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Protein denaturation in wet nanoporous silica gels: Effect of caging and crowding on the dynamics of GFPmut2

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Summary. — Proteins encapsulated in wet, nanoporous silica gels usually maintain their structural and functional properties. However, excluded-volume effects and the perturbation of water structure inside the pores of the gel may influence the kinetics of conformational transitions and the thermodynamics of functionally relevant conformational states. We investigated the unfolding of a mutant of GFP, GFPmut2, in wet nanoporous silica gels. Protein molecules are individually caged in the pores of the gel, avoiding protein aggregation and reproducing some of the effects exerted by molecular crowding and confinement in the cellular environment. Encapsulation in silica gels results in the alteration of the equilibrium distribution of native conformations, so that at least two alternative substates of the protein, spectrally undistinguishable in bulk studies, are significantly populated in the absence of denaturant. The evidence of an altered conformational distribution upon caging indicates that studies in dilute solution can miss functionally relevant structural and dynamic properties, highlighting the importance to carry out experiments under conditions that mimic the intracellular milieu.

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PACS 87.15.He – Dynamics and conformational changes.
PACS 87.64.Ni – Optical absorption, magnetic circular dichroism, and fluorescence spectroscopy.

1. – Introduction

Entrapment of biological macromolecules into solid matrices such as silica gels is a widely exploited tool to study enzymatic reactions and protein dynamics allowing to isolate metastable intermediates and to select and stabilize specific protein conformations of biotechnological and biochemical interest [1-13]. Recently, it has been suggested that the altered microenvironment experienced by proteins encapsulated in wet nanoporous

Fig. 1. – Three-dimensional structure of the Green Fluorescen Protein. The protein folds in a β-barrel structure with an α-helix bearing the 4-(p-hydroxybenziliden)-imidazolin-5-one chromophore in the center of the cavity.

silica gels can mimic some of the physical properties of the crowded intracellular milieu such as excluded-volume effects, and altered microviscosity and activities of solvent and solutes [14-16]. Functional and dynamic properties of proteins in such an environment are expected to be very different from those in dilute solutions. For this reason the characterization of protein structure, function and dynamics in wet nanoporous silica gels can represent a valuable tool to unveil the role of intracellular macromolecular crowding and confinement on proteins stability and biological function. Encapsulation in a confined space has been demonstrated to stabilize many proteins against chemical denaturation [14, 16-28], even though the contrasting effects on protein stability of an altered solvent structure [29] and a reduced entropy of the unfolded state are difficult to predict.

In the present work the effect of encapsulation in wet nanoporous silica gels on the stability and unfolding kinetics of a mutant of the Green Fluorescent Protein (GFP) has been investigated.

GFP is a highly fluorescent protein in which the fluorophore, located at the center of a β-barrel structure (fig. 1), is formed autocatalytically by an internal cyclization in oxidizing conditions of the three adjacent residues Ser65, Tyr66, Gly67. The GFP mutant used in the present study is GFPmut2 [30] carrying the triple substitution S65A, V68L, S72A that confers a high-fluorescence quantum yield, good resistance to photobleaching and high-expression yields at 37 °C.

2. – Material and methods

Protein expression and purification

Protein expression and purification was carried out as previously described [31] using as expression system the pKEN1 vector [32] containing the GFPmut2 sequence (Dr. Brendan P. Cormack, Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA) in XL1blue cells.

Chemicals and buffers

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification.

Experiments were carried out in 20 mM Tris-HCl, *p*H 7.4 or 7.0 at 37 °C. 600 mM NaCl was added to the buffer to prevent partitioning of the positively charged guanidinium ions in the pores of the gel, which, at a *p*H around neutrality, bear a net negative charge [33, 34]. Guanidinium hydrochloride (GdnHCl) was dissolved in 40 mM Tris-HCl, 1.2 M NaCl to obtain a 7 M stock solution [35, 36]. The stock solution was diluted with buffer to obtain the desired denaturant concentration.

Silica gels

GFPmut2-doped nanoporous silica gels were prepared according to the procedure of Bettati and Mozzarelli [4] with some modifications [37]. The sol containing the protein was layered on quartz slides and, after gelation occurred, the silica gels were kept at 4 °C in 100 mM potassium phosphate buffer, *p*H 7.0. Silica gels were tested for the absence of protein leakage in the time needed to complete experiments.

Fluorescence measurements

Fluorescence emission spectra and single-wavelength kinetic traces were acquired with a FluoroMax-3 fluorometer (HORIBA Jobin Yvon, Longjumeau Cedex, France), equipped with a thermostated cell-holder. The protein fluorescence was excited at 485 nm, using a 2 nm excitation slit. The emission of the chromophore was collected in the range 495–600 nm or at 507 nm (emission slit = 2 nm). The experiments were carried out at a protein concentration of 100 nM in solution and 1 μM in silica gel. The slides were fixed inside the optical cuvette at an angle of 45° with respect to the excitation light to minimize the amount of scattered light reaching the emission detector [38].

The estimated dead time due to manual mixing is about 10 s for kinetic experiments in solution, and about 30 s for those in silica gel.

Circular dichroism measurements

Circular dichroism measurements were carried out using a J-715 spectropolarimeter (Jasco, Easton, MD) equipped with a Peltier element for temperature control. Measurements were carried out at a protein concentration of 6 μM and 30 μM for experiments in solution and in silica gel, respectively. Experiments in solution were carried out using a microcuvette with an optical path of 0.1 cm. Spectra were collected in the range 210–260 nm, because Tris-HCl buffer interferes with far-UV light at low wavelengths. Single-wavelength kinetics were collected at 220 nm.

Absorbance measurements

Absorbance spectra were acquired with a Cary 400 Scan spectrophotometer (Varian Inc., Palo Alto, CA) using quartz cuvettes with an optical path of 1 cm. Single-wavelength kinetic traces were collected at 485 nm. The temperature was controlled by a circulating water bath.

Refolding

The reversibility of the unfolding reaction was assessed by measuring the degree of signal recovery upon removal of denaturant. The protein, in solution or encapsulated in silica gel, was fully denatured in 6.0 M GdnHCl. The solution of denatured protein was diluted 15 fold in Tris/NaCl buffer, *p*H 7.4 at 37 °C, to obtain a final GdnHCl concentration of 0.4 M. The gel samples containing the denatured protein were transferred to Tris/NaCl buffer, *p*H 7.0, at 37 °C. In both cases the kinetics of the refolding reaction was followed until the signal reached a constant value.

Data analysis

Protein unfolding kinetic traces were fitted to a single, double or triple exponential equation:

$$I = I_0 + a \cdot e^{-k_1 t} \,, \tag{1}$$

$$I = I_0 + a \cdot e^{-k_1 t} + b \cdot e^{-k_2 t} \,, \tag{2}$$

$$I = I_0 + a \cdot e^{-k_1 t} + b \cdot e^{-k_2 t} + c \cdot e^{-k_3 t} \,, \tag{3}$$

where I is the fluorescence or CD signal intensity, and I_0 is the signal intensity at time $t = 0$; a, b and c are pre-exponential factors accounting for the amplitude of the corresponding kinetic phase; k_1, k_2 and k_3 are the rate constants.

The dependence of the natural logarithm of the unfolding rate constants ($\ln k_{\mathrm{obs}}$) on denaturant concentration was fitted to the linear equation

$$\ln k_{\mathrm{obs}} = \ln k_{\mathrm{obs},0} + m_{k_{\mathrm{obs}}} \cdot [\mathrm{GdnHCl}] \,, \tag{4}$$

where $k_{\mathrm{obs},0}$ is the unfolding rate constant in the absence of denaturant and $m_{k_{\mathrm{obs}}}$ [39] is a parameter that indicates the variation in the protein surface area exposed to the solvent going from the native to the transition state.

The fraction of unfolded protein was calculated from equilibrium experiments at different denaturant concentrations according to the equation

$$f_U = \frac{I - I_{0,N}}{I_{0,U} - I_{0,N}} \,, \tag{5}$$

where I and $I_{0,N}$ are the observed signal intensity at a defined denaturant concentration and in the absence of denaturant, respectively, and $I_{0,U}$ is the signal intensity of the fully denatured species. The equilibrium constants, K_{eq}, and the unfolding free energies, ΔG^0_U, at each denaturant concentration were calculated as

$$K_{\mathrm{eq}} = \frac{f_u}{1 - f_u} \,, \tag{6}$$

$$\Delta G^0_U = -R \cdot T \cdot \ln K_{\mathrm{eq}} \,, \tag{7}$$

$\Delta G^0_{0,U}$, the free energy change in the absence of denaturant, and m, the dependence of the unfolding free energy on denaturant concentration, were calculated with the linear extrapolation method (LEM) [40] using the following equation to fit the dependence of ΔG^0_U on GdnHCl concentration:

$$\Delta G^0_U = \Delta G^0_{0,U} - m \cdot [\mathrm{GdnHCl}] \,. \tag{8}$$

The denaturant concentration at half transition (D_{50}) was calculated as

$$D_{50} = \frac{\Delta G^0_{0,U}}{m} \,. \tag{9}$$

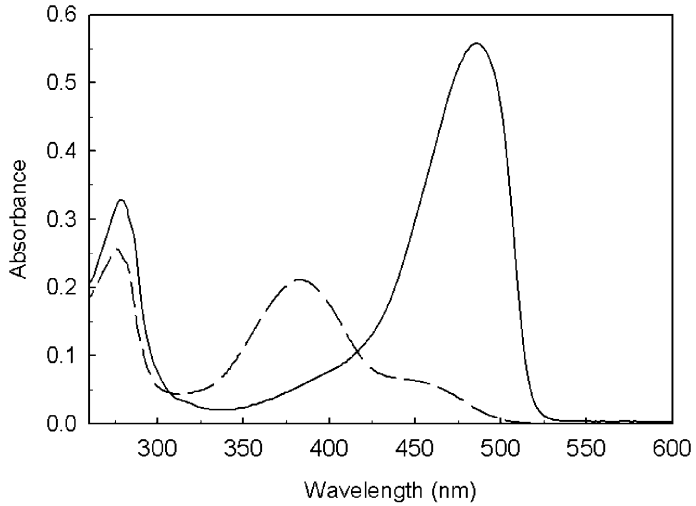

Fig. 2. – Absorbance spectrum of a solution containing 6 μM GFPmut2, 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.4 (solid line) and of a solution containing 6 μM GFPmut2, 20 mM Tris-HCl, 600 mM NaCl, 5 M GdnHCl, *p*H 7.4 (dashed line).

3. – Results and discussion

3˙1. *Thermodynamic stability.* – Encapsulation of GFPmut2 in silica gel does not alter either the protein secondary structure or the chromophore microenvironment, as demonstrated by the overlapping of the far-UV circular dichroism spectra and the fluorescence emission spectra upon excitation at 485 nm of the protein in solution and entrapped in the silica matrix [37].

Treatment of GFPmut2 with high concentrations of GdnHCl leads to the complete abolishment of the secondary structure spectral signatures, both for the protein in solution and encapsulated in silica gel [37]. This finding indicates that the constraints opposed by the gel to conformational transitions do not hamper the unfolding of the protein secondary structure. Denaturation also determines the complete abolishment of the fluorescence emission upon excitation at 485 nm as a consequence of the disruption of the extensive hydrogen bond network that stabilizes the anionic form of the chromophore in the native protein (data not shown). The comparison of the absorbance spectra of the native and unfolded GFPmut2 in 5 M GdnHCl (fig. 2) shows that denaturation of the protein structure does not lead to the disruption of the chromophore itself but rather to its exposure to the solvent, as already pointed out for wild-type GFP [41]. The spectrum of the unfolded protein is structured and characterized by a predominant peak at 385 nm which can be attributed to the protonated form of the chromophore. The absorption maximum is blue shifted with respect to that of the protonated form of the native chromphore by about 10 nanometers, probably as a consequence of the exposure to the solvent.

The dependence on denaturant concentration of the molar ellipticity at 220 nm of GFPmut2 in solution and encapsulated in silica gel (fig. 3A) allows the calculation of the unfolding thermodynamic parameters ($\Delta G^0_{0,U}$, m and D_{50}) and the characterization of the effect of encapsulation on the stability of the protein. As can be better appreciated

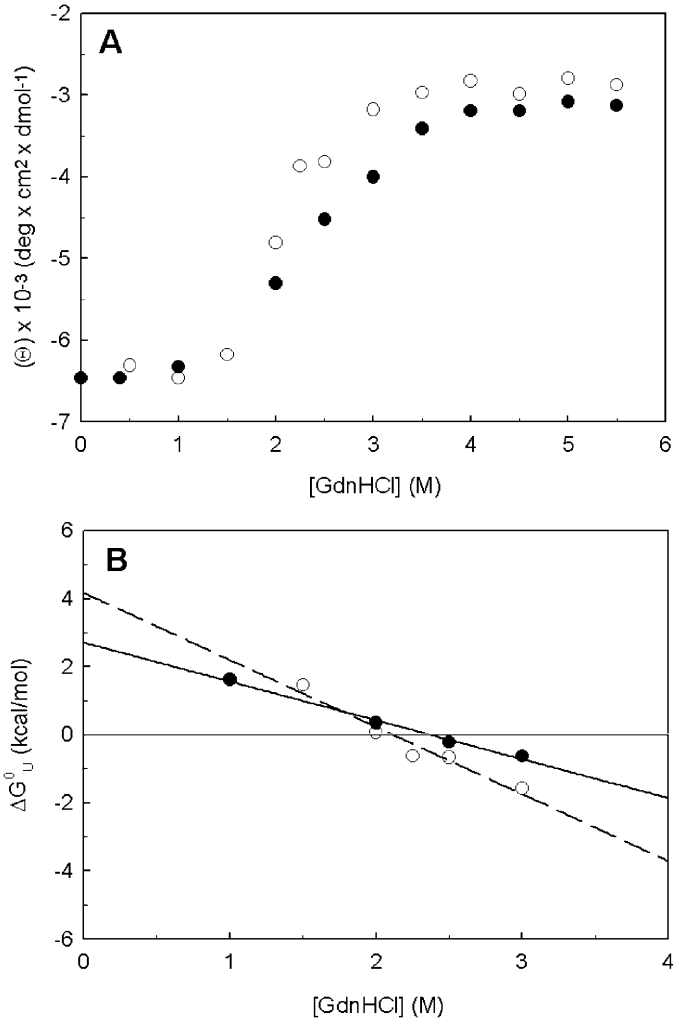

Fig. 3. – A: Dependence on denaturant concentration of the mean residue ellipticity at 220 nm of GFPmut2 in solution (closed circles) and encapsulated in silica gel (open circles). Experiments were carried out in solution in 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.4, and in silica gels in 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.0. B: Dependence on denaturant concentration of the unfolding free energies ΔG_U^0. ΔG_U^0 values were calculated from data in fig. 3A according to eqs. (5), (6) and (7). Lines through data points represent the fitting to eq. (8).

from the linearized plots (fig. 3B), encapsulation does not have any significant effect on the apparent unfolding midpoint (2.4 ± 0.1 M in solution, and 2.1 ± 0.2 M in silica gel), but the increase in the dependence of ΔG_U^0 on denaturant concentration leads to a significant increase in the $\Delta G_{0,U}^0$ for the encapsulated protein. The relevance of this apparent stabilizing effect depends on the reversibility of the observed reaction, *i.e.* on the recovery of the native spectroscopic properties upon refolding.

The time course of the unfolding/refolding reactions of GFPmut2 in solution and in silica gel followed by far-UV circular dichroism at 220 nm is shown in fig. 4. In

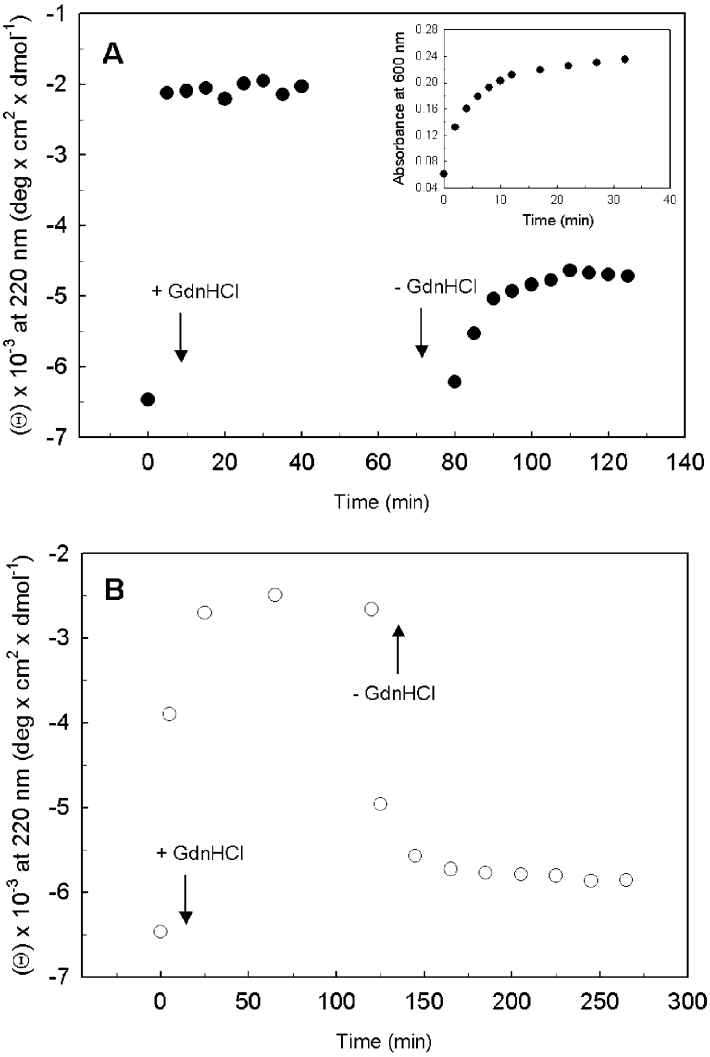

Fig. 4. – Representative unfolding/refolding time courses of GFPmut2 in solution (panel A) and encapsulated in silica gel (panel B) followed by circular dichroism at 220 nm. Experiments in solution were carried out in 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.4; experiments in silica gel were carried out in 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.0. A: The native protein is denatured in 6 M GdnHCl and then refolded by a 15 fold dilution in buffer without denaturant. Inset: time course of the refolding experiment in solution followed by light scattering at 600 nm. B: The native protein encapsulated in silica gel is denatured in 6 M GdnHCl and then refolded by soaking the gel slide in native buffer.

solution (fig. 4A) the unfolding in 6 M GdnHCl is completed in the dead time of manual mixing. Refolding of the protein is initiated with a 15 fold dilution in 20 mM Tris-NaCl buffer, *p*H 7.4 and followed until equilibrium is reached. Immediately after dilution in buffer the protein recovers much of its native signal, which is subsequently slowly lost within 2 hours. The recovery of the signal at equilibrium in distinct experiments was always below 70%. Scattering experiments at 600 nm (fig. 4A, inset) suggest that the decrease in the circular dichroism signal can be attributed to protein aggregation, which could not be prevented by addition of reducing agents, such as DTT, to the refolding buffer. The unfolding kinetics of GFPmut2 in silica gel, in 6 M GdnHCl is much slower than in solution and reaches completion after about 100 minutes (fig. 4B). Upon removal of denaturant, the signal at 220 nm decreases (*i.e.* the secondary structure content increases) and at equilibrium about 90% of the native protein signal is recovered. The quantitative comparison of the stability of GFPmut2 in solution and in silica gel is limited by the incomplete reversibility of the reaction in solution, nevertheless an apparent stabilizing effect of the entrapment on GFPmut2 is observed. The pronounced tendency of GFP towards aggregation, already reported for the wild-type protein [42] and for Cycle3 [42] and S65T [43] mutants, has usually prevented the calculation of significant thermodynamic parameters for the unfolding/refolding reactions. The entrapment of protein molecules inside the pores of the silica gel hampers inter-protein interactions and hence aggregation, thus allowing the determination of thermodynamic parameters under equilibrium conditions.

The unfolding reaction measured by fluorescence emission upon excitation at 485 nm is poorly reversible (less than 50% of native signal is recovered after fast unfolding at high denaturant concentration), both in solution and in silica gel (data not shown). For this reason no stability studies can be carried out using fluorescence emission as a spectroscopic probe. The incomplete reversibility of the unfolding reaction does not affect unfolding kinetics measured at high denaturant concentration, where the refolding rate constant is much lower than the unfolding rate constant, and its contribution to the observed unfolding rate is negligible.

3˙2. *Unfolding kinetics*. – Unfolding kinetics were measured on GFPmut2 both in solution and in silica gels by fluorescence emission at 507 nm upon excitation 485 nm, and by circular dichroism at 220 nm. The kinetic traces collected by fluorescence emission on the protein in solution are well fitted by a biexponential decay up to 4.5 M GdnHCl. A representative kinetic at 4 M GdnHCl is reported in fig. 5A. At higher GdnHCl concentrations only a single kinetic phase is detectable. The fast phase, with a relative amplitude of about 20%, shows a negligible dependence on denaturant concentration (data not shown). Kinetics followed by absorbance measurements at 485 nm are well described by a single exponential equation between 3 M and 5.5 M GdnHCl. A representative kinetic at 4 M GdnHCl is reported in fig. 5B. The rate constants measured by absorbance spectroscopy agree with the rate constants of the slower process monitored by fluorescence (data not shown), even though the contribution of the protonated form of the chromophore to the absorption at 485 nm (see fig. 2) may partially vitiate the quality of the collected data. Both fluorescence emission upon excitation at 485 nm and absorbance at 485 nm are sensitive to the integrity of the network of hydrogen bonds stabilizing the anionic form of the chromophore, and in this regard they can be considered good probes of the protein unfolding [42,44]. Thus the fast unfolding phase monitored by fluorescence does not appear to represent a denaturation event, due to its independence from denaturant concentration and its absence in kinetics monitored by absorbance. As

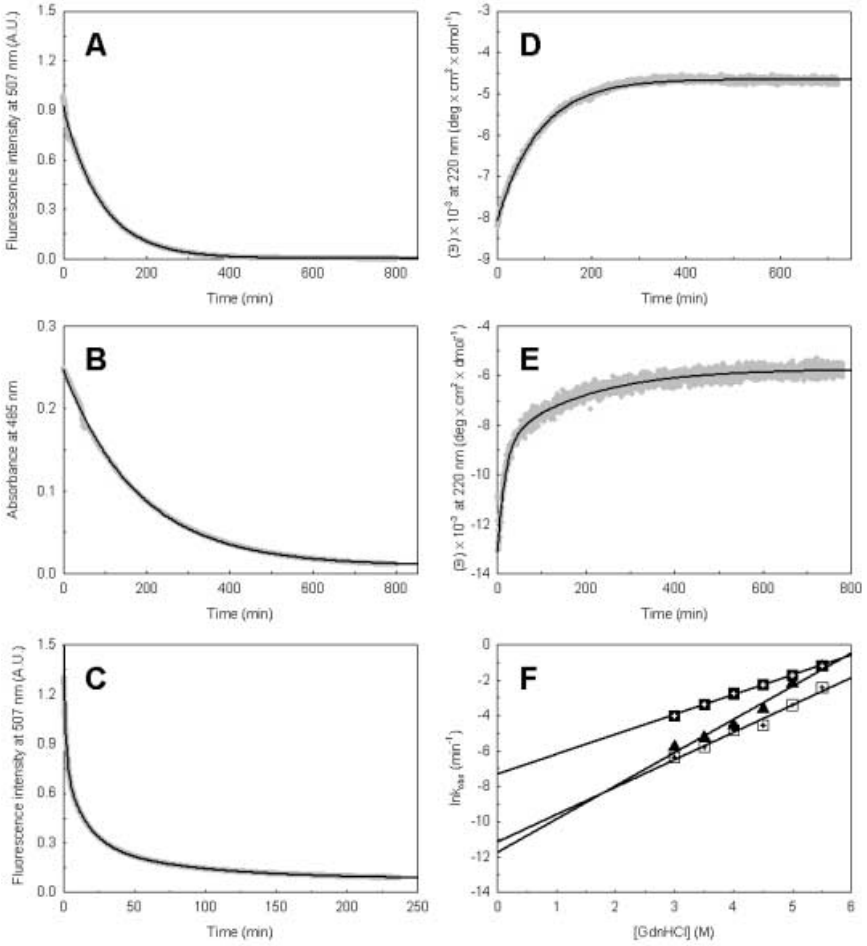

Fig. 5. – A: Representative unfolding kinetics of GFPmut2 in solution in the presence of 4 M GdnHCl, 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.4, monitored by fluorescence emission at 507 nm upon excitation at 485 nm. The line through data points is the fitting to a double exponential decay. B: Representative unfolding kinetics of GFPmut2 in solution in the presence of 4 M GdnHCl, 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.4, monitored by absorbance at 485 nm. The line through data points is the fitting to a single exponential decay. C: Representative unfolding kinetics of GFPmut2 in silica gel in the presence of 4 M GdnHCl, 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.4, monitored by fluorescence emission at 507 nm upon excitation at 485 nm. The line through data points is the fitting to a triple exponential decay. D: Representative unfolding kinetics of GFPmut2 in solution in the presence of 4 M GdnHCl, 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.4, monitored by circular dichroism at 220 nm. The line through data points is the fitting to a single exponential decay. E: Representative unfolding kinetics of GFPmut2 in silica gel in the presence of 4 M GdnHCl, 20 mM Tris-HCl, 600 mM NaCl, *p*H 7.0, monitored by circular dichroism at 220 nm. The line through data points is the fitting to a double exponential decay. F: Semi-logarithmic plot of the dependence on GdnHCl concentration of the unfolding rate contants of GFPmut2 in solution (closed triangles) and encapsulated in silica gels (open and closed squares). Data points are obtained by averaging the rates constants measured by fluorescence emission and circular dichroism. Lines through data points represent the fitting to eq. (4).

TABLE I. – *Natural logarithms of the observed unfolding rates at zero denaturant concentration ($\ln k_{\mathrm{obs},0}$) and dependence of the observed unfolding rates on denaturant concentration ($m_{k_{\mathrm{obs}}}$). Parameters were determined by fitting the dependence on* GdnHCl *concentration of the natural logarithm of the observed unfolding rates according to eq. (4).*

Spectroscopic technique	Experimental conditions	$\ln k_{\mathrm{obs},0}$ (min^{-1})	$m_{k_{\mathrm{obs}}}$
Fluorescence emission	Solution	-11.8 ± 0.7	1.88 ± 0.17
	Silica gel	-7.1 ± 0.2	1.07 ± 0.05
		-10.7 ± 0.5	1.52 ± 0.11
Circular dichroism	Solution	-11.7 ± 0.6	1.88 ± 0.14
	Silica gel	-7.5 ± 0.1	1.17 ± 0.03
		-11.6 ± 0.6	1.58 ± 0.13

the fast kinetic phase observed by fluorescence can be well reproduced by an equal molar concentration of NaCl (data not shown), this fast event appears to be the effect of the increased ionic strength on the flurophore emission properties. The unfolding kinetics of GFPmut2 encapsulated in silica gels, when monitored by fluorescence, are well described by a triple exponential decay. A representative kinetics at 4 M GdnHCl is reported in fig. 5C. The first phase accounts for about 50% of the total amplitude and is due to the effect of the ionic strength as pointed out for the experiments in solution. The rate constants for the unfolding in solution lay between the two rate constants measured in silica gels, with the slower rate constant in the gel overlapping, within the experimental error, with the rate constant in solution at zero denaturant concentration (table I).

The kinetic traces of unfolding of GFPmut2 monitored by circular dichroism in solution are well described by a monoexponential function, whereas biexponential equations are needed to describe the unfolding kinetics of the protein encapsulated in silica gel. Representative kinetics at 4 M GdnHCl are reported in fig. 5D-E. The dependence of the natural logarithm of the unfolding rate constants on GdnHCl concentration is more pronounced in solution than in silica gel, but, as observed for unfolding monitored by fluorescence, the rate of unfolding in solution at zero denaturant concentration overlaps with the slower rate constant measured in silica gel (table I). The unfolding rate constants measured by fluorescence and CD overlap within the experimental error, both for solution and silica gel experiments. The more pronounced dependence on denaturant concentration of the unfolding rates measured in solution with respect to those measured in silica gel (table I and fig. 5F) is indicative of a reduction in the exposure of protein surface area upon unfolding, as a consequence of the steric restrictions opposed by the gel pores. The near perfect overlapping at zero denaturant concentration of the slow unfolding rate in silica gel with that in solution indicates that entrapment does not affect the unfolding reaction of the predominant species populating the solution. The presence of an additional unfolding phase in silica gel, which is faster than the unfolding phase observed in solution is, in principle, compatible both with the stabilization of an unfolding intermediate (in a sequential mechanism like: $\mathrm{N} \rightarrow \mathrm{I} \rightarrow \mathrm{D}$), and with a perturbation of the equilibrium between two native conformations of the protein. Our results are compatible with the stabilization of a native conformation not significantly populated in solution, due to the near perfect overlapping of the unfolding kinetics measured by fluo-

rescence and by circular dichroism, that seems to rule out the presence of an unfolding intermediate. Furthermore, the very similar dependence on denaturant concentration of the two unfolding rates measured in silica gel (table I and fig. 5F) suggests that the reactions are associated with similar changes in the exposed surface area of the protein, a result that can be compatible only with two native states that unfold following parallel paths to the denatured state.

4. – Conclusions

Entrapment of GFPmut2 in silica gels has no effects on the native secondary structure of the protein and on the chromophore microenvironment. The encapsulation in silica gel strongly affects the unfolding kinetics that become biphasic. Our results are compatible with the presence, in the gel under native conditions, of two spectroscopically indistinguishable protein conformers, one of which is not significantly populated in solution. The slow unfolding conformer, which represents the predominant species in solution, shows an unfolding rate in the absence of denaturant which is two orders of magnitude lower than the fast unfolding species. Further studies are needed to unveil the origin of the stabilization of this protein conformation, that might arise from the crowding and confinement effects exerted by the silica matrix on the encapsulated protein. These results point out the importance of studying protein dynamics in experimental conditions that mimic the intracellular milieu.

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