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A single mutation in an SH3 domain increases amyloid aggregation by accelerating nucleation, but not by destabilizing thermodynamically the native state

Lorena Varela, Bertrand Morel, Ana I. Azuaga, Francisco Conejero-Lara *

Departamento de Química Física e Instituto de Biotecnología, Facultad de Ciencias, Universidad de Granada, Campus Fuentenueva, 18071 Granada, Spain

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ABSTRACT

We investigated the relationship between thermodynamic stability and amyloid aggregation propensity for a set of single mutants of the alpha-spectrin SH3 domain (Spc-SH3). Whilst mutations destabilizing the domain at position 56 did not enhance fibrillation, the N47A mutation increased the rate of amyloid fibril formation by 10-fold. Even under conditions of identical thermodynamic stability, the aggregation rate was much higher for the N47A mutant than for the WT domain. We conclude that the N47A mutation does not change the apparent mechanism of fibrillation or the morphology of the amyloid fibrils, and that its amyloidogenic property is due to its effect upon the rate of the conformational events leading to nucleation and not to its overall destabilizing effect.

1. Introduction

Amyloid fibril aggregation is involved in the development of a group of important diseases, generically known as amyloidoses, which include Alzheimer and Parkinson diseases, several systemic amyloidoses, diabetes type II or diverse encephalopathies [1,2]. Effective therapies or methods of prevention for this type of diseases are still very scarce, mainly because little is known about the molecular mechanisms of these aggregation processes. Formation of amyloid structures by globular proteins requires the accumulation of a critical concentration of protein in partially-folded conformations exposing a significant segment of the polypeptide chain to solvent under conditions in which stable intermolecular interactions can be formed [3–5].

A number of studies have reported an inverse correlation between the propensity of proteins to aggregate into amyloid-like structures and the thermodynamic stability of the native state [6–8]. Furthermore, recent studies have proposed that stabilization of the native fold results in the modulation of the conformational ensemble favoring structural cooperativity and

reducing sampling of partially-folded amyloidogenic conformations [5,9]. This has led to promising therapeutic strategies for amyloid-related diseases based on stabilization of the native state by specific drugs [10]. There are however examples in which kinetic stability, rather than thermodynamic stability, controls fibrillation [11] and in some cases the formation of amyloid fibrils is preceded by an assembly of quasi-native or native-like structures into aggregates [12,13]. Thus, a detailed understanding of the thermodynamic and kinetic factors determining accumulation of amyloidogenic species in proteins still remains far from complete.

Src-homology region 3 (SH3) domains are small modules found as part of proteins that mediate transient protein–protein interactions relative to many cellular processes [14,15]. Due to their small size, high solubility, simple structure and uncomplicated folding mechanism, the folding and stability of these modular domains have been extensively studied [16–20]. Moreover, SH3 domains can form amyloid fibrils at acid pH [4,9,21], being among the first examples of proteins unrelated to amyloidoses that form amyloid fibrils. In the case of the SH3 domain of α-spectrin (Spc-SH3), the mutation of asparagine 47 to alanine, placed within the folding nucleus of the domain, strongly accelerates the formation of amyloid fibrils [21]. Here we investigate whether the increased tendency of the N47A mutant to form amyloids relative to the WT domain is exclusively due to a thermodynamic destabilization on the native structure or it is related to other specific effects exerted by the mutation. We have compared the thermodynamic stability of

the native state and the amyloid aggregation propensity between the WT Spc-SH3 domain and several single mutants. In addition, we have compared the kinetics of fibril formation of the WT protein and the N47A mutant under conditions of identical thermodynamic stability. The results demonstrate that thermodynamic destabilization of the native state produced by the mutation is not the main factor favoring fibril formation by this small domain. Instead, the amyloidogenic effect appears to be due to an increase in the rate of conformational events taking place during nucleation of the fibrils.

2. Materials and methods

2.1. Protein samples

The Spc-SH3 domain variants were expressed and purified as described [19]. Protein aliquots were dialyzed extensively against pure water and lyophilized. For aggregation experiments the lyophilized protein was dissolved, unless otherwise stated, in 100 mM glycine, 100 mM NaCl, pH 3.2, at 4 °C, centrifuged for 2 min at 14 000 rpm in a micro-centrifuge and filtered through a 0.2 μm filter. The protein concentration was determined by measurement of absorbance at 280 nm using extinction coefficients of 15 512 $M^{-1} cm^{-1}$ and 15 220 $M^{-1} cm^{-1}$ for the WT and N47A variants, respectively.

2.2. Differential scanning calorimetry

Temperature scans were performed at a protein concentration of 0.8 mg/mL in a VP-DSC microcalorimeter (MicroCal, Northampton, MA, USA) between 5 and 100 °C at a scan rate of 1.5 °C min^{-1}. The reversibility of the thermal unfolding was always checked in a second consecutive scan of the same sample. Instrumental baselines were subtracted to the experimental thermograms of the samples and the time response of the calorimeter was then corrected. The partial molar heat capacity curves (Cp) were calculated and analyzed using Origin 8.0 (OriginLab, Northampton, MA, USA). Cp curves were analyzed according to the two-state unfolding model [22].

2.3. Thioflavin T binding assay

Thioflavin T (ThT) binding assays were performed to monitor amyloid fibril aggregation [23] using a Perkin Elmer LS-55 spectrofluorimeter (Perkin Elmer, Shelton, CT, USA). A 250 μM stock solution of ThT was freshly prepared in 25 mM potassium phosphate buffer (pH 6.0). Protein aliquots (10 μl) were diluted into the phosphate buffer containing 12.5 μM ThT and adjusted to a final volume of 1 mL. Fluorescence emission intensity was measured out at room temperature using a 10 mm path-length cuvette.

2.4. Dynamic light scattering

Dynamic light scattering (DLS) measurements were performed with a DynaPro MS-X instrument (Wyatt Technology Corporation, Santa Barbara, CA, USA) using a 30 μL quartz thermostated sample cuvette. The protein solutions and the buffer were centrifuged and filtered through 0.02 μm Anotop filters (Whatman plc, Brentford, Middlesex, UK) immediately before measurements. Sets of DLS data at constant temperature (37 °C) were acquired every 45 s until saturation of the signal. Laser power was adapted to avoid early saturation of the instrument. Dynamics software (Wyatt Technology Corporation, Santa Barbara, CA, USA) was used in data collection and processing.

2.5. Transmission electron microscopy

Protein samples were diluted 10-fold with buffer and a 15 μL aliquot was placed on a formvar-coated copper grid and left for 4 min. The grid was then washed twice with distilled water and stained with 1% (w/v) uranyl acetate for 1 min. The dried samples were then observed in a Zeiss 902 electron microscope operating at 80 kV.

3. Results

3.1. The thermodynamic stability of single mutants does not correlate with their propensity to form amyloid aggregates

The propensity to form amyloid fibrils of the WT Spc-SH3 and several single-point mutants was analyzed by ThT fluorescence under the conditions described previously for the N47A mutant [21], i.e., at 37 °C in 100 mM glycine buffer pH 3.2, in the presence of 100 mM NaCl and a protein concentration of 8.2 mg/mL (Fig. 1a). The mutations are located at two different regions within the putative folding nucleus of the Spc-SH3 domain [17]. Most of the variants except the A56 K mutant formed amyloid fibrils when incubated at 37 °C for long periods, with morphologies similar to those of the N47A mutant, as observed by transmission electron microscopy (TEM) (not shown). The aggregation rates of the WT domain and the A56G and A56E mutants were similar but they all aggregated considerably slower than the N47A mutant and their
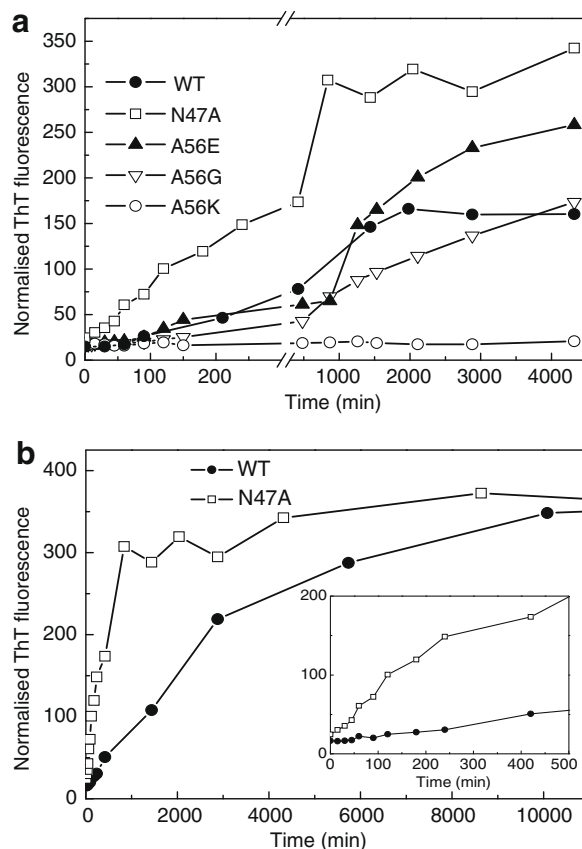

Fig. 1. Kinetics of amyloid fibril growth of variants of Spc-SH3 domain measured by ThT fluorescence. (a) Aggregation was followed at 37 °C in 100 mM glycine buffer pH 3.2, with 100 mM NaCl, at an equal protein concentration of 8.2 mg mL^{-1} for several variants as indicated. (b) Aggregation was followed as in panel (a) but at pH 2.78 for WT and pH 3.20 for N47A. The inset shows an expansion of the first 500 min of incubation.

Table 1
Thermodynamic parameters of the equilibrium thermal unfolding of Spc-SH3 variants determined by DSC.

Variant	pH	T_m (°C)	ΔH_m (kJ mol^{-1})
WT		53.9	165
N47A		50.9	149
A56G	3.2	49.3	148
A56E		50.1	146
A56K		53.3	160
WT	2.0	43.1	125
	2.5	49.5	144
	3.0	52.4	159
	3.2	54.8	168
	3.5	58.9	182
N47A	2.0	36.8	106
	2.5	40.0	114
	3.0	49.5	148
	3.2	51.2	152
	3.5	55.4	170

kinetics presented longer lag phases, suggesting a slower formation of aggregation nuclei.

The thermodynamic stability of all the variants was analyzed by differential scanning calorimetry (DSC) at pH 3.2 and protein concentrations sufficiently low (0.8 mg/mL) to avoid aggregation during the thermal unfolding. The WT and the N47A mutant were also studied by DSC at different pH values between 2.0 and 3.5. Under these conditions, the thermal unfolding of all variants was highly reversible and followed the two-state unfolding model. Identical DSC thermograms were obtained for some of the variants using different scan rates between 1 and 2 °C/min (not shown), indicating that the thermal unfolding occurs under equilibrium during the entire DSC scan. The thermodynamic parameters (Table 1) do not correlate whatsoever with the aggregation propensities of the mutants. For instance, the A56E and A56G mutants, with stabilities similar to the N47A mutant, form amyloid fibrils at slower rate than the latter but at similar rate as the more stable WT domain. In contrast, the A56 K mutant has practically identical stability as the WT domain but it does not form amyloid aggregates within the time period analyzed.

3.2. Under conditions of identical stability the WT and N47A Spc-SH3 domains have different propensity to form amyloids

A plot of the unfolding enthalpies, ΔH_m, versus the unfolding temperatures, T_m, measured for the different variants at pH 3.2 and for the WT and N47A variants at several pH values, shows a single linear dependence (not shown). This indicates that the changes in enthalpy of unfolding are only due, within the experimental error, to the its dependence with temperature due to the heat capacity change of unfolding, $\Delta C_p = d\Delta H/dT$, common to all domain variants [24]. This is consistent with insignificant changes in the native structure produced by the pH or the mutations. Using the heat capacity change of unfolding derived from the slope of the plot (3.6 ± 0.1 kJ K^{-1} mol^{-1}) and the thermodynamic data of Table 1, we calculated the Gibbs energy change of unfolding at 37 °C as described elsewhere [16] as a function of pH and determined that the WT Spc-SH3 domain at pH 2.78 and the N47A mutant at pH 3.20 have the same Gibbs energy change of unfolding, i.e., identical thermodynamic stability. Thus, to exclude the influence of the native state stability, we compared the kinetics of amyloid formation at 37 °C of the WT Spc-SH3 domain at pH 2.78 and the N47A mutant at pH 3.20 (Fig. 1b). The N47A mutant still aggregates much faster than the WT domain, which indicates that the amyloidogenic effect of this mutation is not related to a global destabilization of

the native state. This difference is neither due to the pH difference affecting the net charge of the protein because the rate of fibrillation of the N47A mutant increases with the pH reduction (results not shown).

3.3. The WT and the N47A Spc-SH3 domains form amyloid fibrils of similar morphology

We compared by TEM the morphology of the particles appearing during the aggregation process of WT and N47A Spc-SH3 under conditions of equal thermodynamic stability (Fig. 2). At early times of incubation (30 min) the N47A forms protofibrillar and amorphous aggregates of protein (Fig. 2a), which quickly reorganize after only 60 min of incubation into small curly fibrils with diameter of 6–7 nm and lengths between few tenths and several hundreds of nanometers (Fig. 2b). These fibrils elongate further for longer incubation times. In the case the WT domain, at 30 min of incubation only few small globular and amorphous aggregates were sparsely visible (not shown) but a variety of irregular aggregate clusters form at 60 min of incubation (Fig. 2d), which become reorganized later to form fibrillar structures as observed at 180 min of incubation (Fig. 2e). After long incubation
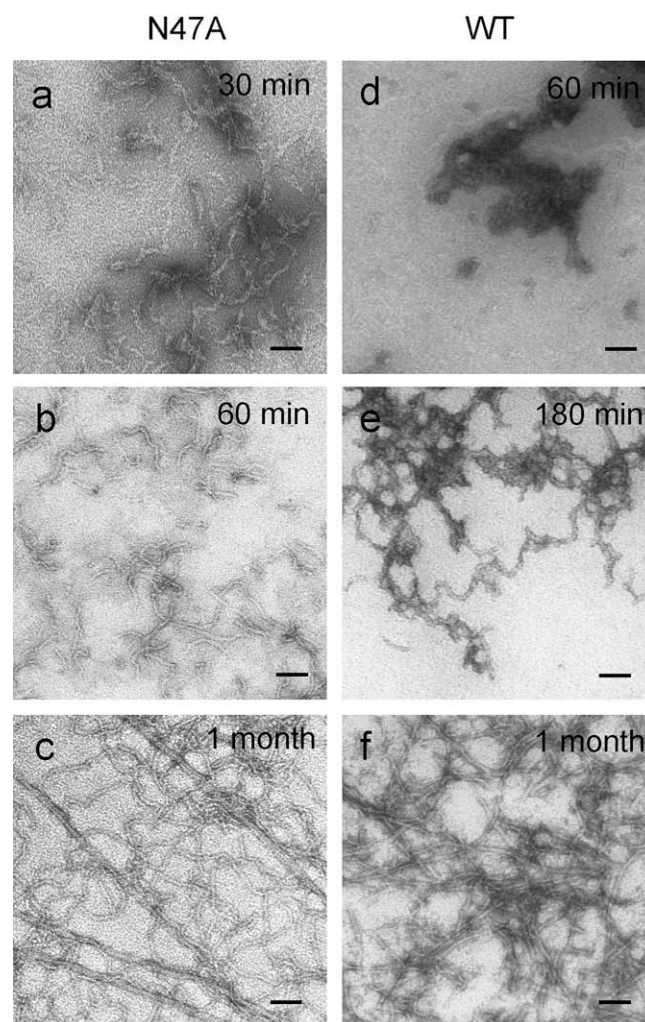

Fig. 2. Electron microscopy images of aggregated N47A (panels a, b and c) and WT (panels d, e and f) Spc-SH3 variants after different times of incubation at 37 °C: (a) 30 min; (b and d) 60 min; (e) 180 min; (c and f) 1 month. Incubation conditions are identical to those of Fig. 1b for each variant. The length of the black segments corresponds to 100 nm in all images.

times both variants presented a tangle of amyloid fibrils with similar apparent curly morphology and diameter (Fig. 2c and f) and few mature amyloid fibrils already appeared in the N47A samples.

It appears that for both protein variants amyloid fibril formation involves similar early events, i.e., an initial formation of amorphous prefibrillar aggregates and a subsequent reorganization of these aggregates into fibrils. Both events occur more rapidly in the N47A mutant than in the WT form.

3.4. The amyloidogenic mutation N47A accelerates formation of early oligomers

We followed by DLS the early stages of aggregation of the WT and N47A variants at 37 °C under conditions of identical thermodynamic stability (Fig. 3). The growth of the scattering signal (Fig. 3a) has a much shorter lag time for the N47A mutant, indicating a faster formation of aggregation nuclei. From the DLS data we calculated the size distribution of particles in the mixture as a function of the incubation time. The time evolution of the apparent hydrodynamic radius, R_h, for the two smallest peaks in the distributions are shown in Fig. 3b. At the start of the incubation, the size distribution of particles shows for both protein variants only particles with an apparent R_h of ≈ 1.7 nm, consistently with the value reported for native Spc-SH3 [20]. This R_h increases from ≈1.7 to ≈3.2 nm in N47A at around 100 min of incubation. In our previous

work we interpreted this observation as indicative of oligomerisation following a conformational change in the protein [21]. In the case of the WT domain this event is delayed more than 300 min of incubation, corresponding approximately to the duration of the lag phase observed by ThT fluorescence.

Simultaneously, within few minutes from the start of the incubation additional species appeared with an apparent R_h starting at ≈7–9 nm and increasing progressively with the incubation time. These particles were identified previously as small protofilaments of 6–7 nm in diameter elongating as the aggregation progresses [21]. The fibril elongation is slightly slower for the WT, as indicated by the slower increase in their average R_h, which reaches ≈40 nm at ≈300 min (≈150 min for the N47A mutant). The apparent R_h of the fibrils stops increasing because of the lack of linear persistence of the fibrils. This apparent R_h would correspond to fibril lengths of about 250–500 nm [25]. Finally, at long incubation times larger particles with apparent R_h reaching up to several micrometers became developed for both proteins corresponding to long amyloid fibrils (not shown).

To test the effect of the presence of preformed nuclei of N47A mutant upon the fibril nucleation of the WT protein, we preincubated an 8.2 mg mL^{-1} sample of N47A mutant at 37 °C for 100 min allowing formation of oligomeric species. Then, we immediately added a 10% of this sample to an identical fresh sample of WT protein and incubated the mixture at 37 °C while measuring the DLS signal (Fig. 3a and c). The presence of N47A nuclei reduced significantly the lag phase of aggregation of WT but did not affect importantly the growth rate of fibrils as observed by the similar slope of the increase in the DLS signal. Importantly, the formation of oligomeric species of WT Spc-SH3 with apparent R_h of ≈3.2 nm was accelerated significantly, indicating that pre-existing N47A oligomers could catalyze formation of WT oligomers and facilitate nucleation.

4. Discussion

Here we have demonstrated that the effect of mutations in Spc-SH3 upon the thermodynamic stability of the native state does not correlate with the changes in the rates of amyloid aggregation. Whilst the destabilizing mutation N47A at the tip of the distal loop of the domain greatly enhances amyloid aggregation, other similarly destabilizing mutations at the 3_{10} helix do not change importantly the aggregation propensity. We have also shown that even under conditions where the WT and the N47A mutant have identical stability their kinetics of aggregation are markedly different. These results demonstrate that an overall thermodynamic destabilization of the native state is not the main factor driving amyloid formation in this small domain.

The fibrillation presents a much shorter lag phase for the N47A mutant than for the WT domain, suggesting a faster formation of aggregation nuclei. In addition, the rate of fibril elongation observed by DLS is roughly 2-fold higher for the N47A mutant than for the WT, in great contrast with a roughly 10-fold difference in the growth of fibril mass observed by ThT fluorescence. This indicates that the amyloidogenic effect of the N47A mutation occurs mainly at the stage of the conformational events previous to nucleation or at the nucleation step itself. In fact, the analysis by DLS shows that formation of early oligomers occurs earlier for the N47A mutant than for the WT and this event appears crucial in the development of fibrillar aggregates, thus conditioning all the subsequent fibrillation process. Indeed, for both variants the duration of the lag phase in fibril formation is very similar to that of formation of oligomers (see Figs. 1b and 3). This suggests that these oligomers may be the competent species of fibril nucleation or may even constitute themselves the aggregation nuclei. The
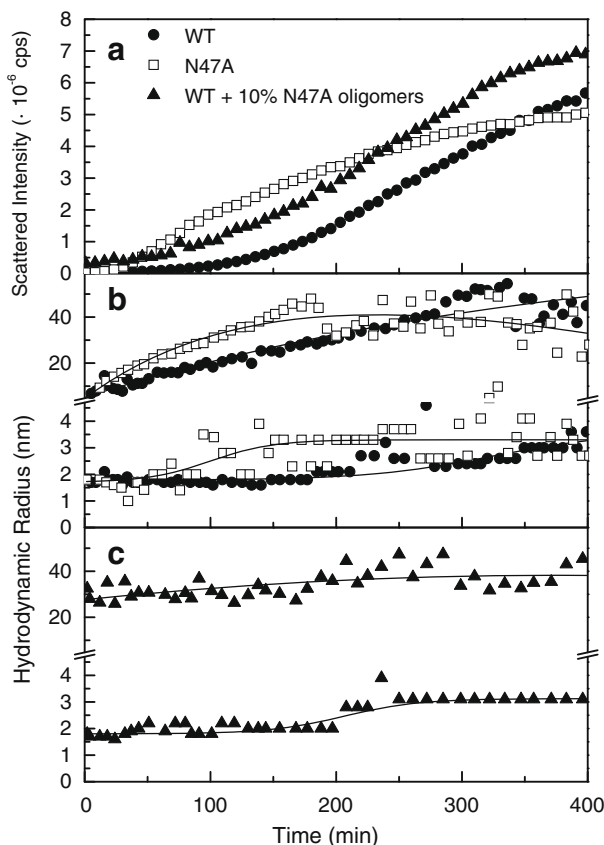

Fig. 3. Aggregation kinetics at 37 °C of WT and N47A Spc-SH3 followed by DLS. Experimental conditions are identical to those of Fig. 1b. (a) Time dependence of the scattering intensity for WT (filled circles), N47A (open squares) and WT in the presence of 10% N47A preincubated for 100 min (filled triangles). (b) Apparent hydrodynamic radius, R_h, determined by DLS for the two smallest species during the course of aggregation at 37 °C observed in the size distributions for WT (filled circles), N47A (open squares). (c) Same as in (b) for WT in the presence of 10% N47A preincubated for 100 min. Symbols (filled triangles) correspond to the maximum of each peak in the size distributions. The lines are drawn only for the sake of clarity.

presence of oligomeric species in rapid equilibrium with the mono-meric form has also been reported as critical for fibril nucleation as for example in Abeta [26] or yeast prion Sup35p [27] and the importance of their characterisation is emphasized by their impli-cation in a number of neurotoxic processes [28].

The observation that preformed aggregation nuclei of the N47A mutant could accelerate significantly nucleation of the WT protein whereas the rate of fibril elongation was not affected is of particu-lar interest because it suggests that nuclei pre-existing in the mix-ture can catalyze formation of additional aggregation nuclei, likely through transient intermolecular interactions.

Our results may appear in conflict with previous studies, which have found a significant inverse correlation between native stabil-ity and the propensity to form amyloids. For instance, several mu-tants of the B1 domain IgG-binding protein G induced amyloid aggregation in inverse correlation with their native stability [8,29]. Similarly, a significant inverse correlation has been found between native stability of a series of acylphosphatase mutants and their susceptibility to amyloid fibrillation induced by TFE [6]. These studies concluded that key requirement for fibril formation was an increase in the population of intermediate folding confor-mations that become favoured by destabilizing the native state. On the other hand, a mutational analysis of the fibrillation of the thermophilic protein S6 has revealed no correlation whatsoever between native stability and fibril formation [12]. Instead, the unfolding rates correlated directly with the lag phases of amyloid aggregation suggesting that the nucleation occurs from a quasi-na-tive state. In this case, certain amino acid residues locally grouped in the structure were found to act as "gate keepers" inhibiting the access to specific states that trigger the aggregation cascade. Addi-tional evidence supporting the importance of local effects has been provided by the study of two amyloidogenic variants of human lysozyme [5,30], in which transient unfolding of a specific region of the protein including the beta domain and the C-helix is fa-voured by the mutations.

Our previous studies by native state hydrogen-deuterium ex-change have revealed that under native conditions at acid pH the Spc-SH3 domain undergoes a variety of conformational fluctua-tions ranging from local distortions of flexible regions to extensive unfoldings [19,31]. Moreover, we reported that single mutations changing the native stability produced redistributions of the con-formational ensemble that differed depending of the mutational position [32,33]. Although both the 47 and 56 positions are located within the putative folding nucleus of the Spc-SH3 domain [17], they differ in conformational flexibility in the native state. A local destabilization at position A56 affects the whole domain's core lowering the energy of highly unfolded states but leaving un-changed the distribution of the most accessible states. By contrast, the N47A mutation destabilizing the flexible distal loop produces a redistribution of highly populated states, which may favor particu-lar states prone to aggregation. This view is consistent with our finding that similarly destabilizing mutations such as N47A and A56G produce disparate amyloidogenic effects.

It appears, therefore, that although transient exposure of certain regions of the polypeptide chain is a common and obligatory step in amyloid fibril formation by globular proteins, the precise details of the mechanism by which this event conducts to nucleation and the subsequent aggregation cascade may strongly differ between proteins and determine whether or not a correlation between sta-bility of the native state and amyloid aggregation propensity is found in a mutational analysis. In the case of the small Spc-SH3 do-main, not all mutations destabilizing the native state are intrinsi-cally amyloidogenic and it seems that the N47A mutation produces a particular redistribution of the conformational ensem-ble of the protein leading specifically to a significant reduction in the energy barrier of nucleation of the fibrillation process.

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