The early stages of protein folding include a rapid collapse of an unfolded coil-like polypeptide chain to a more compact molten globule state. It is commonly admitted that this collapse is driven by hydrophobic interactions between side groups of the chain and is believed to restrict the number of accessible conformations of the molecule. Even for a chain with a rather short number of residues, the number of conformations of a polypeptide chain cannot be sampled exhaustively in times of the order of seconds or less as is observed for the folding of proteins (the Levinthal paradox [1]).

For a compact state a systematic exploration of the phase space can be achieved in a reasonable time to find the minimum energy configuration of the folded biologically active protein. In this view the collapse is a stage participating to solve the Levinthal paradox.

The interior of cells is a very crowded media [2,3], where although each protein is present at a rather low concentration the over all occupied volume fraction can range up to values as high as $\Phi \approx 0.3$. The excluded volume [4] (i.e., the space physically not accessible to a molecule due to the presence of the other ones) affects a number of phenomena when compared to highly dilute concentrations. Recent theoretical works [5,6] suggested that the presence of a crowded media would modify the equilibrium between the unfolded and the folded state of the proteins. The proteins in the unfolded state ($U$) occupy an apparent volume which is larger than the compact folded state ($F$). Thus the more extended conformation of the unfolded state ensemble is preferentially destabilized with respect to the folded state by the excluded volume. It is predicted that these extended conformations are compressed due to macromolecular crowding. This effect induces a shift of the $U \rightleftharpoons F$ equilibrium to the folded state and hence a stabilization of the native protein: in this view the proteins are more stable in cells than in dilute solution. Different groups have now reported experimental evidence of protein stabilization by the presence of an inert macromolecular crowder at high concentration by using different experimental methods [7–10]. But a direct experimental evidence of the physical mechanism responsible for the stabilization remains to be shown. Neutron scattering is a unique tool for such study because it allows to measure the form factor of a small quantity of a molecule in presence of a large amount of background molecules. Using the coherent scattering length differences between hydrogen and deuterium it was originally possible to measure the form factor of a polymer chain deuterated solvent [11]. Using the same property, in a ternary mixture (solvent, test molecule and macromolecular crowder) one can match the signal of one molecule using an appropriate mixture of $H_2O$ and $D_2O$ (for example the crowder) and observe the signal of a test molecule, although at low concentration. With this method we were able to measure the compression of random coils due to macromolecular crowding [12]. The signal of deuterated polyethylene glycol (PEG) at low concentration was measured as a function of the mass fraction of a background macromolecule, the Ficoll 70 ($F70$ [13]), a heavily branched polymer of sucrose. The F70 is a molecule that is commonly used to mimic the interior of the cytoplasm because it is inert and can be diluted in water up to very high concentrations (up to a mass fraction of $\Phi \approx 0.4$). Due to the tendency of the PEG to aggregate at high concentration of $F70$ the results were obtained by extrapolation to zero PEG concentration where only the signal of one chain was observed [12]. The compression of a chain of radius similar to the F70 was observed to be as high as 50% at Ficoll mass fraction of $\approx 0.35$. In this paper, we report the compression of the chain as a function of its mass in order to compare with the prediction of crowding theories and to try to get further information on the conformation of the compressed chain.

In our previous study [12], $F70$ was used as crowding agent: it is highly ramified nonionic synthetic polymer of sucrose, with an almost spherical shape and a radius of gyration $R_g^{F70} = 50$ Å. Poly(ethylene glycol) (PEG) was used as a model of random coil. The first measurements were performed on a deuterated PEG of weight-average molecular weight $M_w = 18$ kDa, purchased from polymer source, having a radius of gyration in dilute solution of $R_g \approx 52$ Å. To study the effect of the ratio between the radii of gyration of the crowder and of the polymer we extended the measurements to PEGs of molecular weights $M_w = 42.8$ kDa and $M_w = 132$ kDa, keeping $F70$ as a macromolecular crowder. In order to mimic the volume fraction occupied in vivo we added $F70$ to the highest possible volume fractions before phase separation was observed.

The neutron scattering experiments were performed on
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Different concentrations of F70. Thus, as we reported in rather low PEG concentration especially for high mass fractions of F70. Mixtures of PEG and F70 have a tendency to segregate at second virial coefficient and dependent molar mass. We obtain the mass of the polymer crowding agent and at F70 mass fractions of 1 for the 3 PEG with different molecular weights without extrapolation the data to zero PEG concentration.

The small-angle neutron scattering (SANS) spectrometer KWS-2 of the Jülich Centre for Neutron Science at FRM II (Germany), and on the SANS spectrometer PACE of the Laboratoire Léon Brillouin (France).

The matching point of F70 corresponds to a mixture of D₂O and H₂O solution with a D₂O mole fraction x=0.41. Mixtures of PEG and F70 have a tendency to segregate at rather low PEG concentration especially for high mass fractions of F70. Thus, as we reported in [12], we measured different concentrations cₚ of PEG per F70 mass fraction and extrapolated the data to zero PEG concentration.

Typical spectra obtained on KWS-2 are presented on Fig. 1 for the 3 PEG with different molecular weights without crowding agent and at F70 mass fractions of Φ=0.15. The spectra were fitted using

\[ I(q) = \frac{c_p (\Delta \rho)^2 \nu_s^2}{N_A} M_a(\Phi) P(q) [1 - 2M_a(\Phi) c_p A_2(M_a, \Phi)] \]

(1)

Δρ is the neutron scattering length density contrast between the deuterated PEG and the solvent, Nₐ is the Avogadro number, P(q)=\( \frac{2}{q} (x - 1 + e^{-x}) \) is the molecular form factor of a Gaussian chain [14] with x=(qRₚ)². A₂ is the second virial coefficient and M_a(Φ) is the concentration dependent molar mass. We obtain the mass of the polymer M_a(Φ=0) by extrapolation to zero PEG concentration. \( v_s \) is the specific volume of the monomer. The molecular weight dependence of the radius of gyration extrapolated to zero PEG concentration in water (Φ=0) follows a scaling law \( R_g(M_w(\Phi=0)) - M_w(\Phi=0)^{\nu} \) with \( \nu=0.48 \), which is close to the theoretical value for a Gaussian chain. This result supports the random coil structure of the PEG in water for this range of molecular weights.

As reported in [12] for the 18 kDa PEG, the extrapolations of apparent masses M_a(Φ) to zero PEG concentration (cₚ=0) converge, for all F70 mass fractions, to the values which are given by the D-PEG supplier. This behavior was also observed for the different polymer weights of M_w =42.8 and M_w=132 kDa, and is a sound signature that by extrapolation to zero PEG concentration (cₚ→0) we do capture the conformation of a single chain.

The evolution of the apparent molecular mass as a function of the polymer concentration c_p can be described by a virial expansion. As far as it remains in the linear regime, the slope of M_a(c_p, Φ) as a function of c_p is proportional to the second virial coefficient which is a direct measure of the excluded volume between pairs of chains. The F70 mass fraction dependence of the virial coefficients a₂(M_w, Φ) =A₂(M_w, Φ)*M_w² is presented on Fig. 2 for the three polymer molecular weights studied (we preferred the representation of a₂ rather than A₂ because a₂ has the dimension of a volume). In the absence of F70, a₂(M_w, 0) is positive, the PEG-PEG interactions are repulsive, the chains strongly repeal and do not interpenetrate each other. This is characteristic of a polymer in a good solvent. a₂(M_w, 0) strongly depends on M_w and is of the order of the volume occupied by the chain, but due to the limited number of molecular weights we did not investigate further this point. When increasing the F70 mass fraction, the second virial coefficients tend to zero and become negative for the three molecular weights. Such a behavior is usually observed in polymers when the quality of the solvent is changed, and the chains either collapse or aggregate, mainly depending on their concentration [15,16].

Our system is a ternary system, and the interactions measured by pairs of polymer are modified by the presence of the

FIG. 1. Spectra measured on KWS-2 for three different polymer molecular weights: 18, 42.8, and 132 kD without Ficoll (upper graph) and with a mass fraction of Ficoll of Φ=0.15 (lower graph). The solvent scattering length density is matched to the F70 signal (see text).

FIG. 2. Apparent second virial coefficient a₂(M_w, Φ) obtained from the slope of the forward intensity as a function of the Ficoll mass fraction Φ. The dependence is presented for the three different molecular weights of PEG.

M_w(Φ=0) by extrapolation to zero PEG concentration. v_s is the specific volume of the monomer. The molecular weight dependence of the radius of gyration extrapolated to zero PEG concentration in water (Φ=0) follows a scaling law \( R_g(M_w(\Phi=0)) - M_w(\Phi=0)^{\nu} \) with \( \nu=0.48 \), which is close to the theoretical value for a Gaussian chain. This result supports the random coil structure of the PEG in water for this range of molecular weights.

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FIG. 3. Ficoll fraction dependence of the radius of gyration of the D-PEG normalized to its value at Φ=0 for the three different molecular weights.

F70, $a_2(M_w, Φ)$ tends to zero at rather low F70 mass fraction, around 5–6% and interestingly this fraction seems to be independent of the polymer molecular weight, although we must remain cautious on this point due to experimental uncertainties and the limited number of molecular weights studied. The F70 mass fraction dependence of the second virial coefficient depends very strongly on the polymer molecular weights, meaning that the aggregation of the polymer strongly increases with increasing the chain length. This is the reason why from $M_w=18$ to $M_w=132$ kDa the stability of the solution strongly decreases, and beyond these values the solution is turbid.

As reported in [12], the Φ dependence of the radius of gyration of the chains was obtained by extrapolation to zero PEG concentration [$R_g(Φ)=lim_{q→0} R_g(Φ, c_p)$]. The evolution of the normalized radii of gyration $R_g(Φ)/R_g(0)$ as a function of the F70 mass fraction for the three molecular weights of PEG is shown on Fig. 3. The compression of the chain is clearly observed whatever the polymer weights, but again due to aggregation of the PEGs, $R_g(Φ)/R_g(0)$ was measured in a limited F70 range for the higher masses. Nevertheless a diminution of the radius of gyration $R_g$ of 20% and 30% were, respectively, observed for the 42.8 and 132 kDa, respectively, at Φ~0.1 and Φ~0.18. The compression occurs thus at smaller crowder fraction when increasing the molecular weights of PEG, which is the relative size of the coil as compared to that of F70.

Whatever the molecular mass up to 132 kDa the PEG adopt a random coil conformation in water as it can be seen by both the form factor and the molecular weight dependence of the radius of gyration. From the repulsion of the chain we can deduce that water is a good solvent for PEG at room temperature. In this case the exponent $κ$ should be closer to $κ=0.588$ rather than $κ=0.5$, the difference probably arises from both the rather limited range of chain lengths and the uncertainty of the data. When we add in the solution a cosolute like the F70, we observe a clear compression of the chains, with a very significant decrease of the radius of gyration. The evolution of $R_g(Φ)/R_g(0)$ depends on the ratio between the molecular weights (i.e., the respective size) of the F70, used as a simple model for a sphere, and the PEG that takes a random coil conformation. What is the change of conformation due to the presence of the macromolecular crowder and what can we learn from the molecular weight dependence? Figure 4 depicts the apparent Flory exponent $ν(Φ)$ between two molecular weights which is obtained by $ν(M_1, M_2, Φ)=ln[R_g(M_1(Φ)/R_g(M_2, Φ)]/ln(M_1/M_2)$ as a function of Φ. From an experimental point of view it is rather difficult to measure different molecular weights at the same Ficoll fraction, therefore we used the refined values for the first weight (18 kDa) to compute the exponent for the other masses. The value of 0.3 is close to the one observed when increasing the size of a compact object. This behavior seems to be contradicted by the form factors because the intensity still decays as $q^{-2}$ at high wave vectors and thus is not compatible to either a sphere of at least a compact shape. If the chains were totally collapsed, the respective radius of gyration for the PEGs with $M_w=18$, 42.8 and 132 kDa would be 14.1, 18.8, and 27.5 Å which is far from the values measured at the highest F70 mass fractions respectively 26.6, 60.7 and 109 Å. The shape of the objects still remains quite open. The picture that emerges is that, up to a certain size ζ, the polymer adopts a random coil structure and, at high F70 fraction, the compressed polymer is formed by a compact addition of these objects. The compression of the chain occurs over long distances. This picture is similar to a compact addition of thermal blobs although the nature of our objects probably differs because of the size of the blobs. Notice that a value of $ν$ smaller than 0.3 can be obtained when increasing the size and changing the conformation simultaneously.

The decrease of the radius of gyration of a polymer in a good solvent when increasing concentration is fairly well understood; theoretical approaches have led to the prediction that it scales with $v^{-1/8}$ in the semidilute regime and this dependence was experimentally supported by small-angle neutron scattering measurements [15,17]. The same behavior was observed in a polymer mixture; the radius of a host polymer decreases when increasing the concentration of guest one [18]. In both cases the changes in the radius of gyration are due to unfavorable interaction between unlike monomers either from the same [15,17] or from a different polymer [18]. The compression we observe here, due to macromolecular crowding, is fundamentally different from the polymer collapse because, in our case, the quality of the solvent is not changed. This was verified by the absence of conformational change of the PEGs in the presence of simi-
in equilibrium of the unfolded state ensemble it is possible to calculate the change between the chemical potential of the native state and the PEG interactions are neither favorable nor unfavorable. The theories developed to study the influence of macromolecular crowding on protein stability, calculate the change in the chemical potential due to macromolecular crowding using the scaled particle theory. From the respective changes in the chemical potential due to macromolecular crowding on protein stability, calculate the change neither in the form factor nor in the radius of gyration of the stabilization is the compression of the unfolded state ensemble which is modeled by random coil-like structure. We calculated the effect of a given volume fraction of added hard spheres on the mean radius of gyration of the distribution and found that the radius of gyration strongly decreases as a function of the volume fraction of added hard spheres, with an order of magnitude similar to that we measured. Moreover this effect strongly depends on the size of the chain that is compressed as we observed experimentally.

Early stages of protein folding [19,20] include the collapse of an unfolded polypeptide coil into a more compact molten globule state. The introduction of excluded volume effects to the equilibrium \( \Phi \Rightarrow F \) between the native state and the unfolded state ensemble leads theoretically to the stabilization of proteins, which was observed experimentally by [21] and others [7–10]. In this paper we reported observation of Gaussian chain compression and scaling effects that support the theoretical prediction of excluded volume effect [5,6].

[13] F70 is a trademark of Pharmacia Inc.