Reversible Aggregation Plays a Crucial Role on the Folding Landscape of p53 Core Domain

Daniella Ishimaru,* Luis M. T. R. Lima,† Lenize F. Maia,* Priscila M. Lopez,* Ana P. Ano Bom,* Ana P. Valente,* and Jerson L. Silva*

*Laboratório de Termodinâmica de Proteínas e Estruturas Virais Gregorio Weber, Centro Nacional de Ressonância Magnética Nuclear de Macromoléculas, Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, and †Departamento de Medicamentos, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil

ABSTRACT The role of tumor suppressor protein p53 in cell cycle control depends on its flexible and partially unstructured conformation, which makes it crucial to understand its folding landscape. Here we report an intermediate structure of the core domain of the tumor suppressor protein p53 (p53C) during equilibrium and kinetic folding/unfolding transitions induced by guanidinium chloride. This partially folded structure was undetectable when investigated by intrinsic fluorescence. Indeed, the fluorescence data showed a simple two-state transition. On the other hand, analysis of far ultraviolet circular dichroism in 1.0 M guanidinium chloride demonstrated a high content of secondary structure, and the use of an extrinsic fluorescent probe, 4,4′-dianilino-1,1′ binaphthyl-5,5′-disulfonic acid, indicated an increase in exposure of the hydrophobic core at 1 M guanidinium chloride. This partially folded conformation of p53C was plagued by aggregation, as suggested by one-dimensional NMR and demonstrated by light-scattering and gel-filtration chromatography. Dissociation by high pressure of these aggregates reveals the reversibility of the process and that the aggregates have water-excluded cavities. Kinetic measurements show that the intermediate formed in a parallel reaction between unfolded and folded structures and that it is under fine energetic control. They are not only crucial to the folding pathway of p53C but may explain as well the vulnerability of p53C to undergo departure of the native to an inactive state, which makes the cell susceptible to malignant transformation.

INTRODUCTION

The wild-type tumor suppressor protein p53C is a nuclear phosphoprotein that plays a key role in cell cycle control (Hall et al., 1996). In normal conditions, p53C has a short half-life, being directed to ubiquitin-mediated degradation by binding to MDM2 protein (Lane and Hall, 1997). The occurrence of cellular stress (ultraviolet radiation, genomic damage, hypoxia) activates p53C and causes it to remain stable in the cell for a longer period of time; this leads to cell cycle arrest or apoptosis. It is the failure of these actions that has been related to tumor progression (Prives and Hall, 1999). In fact, mutations in the *p53C* gene constitute the most frequent genetic alteration in human cancers. More than 50% of all cancers—including lung, colon, bladder, breast, and ovary—reassociated with a mutant, nonfunctional p53C; ~90% of these mutants have a single amino-acid residue altered at p53C's core domain (p53C), making this domain one of the most investigated proteins during the last decade. Therefore understanding of folding/unfolding properties of the native state of p53C is an important prerequisite for directing successful therapeutic approaches.

The classical view of protein folding holds that it occurs through specific pathways, where partially folded intermediate conformations gradually drive the protein to its native state (Kim and Baldwin, 1990). However, several proteins have been shown to fold apparently in a single step, without detectable intermediates. An alternative hypothesis suggests the concept of a smooth or rugged funnel to represent the energy landscape for protein folding without and with intermediate structures, respectively (Bryngelson et al., 1995; Dill and Chan, 1997; Plotkin and Onuchic, 2002).

In accordance with the rugged funnel hypothesis, intermediate structures have been detected for several proteins (Plotkin and Onuchic, 2002). They are important since they can either be related to real intermediates of protein folding or be identified as important precursors along protein misfolding and aggregation pathways. Many intermediate conformers are associated with human diseases such as Alzheimer's, Parkinson's, prion-related encephalopathies, and some types of cancer (Dobson, 1999; Bullock and Fersht, 2001; Cordeiro et al., 2001; Fändrich et al., 2001; Lashuel et al., 2002; Sacchettini and Kelly, 2002; Foguel et al., 2003). Intermediates of protein folding/unfolding processes can be captured by mild denaturing conditions, such as changes in pressure, temperature, small variations in pH, and addition of small amounts of chaotropic agents such as urea and GdmCl

Submitted April 19, 2004, and accepted for publication July 23, 2004.

Daniella Ishimaru and Luis M. T. R. Lima contributed equally to this work.

Address reprint requests to Jerson L. Silva, Universidade Federal do Rio de Janeiro, Departamento de Bioquímica Médica, Instituto de Ciências Biomédicas, CCS Sala E10, Cidade Universitária, Rio de Janeiro, RJ 21941-590 Brazil. Tel.: 55-21-2562-6756; Fax: 55-21-2562-6756; E-mail: jerson@bioqmed.ufrj.br.

Abbreviations used: p53C, core domain of the tumor suppressor protein p53C; GdmCl, guanidinium chloride; CD, circular dichroism; bis-ANS, 4,4′-dianilino-1,1′ binaphthyl-5,5′-disulfonic acid.

0006-3495/04/10/2691/10 $2.00

doi: 10.1529/biophysj.104.044685

(Brems, 1988; Carra and Privalov, 1996; Fersht, 1999; Dobson, 2000; Kuwajima and Arai, 2000; Silva et al., 2001; Ishimaru et al., 2003a). The protein conformational changes induced by these treatments can be accompanied by means of spectroscopic techniques such as fluorescence, CD, and NMR (Sanz and Fersht, 1993; Bullock et al., 1997; Foguel et al., 1998; Fersht, 1999; Mateu et al., 1999; Dobson, 2000; Kuwajima and Arai, 2000; Neira and Mateu, 2001; Silva et al., 2001; Kuwata et al., 2002; Ishimaru et al., 2003a). However, some intermediates are not observable by most usual spectroscopic methods, as in the case of barnase (Sanz and Fersht, 1993).

Kinetic intermediates of p53C have been described only for the folding of the C-terminal region; they are transient, highly structured, but spectroscopically silent (Mateu et al., 1999). We recently described that denaturation by high temperature or high pressure leads to generation of irreversible aggregates (Ishimaru et al., 2003b). The isolation of a monomeric intermediate by high pressure was only attained by the combination of pressure with subzero temperatures (Ishimaru et al., 2003a).

In the context of the cell, the unfolding process participates in several important steps in cellular physiology, such as protein turnover in proteasomes (Fersht and Daggett, 2002). In addition, several diseases related to misfolded proteins are, in fact, diseases of protein unfolding, since the protein is initially correctly folded (Ferrão-Gonzales et al., 2000; Cordeiro et al., 2001). Therefore the detection of intermediate species, in special aggregating ones, during p53C unfolding might be important for the cellular homeostasis since its accumulation could interfere with the proper integration of cellular functions.

Here we describe an intermediate structure during equilibrium and kinetic folding and unfolding of p53C. This intermediate is obtained by low concentrations of GdmCl and has a high tendency to form aggregates. The reversibility of the aggregation sheds light into the mechanism of folding of p53C and its role on the well-known plasticity of this tumor suppressor protein. The elution from the gel filtration chromatography and the light-scattering value indicates that this aggregate is small (10–20 subunits). This intermediate also occurred with the hot-spot mutant R248Q (one of the most prominent somatic mutations in different types of cancer) which points to the medical impact of this finding. The kinetic data show how high concentration of protein favors the aggregation, a condition that would be present in a cellular situation of loss-of-function mutation, such as the R248Q. These polymeric intermediates may be the site where p53 mutants (translated from a single mutant allele) are able to drive wild-type p53 protein (translated from the remaining wild-type p53 allele) into a mutant conformation. Thus, these intermediates may be targets to the development of lead compounds capable of destabilizing them with potential therapeutic action against tumor diseases.

MATERIALS AND METHODS

Chemicals

All reagents were of analytical grade. Distilled water was deionized and filtered through a Millipore (Billerica, MA) water purification system before use. Bis-ANS was purchased from Molecular Probes (Eugene, OR). All solutions were prepared just before use. Stock solutions of GdmCl were checked for exact concentration by refractive measurements (Pace, 1986).

p53C subcloning, expression, and purification

The core domain of the tumor suppressor human protein p53C was obtained as described elsewhere (Ishimaru et al., 2003a). Purification was performed as described in Bullock et al. (1997). Protein samples were stored in 50 mM Tris.Cl, pH 7.2, 150 mM NaCl, 5 mM DTT, and 5% glycerol in liquid nitrogen. p53C comprises amino-acid residues 94–312.

Equilibrium folding and unfolding measurements

p53C at 5 μM was used in all equilibrium measurements. All experiments were carried out at least three times, in buffer Tris.Cl 50 mM, pH 7.2, 150 mM NaCl, 5 mM DTT, and 5% glycerol. Intrinsic fluorescence spectra were obtained by excitation at 278 nm and emission was monitored from 295 to 415 nm in an ISS (Champaign, IL) spectrometer. Because of the high protein concentration used, and since the buffer contributed $<2\%$ to the emission, no correction was required. For light-scattering data, excitation was at 320 nm and emission was collected from 300 to 340 nm. For experiments in the presence of bis-ANS (5 μM) the excitation wavelength was at 360 nm and emission was collected from 400 to 600 nm.

Equilibrium unfolding in GdmCl was performed at 25°C $\pm$ 0.2°C in the presence of the concentrations indicated in the figures. Data were collected after overnight incubation with the denaturing agent at 25°C.

Circular dichroism

Experiments were carried out at least three times with 5 μM p53C in 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM DTT, and 5% glycerol. Far-ultraviolet spectra were monitored from 200 to 260 nm in a 2.00 mm quartz cuvette and recorded in a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) at 25°C $\pm$ 0.2°C.

Kinetic measurements

Kinetic unfolding experiments were performed using a SX18MV stopped-flow apparatus (Applied Photophysics, Leatherhead, UK). All experiments were performed at 25°C $\pm$ 0.2°C, with 5 μM p53C, unless otherwise stated. Intrinsic fluorescence was followed by setting excitation to 278 nm, collecting emission through cut-off filter (WG320, with 50% transmittance at 320 nm).

All data presented are an average of 5 to 10 runs, and all concentration refers to those resulting from mixing equal volumes of protein and GdmCl from each syringe (both in 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM DTT, and 5% glycerol). Data were fitted to obtain the rates using nonlinear least-squares fitting softwares provided by the manufacturer, using single exponential equation. Kinetic refolding experiments were performed incubating 50 μM p53C in GdmCl for at least 2 h. Later, samples were diluted 10 times with buffer and different amounts of GdmCl to provide indicated GdmCl final concentration. Aggregation was immediately followed in a spectrofluorimeter (Cary Eclipse Spectrofluorimeter, Varian, Palo Alto, CA) over time by light-scattering measurements with excitation and emission set at 320 nm. All experiments were performed at 25°C $\pm$ 0.2°C, in 50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM DTT, and 5% glycerol.

Gel filtration chromatography

Gel filtration chromatography was performed using a GPC100 column (SynChropak, Synchrom, Lafayette, IN) attached to a high-pressure liquid chromatography system (Shimadzu, Tokyo, Japan) with absorbance recorded at 280 nm. The system was equilibrated with 50 mM sodium phosphate, pH 7.2, 5 mM DTT, 150 mM NaCl, and 5% glycerol, in the absence or presence of the indicated GdmCl concentration, with a flow rate of 0.6 mL/min.

NMR data

For 1D-NMR experiments, 10% D_2O (Isotec., Miamisburg, OH) was added to purified p53C samples. ^{1}H-NMR measurements were performed at 25°C on a Bruker Avance DRX 600 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany), equipped with a 5-mm inverse detection triple resonance probe with z gradient. Water suppression was achieved using the watergate technique (Piotto et al., 1992).

RESULTS

The presence of one tryptophan and eight tyrosine residues in p53C allowed us to follow the unfolding process through the fluorescence emission spectra based on these intrinsic probes (Fersht, 1999; Dobson, 2000; Nichols and Matthews, 2001; Silva et al., 2001; Ishimaru et al., 2003a,b). From each spectrum, we calculated the spectral center of mass, an index of the average energy value of the spectrum. Since aromatic amino-acid residues are sensitive to the polarity of their immediate environment, changes in center of mass will reflect conformational changes induced in the protein. To investigate the structural stability of p53C, we performed isothermal unfolding experiments by employing GdmCl as denaturant, at 25°C (Fig. 1). GdmCl has been widely used for protein denaturation studies providing a more pronounced effect as compared with urea, but in general similar results with both are obtained (Pace, 1986). The fluorescence emission spectra of p53C at high GdmCl concentrations (from 3 M to 6 M), clearly showed both Tyr and Trp contributions (see 6 M spectrum in inset to Fig. 1), suggesting that aromatic residues were highly exposed to the aqueous environment and allowing us to consider them as completely unfolded. Fitting the data shown in Fig. 1, a $[GdmCl]_{50\%}$ value equal to 1.00 ± 0.03 M was obtained, which is similar to previous reports (Bullock et al., 2000; Bell et al., 2002), presenting no apparent accumulated intermediate.

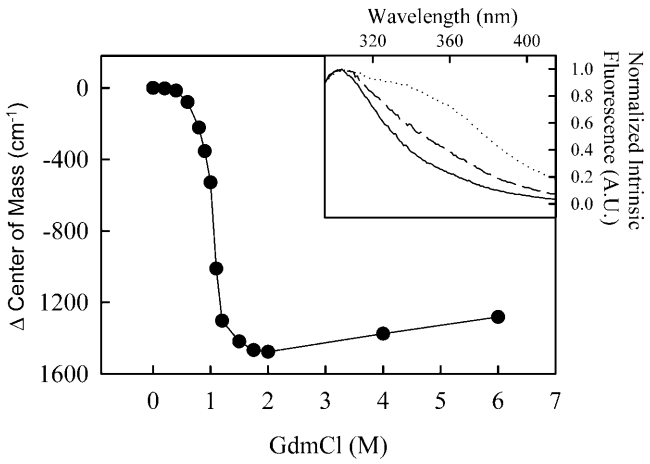

FIGURE 1 Effects of GdmCl-induced denaturation on the tertiary structure of p53C. p53C samples (5 μM) were exposed to 0–6 M GdmCl at 25°C (15 h in each case) in a buffer containing 50 mM Tris.Cl, pH 7.2, 150 mM NaCl, 5 mM DTT, and 5% glycerol. Data were analyzed as center of spectral mass. Line in the main panel corresponds to fitted data as described in Materials and Methods. (*Inset*) Self-normalized spectra obtained in 1 M GdmCl (*dashed line*), 6 M GdmCl (*dotted line*), and no GdmCl (*solid line*). Excitation wavelength was 278 nm and emission was collected from 295 to 415 nm.

Intrinsic fluorescence probes the local environments of the Trp and Tyr, providing a measure of the tertiary conformation. A well-known test of a two-state transition is to examine whether changes in secondary structure occur in parallel with the changes in fluorescence. When we examined the secondary structure of p53C by far ultraviolet circular dichroism (Fig. 2) as a function of GdmCl, the changes were not coincident with the intrinsic fluorescence data. When we followed β-sheet structure at 218 nm (Fig. 2 *A*), we observed a slight loss of secondary structure up to 0.4 M GdmCl, followed by an increase around 0.9–1.2 M GdmCl and a second and abrupt loss of structure above 1.5 M. This result directly entails the existence of an intermediate conformation with a significantly higher content of secondary structure around 1 M GdmCl. The ellipticity at 1 M GdmCl increased about twice (from −25 to −50 mdeg). Examples of spectra at 0, 1, and 6 M GdmCl are shown in Fig. 2 *C*. When a hot-spot mutant (R248Q) was utilized, we observed the same pattern (Fig. 2 *B*), where the protein presented the higher degree of secondary structure at 1 M GdmCl.

Because intrinsic fluorescence and CD data are noncoincident, a two-state transition can be ruled out. To further characterize the properties of this intermediate, we utilized an extrinsic fluorescent probe (bis-ANS) for analysis of GdmCl-induced denaturation of p53C (Fig. 3). The hydrophobic probe bis-ANS binds noncovalently to hydrophobic regions of proteins. It is a sensitive probe for structural changes promoted by different treatments (Silva et al., 1992a; Ishimaru et al., 2003a) because it undergoes a large increase in the fluorescence quantum yield when bound to proteins. Fig. 3 *A* shows bis-ANS spectra in the presence of p53C and no GdmCl (*solid line*), 1 M (*dashed line*) and 6 M (*dotted line*) GdmCl. At 1 M GdmCl, we can observe a large increase in bis-ANS fluorescence, which can be better visualized in Fig. 3 *B*. The binding of this extrinsic fluorescent probe is eightfolded increased at 1–1.1 M GdmCl and decreases as the GdmCl concentration is further raised. This large increase in bis-ANS binding by the intermediate indicates that it exposes structured hydrophobic pockets to the solvent around 1 M GdmCl. The increase in bis-ANS binding coincides with the gain in secondary structure,

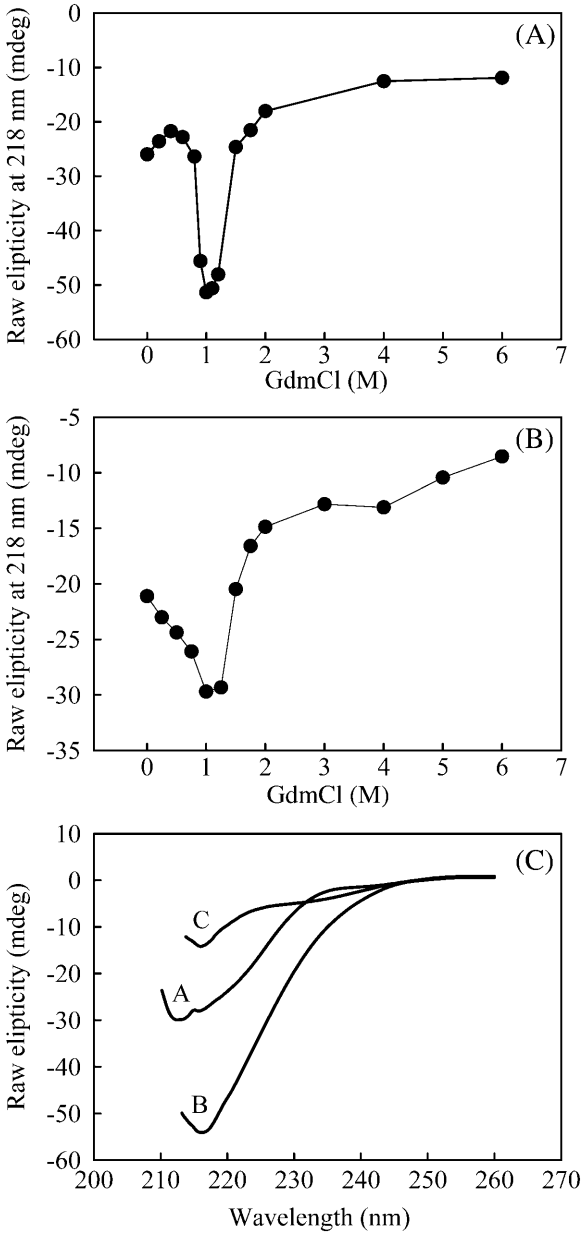

FIGURE 2 Analysis of GdmCl effects on the secondary structure of p53C. p53C samples (5 μM) were incubated with or without GdmCl for 15 h at 25°C, as described in Materials and Methods. (*A* and *B*) CD signal was monitored at 218 nm after incubation with 0–6 M GdmCl. (*A*) Wild-type p53C. (*B*) p53C mutant R248Q. (*C*) Far-ultraviolet CD spectra of p53C at 25°C. (*Curve A*) spectrum of p53C without GdmCl; (*curve B*) p53C at 1 M GdmCl; (*curve C*) p53C at 6 M GdmCl. Ellipticity values were omitted when the gain of the photomultipliers was too high.

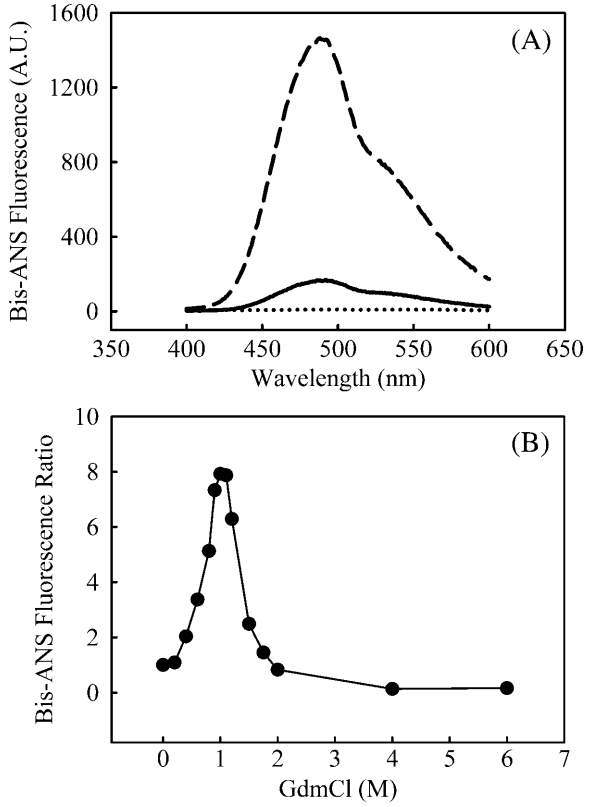

FIGURE 3 Bis-ANS fluorescence: Evidence for loosely packed hydrophobic cores. p53C (5 μM) was incubated with increasing concentrations of GdmCl (0–6 M) for 15 h at 25°C. Then, 5 μM bis-ANS was added to the medium and 60-min incubation period was allowed before acquisition of each fluorescence spectrum. Data were collected at 25°C with excitation wavelength at 360 nm and emission collected from 400 to 600 nm. (*A*) Bis-ANS fluorescence spectra at 0 M (*solid line*), 1 M (*dashed line*), or 6 M (*dotted line*) GdmCl. (*B*) Ratio of the area of bis-ANS fluorescence against increasing concentrations of GdmCl.

corroborating the presence of a nonnative, partially structured conformation that may resemble a molten globule. Molten globules are intermediate conformations usually achieved by mild denaturing conditions, which have a compact conformation, although not yet as compact as the native one (Baldwin, 1996; Creighton, 1997; Arai and Kuwajima, 2000; Kuwajima and Arai, 2000). In addition, these partially folded states are usually characterized by having less tertiary structure, a substantial content of secondary structure, and an increase in the binding of ANS dyes because of the presence of loosely packed hydrophobic cores (Fersht, 1999; Dobson, 2000; Kuwajima and Arai, 2000).

The unfolding of p53C can also be monitored directly by a series of one-dimensional (^{1}H) NMR spectra. In Fig. 4 *A*, the native spectrum of p53C has a good dispersion of chemical shifts of amidic and aromatic protons, indicating a structured protein. Addition of 0.8 M GdmCl produces enlargement of some peaks and disappearance of others (Fig. 4 *B*). Moreover a strong signal from 5.5 to 7.5 ppm appears, probably corresponding to the denaturant itself. At 1.0 M GdmCl (Fig. 4 *C*), the concentration at which the intermediate was detected in Figs. 2 and 3, we observe a loss of sharp ^{1}H peaks. However, when p53C was incubated with 4 M GdmCl, we were able to see reappearance of signals including a clear signal corresponding to the single Trp residue (Trp^{146}) between 9.5 and 10.0 ppm (Fig. 4 *D*). Clustering of peaks around 7.5 and 8.5 ppm suggests that

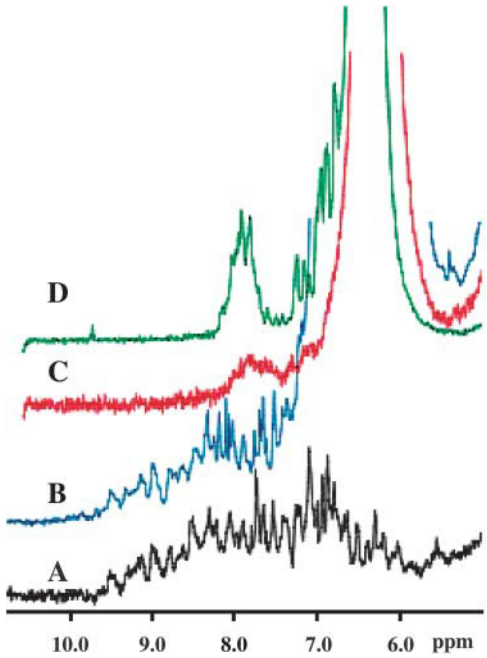

FIGURE 4 ^{1}H-NMR spectra of the aromatic region of p53C. (*A*) The native state of p53C. (*B*) p53C incubated for 15 h with 0.8 M GdmCl. (*C*) The intermediate, aggregated state in 1 M GdmCl. (*D*) The unfolded state, in 4 M GdmCl. All samples were buffered in 50 mM sodium phosphate, pH 7.2, 5 mM DTT, 150 mM NaCl, and 10% D_2O at 25°C ± 0.2°C. A peak between 5.5 and 7.5 ppm correspond to GdmCl signal.

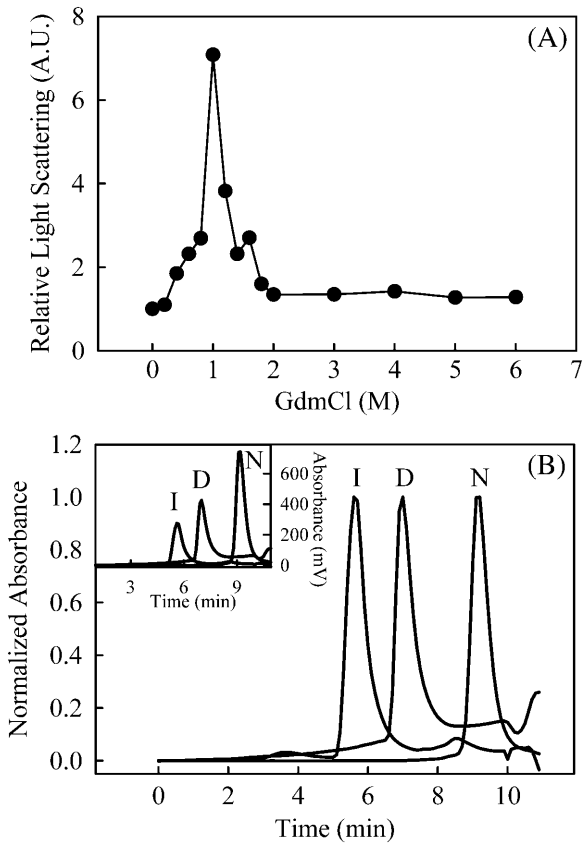

FIGURE 5 Investigation of the oligomeric state of p53C. (*A*) p53C samples were incubated with 0–6 M GdmCl and sample's average size was analyzed by light scattering ($LS_{obs}/LS_{no\ GdmCl}$) from 300 to 340 nm (incident light at 320 nm) at 25°C ± 0.2°C. (*B*) Normalized absorbance at 280 nm detected during a gel filtration chromatography of p53C native sample (*N*), with 1 M GdmCl (*I*), or with 3 M GdmCl (*D*). (*Inset*) Raw absorbance of p53C samples.

different protons are in a similar chemical environment, typical of a fully unfolded conformation.

The exposure of hydrophobic regions generated by low concentrations of GdmCl and detected by the increase in bis-ANS binding could induce the formation of aggregates. Besides, disappearance of ^{1}H signals could also indicate the presence of an aggregated protein, since NMR can only detect signals of relatively small soluble proteins. Aggregates formed by partially folded conformations have been described for several proteins (Brems, 1988; St. John et al., 2001; Ishimaru et al., 2003b). To evaluate changes in p53C average size, we measured light scattering at increasing concentrations of GdmCl (Fig. 5 *A*). p53C showed an increase in light scattering up to 1 M GdmCl, returning to low values at higher GdmCl concentrations. We infer that p53C aggregates in 1 M GdmCl and then unfolds at higher concentrations of the denaturant.

Aiming to further characterize the oligomeric state of p53C, we performed gel filtration chromatography by high-performance liquid chromatography. Fig. 5 *B* shows a retention time of 9.2 min for the native conformation (*N*), whereas the completely denatured protein (*D*), which presents an expected greater hydrodynamic volume due to a more open conformation of the denatured state, exhibited a retention time of 7 min. However, the conformation obtained after p53C incubation with 1 M GdmCl showed a retention time of 5.6 min, corroborating that this intermediate is indeed multimeric. Nevertheless, the elution of the aggregate in the high-performance liquid chromatography gel filtration column does indicate that it has a limited microscopic size. Indeed, the increase in light scattering (Fig. 5 *A*) was <10-fold. We tested binding of thioflavin T to these aggregates and the increase in fluorescence was not very large (data not shown) which is in agreement with the presence of a small aggregate. At GdmCl concentrations between 0 and 1 M and between 1 and 3 M, gel-filtration chromatograms indicated that the p53C intermediate was capable of existing in equilibrium with the native or denatured states, respectively (data not shown).

To evaluate the reversibility of this aggregate, we subjected the sample to 290 MPa at 25°C and followed its oligomeric state as a function of time (Fig. 6 *A*). Immediately after pressurization, the light-scattering value decreased 50% in comparison with the one obtained with the aggregated p53C. These values continued to decrease for another 60 min, when the light-scattering data were similar to the one detected with the native, monomeric protein (*triangle* in Fig.

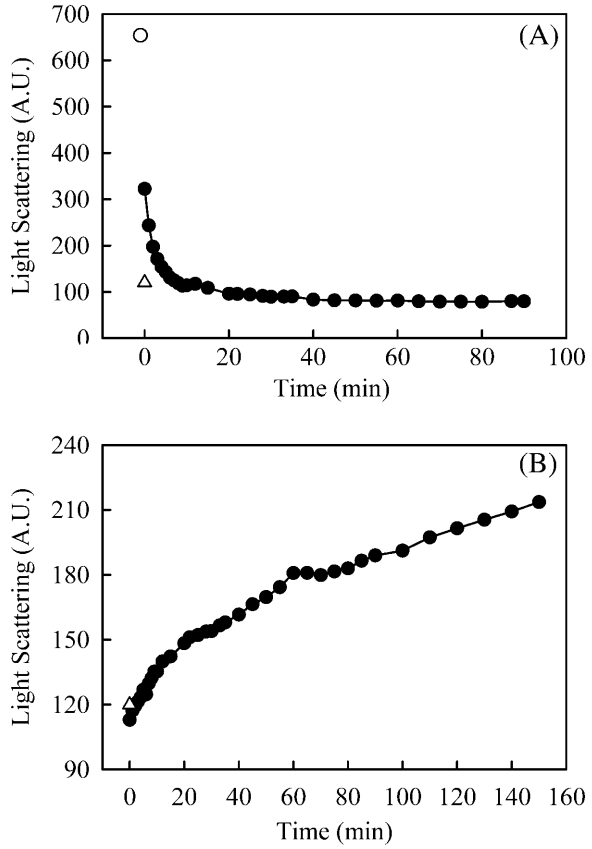

FIGURE 6 Investigation of the reversibility of the p53C aggregates. (*A*) p53C at 5 μM was allowed to aggregate through incubation with 1 M GdmCl at 25°C ± 0.2°C for 24 h. The aggregated sample was subjected to 2.9 kbar at 25°C and light-scattering values from 300 to 340 nm (incident light at 320 nm) were accompanied over time. (*B*) Light-scattering values after return to atmospheric pressure.

6). When we returned to atmospheric pressure, the p53C began to aggregate again (Fig. 6 *B*), indicating that the p53C aggregates induced by incubation with 1 M GdmCl were highly specific. The susceptibility to pressure of these aggregates indicates that they have a structure that excludes water as found with some aggregates (Ferrão-Gonzales et al., 2000; Silva et al., 2001; St. John et al., 2001; Foguel et al., 2003). The liability to pressure is the characteristic of some aggregates. However, there are some fibrillar structures that are highly resistant to pressure. For example, at some conditions, PrP fibrils become insensitive to pressure (Cordeiro et al., 2004; Torrent et al., 2004) because of very tight packing. Aggregates obtained by combined thermal and pressure denaturation of p53C are also resistant to pressure (Ishimaru et al., 2003b). As pointed out by Torrent et al. (2004), different pathways of aggregation and amyloid formation can lead to a different degree of compactness of the final structures. It seems that early aggregates tend to be more susceptible to pressure as recently shown by Niraula et al. (2004) for the multimeric precursor of amyloid fibrils of the disulfide-deficient mutant of hen lysozyme.

To further characterize the formation of the aggregates, we performed kinetic unfolding measurements. Using a stopped-flow fluorimeter, p53C was mixed with different amounts of GdmCl and changes in intrinsic fluorescence emission were followed over time. We observed an increase of the emission, which can be well adjusted to a single exponential profile (Fig. 7 *A*). There are no further changes in fluorescence even up to 15 min (data not shown). As generally expected, unfolding rate constants present a dependence on GdmCl concentration (Fig. 7 *B*). In principle, a single exponential behavior indicates no apparent kinetic unfolding intermediate. However, measurements of p53C unfolding by GdmCl show a dependence of the rate constant on protein concentration (Fig. 8). These data indicate the existence of at least one intermediate step in

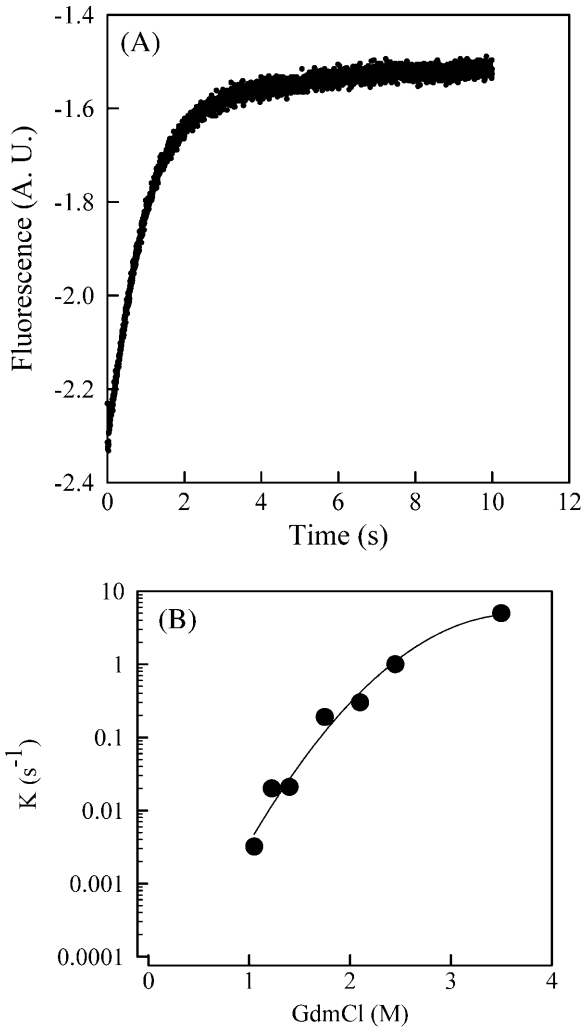

FIGURE 7 Kinetic unfolding of p53C. (*A*) p53C (5 μM final concentration) was mixed with GdmCl (3.5 M final concentration) and intrinsic fluorescence was followed. Signal shows a simple exponential dependence on time. (*B*) Kinetic fluorescence changes rate upon p53C unfolding in the indicated concentration of GdmCl. Experiments performed at 25°C ± 0.2°C. Details in Materials and Methods.

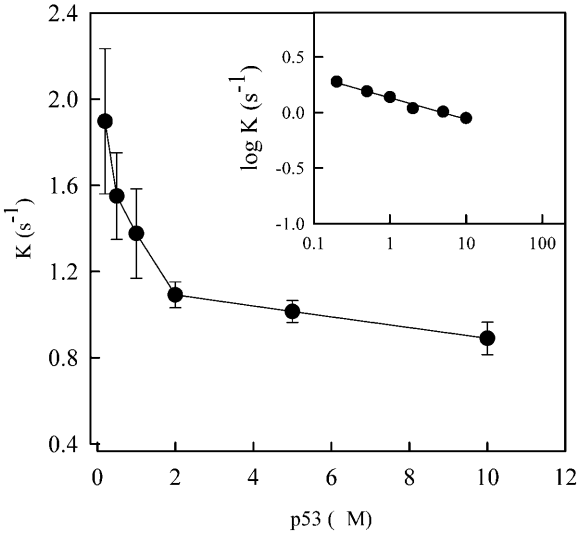

FIGURE 8 Dependence of kinetic unfolding on p53C concentration. p53C (final concentration as indicated) was mixed with GdmCl (to give 3.5 M final concentration) and data were analyzed as described in Materials and Methods. (*A*) Kinetic rate constant versus protein concentration. (*Inset*) Double log plot. Experiments performed at 25°C ± 0.2°C.

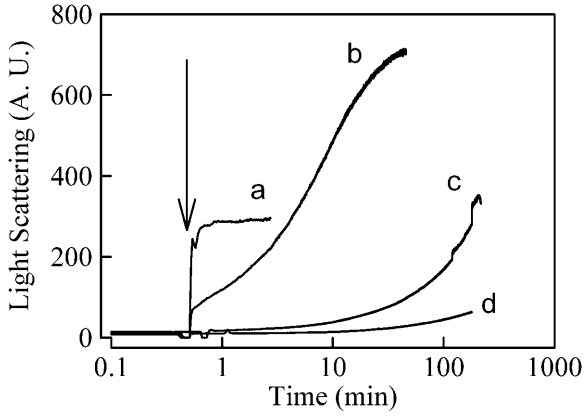

FIGURE 9 Formation of intermediate p53C aggregates from unfolded protein. p53C unfolded in 3.0 M GdmCl was diluted into buffer to 5 μM p53C (final concentration), containing GdmCl for (final concentrations) 0.050 M (*a*), 0.10 M (*b*), 0.20 M (*c*), and 0.40 M (*d*), and light scattering was followed over time. All experiments were performed at 25°C ± 0.2°C. Details in Materials and Methods.

which protein association occurs. The fact that increasing protein concentration leads to a slower kinetic indicate that p53C association/collapse is a step that anticipates subsequent protein unfolding. The existence of a protein-concentration dependence on the kinetics of p53 unfolding is indicative of a high-order (bimolecular or higher) association event. Thus, at low protein concentration, the unfolding is faster because it occurs with no concurrent association. In contrast, at high protein concentration the association competes with the unfolding.

The kinetic data seem to corroborate the finding at equilibrium conditions of an association/aggregation process during unfolding. However, we could not identify any change in light scattering during the unfolding kinetics, indicating that the lifetime of the aggregated intermediate is very short. Nevertheless, aggregation occurs and the intermediate accumulates in the refolding pathway (Fig. 9). Under refolding conditions, the kinetic data show the accumulation of aggregated material when the protein is diluted from the unfolding condition (3 M GdmC) to low concentrations of the denaturant (Fig. 9).

DISCUSSION

One of the first established principles for protein folding was that the process should not be random (Levinthal, 1969). Therefore, a number of theories have been proposed to explain the protein folding and several of them are based on the formation of intermediates (Kim and Baldwin, 1990; Plotkin and Onuchic, 2002). Bovine growth hormone has been shown to undergo a multistate denaturation process with stable intermediates (Brems, 1988); human recombinant γ-interferon has an expanded intermediate at 0.9 M GdmCl (Kendrick et al., 1998); Arc repressor protein has a molten-globule conformation in its pressure-induced monomeric state (Silva et al., 1992b). Nevertheless, each intermediate is characteristic for a specific condition and a specific protein. For instance, whereas p53C showed an increase in secondary structure (a more negative CD signal) for the intermediate state (Fig. 2), the recombinant human growth hormone had much less intermolecular β-sheet in the aggregates prepared in 0.75 M GdmCl compared with those formed in buffer alone under high pressure (St. John et al., 2001).

The relation structure-function is a fundamental subject for the study of proteins. In this view, intermediates constitute an issue of intense debate as to whether they represent conformations essential for directing the protein folding to the native state, or misfolded structures trapped in physiologically nonrelevant local energy minima (Kim and Baldwin, 1990; Bryngelson et al., 1995; Carra and Privalov, 1996; Creighton et al., 1996; Dill and Chan, 1997; Wagner and Kiefhaber, 1999). Several experimental data have shown that some intermediates are indeed specific folding intermediates. Besides, studies of transition states have demonstrated that intermediate conformations are very close to the native state, although somewhat distorted (Oltzen et al., 1994). On the other hand, some intermediate conformations could represent energetically trapped, misfolded structures.

In the case of p53C, protein-engineering studies have detected important kinetic intermediates for the proper folding of the tetrameric C-terminal (Mateu et al., 1999). For this domain, a transient, highly structured dimeric intermediate was observed, although the transition from the monomeric intermediate to the tetrameric native protein was undetectable spectroscopically. The initial step of folding behaved as a nucleation-condensation mechanism with an

early transition state, whereas the spectroscopically silent step followed the framework mechanism (Mateu et al., 1999). Still, hydrogen exchange studies probed by chemical denaturant and temperature have shown that no particular folding intermediate is populated for this same domain (Neira and Mateu, 2001).

A denaturing midpoint of 1.00 ± 0.03 M GdmCl at 25°C and a two-state transition for the p53C denaturation process are consistent with a previous report (Bullock et al., 1997). However, the CD signal and the bis-ANS binding both reached a minimum and a maximum, respectively, at 1.0 M GdmCl indicating that an intermediate structure exists at a GdmCl concentration coincident with the midpoint value obtained by intrinsic fluorescence. Intermediate conformations of p53C have been previously achieved by high pressure (Ishimaru et al., 2003a,b), high temperatures (Ishimaru et al., 2003b), and low pH (Bullock et al., 2000). The conformation achieved by pH values below 5.5 has been described as having large increases in tryptophan as well as in ANS fluorescence, indicating a transition to an acid molten-globule state (Bullock et al., 2000). However the species obtained by high pressure at subzero temperatures resembles that of the hot-spot mutant R248Q and exists in a preaggregating state (Ishimaru et al., 2003a). In contrast, those achieved by high pressure at 37°C and high temperatures (up to 55°C) are highly aggregating conformations (Nichols and Matthews, 2001). In addition, a recent report by Friedler and colleagues (Friedler et al., 2003) showed that the wild-type p53C is a kinetically unstable protein at 37°C exhibiting an in vitro unfolding rate of 1.9×10^{-5} s^{-1}. Moreover, they also showed that p53C unfolding induced by this temperature was accompanied by the formation of large aggregates.

Interestingly, under GdmCl conditions where the intermediate was detected, the extension of aggregation was intense, as demonstrated by light-scattering data and gel-filtration chromatography (Fig. 5), and almost no peaks could be detected by NMR (Fig. 4). On the other hand, increasing the GdmCl concentration up to 4.0 M reversed the aggregation and a soluble, although denatured, protein was observed. A similar behavior was described with urea-induced denaturation (Bullock et al., 1997). Therefore, p53C denaturation by GdmCl occurred in two stages: formation of an intermediate, partially folded, and aggregating structure at 1.0 M GdmCl, and further denaturation of the protein at higher GdmCl concentrations.

Partially folded denatured structures are generally the conformers responsible for protein aggregation (Brems, 1988; Sanz and Fersht, 1993), and aggregation is usually considered to be an irreversible process (Kendrick et al., 1998; Friedler et al., 2003). Consequently, the nature of the intermediate state is essential for the understanding of the aggregation pathway (Kendrick et al., 1998). Interestingly, some human diseases, as senile systemic amyloidosis and some types of cancer, involve wild-type proteins prone to aggregate (Moll et al., 1996; Ostermeyer et al., 1996; Ferrão-Gonzales et al., 2000). In the case of senile systemic amyloidosis, aggregation of wild-type protein occurs because of an intermediate, alternative conformation of the protein (Ferrão-Gonzales et al., 2000). Exact protein folding in vivo is assisted by a more complex system, which obviously is not obtained in any in vitro condition, even for a cell-free pull-down preparation. However, our equilibrium and kinetic data strongly indicate that this off-pathway oligomer is a stable intermediate, that once formed has a high energetic barrier to be overcome. This behavior resembles that of the prion protein, which gives origin to an aggregated amyloid structure after incubating the native protein for a period of time with different subdenaturing concentration of urea (Baskakov et al., 2001).

Our results suggest that the equilibrium denaturation processes of p53C induced by GdmCl is not a simple two-state transition, but one that occurs via a partially folded conformation, indicating the presence of an intermediate state on the p53C folding/unfolding pathway. This intermediate has the propensity to undergo aggregation into a state whose most interesting feature is the high content of secondary structure. Assembly into aggregates in the folding/unfolding pathway may contribute to restrict the folding landscape. The reversible dissociation of these aggregates by pressure is a clear indication that they have water-excluded cavities (Silva et al., 2001; Foguel et al., 2003). The limited changes in light scattering (~7-fold) also indicate a prominent role of this aggregate to the folding pathway. Small angle x-ray scattering are currently being performed to characterize the precise size and shape of this aggregate.

The reaction scheme in Fig. 10 is based on the equilibrium and kinetic data described here. The aggregate is formed off-pathway from the intermediate and imposes a constraint in the folding landscape of the protein. The kinetic data clearly demonstrate how high concentration of protein favors the aggregation, a condition that would be present in a cellular situation of loss-of-function mutation, such as that present for the hot-spot mutant R248Q. The small aggregate in

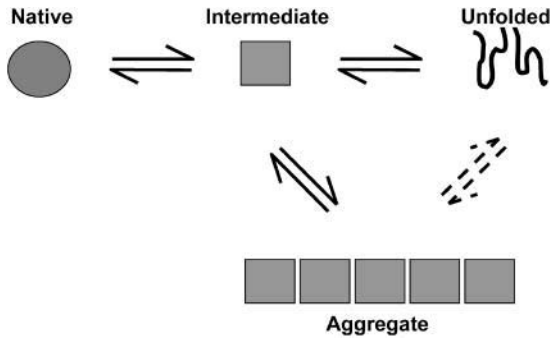

FIGURE 10 Reaction scheme for p53C folding/unfolding pathway with concurrent formation of aggregates from the intermediate. Although less likely, the aggregates can also be formed from the unfolded protein (*dashed arrows*).

equilibrium with an on-pathway intermediate—as described here—may be the site where p53 mutants (translated from a single mutant allele) are able to drive wild-type p53 protein (translated from the remaining wild-type p53 allele) into a mutant conformation. These intermediates may be targets to the development of lead compounds capable of destabilizing them with potential therapeutic action against tumor cancer.

The detection of an intermediate conformation prone to aggregate during p53C unfolding has physiological meaning if one recalls that protein unfolding is an essential step during protein degradation in proteasomes (Fersht and Daggett, 2002). Moreover, these aggregates are not only crucial to the proper folding pathway of p53C but may explain as well the vulnerability of p53C to undergo departure of the native to an inactive state, which makes the cell susceptible to malignant transformation.

We are grateful to Prof. Martha M. Sorenson for critical reading of the manuscript; Profs. Hector Barrabin, Helena Scofano, and Julio A. Mignaco for using their stopped-flow apparatus; Prof. Pedro Lagerblad de Oliveira for the use of his Cary Spectrofluorimeter; and Emerson R. Gonçalves for competent technical assistance.

This work was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Programa de Núcleos de Excelência (PRONEX), Fundação de Amparo à Pesquisa no Estado do Rio de Janeiro (FAPERJ) of Brazil to J.L.S and by an international grant from the International Centre for Genetic Engineering and Biotechnology (ICGEB) to J.L.S..

REFERENCES

Arai, M., and K. Kuwajima. 2000. Role of the molten globule state in protein folding. *Adv. Protein Chem.* 53:209–282.

Baldwin, R. L. 1996. On-pathway versus off-pathway folding intermediates. *Fold. Des.* 1:R1–R8.

Baskakov, I. V., G. Legname, S. B. Prusiner, and F. E. Cohen. 2001. Folding of prion protein to its native α-helical conformation is under kinetic control. *J. Biol. Chem.* 276:19687–19690.

Bell, S., C. Klein, L. Müller, S. Hansen, and J. Buchner. 2002. p53C contains large unstructured regions in its native state. *J. Mol. Biol.* 322:917–925.

Brems, D. N. 1988. Solubility of different folding conformers of bovine growth hormone. *Biochemistry.* 27:4541–4546.

Bryngelson, J. D., J. N. Onuchic, N. D. Socci, and P. G. Wolynes. 1995. Funnels, pathways, and energy landscape of protein-folding: a synthesis. *Proteins.* 21:167–195.

Bullock, A. N., J. Henckel, B. S. Dedecker, C. M. Johnson, P. V. Nikolova, M. R. Proctor, D. P. Lane, and A. R. Fersht. 1997. Thermodynamic stability of wild-type and mutant p53C core domain. *Proc. Natl. Acad. Sci. USA.* 94:14338–14342.

Bullock, A. N., and A. R. Fersht. 2001. Rescuing the function of mutant p53C. *Nat. Rev. Cancer.* 1:68–76.

Bullock, A. N., J. Henckel, and A. R. Fersht. 2000. Quantitative analysis of residual folding and DNA binding in mutant p53C core domain: definition of mutant states for rescue in cancer therapy. *Oncogene.* 19:1245–1256.

Carra, J. H., and P. L. Privalov. 1996. Thermodynamics of denaturation of staphylococcal nuclease mutants: an intermediate state in protein folding. *FASEB J.* 10:67–74.

Cordeiro, Y., J. Kraineva, R. Ravindra, L. M. T. R. Lima, D. Foguel, R. Winter, and J. L. Silva. 2004. Hydration and packing effects on prion folding and β-sheet conversion: HIGH pressure spectroscopy and pressure perturbation calorimetry studies. *J. Biol. Chem.* 279:32354–32359.

Cordeiro, Y., F. Machado, L. Juliano, M. A. Juliano, R. R. Brentani, D. Foguel, and J. L. Silva. 2001. DNA converts cellular prion protein into the β-sheet conformation and inhibits prion peptide aggregation. *J. Biol. Chem.* 276:49400–49409.

Creighton, T. E. 1997. How important is the molten globule for correct protein folding? *Trends Biochem. Sci.* 22:6–10.

Creighton, T. E., N. J. Darby, and J. Kemmink. 1996. The roles of partly folded intermediates in protein folding. *FASEB J.* 10:1110–1181.

Dill, K. A., and H. S. Chan. 1997. From Levinthal to pathways to funnels. *Nat. Struct. Biol.* 4:10–19.

Dobson, C. M. 1999. Protein misfolding, evolution and disease. *Trends Biochem. Sci.* 24:329–332.

Dobson, C. M. 2000. The nature and significance of protein folding. *In* Mechanism of Protein Folding. R.H. Pain, editor. Oxford University Press, New York. 1–33.

Fändrich, M., M. A. Fletcher, and C. M. Dobson. 2001. Amyloid fibrils from muscle myoglobin. *Nature.* 410:165–166.

Ferrão-Gonzales, A. D., S. O. Souto, J. L. Silva, and D. Foguel. 2000. The preaggregated state of an amyloidogenic protein: hydrostatic pressure converts native transthyretin into the amyloidogenic state. *Proc. Natl. Acad. Sci. USA.* 97:6445–6450.

Fersht, A. 1999. Protein stability. *In* Structure and Mechanism in Protein Science. A Guide to Enzyme Catalysis and Protein Folding. W. H. Freeman & Co., New York. 508–539.

Fersht, A. R., and V. Daggett. 2002. Protein folding and unfolding at atomic resolution. *Cell.* 108:573–582.

Foguel, D., J. L. Silva, and G. de Part-Gay. 1998. Characterization of a partially folded monomer of the DNA-binding domain of human papillomavirus E2 protein obtained at high pressure. *J. Biol. Chem.* 273:9050–9057.

Foguel, D., M. C. Suarez, A. D. Ferrão-Gonzales, T. C. Porto, L. Palmieri, C. M. Einsiedler, L. R. Andrade, H. A. Lashuel, P. T. Lansbury, J. W. Kelly, and J. L. Silva. 2003. Dissociation of amyloid fibrils of α-synuclein and transthyretin by pressure reveals their reversible nature and the formation of water-excluded cavities. *Proc. Natl. Acad. Sci. USA.* 100:9831–9836.

Friedler, A., D. B. Veprintsev, L. O. Hansson, and A. R. Fersht. 2003. Kinetic instability of p53C core domain mutants. Implications for rescue by small molecules. *J. Biol. Chem.* 278:24108–24112.

Hall, P. A., D. Meek, and D. P. Lane. 1996. p53C–integrating the complexity. *J. Pathol.* 180:1–5.

Ishimaru, D., L. F. Maia, L. M. Maiolino, P. A. Quesado, P. C. M. Lopez, F. C. L. Almeida, A. P. Valente, and J. L. Silva. 2003a. Conversion of wild-type p53C core domain into a conformation that mimics a hot-spot mutant. *J. Mol. Biol.* 333:443–451.

Ishimaru, D., L. R. Andrade, L. S. P. Teixeira, P. A. Quesado, L. M. Maiolino, P. C. M. Lopez, Y. Cordeiro, L. T. Costa, W. M. Heckl, G. Weissmüller, D. Foguel, and J. L. Silva. 2003b. Fibrillar aggregates of the tumor suppressor p53C core domain. *Biochemistry.* 42:9022–9027.

Kendrick, B. S., J. F. Carpenter, J. L. Cleland, and T. W. Randolph. 1998. A transient expansion of the native state precedes aggregation of recombinant human interferon-γ. *Proc. Natl. Acad. Sci. USA.* 95:14142–14146.

Kim, P. S., and R. L. Baldwin. 1990. Intermediates in the folding reactions of small proteins. *Annu. Rev. Biochem.* 59:631–660.

Kuwajima, K., and M. Arai. 2000. The molten globule state, the physical picture and biological significance. *In* Mechanism of Protein Folding. R. H. Pain, editor. Oxford University Press, New York. 139–174.

Kuwata, K., H. Li, H. Yamada, G. Legname, S. B. Prusiner, K. Akasaka, and T. J. James. 2002. Locally disordered conformer of the hamster prion protein: a crucial intermediate to PrPSc? *Biochemistry.* 41:12277–12283.

Lane, D. P., and P. A. Hall. 1997. MDM2—arbiter of p53C's destruction. *Trends Biochem. Sci.* 22:372–374.

Lashuel, H. A., D. Hartley, B. M. Petre, T. Walz, and P. T. Lansbury, Jr. 2002. Neurodegenerative disease: amyloid pores from pathogenic mutations. *Nature*. 418:291.

Levinthal, C. 1969. How to fold graciously. *In* Proceedings of a Meeting Held at Allerton House. I. L. Monticello, editor. University of Illinois Press, Champaign, IL. 22–24.

Mateu, M. G., M. M. S. del Pino, and A. R. Fersht. 1999. Mechanism of folding and assembly of a small tetrameric protein domain from tumor suppressor p53C. *Nat. Struct. Biol.* 6:191–198.

Moll, U. M., A. G. Ostermeyer, R. Haladay, B. Winkfield, M. Frazier, and G. Zambetti. 1996. Cytoplasmic sequestration of wild-type p53C protein impairs the G1 checkpoint after DNA damage. *Mol. Cell. Biol.* 16:1126–1137.

Neira, J. L., and M. G. Mateu. 2001. Hydrogen exchange of the tetramerization domain of the human tumor suppressor p53C probed by denaturants and temperature. *Eur. J. Biochem.* 268:4868–4877.

Nichols, N. M., and K. S. Matthews. 2001. p53C unfolding detected by CD but not by tryptophan fluorescence. *Biochem. Biophys. Res. Commun.* 288:111–115.

Niraula, T. N., T. Konno, H. Li, H. Yamada, K. Akasaka, and H. Tachibana. 2004. Pressure-dissociable reversible assembly of intrinsically denatured lysozyme is a precursor for amyloid fibrils. *Proc. Natl. Acad. Sci. USA.* 23:4089–4093.

Oltzen, D. E., L. S. Itzhaki, N. F. El Masry, S. E. Jackson, and A. R. Fersht. 1994. Structure of the transition state for the folding/unfolding of the barley chymotrypsin inhibitor 2 and its implications for mechanisms of folding. *Proc. Natl. Acad. Sci. USA.* 91:10422–10425.

Ostermeyer, A. G., E. Runko, B. Winkfield, B. Ahn, and U. M. Moll. 1996. Cytoplasmically sequestered wild-type p53C protein in neuroblastoma is relocated to the nucleus by a C-terminal peptide. *Proc. Natl. Acad. Sci. USA.* 93:15190–15194.

Pace, C. N. 1986. Determination and analysis of urea and guanidine hydrochloride denaturation curves. *Meth. Enzymol.* 131:266–280.

Piotto, M., V. Saudek, and V. Sklenar. 1992. Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. *J. Biomol. NMR.* 2:661–665.

Plotkin, S. S., and J. N. Onuchic. 2002. Understanding protein folding with energy landscape theory. Part I: Basic concepts. *Q. Rev. Biophys.* 35:111–167.

Prives, C., and P. A. Hall. 1999. The p53C pathway. *J. Pathol.* 187:112–126.

Sacchettini, J. C., and J. W. Kelly. 2002. Therapeutic strategies for human amyloid diseases. *Nat. Rev. Drug Discov.* 1:267–275.

Sanz, J. M., and A. R. Fersht. 1993. Rationally designing the accumulation of a folding intermediate of barnase by protein engineering. *Biochemistry.* 32:13584–13592.

Silva, J. L., D. Foguel, and C. A. Royer. 2001. Pressure provides new insights into protein folding, dynamics and structure. *Trends Biochem. Sci.* 26:612–618.

Silva, J. L., L. Peng, M. Glaser, E. W. Voss, and G. Weber. 1992a. Effects of hydrostatic pressure on a membrane-enveloped virus: high immunogenicity of the pressure-inactivated virus. *J. Virol.* 66:2111–2117.

Silva, J. L., C. F. Silveira, A. Correia Jr., and L. Pontes. 1992b. Dissociation of a native dimer to a molten globule monomer. *J. Mol. Biol.* 223:545–555.

St. John, R. J., J. F. Carpenter, C. Balny, and T. W. Randolph. 2001. High pressure refolding of recombinant human growth hormone from insoluble aggregates. *J. Biol. Chem.* 276:46856–46863.

Torrent, J., M. T. Alvarez-Martinez, M. C. Harricane, F. Heitz, J. P. Liautard, C. Balny, and R. Lange. 2004. High pressure induces scrapie-like prion protein misfolding and amyloid fibril formation. *Biochemistry.* 43:7162–7170.

Wagner, C., and T. Kiefhaber. 1999. Intermediates can accelerate protein folding. *Proc. Natl. Acad. Sci. USA.* 96:6716–6721.