Eur Biophys J (2007) 36:795–803
DOI 10.1007/s00249-007-0160-4

ORIGINAL PAPER

Environment effects on the oscillatory unfolding kinetics of GFP

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Received: 6 December 2006 / Revised: 13 March 2007 / Accepted: 20 March 2007 / Published online: 12 April 2007

Abstract The chromophore of a green fluorescent protein (GFP) mutant engineered to enhance emission and stability is known to display erratic switchings among a few of its chemical substates and, in particular, between the anionic A and the neutral N substates, whose difference is associated with a proton exchange and a consequent conformation rearrangement. However, when close to unfolding, the A–N switchings suddenly become very regular as shown by fluorescence oscillations that have been recently observed for molecules embedded in wet silica gel. In order to establish whether the matrix hosting the protein is responsible for these oscillations, we investigated the effect of another medium (silanized surfaces), of a different denaturant (urea) and of cosolvents (D_2O and glycerol). The occurrence of periodic A–N switchings, in the last milliseconds before GFP unfolding, is observed under all investigated conditions, together with three specific frequency values that characterize the pre-unfolding fluorescence. Urea and guanidinium, the denaturants employed in order to unfold GFP, do not lead to appreciable differences in the observed switching parameters, whereas the different media embedding the protein give rise only to frequency shifts that scale with the viscosity of the host. The periodicity of the GFP A–N switchings and their dependence on cosolvents suggest that they could be associated with oscillatory motions between meta-stable conformations of the β-barrel surrounding the chromophore near protein unfolding.

Keywords GFP · Single molecule · Protein unfolding · Periodic A–N switching · Wet silica gel · Silanized surface

Abbreviations

GFP	Green fluorescent molecole
GFPmut2	Green fluorescent protein carrying the triple substitution S65A, V68L, S72A
A	Anionic state of the GFP chromophore
N	Neutral state of GFP chromophore
DIEA	*N*,*N*-diisopropylethylamine
DCM	Dichlorodimethylsilane
GuHCl	Guanidinium hydrochloride

Introduction

Until a decade ago the presence of heterogeneous molecular chemical states in biopolymers was mainly inferred from indirect analysis of data on ensemble measurements. Recently, however, more direct experimental investigations have become possible due to great advances in single-molecule observation and handling. High-resolution spectroscopy and microscopy have paved the way to detailed examinations of individual molecules in the course of chemical reactions (Schuler 2005; Bohmer and Enderlein 2003; Weiss 2000). As a significant example we recall the monitoring, in real time, of the kinetics of single molecules folding–unfolding processes (Xie et al. 2004; Rhoades

Proceedings of the XVIII Congress of the Italian Society of Pure and Applied Biophysics (SIBPA), Palermo, Sicily, September 2006.

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et al. 2003; Lipman et al. 2003; Liphardt et al. 2001; Talaga et al. 2000; Eaton et al. 2000). Since long-lasting measurements on individual protein molecules are often necessary, for hours and even days, this requires that single molecules must be prevented from diffusing during the observation time and that photobleaching be negligible. Therefore, embedding biopolymers in suitable media, such as wet silica gels (Bettati and Mozzarelli 1997) and aminosilanized glasses, has been a successful approach to single molecule investigation (Jia et al. 1999). The absence of covalent interactions with the highly hydrated silica gel microenvironment of the trapped molecules, allows the retention of native structure and function. Furthermore, the chemically inert matrix prevents protein aggregation, restricts protein mobility due to the narrow pore size, and it also constitutes the ideal host as shown by the low background fluorescence. Recent observations of protein unfolding reactions employing gel embedded single GFP molecules have shown new features that were not previously accessible (Baldini et al. 2005; Cannone et al. 2005). It should be added that also silanized glasses are a good medium for protein kinetics investigations as proven in a few studies (Jia et al. 1999).

In this paper, the attention is focused on the properties of the switching between the anionic A (deprotonated) and the neutral N (protonated) chemical states of the GFPmut2 chromophore (Zimmer 2002), inferred from the fluorescence emission jumps between blue (460 nm) and green (508 nm), respectively (Cannone et al. 2005). Fluorescence from either state is known to be a marker of the structural integrity of the protein, whereas upon disruption of the native structure no fluorescence occurs (Saxena et al. 2005; Scharnagl et al. 1999). As recently reported, the A–N switching is a process occurring at random but, when in proximity of the unfolding event, the switching becomes very regular and is characterized by a few discrete frequency/period values (Baldini et al. 2005). The periodic regime of the pre-unfolding oscillations is displayed by all the examined molecules and lasts only some tens of milliseconds before full unfolding is achieved, as monitored by loss of fluorescence. Here the occurrence of pre-unfolding oscillations under different conditions is shown to be a regular feature of GFPmut2, independent of the denaturant agent employed to unfold the protein, whereas the chosen host matrix shifts the value of the oscillation frequencies. A correlation found between the values of the pre-unfolding oscillation frequencies and those of the unfolding times appears as a further support to the existence of conformational substates and discrete unfolding pathways (Baldini et al. 2005). Such information should allow a more detailed description of the heterogeneities hidden in biological processes.

Materials and methods

Single molecules fluorescence apparatus

The home-built inverted scanning two-photon microscope employed here has been described elsewhere (Malengo et al. 2004). A 80 MHz mode-locked titanium sapphire laser at 860 nm (Tsunami 3960, Spectra Physics, CA, USA) was used to prime the single molecule's fluorescence (Diaspro 2001). Operating at this wavelength makes it possible to excite the anionic and the neutral state of the GFPmut2 chromophore (Abruzzetti et al. 2005; Cormack et al. 1996) simultaneously (Baldini et al. 2005). An average excitation intensity of 10 kW/cm^2 was fed into the microscope by a dichroic mirror (550 DCLP, Chroma Technology, Rockingham, VT, USA) and the light falling on the sample was circularly polarized by a $\lambda/4$ wave plate. The protein fluorescence collected by the objective (Oil 100 × NA = 1.3, Plan Fluor, Nikon), was separated from the excitation light by a dichroic mirror (495DCLP, Chroma Technology, Rockingham, VT, USA). Blue and green emissions of GFPmut2 were selected with two band-pass filters (460/30LP, 515/30LP, Chroma Technology, Rockingham, VT, USA) and simultaneously detected by two SPAD photodiodes (SPCM 15, EG & G, Salem, MA, USA). All the experiments were performed at room temperature.

Fluorescence acquisition of single molecule images

Single molecule fluorescence images and traces were collected with the above-described apparatus, which employs a tightly focused laser spot (≅200 nm) to excite one molecule at a time (Cannone et al. 2005). The acquisition of the images (100 × 100 pixels) with 1 ms residence time per pixel takes ≅1 s. The employed field of view was 5 × 5 μm^2 and the excitation power on the sample (λ_{exc} = 860 nm) was ≅10 kW/cm^2. The image acquisition was performed by a home-coded software developed with Labwindows/CVI (National Instruments, Milano, Italy) (Malengo et al. 2004). The two-photon fluorescence dynamics of single molecules was acquired at 100 μs time resolution by pointing the excitation beam onto the spot ascribed to each single molecule. In order to record the fluorescence emission of a single GFPmut2 molecule during the unfolding process, a sequence of fluorescence traces were acquired for each spot appearing in the fluorescence image. Each fluorescence trace lasts 8 s in both the emission channels. Obviously, since a single GFPmut2 molecule can be found either in the anionic or in the neutral state, the simultaneous acquisition of fluorescence in the neutral and anionic channels allows the detection of transitions between the two states

at 100 μs time resolution. In particular, attention was paid to the transitions from the anionic to the neutral state since the fluorescence emission of single GFPmut2 molecules in the anionic state is stronger than that in the neutral one.

Wet silica gels

Encapsulation of GFPmut2 in silica gels was carried out according to a modified version of a previously reported procedure (Bettati and Mozzarelli 1997). The stock protein solution was diluted in 50 mM phosphate buffer, pH 7.5. Upon gelation, protein-doped silica gels were covered with 100 mM phosphate buffer, pH 7.0, and stored at 4°C for at least 12 h before use. The final GFPmut2 concentration was approximately 100 nM. A droplet of the mixture was deposited in a circular micro-chamber, ≅5 mm in diameter and ≅400-μm thick, built on a glass slide. The resulting gels are transparent in the visible range, with optical qualities that allow spectroscopic measurements. Since the silica gel contains a significant amount of water, it is easy to add denaturants and/or viscogenic agents, such as glycerol (up to 10% w/w), and to exchange the buffer solution. For example, measurements in the presence of D_2O were obtained after a quick rinsing of the gel with a D_2O solution.

Amminopropyl silanization of clean glass

GFPmut2 molecules have been linked to a glass through silanization technique. Clean glasses were amino-silanized by a reaction with 0.1% (v/v) (3-aminopropyl) trimethoxysilane in chloroform, providing a surface that is positively charged at neutral pH or below. The reaction was allowed to proceed for 1 h at room temperature. The silanized glasses were repetitively washed with chloroform and water. A drop of 10 nM GFPmut2 solution was deposited on the silanized glass and drops of buffer or denaturant were added when needed.

Unfolding-refolding experiments

Drops of the concentrated denaturant solution, 6 M guanidinium hydrochloride (GuHCl) or 6 M Urea (Sigma-Aldrich, St. Louis, MO, USA), were added to the gel or silanized surface and the acquisition of the fluorescence signal from a single molecule was started immediately (Fig. 1a). GFPmut2 refolding was obtained by rapidly rinsing the sample with denaturant-free solutions, at pH 6.8. The focal plane position was maintained by micro-adjustment of the piezo control during the denaturation experiments.

Results and discussion

Fluorescence traces during unfolding

A typical fluorescence image of a gel hosting several isolated GFPmut2 molecules is reported in Fig. 1. Each spot corresponds to a single molecule in the anionic state A. The time resolved fluorescence trajectories of single molecules were acquired simultaneously in the neutral (N) and anionic (A) channels beginning when a drop of 6 M GuHCl solution was added to the gel (Fig. 1a), and ending when complete unfolding occurred (Fig. 1c, d, e). Fluorescence was recorded with a succession of acquisitions, each corresponding to excitation lasting 55 s, that were intercalated by 5-s excitation interruptions in order to prevent GFP bleaching. Few seconds after the addition of the GuHCl solution, a sudden decrease (about 20%) in the fluorescence intensity was observed (Fig. 1a). This effect, ascribed to the increase in chloride ions concentration, is known to affect the chromophore fluorescence yield (Chirico et al. 2006; Cannone et al. 2005). This initial fluorescence drop was not observed when unfolding was caused by urea. Simultaneous observations in both channels reveal A–N switching transitions, which appear randomly distributed in the 55 s recording of each trace (Fig. 1b). The on- and off-times of the A channel fluorescence emission under $I_{exc} = 10$ kW/cm^2 lie in the second and the millisecond range, respectively, with an average value of $\langle t_{on} \rangle = 10 \pm 2$ s and $\langle t_{toff} \rangle = 140 \pm 20$ μs. Both values show a random distribution well described by exponential decays (Baldini et al. 2005). Full unfolding is achieved when the fluorescence vanishes in both channels (Fig. 1c, d, e), at T_{UN}, the time elapsed after the addition of denaturant (Baldini et al. 2005; Cannone et al. 2005). At 6 M denaturant concentration, unfolding may require from several minutes to hours (Campanini et al. 2005; Cannone et al. 2005).

Pre-unfolding fluorescence oscillations

As recently reported (Baldini et al. 2005), the A–N switching kinetics, just before the unfolding event, displays a peculiar behavior: rather regular and faster rate switchings take place (Fig. 1c, d, e). This ''ordered'' regime, that is observed in all the examined molecules trapped in wet silica gel, lasts some tens of milliseconds, $\Delta\tau_0 \sim 42 \pm 2$ ms. Then, at unfolding completion, the signal suddenly vanishes. In particular, in the first half of the track (0–20 ms) the switching oscillations appear much more regular than those in the second half (20–40 ms). In addition, the pre-unfolding A–N switchings are found to possess only three well-defined values of the switching period $\Delta T = t_{on} + t_{off}$ (frequencies ν_i)

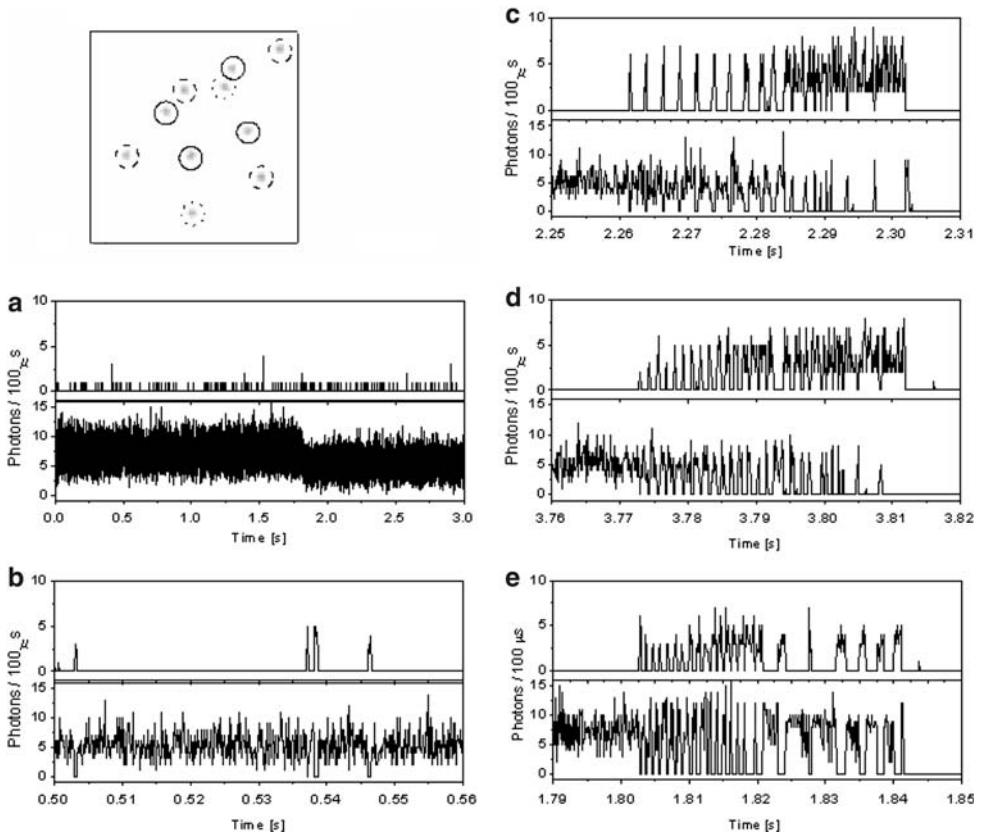

Fig. 1 *Top left* Fluorescence image (5 × 5 μm field of view with 1 ms residence time per pixel) of eight single GFPmut2 molecules in wet silica gel. All spots, recorded in the anionic channel, at λ_{exc} = 860 nm and λ_{em} = 515/30 nm, are ascribed to single molecules. The *circles* around the spots indicate the frequency values that each single GFPmut2 molecule will assume just before unfolding. (*Solid line* ν = 450 Hz, *dashed line* ν = 725 Hz and *dotted line* ν = 969 Hz). **a** Fluorescence emission of randomly selected single GFPmut2 molecules just after the addition of a 6.0 M GuHCl solution. Emission is simultaneously recorded in two channels. *Bottom trace* anionic channel, *top trace* neutral channel. **b** Typical section of the fluorescence traces of the same single GFPmut2 molecules of **a**, in the presence of 6.0 M GuHCl, recorded in the neutral (*top*) and anionic (*bottom*) channels. Four anticorrelated A–N fluorescence switchings are reported. **c**, **d**, **e** Data show the pre-unfolding oscillations that take place just before the unfolding event. The unfolding is indicated by the vanishing of fluorescence in both channels, neutral (*top*) and anionic (*bottom*). In **c** the emission is seen to oscillate between the two channels at ν = 450 Hz, in **d** at 725 Hz and in **e** with the highest pre-unfolding frequency value ν = 969 Hz. All fluorescence traces are recorded with a gate rate of 10 kHz under an excitation intensity of 10 kW/cm^2

at 2.3 ± 0.2, 1.3 ± 0.1 and 1.1 ± 0.1 ms, as confirmed also by Fourier transform of the initial 0–20 ms half of the fluorescence traces of 100 single molecules. The frequency values are: ν_1 = 450 ± 18 Hz, ν_2 = 725 ± 20 Hz and ν_3 = 933 ± 26 Hz (Fig. 2a, Table 1). It should be noticed that each molecule is found to oscillate at one and only one of the three observed frequencies, and that the fluorescence signal in the two channels is strictly anti-correlated, thus proving that the chromophore switches between A and N states.

Pre-unfolding N–A switching kinetics with different denaturants

The switching rate values are independent of the denaturant chosen to induce the GFPmut2 unfolding. Urea gives rise to pre-unfolding oscillation frequencies (Fig. 2c) displaying the same values observed in the presence of GuHCl (Fig. 2b). The independence from the denaturant choice can also be seen in Fig. 3, where the pre-unfolding A–N switching frequencies of four single GFPmut2 molecules that have undergone five consecutive unfolding-refolding cycles are shown. In particular, three of these molecules have been treated by alternating GuHCl (cycles 1, 3, 5) to urea (cycles 2, 4) and one with GuHCl only. To be noted that in two consecutive cycles a protein exhibits the same pre-unfolding oscillation frequency (here ≅725 Hz) although the unfolding is induced by different chemical agents. At the same time, toward the end of each denaturation cycle, one and only one of the three reported oscillation frequency values is observed for a given molecule.

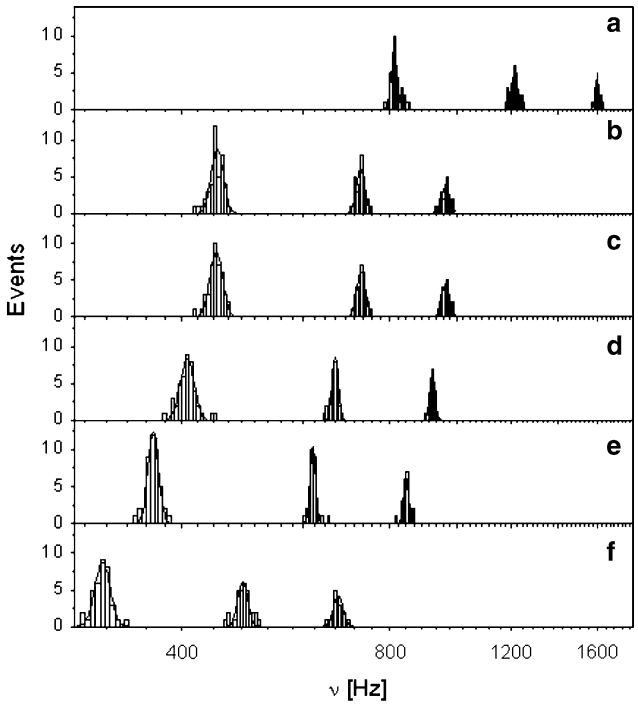

Fig. 2 Fourier transforms of the periodic section, first half approximately, of the pre-unfolding N–A switchings of GFPmut2 in different hosts. All data are taken at 6.0 M GuHCl with the exception of *c*. *a* amminopropyl silanized surface, *b* wet silica gel, *c* wet silica gel at 6.0 M urea, *d* wet silica gel at 5% glycerol, *e*, wet silica gel at 10% glycerol, *f* wet silica gel in D_2O. The data refer to 100 single GFPmut2 molecules observed during the last ≅40 ms just before unfolding. *Solid lines* are Gaussian fits to the data (the Gaussian fit parameters are reported in Table 1)

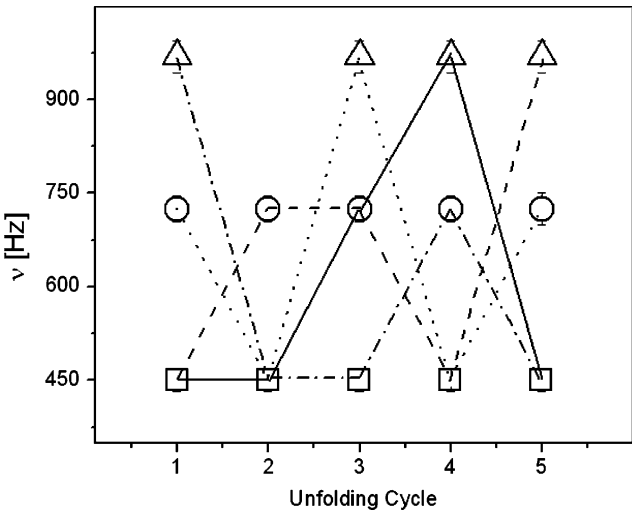

Fig. 3 Pre-unfolding *v* values recorded during five consecutive unfolding-refolding cycles of four GFPmut2 single molecules trapped in wet silica gel. The data refer to three molecules that were unfolded by alternating denaturants (6.0 M GuHCl in cycles 1, 3, 5 and 6.0 M urea in cycles 2 and 4, *broken lines*) and to one molecule that cycled under the action of GuHCl, only (*continuous line*). The *symbols* refer to the three pre-unfolding oscillation frequency values as given by the *vertical axis scale*

Pre-unfolding N–A switching kinetics in different matrices

Figure 1 shows a typical fluorescence image, where each spot represents a single GFPmut2 molecule immobilized in wet silica gel. The differently hatched circles around the

Table 1 The pre-unfolding oscillation values in the different experimental conditions and the relative unfolding times

Silanized surface				
GuHCl = 6.0 M	Pre-unfolding oscillations	v_{01} = 811 ± 18 Hz	v_{02} = 1,211 ± 27 Hz	v_{03} = 1,598 ± 21 Hz
	Pre-unfolding frequency	44%	30%	26%
	Unfolding time windows	3–18 min	12–36 min	37–62 min
Wet silica gels				
GuHCl = 6.0 M	Pre-unfolding oscillations	v_1 = 450 ± 18 Hz	v_2 = 725 ± 20 Hz	v_3 = 969 ± 26 Hz
No glycerol	Pre-unfolding frequency	43%	30%	28%
	Unfolding time windows	5–33 min	34–59 min	60–111 min
GuHCl = 6.0 M	Pre-unfolding oscillations	v_1 = 407 ± 19 Hz	v_2 = 667 ± 13 Hz	v_3 = 922 ± 17 Hz
5% Glycerol	Pre-unfolding frequency	42%	30%	27%
	Unfolding time windows	8–32 min	30–66 min	70–92 min
GuHCl = 6.0 M	Pre-unfolding oscillations	v_1 = 363 ± 12 Hz	v_2 = 619 ± 11 Hz	v_3 = 849 ± 11 Hz
10% Glycerol	Pre-unfolding frequency	41%	31%	27%
	Unfolding time windows	8–31 min	33–62 min	64–108 min
GuHCl = 6.0 M	Pre-unfolding oscillations	v_1 = 308 ± 19 Hz	v_2 = 494 ± 20 Hz	v_3 = 698 ± 18 Hz
D_2O	Pre-unfolding frequency	39%	30%	28%
	Unfolding time windows	10–50 min	52–90 min	91–141 min

The frequency values are obtained from the multi Gaussian fit reported in Fig. 2. The unfolding time window is the time interval of unfolding of the single GFPmut2 molecules exhibiting the same pre-unfolding oscillation value

spots correspond to the three frequency values of the pre-unfolding oscillations found when denaturing the proteins, as illustrated above. The values are found to be independent of the molecule location in the gel, thereby excluding heterogeneity effects on the switching. This is in agreement with time-resolved fluorescence anisotropy measurements on gels doped with fluorescent dyes, indicating that the local microenvironment of the molecules is not appreciably heterogeneous, as confirmed also from the absence of dispersion on the rotational diffusion values of trapped molecules (Narang et al. 1994, 1995). Different matrices, however, induce different oscillation frequency values. In order to investigate the role played by the trapping matrix on the oscillations, GFPmut2 has also been denatured with 6.0 M GuHCl when bound to an amino-silanized glass surface. Although characterized by faster unfolding kinetics (see Table 1 and the further discussion on this point), in this case periodic pre-unfolding switchings are also observed. Again three frequency values were found and the relative Fourier transforms are shown in Fig. 2f. The preunfolding oscillations are shifted to higher frequencies by a factor of 1.7 ± 0.1, as reported in Table 1. As already observed in the case of gels, microenvironment heterogeneity of the silanized glass does not seem to affect the switching rates. GFPmut2 pre-unfolding oscillations occur at three distinct frequency values in both the hosting matrices.

Pre-unfolding N–A switching kinetics versus glycerol concentration

In order to evaluate the effect of viscosity on the pre-unfolding switching rates, media with different viscosity have been used for GFPmut2. Silanized wet glass surfaces are taken as a reference condition since in this case the protein molecules can be considered as almost fully hydrated. The role of viscosity in silica gels has also been investigated by soaking them with 5 and 10% w/w glycerol solutions (Sottini et al. 2004; Uribe and Sampedro 2003; Gavish 1980). Higher glycerol concentrations cannot be used since strong quenching of the GFPmut2 fluorescence would occur in both channels, thereby hampering the observation of the molecules. Also in this case, when unfolding was induced by 6 M GuHCl, the oscillations occurring in the last tens of milliseconds before unfolding were analyzed by Fourier transform. As shown in Fig. 2d, e, the effect of glycerol on the pre-unfolding oscillations is that of shifting the frequencies to lower values with increasing glycerol concentration (by a factor of 0.84 ± 0.04 at 10% glycerol). In Fig. 4a, the reciprocals of the pre-unfolding oscillation frequency values, normalized to those in silanized glasses, ν_{0i}/ν_i, are plotted versus the normalized viscosity, η/η_0, where $\eta_0 = 1$ cP is the viscosity of water. The microviscosity inside the silica gel is taken equal to ≅2 cP (Narang et al. 1994). The plot shows a linear behavior described by the expression: $\frac{\nu_{0i}}{\nu_i} = 1 + \varepsilon\frac{\eta}{\eta_0}$, with $\varepsilon = 0.85 \pm 0.04$ for ν_1, $\varepsilon = 0.73 \pm 0.02$ for ν_2 and $\varepsilon = 0.65 \pm 0.02$ for ν_3. The observed linear trend agrees with the Kramers theory that implies a linear dependence of the reciprocal of chemical reaction rates (here A–N switching) on viscosity (Saxena et al. 2005; Gavish and Yedgar 1995; Kramers 1940).

Pre-unfolding N–A switching kinetics in D_2O

Since the A–N switching reaction involves a protonation–deprotonation process, the mechanisms underlying the kinetics of the pre-unfolding oscillations have been investigated replacing water as a solvent in the unfolding experiments with D_2O (Haupts et al. 1998). Here, again, the fluorescence emission, starting nearly 40 ms before unfolding, displays a train of regular oscillations associated with the A–N switching of each molecule. From the Fourier analysis of data in D_2O wet silica gel (Fig. 2f), one finds that the three values of the switching rate have become much lower than those in silanized glasses (by a ~0.68 factor, Table 1). When the frequencies are normalized to the corresponding values in silanized glasses and a relative viscosity of 3.2 is assumed for D_2O gels,[1] then the data fall on the straight lines of Fig. 4a. Preliminary data on D_2O wet silanized glasses show that also in this case the frequencies are shifted to lower values. Therefore it appears that the overall effect of D_2O consists of an increase in the effective,[2] gel viscosity when compared to H_2O (Haupts et al. 1998).

Pre-unfolding oscillations and unfolding time

As shown in Table 1 and Fig. 5, the values of the pre-unfolding frequencies are clearly related to the range of the observed unfolding time values T_{UN}, defined as the time taken by the protein to lose its fluorescence after denaturant addition. This correlation supports the assumption that a close connection exists between pre-unfolding oscillations and unfolding kinetics. The different trapping media, besides affecting the values of the pre-unfolding oscillation frequencies, also modify the time required to unfold, T_{UN}. The increase of T_{UN} with viscosity displays the same trend as that of the pre-unfolding frequencies (Table 1, Fig. 4b). In particular, when the protein is immobilized on a

[1] The viscosity has been estimated by summing the viscosity of the wet silica gel and the viscosity of D_2O: 2 + 1.2 cP.

[2] The protein motion inside the gel is expected to be slowed down by some constraint exerted by the gel cavities resulting in an effective viscosity larger than in water.

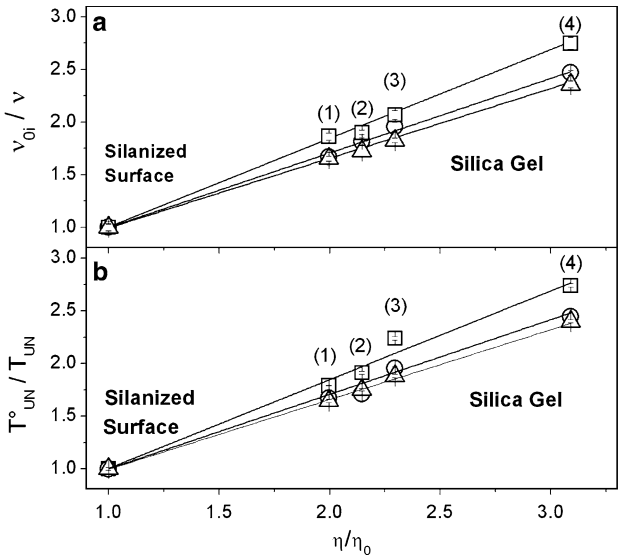

Fig. 4 Plot of the reciprocal of **a** the pre-unfolding oscillation frequency ratio ν_{0i}/ν_i (*triangles* ν_1, *circles* ν_2, *squares* ν_3) and **b** of the average unfolding time ratio T_{UN}/T°_{UN} versus η/η_0, where η is the viscosity of the hosting medium and η_0 is the viscosity of water. The *continuous lines* are linear data fits versus η/η_0. ν_{0i} are the oscillation frequency values in H_2O silanes and T°_{UN} are the corresponding unfolding times. *1* wet silica gel, *2* wet silica gel at 5% glycerol, *3* wet silica gel at 10% glycerol, *4* wet silica gel in D_2O

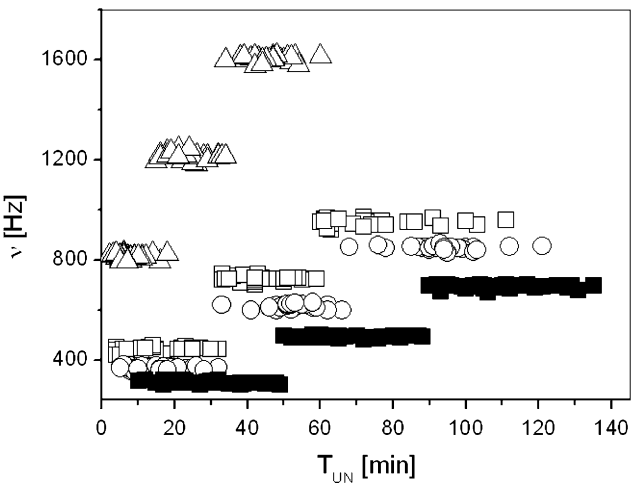

Fig. 5 Pre unfolding frequencies (ν) of 100 GFPmut2 molecules in different hosts versus T_{UN}, unfolding time (*open square* wet silica gel, *open circle* wet silica gel with 10% of Glycerol, *filled square* wet silica gel with D_2O and *open triangle* H_2O silanized surface)

silanized surface, the unfolding time decreases if compared to that in gels (Table 1). On the contrary, when increasing the glycerol concentration in silica gel, a rise of the unfolding time is recorded. The same trend is found in D_2O (Table 1). Under all examined conditions, the single protein molecules characterized by the highest frequency values exhibit the slowest unfolding process and vice versa. As an example, native molecules in wet silica gel displaying $\nu_1 = 450$ Hz unfold quickly ($T_{UN} < 10$ min), whereas those switching at $\nu_3 \sim 970$ Hz unfold slowly ($T_{UN} > 70$ min). Similarly, the GFPmut2 molecules on silanized surfaces that switch at $\nu_1 \sim 810$ Hz unfold with $T_{UN} < 7$ min, whereas those with $\nu_3 \sim 1{,}600$ Hz unfold at $T_{UN} > 40$ min (Fig. 5). None of the observed GFPmut2 molecules violates this correlation, i.e., not a single molecule displaying a long unfolding time is found to exhibit a slow pre-unfolding frequency, or, in other words slow pre-unfolding frequency always implies fast unfolding time.

Nature of the pre-unfolding oscillations

According to the literature, the A–N switching involves a two-step reaction: fast protonation–deprotonation process of the GFP chromophore and a much slower local conformational change coupled to a cooperative movement of the β-sheet backbone (Scharnagl et al. 1999). In particular, the proton transfer process that is involved in the A–N switching is attributed, according to Saxena et al. (2005), to a sequence of ''elementary'' steps: proton diffusion in the bulk solvent with subsequent transfer to the protein–water interface and proton migration from the interface to the chromophore site via an H-bonded network followed by rearrangement of the side chains around the chromophore. Unfolding is known to be accompanied by the protein beta structure disruption as suggested by the chromophore fluorescence loss (Campanini et al. 2005; Cannone et al. 2005; Zimmer 2002).

The onset of periodic switchings between two protonation states of the chromophore at specific frequencies suggests that regular fluctuations may also affect the β-barrel structure just before a drastic disruption of the protein structure occurs. There is no doubt that the oscillatory behavior is a property of the GFP during unfolding since it occurs both when the protein is hosted in different matrices and when denatured by different chemicals. However, the observed switching rates are found to be influenced by the medium (Sottini et al. 2004). In particular the A–N switching rates in wet silica gel are slower than those on a silanized surface, and a further slowing down of the oscillations is found in high viscosity media (glycerol). These results indicate that the periodic protein kinetics is affected by the matrix viscosity (Fig. 4), either intrinsic or induced by cosolvents. The two main steps in the protonation/deprotonation process are recognized: a diffusion of the proton within the protein scaffold, that should be highly dependent upon the micro-environment viscosity, and a some conformational change in the chromophore pocket, less affected by the protein-solvent interactions. Interestingly the oscillation frequencies show a reduced dependence on the relative viscosity, $\partial\frac{\nu_{0i}}{\nu_i}\Big/\partial\frac{\eta}{\eta_0} < 1$. This can be

taken as an indication that the observed oscillations are substantially related to the tiny conformational change of the beta-barrel protein structure in the vicinity of the chromophore.

Conclusions

Previous investigations of the pre-unfolding oscillations of single GFPmut2 molecules embedded in silica gel have been extended also to protein molecules bound to silanized surfaces. In order to ascertain whether the coupling to the environment was responsible for the peculiar behavior of GFPmut2, different conditions of the two protein hosts have been explored. The main results can be summarized as follows.

1. Under all investigated conditions the final instants of an unfolding single protein molecule are accompanied by one of three specific A–N switching frequency values.
2. Two well-known denaturants, GuHCl, charged, and urea, uncharged, give rise to undistinguishable frequency values if the protein is hosted in the same medium.
3. The action of different hosts, silica gels (in H_2O, H_2O–glycerol and D_2O) and a silanized glass surface (soaked in H_2O and D_2O), is that of shifting the three switching frequency values according to the host viscosity, but no change of their relative occurrence fractions are apparent.
4. The unfolding time is correlated to the pre-unfolding frequency.

The observed periodic A–N switchings in close proximity of GFPmut2 unfolding under all observed conditions suggest that the protonation/deprotonation dynamics, which occurs in the ms time range, is probably coupled to protein structure's regular motions that are likely those of the β-barrel, the scaffold that protects the chromophore from the quenching role of the solvent, as long as the barrel is nearly intact.

Acknowledgments Partially supported by INFM-CNR Fondo Integrativo per la Ricerca di Base, Cofin Ministero dell'Istruzione Universita' e Ricerca Scientifica.

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