Spectrin domains R15, R16 and R17 are found in tandem arrays in proteins of the cytoskeleton, and spectrin itself is believed to be responsible for maintaining the shape of red blood cells by imparting elasticity to the cell membrane. In spite of very similar thermodynamic stability and transition state structure (as inferred through phi-value analysis) (1-2), R16 and R17 reach their native state (i.e. they "fold") over a thousand times slower than R15 (3). The intriguing piece of evidence that triggered this project was the finding that such behaviour is likely to reflect differences in the pre(-exponential) factor of the equation describing the folding reaction (4). In a Kramers'-like formulation of folding kinetics this pre-factor is essentially dependent on the intrachain diffusion coefficient (D) of the denatured polypeptide chain, a number which is now possible to measure with a combination of single-molecule Förster resonance energy transfer (smFRET) and fluorescence correlation spectroscopy (FCS), and which reflects the fine structure of the energy landscape underpinning the folding reaction (5). In this framework, a "smooth" energy landscape would be associated with fast folding, while a "rough" landscape would probably originate slower folding kinetics, due to the presence of the so called "internal friction" slowing down the folding process. The term "internal friction" indicates a general ensemble of intra-chain interactions, non-productive for the folding reaction, whose detectable effect is indeed a deceleration of folding (6). It is plausible that slow-folding spectrin domains R16 and R17 have rough energy landscapes and a non-negligible internal friction, whereas the fast folding domain R15 has a smooth energy landscape and no internal friction. I planned to study these spectrin domains using a combination of smFRET and FCS, in conjunction with newly developed data analysis methods and a diffusive description of the folding reaction from Kramers theory (7), in order to investigate the energy landscape that underpins the folding reaction of these proteins. The aim was to highlight and quantify the roughness in the landscape of these proteins, trying to clarify the intriguing features of their folding mechanism and, in turn, deepening our knowledge of the elementary properties of the protein folding reaction.

In order to perform the proposed investigation it is necessary to prepare the proteins by labelling them with fluorescent molecules, namely to covalently attach suitable fluorophores to the polypeptide chain: such fluorophores will be the reporters of protein dimensions and movements (the dynamics). To do so, appropriate amino acid residues ("X") of the polypeptide chain have to be identified and mutated into Cysteines. Single and double X→Cysteine mutants of R15, R16 and R17 domains were designed, produced, lebelled with the appropriate fluorophore(s) Alexa 488 and Alexa 594 (Invitrogen) and purified.
Thermodynamic stability, as well as folding and unfolding kinetics of the double mutants 6-99 and 39-99 of R15, R16 and R17, has been assessed through guanidinium chloride (GdmCl)-induced equilibrium and stopped-flow denaturation experiments, monitoring the fluorophores' fluorescence signal or the intrinsic triptofan fluorescence, when possible.
These same variants and the singly-labelled variants of the same domains have been tested for possible anisotropy of the attached dyes. All of the variants were found to be suitable for the proposed spectroscopic investigation, with exclusion of the doubly-labelled R16 39-99, which was therefore was not included in the following study.

The distance-dependent transfer efficiency of the six doubly-labelled protein variants in different GdmCl concentration ([GdmCl]) was measured with smFRET to determine the compactness of the unfolded states. In parallel, FCS experiments on the same proteins and at the same [GdmCl] were performed, in order to measure the reconfiguration time of the unfolded proteins, which give information into the

polypeptide chain dynamics and how this depends on compactness. These two pieces of information, one spatial and the other temporal, represent the two fundamental pieces of information for calculating the intrachain diffusion coefficient D (8-9), a parameter which reflects the intrinsic mobility of the polypeptide chain, and therefore reflects the nature, smooth or rough, of the folding energy landscape. smFRET and FCS data have been analyzed and combined to calculate D for the two polypeptide chains at several [GdmCl]. Interestingly, the values obtained after the initial analysis did not show a significantly different D between R15 and R16, while R17 displayed a higher D at all [GdmCl]. It has been now been observed for many proteins that the average dimensions of the denatured state increase with increasing denaturant. Since it was plausible to assume that roughness in the denatured state would be most evident when the polypeptide chain was very compact we resorted to investigate R17's denatured state in its most compact state, i.e. in absence of denaturant. For this reason, we resorted to perform FCS measurements in a microfluidic device (10), combining two techniques in an unprecedented experiment. The fast mixing achievable in the microfluidic device allowed us to measure the size distribution and reconfiguration time of the polypeptide chain while it is still in the unfolded state but in virtual absence of denaturant, where it is maximally compact. Such analysis could only be performed on R17, the slowest folding domain, because R16 and R15 fold within the dead time of the microfluidic device. Experiments have been carried out at a concentration of GdmCl from 0.1 M to as low as 0.04M, and single-molecule transfer efficiency histograms and FCS have been recorded in these conditions. At these [GdmCl], the R17 variants showed the greatest compaction and the slowest reconfiguration time, as expected, but maintained a behaviour coherent with the observed trend at higher [GdmCl], without evidence for a particular enhancement of roughness. Nonetheless, these results represent the first example of intrachain diffusion coefficient measured on an unfolded state in native conditions.

Intriguingly, the R15 variants displayed a lower intrachain diffusion coefficient, suggesting a greater energy landscape roughness, than the analogous R17 ones at every [GdmCl], showing convergence to a limiting value, reached only at the highest [GdmCl] (7M). One of the hypotheses that we wanted to test was that the roughness previously identified at the rate-limiting transition state for folding of R17 were present over the whole folding energy landscape. The values of the intrachain diffusion coefficient measured over a wide range of denaturant seem to indicate that the denatured states of R15, R16 and R17 are not too dissimilar from those of other proteins studied in our laboratory (e.g. Cold Shock Protein from *Thermotoga maritima* and human Cyclophillin), suggesting that roughness in R16's and R17's energy landscape is limited to the rate-limiting folding transition state, probably due to the particular folding mechanism of these proteins (4). It is somehow interesting that R15, the fastest folding of the three spectrin domains, for which no roughness has been observed at the rate-limiting transition state, displays a slightly lower intrachain diffusion coefficient in the denatured state compared to R17, which is the slowest folder.

In order to obtain an accurate estimate of the relative amount of roughness in the energy landscape of these proteins, a dependence of the intrachain diffusion coefficient on the solvent viscosity has been measured, in an analogous way as that performed on the same domains for the folding reaction (4). The rationale of this analysis is that the greater the roughness, the more significant the internal friction, the lower the sensitivity of the polypeptide chain dynamics to changes in the external viscosity (or external friction), because the internal component will dominate on the external and dictate the chain dynamics. The results of this analysis indicate that R15 is indeed less sensitive than R16 and R17 to solvent viscosity, indicating that the latter is

characterized by a greater internal friction than the former in the denatured state, and confirming the results from the intrachain diffusion coefficient measurements at different [GdmCl].

Phase two of the proposed project has changed from the investigation of the energy landscape of mutant proteins to a deeper analysis of the wt variants of the three spectrin domains. We didn't observe the hypothesized relationship between folding rate and presence of roughness in the denatured state, but, concentrating our attention towards investigating the specific features of the folding energy landscape of R15, R16 and R17, we completed, for the first time, an experimental characterization of the whole accessible energy landscape for folding of three protein domains from the denatured to the folded state. A manuscript describing the work carried out under MC sponsorship is in preparation, but please note that other papers closely connected to this project have already been published with the fellow's contribution.

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