fmoc chemistry as described (8, 12). The peptide was labeled with 7-methoxycoumarin (Mca) using the amino acid derivative Fmoc-Lys (Mca)-OH (Novabiochem). Binding of CDB38 to the p53 core was determined using fluorescence anisotropy as described (8, 12).

**Time-dependent Fluorescence Studies**—Time-dependent fluorescence measurements were made at 30 or 37 °C using a PerkinElmer LS-50b luminescence spectrofluorometer controlled by a laboratory software or the original Winlab™ software. The buffer was 50 mM Heps, pH 7.2, 1 mM Tris-2-carboxyethylphosphine (TCEP). The protein was buffer exchanged on ice to a final concentration that varied between 1–20 μM and immediately transferred to 30 or 37 °C. The fluorescence emission spectra between 250–400 nm were recorded over time (usually 16–48 h). The unfolding rate constant $k_{unf}$ was calculated from fitting of the fluorescence at 300-nm ($F_{300}$) data using Kaleidagraph™ software (Synergy software). In most cases, an exponential fit was used in accordance with Equation 1. When a subsequent linear phase was observed, caused by aggregation, a linear term was added to the function as seen in Equation 2. In Equations 1 and 2, shown below,

$$F_{300} = A \exp(-k_{unf}t) + C$$

(Eq. 1)

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1 The abbreviations used are: FL-CDB3, fluorescein-labeled core domain binding; Fmoc, N-(9-fluorenylmethoxycarbonyl; Mca, 7-methoxycoumarin; TCEP, Tris-2-carboxyethylphosphine.

2 D. B. Veprintsev and A. R. Fersht, unpublished data.
parallel, there was an exponential increase in $F_{300}$ slope of the linear aggregation phase, and immediately to 30 or 37 °C, and monitoring core domain by mixing it with urea solutions on ice, transferring it to follow the transition between the native, denatured and aggregated forms of the p53 core domain. The protein was unfolded with dithiothreitol as a reducing agent resulted in the minimal unfolding scheme of p53 at 30 and 37 °C was fitted to Reaction 1. Based on this reaction, shown below, the measured unfolding rate constant ($k_1$) for the p53 core domain variants, we fitted the $F_{300}$ curves to an exponential function with a term for linear drift. The wild-type p53 core domain unfolded with $k_1 = 1.9 \times 10^{-5} \text{sec}^{-1}$ (Table I). Replacing TCEP with dithiothreitol as a reducing agent resulted in $k_1 = 1.4 \times 10^{-8} \text{sec}^{-1}$ (Table I), which is somewhat slower but still in the same range as the measured rate for TCEP. Similar experiments performed with tetrameric wild-type p53 (p53 core + tet, residues 94–360) resulted in $k_1 = 6 \times 10^{-10} \text{sec}^{-1}$.

The minimal unfolding scheme of p53 at 30 and 37 °C was fitted to Reaction 1. Based on this reaction, shown below,

$$k_1 \xrightarrow{\text{p53native}} p53_{\text{denatured}} \xrightarrow{\text{small aggregate}} \text{larger aggregates}$$

**REACTION 1**

the measured unfolding rate constant ($k_{\text{obs}}$) for the wild-type p53 core domain equals $k_1 k_2 / (k_2 + k_{-1})$. We found that $k_{\text{obs}} - k_1$ for the wild-type by studying the urea-dependence of unfolding. Urea induces the denaturation of many proteins according to equation 3 (14),

$$\log k_{\text{obs}} = \log k_{\text{obs}} \text{H}_2O + m [\text{urea}]$$

where $k_{\text{obs}} \text{H}_2O$ is the unfolding rate constant in water and $m$ is a parameter that depends on the change in the accessible surface area. In the presence of higher concentrations of urea, $k_1 \gg k_{-1}$, because $k_{-1}$ decreases with increasing concentrations of urea, whereas $k_1$ increases for such reaction (14). A plot of $\ln k_{\text{obs}} \text{versus} [\text{urea}]$ for the wild-type p53 core domain extrapolated to the value of $k_{\text{obs}}$ in the absence of urea, confirming that $k_{\text{obs}}$ in water was indeed $k_1$ (Fig. 3) and showing that
2) a fast linear increase upon the formation of larger aggregate and lasted until a critical mass of unfolded protein was formed; the half-life values ($t_{1/2}$) were calculated using $t_{1/2} = \ln 2/k_2$. WT, wild-type; ND, not determined.

| p53 variant | $k_{obs}(30 \, ^\circ \text{C})$ | $k_{obs}(37 \, ^\circ \text{C})$ |
|-------------|-------------------------------|-------------------------------|
| WT          | $(1.90 \pm 0.06) \times 10^{-5}$ | $(1.30 \pm 0.01) \times 10^{-3}$ |
| WT (DTT)    | $(1.40 \pm 0.02) \times 10^{-5}$ | ND                           |
| V143A       | $(1.80 \pm 0.05) \times 10^{-4}$ | ND                           |
| I195T       | $(5.7 \pm 0.4) \times 10^{-4}$   | $\geq 2.2 \times 10^{-2}$    |
| C242S       | $(1.8 \pm 0.1) \times 10^{-4}$   | ND                           |
| G245S       | $(2.40 \pm 0.05) \times 10^{-5}$ | $(2.50 \pm 0.02) \times 10^{-3}$ |
| R248A       | $(1.30 \pm 0.02) \times 10^{-4}$ | $(6.3 \pm 0.2) \times 10^{-2}$ |
| R249S       | $(5.1 \pm 0.1) \times 10^{-3}$   | ND                           |
| R273A       | $(2.30 \pm 0.05) \times 10^{-5}$ | $(9.7 \pm 0.3) \times 10^{-3}$ |
| R273H       | $(1.40 \pm 0.07) \times 10^{-6}$ | $(1.80 \pm 0.02) \times 10^{-3}$ |
| WT-(94–360) | $(6.0 \pm 0.1) \times 10^{-6}$   | $(3.10 \pm 0.02) \times 10^{-4}$ |
| WT-(1–393)  | ND                            | $(3.3 \pm 0.1) \times 10^{-4}$ |

* The unfolding rate constant with 1 mM dithiothreitol in the buffer instead of TCEP.

* Owing to the different spectral properties of full-length p53, the fluorescence at 350 nm and not at 300 nm was followed.

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Fig. 3. Urea dependence of unfolding rate of wild-type p53 core domain. The wild-type p53 core domain was mixed with various urea concentrations on ice, transferred to 30 °C, and the fluorescence at 300 nm was measured. In $k_1$, versus [urea] for wild-type p53 core domain is shown (circles). The curve was fit to a linear function using Kaleidagraph. In $k_1$, $k_{obs}$ is also shown (square), and its value is $10.87$, which is in close agreement with the $y$-axis intercept value of $10.6 \pm 0.2$.

$k_1$ is the rate determining step for the unfolding reaction. The intercept at 0 mM urea was $k_{obs} = 2.6 \pm 0.5 \times 10^{-5} \text{ sec}^{-1}$, in close agreement with the experimental value of $k_{obs} = 1.9 \pm 0.6 \times 10^{-5} \text{ sec}^{-1}$ at 0 mM urea (Table I). The slope, corresponding to the $m$ value for unfolding (m) (14) was $2.5 \text{ M}^{-1}$. For destabilized mutants, $k_2 \gg k_{1/2}$, and thus $k_{obs} = k_1$.

The aggregation process represented by $k_2$ was suppressed by urea at higher concentrations, and only the first exponential phase was observed. Fluorescence spectra taken after the unfolding experiments were finished confirmed that, with an increasing urea concentration, as the fraction of unfolded p53 core domain increased, $F_{340}$ became higher while $F_{300}$ decreased significantly.

Aggregation of Denatured p53 Core Domain—We tentatively analyzed the aggregation in the absence of urea. Typically, $LS_{270}$ could be divided into four phases (Fig. 2b) as follows: 1) a lag phase, which occurred during the formation of an unfolded protein that presumably forms “seeds” for aggregation and lasted until a critical mass of unfolded protein was formed; 2) a fast linear increase upon the formation of larger aggregate particles that scattered light more; 3) a saturation level that was reached when the aggregates were fully formed, which led to 4) a drop in the intensity as the aggregates precipitated. The time course of $LS_{270}$ was concentration dependent (Fig. 2b); aggregation got faster and the lag phase got shorter until it was impossible to detect with increasing protein concentration. We were unable to analyze the data quantitatively.

Comparison of the light scattering and $F_{300}$ data showed that the major unfolding event takes place during the observed lag phase. This means that the aggregation process is initiated only after a sufficient amount of unfolded protein is formed. Thus, p53 core domain unfolding occurs in a two-step mechanism, i.e. a rate-determining step of equilibrium unfolding followed by irreversible aggregation, as described in Reaction 1.

Destabilized p53 Core Domain Mutants Denature Rapidly—We have studied the unfolding kinetics of p53 core domain mutants at 37 °C (Fig. 4). The mutants studied have mutations located in different sites within the protein and different thermostability mutations (5, 6, 11). These were as follows: 1) the DNA contact “hot-spot” mutants R248A, R273H, and R273A, which have a stability similar to that of wild type; 2) the weakly destabilized G245S, which is located at the DNA binding loop 3, but the mutated residue does not interact directly with the DNA; 3) the highly destabilized $\beta$-sandwich mutants V143A and I195T; 4) the locally distorted loop 3 mutant R249S (15); and 5) the zinc-binding residue mutant C242S.

The wild-type p53 core domain unfolded at 37 °C with $k_{obs} = 1.3 \times 10^{-5} \text{ sec}^{-1}$, corresponding to a half-life time ($t_{1/2}$) of 9 min (Table I). The hot-spot mutants G245S and R273H, which are of similar stability to wild type, had $t_{1/2}$ values of 4.6 and 6.4 min, respectively (Table I). The highly destabilized mutant I195T had $k_{obs} > 2.2 \times 10^{-2} \text{ sec}^{-1}$, corresponding to $t_{1/2}$ less than 1 min. In general, highly destabilized mutants unfold in less than 1–2 min at 37 °C and aggregate immediately after, so it was not possible to obtain quantitative data for them.

We measured the unfolding rate constants of the destabilized mutants at 30 °C. $k_{obs}$ was 40–130 times lower than at 37 °C, with $t_{1/2} = 8–14$ h for wild type, G245S, and R273H, and 20–240 min for the highly destabilized mutants V143A, I195T, C242S, R249A, and R249S (Table I). There was a linear relationship between log $t_{1/2}$ for unfolding and the free energy of denaturation (Fig. 5). The more destabilized mutants unfold faster both at 30 and 37 °C.

In addition to faster unfolding, the more destabilized mutants also aggregated faster both at 30 and 37 °C. The R273 mutants, which have similar stability to that of the wild-type, aggregated only to a small extent and in a pattern similar to that of the wild-type. The loop 3 mutants in residues G245, R248, and R249, which are destabilized by 1–2 kcal/mol, aggregate faster. The highly destabilized mutants, such as C242S, V143A, and I195T, aggregated fully in less than 10 min.

**Tetramerization Increased the Kinetic Stability of Wild-type p53**—We repeated the unfolding experiments for the tetrameric p53 construct (residues 94–360), which contains the core and tetramerization domains, and for the full-length pro-
tein (residues 1–393). At 37 °C, wild-type p53-(1–393) and p53-(94–360) were four times kinetically more stable than core domain only (residues 94–312), with \( t_{1/2} = 35–37 \) min (compared with 9 min for p53 core domain). Similar results were obtained at 30 °C, where tetrameric wild-type p53-(94–360) was 3–4 times more stable than core only.

A p53-stabilizing Peptide Slowed Down the p53 Unfolding Rate—The peptide CDB3 (REDEDEIEW-NH₂) and its derivatives, which bind the native but not the denatured state of p53 core domain, can restore the activity of unfolded p53 mutants by shifting the equilibrium toward the native state (8). The most potent CDB3 derivative is the fluorescein-labeled peptide, FL-CDB3. However, we could not test the effect of FL-CDB3 on p53 core unfolding, because the presence of fluorescein and tryptophan causes its fluorescence spectrum to mask that of p53 core. We synthesized a new CDB3 derivative in which we replaced the tryptophan with methoxycarbonyl (Mca). The peptide, CDB38, had the sequence Lys (Mca)-REDEDEIE-NH₂. Fluorescence anisotropy experiments have shown that CDB38 bound the p53 core with \( K_d \) = 15 μM at 10 °C.

CDB38 slowed down the unfolding of p53 core domain at 37 °C in a concentration-dependent manner (Fig. 6). In the presence of 105 μM CDB38, the unfolding rate of p53 core was \( k_{obs} = 3.5 \times 10^{-4} \), corresponding to a half-life of 33 min, which is 3.7 times longer than in the absence of the peptide. Increasing the concentration of CDB38 to 250 μM resulted in a half-life of 50 min, which is 5.7 times longer than that of the p53 core alone. In theory, the data should fit Equation 4, shown below.

\[
\log k_{obs} = \log k_0 + \log K_d + \log [CDB38] + \log K_d[\text{Mca}]
\]

Where \( k_0 \) is the unfolding rate constant in the absence of peptide and \( K_d \) is the binding constant for CDB38 binding to p53 core at 37 °C. Fitting the data to this equation resulted in \( K_d = 43 \) ± 7 μM (Fig. 6).

**DISCUSSION**

**Kinetics of Unfolding of p53 Core Domain**—The wild-type p53 core domain unfolded with a half-life of 9 min at 37 °C and ~10 h at 30 °C. This step was followed by a concentration-dependent rapid aggregation phase. There was a lag phase in the aggregation process during the initial unfolding process that lasted, presumably, until a critical mass of unfolded p53 was formed to produce seeds for aggregation, which grew rapidly to high molecular weight aggregates. A similar aggregation pattern was recently reported for zinc-free p53 (16).

**Thermodynamically Destabilized p53 Core Domain Mutants Are Also Kinetically Unstable**—p53 mutants that are thermodynamically unstable (6, 11) unfolded more rapidly at body temperature (Figs. 4 and 5). At 37 °C, the estimated degree of unfolding of the mutants used in this study (6) ranges from nearly completely native proteins (R273H, 2% unfolded and G245S, 5% unfolded), through mutants like R249S (15% unfolded) and C242S (53% unfolded), to highly unfolded mutants (I195T, 86% unfolded and V143A, 70% unfolded). Mutants that are less stable than wild-type by >2 kcal/mol, such as V143A, I195T, C242S, and R249S (6), unfolded more than 10 times faster (Fig. 5). Mutants such as I195T, which are highly destabilized, had very short half-lives, which were sometimes too short to measure at 37 °C. Less destabilized mutants, such as R273H and G244S, unfolded only slightly faster than wild-type. The fact that mutants with a higher unfolding degree aggregate faster implies that aggregation occurs from a dena-

**Fig. 5.** Correlation between the kinetic stability (\( \log k_{obs} \)) and thermodynamic stability relative to the wild type (\( \Delta G \) in 3 M urea). Values of \( \Delta G \) for the p53 core domain mutants at 37 °C (a) and 30 °C (b) were taken from Ref. 6.

**Fig. 6.** CDB38 slows down the unfolding of wild type p53 core domain at 37 °C. p53 core (1.6 or 2 μM) was incubated in the presence of various CDB38 concentrations, and \( F_{ave} \) was monitored and fitted as described above. \( k_{obs} \) is plotted versus [CDB38], and the curve was fitted as described in the text to determine \( K_d \) at 37 °C for CDB38 binding to the p53 core domain.

**Fig. 7.** A model for the action of p53 stabilizing drugs. Newly synthesized mutant p53 is released from the ribosome (circle) in its unfolded form. Then, two pathways are possible. 1) In the presence of a stabilizing drug such as FL-CDB3 (small square), the protein refolds to its native conformation (large square), is transported into the nucleus as a complex with the drug, and then binds its target DNA. 2) In the absence of the drug, the unfolded protein aggregates and cannot be transported into the nucleus and bind DNA.
tured state. For wild type p53, this denatured state could be monitored using urea, as shown here, or by looking at the zinc-free form (16).

Implications for Rescue by Small Molecules—The kinetic stability and thermodynamic stability of p53 decrease in parallel. Many destabilized mutants have melting temperatures that are below body temperature and, therefore, denature very rapidly after biosynthesis and before they even become functional because of their short half-lives at body temperature. The kinetic instability may be the underlying reason for their inactivation. Our results for the peptide CDB38 demonstrate that the peptides that bind the native but not the denatured state of p53 core domain (7, 8) can increase the half-life of the p53 core domain and thus stabilize it. Such drugs, which rescue the conformation of unstable mutants of p53 (e.g. FL-CDB3 (8) or possibly PRIMA-1 (9)) must have to act during biosynthesis, before the rapid unfolding of the mutant protein takes place (see model in Fig. 7). These drugs will maintain the newly synthesized protein in its native conformation, prevent its unfolding and aggregation, and allow its localization to the nucleus, where it will be able to bind its target DNA. Once the mutant p53 reaches its target location or is bound to other proteins, it can be stabilized by the specific DNA or the p53 binding proteins that will displace the drug, which will be free to bind another newly synthesized mutant p53. This is the basis of a chaperoning strategy to rescue mutant p53 (8). It is important for the chaperoning strategy that the drug not prevent the nuclear import of p53. The binding of FL-CDB3 is balanced so that it binds tightly enough to stabilize p53 but not so tightly that it cannot be displaced. Indeed, preliminary results with living cancer cells show that FL-CDB3 is effective in vivo.3

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