Folding specificity induced by loop stiffness

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Abstract

To test the importance of loop stiffness in restricting the heterogeneity of transition state ensemble, we relaxed the distal loop of 10 unstable redesigned hydrophobic core mutants of α -spectrin SH3 domain. This was achieved by replacing Asp48 by Gly at the tip of the distal hairpin. Although the change was local in nature, the effect on stabilization was not uniform across the core mutants tested. There is an inverse rough correlation between the stabilization and the increase in buried hydrophobic volume, with respect to the wild type. Interestingly enough, proteins that although unstable are properly folded become molten globule-like after relaxation of the distal loop. These results highlight the importance of stiffness in restricting the conformational heterogeneity of a protein during the folding reaction. An interplay between unspecific hydrophobic interactions and constraint induced by polar interactions, or in this case local stiffness, is essential to achieve a well-ordered folded structure.

Keywords: Design; proteins; hydrophobic core; kinetics

One of the key features of folded proteins is the fact that they do possess a single main conformation (with small fluctuations) under native conditions (Dill 1990). This property is not easy to achieve, as illustrated by the fact that in the majority of cases in which new proteins have been designed the final result has been a molten globule conformation (Desjarlais and Handel 1995; Finucane and Woolfson 1999). In fact, it has been shown in some cases that molten globule-like proteins could be thermodynamically more stable than their correctly folded counterpart (Lumb and Kim 1995). In a particular case, the GCN4 dimer, replacement of a buried polar hydrogen pair by a hydrophobic pair stabilizes the protein and eliminates a single well-defined conformation. Thus, it seems that natural proteins pay a stabilization price in order to achieve a well-defined and ordered structure (Klimov and Thirumalai 2002).

Hydrophobic effect is widely believed to be the main force driving protein folding (Dill 1990). Tight packing of the hydrophobic core has been found to have an important

role in the stability of proteins (Chen and Stites 2001): Destabilizing effects of substitutions in the core can arise from loss of hydrophobicity and disruption of its tight packing. On the other hand, there is evidence that hydrophobic interactions do not necessarily confer folding specificity to protein structures (Lumb and Kim 1995). Thus they need to be balanced by polar interactions conferring the necessary conformational specificity (Lumb and Kim 1995).

It has been recently theoretically proposed that stiffness of loops in proteins could determine transition state ensemble (TSE) heterogeneity in proteins (Klimov and Thirumalai 2002). This should be truer if the loop is fully folded in the TSE, as is the case for spectrin SH3 domain (Martinez et al. 1998). One way to test this hypothesis could be to increase the hydrophobicity of the folding nucleus in a protein having a fully folded loop at TSE and then relax the stiffness of the loop. In this case, one would expect to see an increase in heterogeneity during the folding reaction because of a larger number of stabilizing conformations and consequently in the possible appearance of stable folding intermediates. In a recent work, we have analyzed the folding features of the Spectrin-SH3 domain (Musacchio et al. 1992; Martinez and Serrano 1999) with a redesigned hydrophobic core having amino-acid sequences not found in the SH3 family. This 62-residue polypeptide folds into an orthogonal β -sandwich (Musacchio et al. 1992) and follows a two-state transition at low ionic strength (Viguera et al.

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1994b). In total, 13 multiple mutants were expressed and analyzed. All these sequences bury more hydrophobic volume than the wild-type protein. The three, which had less buried hydrophobic volume (Table 1), had similar stabilities as the wild-type protein and faster unfolding and refolding rates, suggesting a preferential stabilization of the TSE. This is because of conformational strain introduced by the design algorithm as shown by deletion of single methyl groups (Ile into Val) and X-ray analysis of the corresponding mutants (Ventura et al. 2002). The remaining 10 mutants had even a larger hydrophobic volume buried in the core and were found to be quite unstable (Table 1).

A feature of SH3 domains, as Klimov and Thirumalai's paper refers, is the existence of a correlation between distal loop curvatures and loop hydrophobicity, defined as the fraction of hydrophobic residues in a loop. In fact, among the 29 PDB entries they considered, distal loop curvatures over k_1 0.5 correspond roughly to 25% hydrophobic residues content in the loop, the exception being adaptor protein's SH3 domain (PDB entry 1gfd; Klimov and Thirumalai 2002). Spectrin SH3 domain is indicated to have a high distal loop curvature, meaning a high bending rigidity compared for instance to src, Fyn, or PI3 SH3 domain's counterparts at native state, thus constituting a good example to test the role of the interplay between hydrophobic burial and loop stiffness, to achieve a defined conformation. To do so, we have chosen the 10 mutants having a very large hydrophobic volume buried in the core (Table 1), and we have tried to stabilize them through local relaxation of the SH3 distal loop. The spectrin SH3 has a conformational strain at the distal loop produced by having the solvent exposed Asn47 at position I of a type II' β -turn in a high energy region of the Ramachandran plot (Musacchio et al. 1992). Mutation of the neighboring solvent-exposed Asp48 to Gly changes

the type II' β -turn to a type I' β -turn, relaxing the strain, and stabilizes the protein by around 1.7 Kcal/mole (Martinez et al. 1998). By introducing this mutation we expected to rescue the stability of the unstable hydrophobic core mutants, without disrupting their structure, as well as increasing the heterogeneity of the TSE.

The core sequences containing the Asp48 into Gly mutation are stabilized with respect to their wild type counterpart, but to a different extent. The degree of stabilization seems to be inversely related to the induction of a molten globule conformation in the structure. It seems that relaxation of the conformational strain at the distal loop favors molten globule formation in those mutants with an excessively buried hydrophobic volume.

Results

Conformational strain and loop stiffness

As indicated in the introduction, it has been found that all SH3 domains have stiff loops, although the degree of stiffness is higher for the spectrin SH3 domain. The spectrin SH3 has a high-energy conformationally constrained β -turn at the tip of the distal loop. The reason is the presence of a non-Gly residue in a region of the Ramachandran plot that is generally forbidden for non-Gly residues. This implies a large energy penalty for folding the corresponding β -hairpin, which is translated in a significant slowdown of the folding reaction, as this hairpin is part of the folding nucleus. As this high-energy, β -turn needs to fold earlier, it forces a significant restraint on the conformation of the rest of the hairpin. In principle, energy relaxation of the turn by the D48G mutation will allow more flexibility in the conformation of the hairpin in the folding reaction, thus increas-

Table 1. Equilibrium free energy analysis of the WT and core mutants

Protein	ΔG^a (Kcal.mole ⁻¹)	m^b (Kcal.mole ⁻¹ .M ⁻¹)	$\Delta G(D48G)^a$ (Kcal.mole ⁻¹)	m^b (Kcal.mole ⁻¹ .M ⁻¹)	$\Delta\Delta G^c$ (Kcal.mole ⁻¹)	Δm^d (Kcal.mole ⁻¹ .M ⁻¹)	Relative volume ^e
WT	3.6 ± 0.10 [1]	0.74 ± 0.03				1.00	
D48G	5.3 ± 0.07 [2]	0.74 ± 0.02					1.00
MaxI	2.1 ± 0.02 [1]	1.01 ± 0.02	2.2 ± 0.09	0.84 ± 0.02	0.07	0.17	1.28
Best	1.6 ± 0.04 [1]	1.03 ± 0.01	2.0 ± 0.08	0.74 ± 0.02	0.41	0.29	1.25
Best2	1.2 ± 0.02 [1]	0.80 ± 0.01	1.2 ± 0.05	0.67 ± 0.01	0.00	0.13	1.25
Best7	1.1 ± 0.09 [1]	0.84 ± 0.01	2.1 ± 0.02	0.70 ± 0.01	1.00	0.14	1.21
MaxF	1.0 ± 0.03 [1]	0.97 ± 0.01	1.9 ± 0.06	0.64 ± 0.01	0.86	0.33	1.27
MaxL	0.8 ± 0.05 [1]	0.87 ± 0.03	1.3 ± 0.03	0.55 ± 0.01	0.51	0.32	1.24
Best9	0.5 ± 0.03 [1]	0.70 ± 0.02	2.5 ± 0.10	0.75 ± 0.03	1.98	-0.07	1.19
MaxW	-0.5 ± 0.3 [1]	0.70 ± 0.05	1.5 ± 0.05	0.75 ± 0.04	1.95	0.05	1.22

^a Free energy of unfolding of the mutants.

^b Dependence of the free energy of unfolding with urea.

^c Difference in unfolding free energy between the mutants with Asp48 and those having a Gly at this position.

^d Difference in the dependence of the free energy of unfolding with urea between the mutants with Asp48 and those having a Gly at this position.

^e Relative increase in hydrophobic volume buried in the core mutants with respect to the wild-type spectrin SH3 domain.

[1] Ventura et al. 2002.

[2] Martinez et al. 1998.

ing the heterogeneity of the TSE and probably of the folded state (Klimov and Thirumalai 2002).

Thermodynamic analysis of hydrophobic core mutants

The core mutants were characterized by means of urea-induced denaturation experiments, monitoring the change in

intrinsic Trp fluorescence (see Fig. 2A,B below). Table 1 shows the equilibrium thermodynamic parameters, ΔG (Gibbs free energy) and m (dependence of the log of the equilibrium constant on urea; related to the difference in solvent accessibility between the folded and unfolded states). Introduction of the Asp48Gly mutation resulted in a general stabilization of the unstable core variants. However,

Figure 1. Kinetic and equilibrium analysis of the SH3 core mutants. (A) Stereo representation of α -spectrin SH3 domain. Residues forming the hydrophobic core are colored blue; Asn47 and Asp 48 belonging to the distal loop are colored orange. Figure prepared using the program MOLMOL (Koradi et al. 1996). (B and C) Equilibrium denaturation. (B) Hydrophobic core mutants having Asp at position 48; (C) core mutants having Gly at position 48. Unfolding was followed by fluorescence spectroscopy with excitation at 268 nm; the emitted signal was recorded above 340 nm. The buffer was 50 mM sodium phosphate, pH 7.0, and protein concentration 20 μ M. (D and E) Kinetic measurements of the unfolding and refolding reaction of the core mutants, Best9 (D) and Max-L (E): urea concentration (molar) versus natural logarithm of the rate constants for refolding and unfolding. Empty circles are wild-type data and filled circles the D48G mutant ones. The solid lines represent the best fit of the whole data set to equations 2 and 5 described in Materials and Methods.

Figure 2. (Continued on next page)

the stabilizing effect was not uniform. On one extreme we had Best9 and MaxW, where all core positions were mutated, that gained around 2.0 Kcal/mole (similar to the wild-type protein). At the other extreme the increase in stability was almost negligible: 0.52 Kcal/mole in MaxL-D48G, 0.05 Kcal/mole in MaxI-D48G. These differences in $\Delta\Delta G$ are accompanied by significant decreases in the m values for the majority of the mutants (exceptions being again Best9 and MaxW).

This variability among the $\Delta\Delta G$ values is indeed unusual because the D48G mutation is affecting a fully solvent exposed region of the protein (Fig. 1A) and is mainly local in character. There are different reasons that could be invoked to explain this behavior: (1) The estimation of the m values for the core mutants is not very accurate in the case of the reference proteins without the D48G mutation. This could be the case for all core mutants except MaxI. However, the fitting was done without introducing any constraint and variation on the initial and final slopes in the fitting equation and did not vary significantly the m and ΔG values; (2) the structure of some core mutants at the distal loop could be quite different from that in the wild type, then mutation of D48G will not alleviate a conformational strain. This option cannot be excluded without knowing the mutants' 3D struc-

ture. However, the fact that the far-UV circular dichroism (CD) spectra of the core mutants (with the exception of MaxW that has an extra Trp and Best8) resembles that of the wild-type (data not shown) suggests that this should not be the case for all proteins. Also, this does not explain the changes in m values upon mutating Asp48 into Gly; (3) the denatured state of the mutants becomes much more compact and stable when introducing the D48G mutation. Since we introduced larger hydrophobic residues in the core, the mutation could allow formation of a stable β -hairpin in the denatured state, with its subsequent stabilization; (4) formation of a folding intermediate breaking the two-state assumption in the energy evaluation. Regarding options (3) and (4), both require a decrease in equilibrium m -value as we observed. In the presence of an equilibrium intermediate, there is an apparent decrease in m value because three transitions take place instead of two. The only way to discriminate between possibilities (3) and (4) is performing a kinetic analysis of the folding reaction. Changes in the refolding slope without curvature, or new phases will argue in favor of a compact denatured state; the opposite will favor the hypothesis of the existence of a folding intermediate. Also, in this case we could rule out explanations (1) and (2). To do the kinetic analysis as well as for further studies, we

Figure 2. Spectroscopic characterization of the Best9 and MaxL mutants in 50 mM phosphate buffer, pH 7.0. (A and B) Fluorescence excitation spectra of Best9 (A) and Max-L (B). (C and D) Far-UV CD spectra of Best9 (C) and Max-L (D). (E and F) Near-UV CD spectra of Best9 (E) and Max-L (F). Wild types (continuous line); D48Gs (dotted line). (G) ANS binding assay.

selected two mutants (Best9 and Max-L), exhibiting very different behaviors but similar stability in the absence of the D48G mutation.

Folding kinetics analysis

The D48G mutation in the wild-type α -spectrin SH3 domain results in a dramatic increase in the refolding rate constant of the D48G mutant compared to the wild type (Martinez et al. 1998); acceleration in the folding kinetics of the D48G core mutants was therefore expected. This is indeed what we found among the selected mutants. The wild type and Best9-D48G's kinetics are characterized by a V-shaped Chevron plot, typical of two-state transition kinetics (Viguera et al. 1994b; Fig. 1D). In Best9-D48G, the unfolding rate is not affected by the mutation, while the refolding is largely accelerated, as it happened in the wild-type SH3 (Martinez et al. 1998). Under all conditions, both the refolding and unfolding traces followed by fluorescence fit well to single exponentials, indicating a lack of intermediates in the folding reaction. The kinetic parameters confirm the stability measured in the thermodynamical analysis

(Table 2). Regarding the m values, we could not conclude anything because of the large uncertainty in the refolding slope of the Best9-D48G mutant resulting from its high refolding rate.

In the other case, the D48G mutants behaved in a peculiar way. MaxL-D48G's folding rate increases compared to the protein without the D48G mutation, as postulated (Fig. 1E). Also, there is an increase in the unfolding rate as seen with some of the more stable core mutants (Best4, Best5, and C8A [Ventura et al. 2002]). However, a curved Chevron plot was observed, while this didn't happen in the reference protein. It was possible to fit MaxL's D48G kinetic data to a three-state model (Table 2). This indicates that in this mutant the hypothesis of the transient accumulation of intermediate at low denaturant concentration is more appropriate than a compact denatured state.

Comparison of the free energy values obtained from equilibrium and kinetics in the case of Best9 shows a good agreement within the error. This is not the case for the MaxL-D48G protein, where a large discrepancy exists between the unfolding free energy determined by the two methods. This discrepancy is not because of a large error in

Table 2. Kinetic parameters for the wild type and selected mutant proteins

Protein	$m_{\ddagger-F}$ (Kcal.mole ⁻¹ .M ⁻¹)	$m_{\ddagger-U}$ (Kcal.mole ⁻¹ .M ⁻¹)	$k_{\ddagger-F}$ (s ⁻¹)	$k_{\ddagger-U}$ (s ⁻¹)	m_{F-U} (Kcal.mole ⁻¹ .M ⁻¹)	K_{I-U}	ΔG_{F-U} (Kcal.mole ⁻¹)	$\Delta\Delta G_{F-U}$ (Kcal.mole ⁻¹)
WT	0.44 ± 0.07	-0.87 ± 0.01	0.005 ± 0.001	3.74 ± 0.02	0.76	—	3.9	—
D48G	0.47	-0.78 ± 0.01	0.01 ± 0.001	63.63 ± 1.2	0.74	—	5.2	1.3
Best9	0.41 ± 0.01	-1.1 ± 0.3	11.4 ± 0.7	27 ± 4	0.89	—	0.5	—
Best9-D48G	0.41 ± 0.10	-0.78 ± 0.2	12.9 ± 2.0	404 ± 28	0.71	—	2.0	1.5
MaxL	0.45 ± 0.01	-0.78*	0.19 ± 0.01	0.13 ± 0.02	—	—	0.1	—
MaxL-D48G	0.47*	-0.78*	7.5 ± 0.3	1236 ± 134	—	0.079	3.0	2.9

The experimental conditions and analysis are described in the Materials and Methods section. The errors shown correspond to the fitting errors. $m_{\ddagger-F}$ dependence of the natural logarithm of unfolding with urea. $m_{\ddagger-U}$ dependence of the natural logarithm of refolding with urea. $k_{\ddagger-F}$ unfolding rate constant in water. $k_{\ddagger-U}$ refolding rate constant in water. m_{F-U} dependence of the natural logarithm of the equilibrium constant with urea, obtained from the kinetic parameters $m_{\ddagger-F}$ and $m_{\ddagger-U}$. ΔG_{F-U} Free energy of unfolding determined from the kinetic parameters. $\Delta\Delta G_{F-U}$ difference in free energy of unfolding between the mutants with Asp48 and those having a Gly at this position determined from the kinetic parameters.

* Value fixed in the fitting equation.

the fitting of the kinetic data (because of the very large folding and unfolding rate constants), but to the existence of a folding intermediate that is kinetically detected.

Spectroscopic analysis

Wild-type SH3 domain's fluorescence emission spectrum is consequent with a partial exposure of the tryptophan residues to the solvent (Burstein et al. 1973; Viguera et al. 1994a). Mutation D48G does not perturb the emission spectrum of the wild-type protein as expected for a local change (data not shown). A similar result is found for Best9 upon mutation D48G (Fig. 2A). However, this is not the case for the other mutant MaxL: MaxL-D48G exhibits a red shift compared to its reference, indicating a higher degree of exposure of the indole rings (Fig. 2B; Burstein et al. 1973).

The analysis of these mutants by means of CD spectroscopy confirmed the fluorescence data. The far-UV CD spectrum of the wild-type SH3 is dominated by the aromatic contribution of Trp41 and Trp42. The maximum at around 218 nm is characteristic of the folded protein and disappears upon unfolding (Viguera et al. 1994a). In the case of the Best9 mutants this maximum slightly increases in the D48G variant as expected for a more stable protein (Fig. 2C). For MaxL, we observed the opposite effect, a decrease in the maximum at 218 nm indicating partial denaturation of the protein (Fig. 2D). Also, we found different behaviors in the near-UV region, where tertiary interactions are investigated. In Best9, there is a very small change in the spectral pattern and in signal intensity (Fig. 2E). Opposite to this in MaxL-D48G the signal decreases considerably with respect to its reference (Fig. 2F). This could be compatible with a loss of specific tertiary structure as it is found in a molten globule conformation, with natively like secondary structure, but lacking specific tertiary interactions (Ptitsyn 1992), following relaxation in the distal loop.

A typical attribute of a molten globule is the binding of ANS (Semisotnov et al. 1991) as a result of the exposure of

hydrophobic patches to the solvent. Denaturation of a molten globule by addition of a denaturant eliminates ANS binding. In Figure 2G, we show the changes in ANS fluorescence upon increasing urea concentration for the wild type and the MaxL and Best9 core variants. ANS fluorescence increases significantly in the presence of MaxL-D48G, and it reaches near background above 1M urea. On the other hand, MaxL shows no significant binding. Regarding Best9, we find the opposite effect: a weak binding in the case of Best9 and no binding in the Best9-D48G mutant. These results suggest that the D48G mutation induced a molten globule formation in the MaxL mutant.

NMR analysis

A key proof to assess a molten globule is NMR (Baum et al. 1989). The wide dispersion of chemical shifts, distinctive of globular proteins, reflects the extremely specific interresidue interaction within the packed folded structure. Molten globule's NMR spectra exhibit limited chemical-shift dispersion, though appreciably greater than that expected for a random coil conformation (Baum et al. 1989; Alexandrescu et al. 1993). As ¹H-NMR spectra show (Fig. 3), both Best9 and Best9-D48G are properly folded species: In this case the point mutation does not provoke band broadening or peak collapse. Regarding MaxL, the NMR spectra shows better dispersion and sharper peaks in the reference than in the D48G counterpart. In this case, the polyleucine core determines a compact wild-type species, which all the performed experiments indicate as fully folded, while the D48G mutation apparently induced a "melting" of the inner side chains. However, careful examination of the 1D spectra of the MaxL-D48G proteins shows some signals in the 1–0 ppm range that are characteristic of a folded SH3 and that indicate the existence of some residual folded protein, or some residual specific tertiary structure in the molten globule.

Figure 3. ^1H -NMR spectra of the mutants Best9WT (A), Max-LWT (B), Best9D48G (C), MaxLD48G (D) in 20 mM phosphate buffer. pH 7.0.

Discussion

In this work, we have analyzed the relationship between loop stiffness (Klimov and Thirumalai 2002) and conformational specificity using core redesigned versions of the spectrin SH3 domain.

Thermodynamic, kinetic and conformational effects of the Asp48 into Gly mutation

Opposite to what was described for the wild-type protein and from a mutant form of it with an elongated hairpin

(Viguera and Serrano 2001), mutation of Asp48 into Gly does not produce a large stabilization of the redesigned core mutants, with two exceptions: Best9 and MaxW. This effect is accompanied by a general decrease in the m -values (with the above exceptions), thus suggesting either a conformational change in the folded or unfolded states, or the presence of a stable folding intermediate. ANS binding, intrinsic fluorescence, UV, CD, and NMR spectroscopy of two selected mutants (Best9 and MaxL) indicates that in those mutants in which we observe a smaller than expected increase in stability there could be a folding intermediate with

properties of a molten globule at low urea concentrations. We also analyzed another of the core mutants (MaxI) that exhibited a small increase in stability upon mutation D48G. This mutant behaves like MaxL under all spectroscopic analysis. The reason why we did not show the data here is because the D48G form has a strong tendency to aggregate and thus we could not analyze it by NMR.

Folding intermediates and molten globules

All the different analyses we have done on the MaxL-D48G protein indicate the existence of a molten globule state present under equilibrium conditions. This state either coexists with a folded conformation or rather retains some cooperativity as has been described for other proteins (Bhattacharjya and Balaram 1997; Wang et al. 1998). Kinetic analysis, on the other hand, reveals the existence of a folding intermediate that is eliminated at high urea concentration. We cannot discriminate whether this folding intermediate coincides with the molten globule we detected at equilibrium under native conditions. An open question is how a folding intermediate would fold further into a molten globule. One possible explanation is that the intermediate only involves folding of part of the structure. In a second stage, the rest of the protein could collapse, keeping part of the structure folded (which could explain the weak folded signals in the NMR spectrum), but adopting many conformations.

Conformational strain, folding specificity, and molten globules

Asn in position 47 is in an unfavorable region of the Ramachandran plot surrounded by ϕ , ψ conformations with a very high energy (Vega et al. 2000). In a previous work, we showed that by increasing the volume buried in the core of the spectrin SH3 we introduced some conformational strain. This results in small conformational changes at the distal (residues 46 to 49) and RT (residues 25 to 31) loops, resulting in a small expansion of the β -sheet barrel (Ventura et al. 2002). The conformational changes in the distal loop involved a local change at residues 45 and 49, pushing aside the β -turn at the tip. However, the local conformation of the β -turn containing Asn47 remained the same. Mutation of Asp48 to Gly relaxes the distal loop β -turn, that adopts a type I' β -turn conformation and stabilizes the protein by more than 1 Kcal/mole (Martinez et al. 1998).

Conformational specificity refers to the ability of a protein sequence to confer a unique tertiary fold with a correctly packed hydrophobic core, which is an attribute of natural proteins (Walsh et al. 2001). The molten globule features in some of our unstable core mutants after the D48G mutation could be due to the fact that relaxation of the distal loop is combined with the release of the conformational strain as a result of overpacking, with a loss in

folding specificity. We suggest that, in order to release the existing overpacking strain, some unstable core mutants go through an expansion of the β -barrel with a consequent higher degree of exposure of their hydrophobic patches. This expansion of the molecule is constrained by distal loop's stiffness. Mutation to D48G relaxes the distal loop allowing a further expansion of the core with the subsequent loss of specific packing in some mutants. The outcome of this conformational rearrangement resembles a "melting" of the hydrophobic core, not influencing the secondary structure of the proteins, but affecting its specific tertiary interactions (Ptitsyn 1992).

Materials and methods

Cloning, mutagenesis, and expression

Mutants were obtained by the polymerase chain reaction (PCR) method and cloned in pBAT-4 (Peranen et al. 1996). Plasmids harboring the mutations were transformed in BL21 (DE3) cells and proteins expressed and purified as previously described (Viguera et al. 1996). All mutants were sequenced and protein identity was confirmed by mass spectrometry.

Thermodynamic analysis

Equilibrium denaturation was followed by fluorescence spectroscopy on a Jasco-710 instrument thermostated to 298 K. Fluorescence excitation was at 268 nm and the emitted signal was recorded above 340 nm. Experimental data were fitted to a two-state transition curve for which the fluorescence of the folded and unfolded states is dependent on the denaturant concentration. Fittings were done using the nonlinear least-squares algorithm provided with Kaleidagraph (Abelbeck Software).

Determination of the kinetic and thermodynamic parameters

In spectrin SH3 domain, fitting of several of these unstable mutants has shown that the curvature of the unfolding data follows the next equation (Martinez and Serrano 1999):

$$\ln k_{\text{F} \rightarrow \text{U}}[\text{urea}] = \ln k_{\text{F} \rightarrow \text{U}}^0 + m_{\text{F} \rightarrow \text{U}}^*[\text{urea}] - 0.014 * [\text{urea}]^2 \quad (1)$$

Accordingly, the kinetic data were fitted to the equation

$$\ln k[\text{urea}] = \ln (k_{\text{F} \rightarrow \text{U}}^* \exp(m_{\text{F} \rightarrow \text{U}}^*[\text{urea}]) + k_{\text{U} \rightarrow \text{F}}^* \exp(m_{\text{U} \rightarrow \text{F}}^*[\text{urea}] - 0.014 * [\text{urea}]^2) \quad (2)$$

where $k_{\text{F} \rightarrow \text{U}}^*$ and $k_{\text{U} \rightarrow \text{F}}^*$ are the refolding and unfolding rate constants in water, respectively. $m_{\text{F} \rightarrow \text{U}}^*$ and $m_{\text{U} \rightarrow \text{F}}^*$ are the slopes of $\ln k$ versus $[\text{urea}]$ in the refolding and unfolding reactions, respectively. With these values, $\Delta\Delta G_{\text{F} \rightarrow \text{U}}$ (the destabilization energy induced by the mutation) and $\Delta\Delta G_{\text{U} \rightarrow \text{F}}$ (its components in the re* folding and unfolding semi-reactions) can be calculated:

$$\Delta\Delta G_{F-U} \leftarrow \Delta G_{F-U,ref} - \Delta G_{F-U,mu} = \Delta\Delta G_{F-U} - \Delta\Delta G_{F-F} \quad (3)$$

$$\begin{aligned} \Delta\Delta G_{F-U} &= RT \ln (k_{F-U,ref}/k_{F-U,mu}) \\ \Delta\Delta G_{F-F} &= RT \ln (k_{F-F,ref}/K_{F-F,mu}) \end{aligned} \quad (4)$$

Where “ref” refers to the D48G mutant and “mut” to each one of the mutations done upon the former. Calculation of the Gibbs energy of unfolding using a nonlinear term in the unfolding reaction produces a good agreement with the calorimetric data, as it happened with barnase.

Max-L’s kinetic trace, showing a curvature in the $\ln k$ versus [urea] plots was fitted to equation 5, which contains a term dependent on the stability of the intermediate and its m value with respect to the unfolded state (m_{I-U}):

$$\ln k [urea] = \ln (k_{F-F} * \exp (m_{F-F} * [urea] - 0.014 [urea]^2) + k_{F-U} * \exp (m_{F-U} * [urea]) * (1/(1 + 1/K_{I-U} \exp m_{I-U} * [urea])) \quad (5)$$

Common parameters have the same meaning as in equations 1–4. K_{I-U} represents the equilibrium constant for the intermediate ($[I]/[U]$) and m_{I-U} the dependence of ΔG_{I-U} with respect to denaturant concentration.

Fluorescence emission and ANS-binding assay

Fluorescence emission spectra of the mutants were acquired with an Aminco Bowman spectrofluorimeter at 25°C. The excitation wavelength was set at 280 nm; emission was recorded between 300 and 430 nm.

ANS fluorescence emission spectra were measured in an Aminco Bowman spectrofluorimeter at 25°C. Protein samples (100 μ M) in 50 mM phosphate, pH 7.0, were diluted 10-fold into the corresponding buffer containing ANS (250 μ M). Solutions with different urea concentration were prepared by adding different volumes of a 10 M urea, 50 mM phosphate, pH 7.0 solution. Samples were equilibrated for 1 h at 20°C before acquiring fluorescence spectra. The sample was excited at 365 nm and emission measured between 400 and 600 nm.

Circular dichroism

CD spectra were recorded at 25°C on a Jasco-710 instrument calibrated using D-10-camphorsulphonic acid. Spectra shown in the text are an average of 20 scans, which were corrected for the baseline signal and normalized.

¹H NMR spectroscopy

NMR samples were prepared at different concentrations, depending on protein solubility, in 600 mL of H₂O²/H₂O, 9:1 (v/v). TSP was used as an internal reference at 0.00 ppm. NMR experiments were performed on a Bruker DRX 500 MHz spectrometer, at 25°C. Monodimensional spectra were acquired with 32,000 data points, which were zero-filled to 64,000 data points before Fourier trans-

formation. Data were processed with the program XWINNMR from Bruker on silicon graphics workstations.

Perla design and selection

Novel core sequences were sampled with Perla, our protein design algorithm (Fisinger et al. 2001; Lopez de la Paz et al. 2001), modeling selected amino acids using side-chain rotamer conformations and quantifying the quality of overall sequence-structure fit, as previously described (Ventura et al. 2002).

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