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Molecular size determination of a membrane protein in surfactants by light scattering

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Abstract

The molecular size of an outer surface protein from the photosynthetic bacterium *Chlorobium tepidum* was studied by dynamic light scattering (DLS) and HPLC gel filtration. For that purpose, the membrane protein was isolated and studied in four different nonionic surfactants, namely *t*-octylphenoxypolyethenoxyethanol (Triton X-100), (methyl-6-*O*-(*N*)-heptyl-carbamoyl)-α-D-glucopyranoside (Hecameg), dodecyl-β-D-maltoside (DDM) and *n*-octyl-oligo-oxyethylene (Octyl-POE). The protein was isolated by solubilization of the membranes with Triton X-100. The final purification step was a gel filtration, which was also used for surfactant exchange. Light scattering reveals the simultaneous presence of particles of different sizes in the 3–6 and 20–110 nm range, respectively. The smaller size is related to the hydrodynamic radius of the individual protein/surfactant complexes, whereas the larger size is associated with the presence of complex aggregates.
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Keywords: *Chlorobium tepidum*; Outer surface protein; Light scattering; Molecular size

1. Introduction

Membrane proteins are amphiphilic macromolecules incorporated vectorially in the lipid membrane. Part of their surface that is in contact with the lipid bilayer core is hydrophobic, while other parts that are exposed to the aqueous environment on either site of the membrane are hydrophilic. Membrane proteins can be solubilized in aqueous solutions, when the membrane lipids are replaced by detergents [1,2] Solubilized membrane proteins form so-called mixed micelles with the detergent. Depending on the size of the protein and the organization of the exposed amino acids, mixed micelles may therefore have many different geometries ranging from almost unperturbed micelles (4–6 nm diameter) into which the protein is embedded and from where it may or may not protrude, to more complex objects with surfaces made up of protein and detergents coats (2–3 nm thickness) [3,4].

It is well known that the process of crystallization still represents the limiting step for the structural analysis of biological macromolecules carried out by X-ray or electron diffraction techniques [1,5–8]. The crystallization of membrane proteins in crystalline arrays requires membrane proteins, which are solubilized and purified to homogeneity. Although gel filtration is a widely used method to study the homogeneity of protein, this method is time-demanding and large amounts of protein and detergents are commonly required. However, a few years ago, it was suggested based on dynamic light scattering (DLS), that monodisperse macromolecule solutions crystallize readily, whereas polydisperse systems rarely do so [9].

DLS is a technique [10] used to measure the translational diffusion coefficient (*D*) of a macromolecule un-

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; DLS, dynamic light scattering; Triton X-100, *t*-octylphenoxypolyethenoxyethanol; Hecameg, (methyl-6-*O*-(*N*)-heptyl-carbamoyl)-α-D-glucopyranoside; DDM, dodecyl-β-D-maltoside; Octyl-POE, *n*-octyl-oligo-oxyethylene

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dergoing Brownian motion in solution. Monochromatic light scattered by moving protein molecules display intensity fluctuations according to their motion. Therefore, an analysis of the light scattering signal provides quantitative information about the behavior of a protein in solution. As additional advantages, a typical DLS experiment takes just a few minutes to be performed, it is a non-destructive procedure and requires a minimum amount of pure sample.

Recently, the isolation and characterization of an Hemagglutinin-related protein (Q8KGA0, primary accession number in SWISS-PROT/TrEMBL data bank) from *Chlorobium tepidum* has been reported [11]. Based on the amino acid sequence, a molecular weight of 22,146 Da can be calculated. In this work, we report the molecular size estimation of this membrane protein using HPLC gel filtration in combination with DLS.

2. Materials and methods

2.1. Isolation of the membrane protein

The protein was isolated from membranes from *C. tepidum* as described in Ref. [11]. Membranes were solubilized using 3% Triton X-100 under anaerobic conditions and the extracts were ultracentrifuged for 1 h at 136,000 × *g*. The resulting supernatant was applied to a Mono Q (Pharmacia) column equilibrated with 20 mM Tris–HCl, pH = 9, buffer containing, 1 mM benzamidine, 1 mM EDTA (buffer A) and 0.03% Triton X-100. The protein was eluted using a linear concentration gradient of NaCl from 0 to 1 M. The flow rate was 1 ml/min. The fractions containing the protein were pooled and after dialysis against buffer A, they were loaded to a subsequent Mono Q column. The column was equilibrated with buffer A containing 100 mM NaCl and 0.03% Triton X-100. The protein was eluted using a linear concentration gradient of NaCl from 100 mM to 1 M and the fractions containing the protein were pooled and concentrated using a Gelmann concentrator with a 10 kDa cut-off membrane. A TSK-SW 55 gel filtration column (37 cm length and 1 cm diameter, Merck) attached to a HPLC system was used for gel filtration. The column was equilibrated with buffer A containing the different surfactants (see Table 1) at a flow rate of 1 ml/min. To detect the eluted proteins the absorption at 280 nm wavelength was monitored.

Table 1
Elution time of the purified hemagglutinin-related protein (Q8KGA0, primary accession number in SWISS-PROT/TrEMBL data bank) in buffer A containing the different surfactants

Surfactant	(%)	Protein concentration (μg/ml)	Retention time (min)
Octyl-POE	1.0	95	20.48 ± 0.69
Hecameg	0.8	61	20.25 ± 0.60
Triton X-100	0.03	149	18.00 ± 0.34
DDM	0.02	168	17.50 ± 0.51

Flow rate, 1 ml/min on a gel filtration column TSK-SW 55 attached to a HPLC system.

2.2. Static and dynamic light scattering

The beam from a solid state diode laser (Coherent Model 2020), operating at 532 nm with vertically polarized light, was focused onto the sample cell through a temperature-controlled chamber (temperature controlled to within ± 0.01 °C) filled with refractive-index-matching toluene. The incident and scattered beams were polarized with Glan and Glan-Thompson polarizers with extinction coefficients higher than 10^{-6} and 10^{-7}, respectively. The sample solutions were filtered through 0.45 μm filters (Millipore) directly into pre-cleaned light scattering cells of highest quality.

The light scattering process defines a wave vector $q=(4\pi n/\lambda)\sin(\theta/2)$, where λ is the wavelength of the incident light in a vacuum, θ is the scattering angle, and n is the refractive index of the medium. In this study the full homodyne intensity autocorrelation function $g(q,t)$ was measured at room temperature at different scattering angles ranging between 15° and 150° with an ALV-5000 multibit, multi-τ full digital correlator that covered a broad dynamic range of about 10 decades [12].

The static light scattering data were obtained according to the simplified Zimm procedure, where the intensity of the scattered light is related to the weight average molecular weight M_W, the second virial coefficient A_2 and the radius of gyration R_g.

$$Kc/R(\theta,c) = \frac{1}{M_W}\left[1+\frac{(R_g q)^2}{3}\right] + 2A_2 c \quad (1)$$

In Eq. (1) $K=4\pi^2 n_o^2 (dn/dc)^2/(N_A\lambda_o^4)$ is the optical constant with n_o the solvent refractive index, dn/dc the refractive index increment and R_θ the Rayleigh ratio as obtained by calibration measurements. The measured intensity autocorrelation function $G(q,t)$ is related to the desired normalized field correlation function $g(q,t)$ by:

$$G(q,t) = A[1+f\,|\,ag(q,t)\,|^2] \quad (2)$$

where f is the instrumental factor, calculated by means of a standard, a is the fraction of the total scattered intensity associated with density fluctuations with correlation times longer than 10^{-6} s and A is the baseline. The correlation functions were analyzed using the inverse Laplace transformation (ILT) of the time correlation functions with the REPES algorithm [13], which minimizes the sum of the squared differences between the experimental and calculated

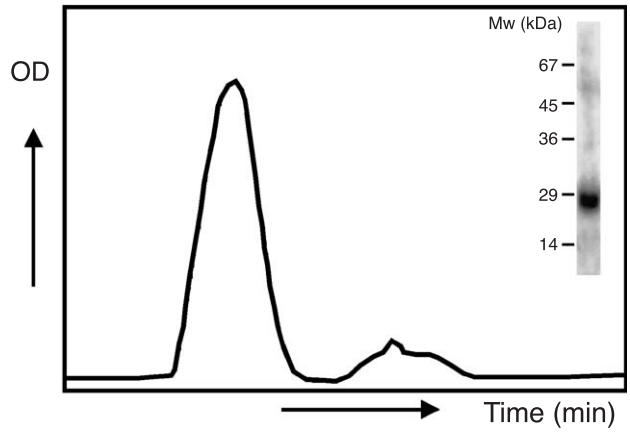

Fig. 1. Elution profile at 280 nm of the purified protein complex in 20 mM Tris–HCl, 1 mM EDTA, 1 mM benzamidine, 1.0% Octyl-POE, pH = 9. Flow rate 1.0 ml/min on a gel filtration column (Merck) attached to a Dionex HPLC system. Insert shows silver-stained SDS-PAGE of the first eluted fraction.

intensity–intensity autocorrelation functions using nonlinear programming.

$$ag(q,t) = \int_0^\infty A(\tau)\exp(-t/\tau)d\tau = \int_0^\infty \tau A(\tau)\exp(-t/\tau)d\ln\tau \quad (3)$$

Thus, relaxation time distributions are given in the form of $\tau A(\tau)$ versus $\log\tau$ plots. Relaxation rates are obtained from the moments of the peaks in the relaxation time distribution.

3. Results

3.1. Characterization of the purified protein by SDS-PAGE and HPLC gel filtration

The protein was isolated from *C. tepidum* using Triton X-100 to solubilize the membranes. After two-anion exchange chromatography steps, a subsequent gel filtration allowed the isolation of an outer surface protein from this organism [11]. In addition, gel filtration experiments were performed to exchange the detergent and to obtain information about the oligomeric state of the isolated protein complexes in different detergents. Fig. 1 shows the elution profiles recorded at 280 nm for the isolated protein in the presence of *n*-octyl-oligo-oxyethylene (Octyl-POE) in buffer A. The isolated protein particles eluted as a main fraction with an additional small fraction. Similar elution profiles were obtained in the presence of DDM, Hecameg and Triton X-100 (data not shown) indicating that the change of the surfactant had no effect on the aggregation state of the purified protein. Table 1 shows the elution time of the protein/surfactants complexes in the presence of the four different detergents. The elution time varies from 17.50 min for DDM to 20.48 min for the Hecameg, indicating size variations of the protein/surfactant complexes. The protein composition of the main fraction is shown in Fig. 1 (insert). The SDS gel shows a protein band at 30 kDa and is close to the molecular weight obtained from the analytical ultracentrifugation [11].

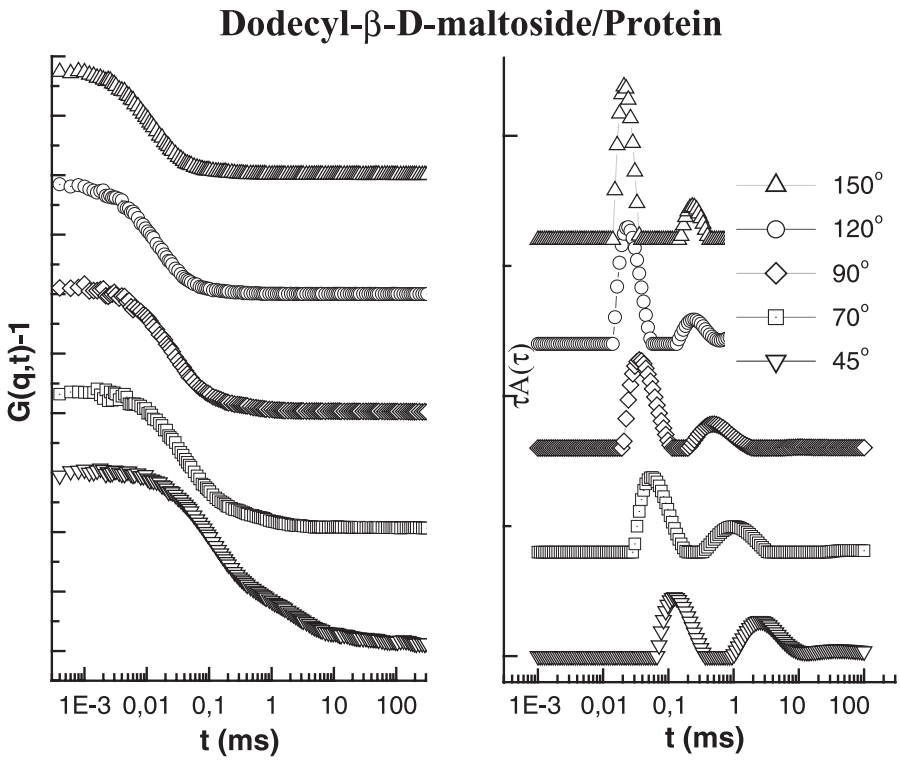

Fig. 2. Experimental correlation functions for the DDM-protein sample at different scattering angles accompanied by the distributions of the experimental correlation functions using the REPES analysis.

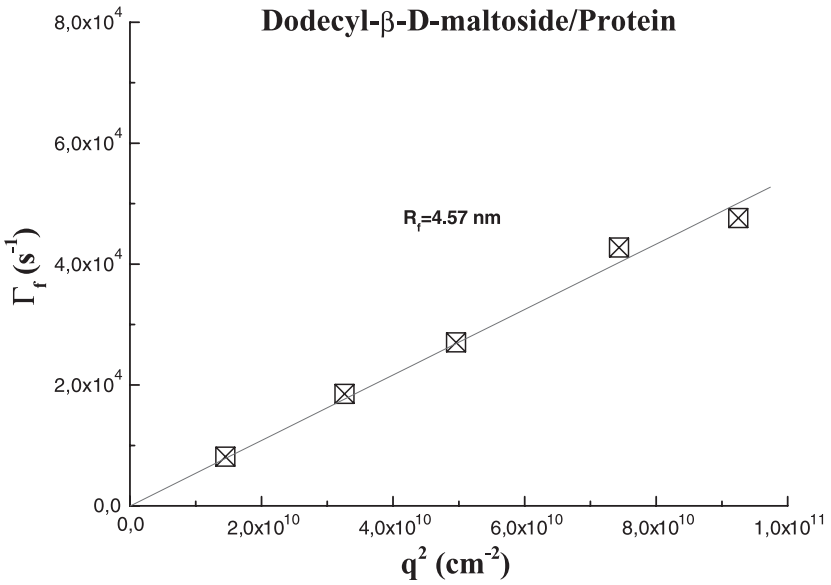

Fig. 3. The angular dependence of the protein/detergent diffusion data yields the linear dependence of the characteristic fast relaxation rate Γ_f ($\Gamma_f = 1/\tau_f$) on q^2. The slope of the linear fit to the data gives the diffusion coefficient which is related to the hydrodynamic radius R_f of the fast diffusion process.

3.2. Static and dynamic light scattering

We investigated the structures and behavior of the membrane protein complexes in the aqueous environment of the four different nonionic surfactants. The DLS experimental correlation functions were treated in the homodyne limit. Fig. 2 shows experimental correlation functions for the DDM/protein sample at different scattering angles accompanied by the distributions of the experimental correlation functions. This analysis revealed several relaxation modes. The faster one corresponds to the same dynamic process and is related to the diffusion dynamics of the solubilized single membrane protein chain in solution. This mode is diffusive since the characteristic relaxation rates Γ ($\Gamma = 1/\tau$) depend linearly on q^2 (see Fig. 3). From the characteristic relaxation rates one can obtain the translational diffusion coefficient D ($D = \Gamma/q^2$) and hence the equivalent hydrodynamic radius R_h using the Stokes–Einstein equation for a sphere:

$$R_h = \frac{k_B T}{6\pi\eta_0 D} \tag{4}$$

where η_0 is the solvent shear viscosity and k_B is the Boltzmann constant at the absolute temperature T. At infinite

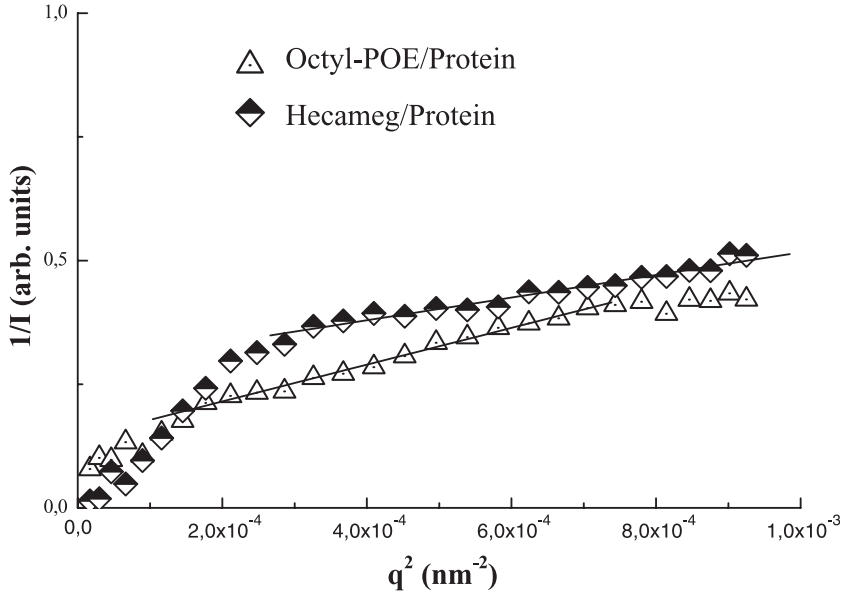

Fig. 4. Static light scattering Ornstein–Zernicke plot of the inverse of the scattering intensity versus the square of the scattering wave vector. Here the intensity of the scattered light is used for the estimation of the radius of gyration R_g.

dilution, all solutions show diffusion coefficients that plateau to a constant value, from which the hydrodynamic radius is calculated using the above Stokes–Einstein equation.

In addition, the existence of the aggregates becomes obvious through the simultaneous appearance of small angle excess scattering in the static light scattering plot from which we can extract their size, which is well-defined. In the static light scattering Ornstein–Zernicke plot (Fig. 4), the inverse of the scattering intensity varies linearly with the square of the scattering wave vector showing the downwards curvature characteristic of the presence of large molecular species (aggregates) besides small ones with a radius of gyration value R_g of 37 nm for a DDM/protein solution (data not show), 40 nm for a Hecameg/protein solution, 89 nm for an Octyl-POE/protein solution and 97 nm for a Triton X-100/protein solution (data not show).

An inherent characteristic of the hydrodynamic radius (R_h) and the root mean square radius (R_g) is the fact that each gives just a mean one-dimensional and time-averaged impression of the true anisotropic extension of a fluctuating polymer chain [14]. Accordingly, it has been acknowledged that macromolecules cannot be described properly by just one of these radii. On the contrary, a combination of both quantities enables a detailed insight into the molecular architecture. For this purpose, the shape-factor or asymmetry factor $\rho = R_g/R_h$ is defined. Values of ρ vary from 0.78 for ideal homogeneous spheres to >2 for extended coils and prolate ellipsoids. Thus, the value of ρ for DDM, Octyl-POE, Hecameg and Triton X-100 large aggregates is around 0.75 and can be correlated with the effective shape of a sphere. It should be emphasized that, although the aggregate species dominate the scattered intensity due to their heavy weighting, they constitute a very low number fraction.

4. Discussion

The basic requirement for nucleation and growth of protein crystals is their presence in a monodisperse state [1,5–8,15]. Membrane proteins are present in solution in complex with detergents and the monodisperse nature of a specific protein preparation must be assessed experimentally. Gel filtration is a widely used method to study the homogeneity of a protein, but it is time-demanding and large amounts of protein and detergents are commonly required. Additionally, the polar group of the detergents interacts in different ways with the support material and leads to false molecular size of membrane proteins.

In the present work, we use DLS to study the effect of different nonionic detergents on the molecular shape of a membrane protein from the outer surface from *C. tepidum*. The mean intensity of the light scattered by a solution of macromolecules contains information on the molecular weight of the macromolecules. On the other hand, the intensity of the scattered light continuously fluctuates around a mean value due to the Brownian movement of the macromolecules. The DLS experiments make use of the fact that the time dependence of these fluctuations can be related to the translational diffusion coefficient of the macromolecules.

The membrane protein was solubilized in Triton X-100 and after anion exchange chromatography, gel filtration has been used for final purification and detergent exchange. Although the length and the nature of the tail (hydrophobic or hydrophilic) is different between the four surfactants, similar retention times were obtained for the protein in the presence of Triton X-100 and DDM and for Hecameg and Octyl-POE, respectively (Table 1). In contrast, in the DLS experiments, the analysis revealed several relaxation modes, of which the faster one corresponds to the same dynamic process and is related to the diffusion dynamics of the solubilized single membrane protein chain in solution. In between the fast and the slow relaxation regime, there is an intermediate q^2 dependent mode visible only in the Octyl-POE/protein and Hecameg/protein spectra with an apparent hydrodynamic radius of about 20 nm.

The retention time of the Octyl-POE/protein and Hecameg/protein complexes correlate with the obtained size from the DLS experiment. In contrast, a discrepancy is obtained for the DDM/protein and Triton X-100/protein complexes. The DMM/protein complexes has a radii of 4.6 nm and a retention time of 17.5 min. In contrast, the Triton X-100/protein complexes has a radii of 5.3 nm and a retention time of 18.0 min. This indicates that the interaction of the protein/surfactant complex with the gel filtration matrix have influence in the resulting retention time and don't reflect the size of the protein/surfactant complex.

The angular dependence of the slow characteristic relaxation rate Γ_s versus q^2 illustrates the presence of two extra distinct diffusional modes dependant on the scattering angle range analyzed. For angles less than 70°, zero angle diffusion could be extrapolated from a linear fit of rate Γ_s versus q^2. At scattering angles between 70 and 150° a second linear region in Γ_s versus q^2 is found, suggesting that another diffusional mode is being observed. The q dependence of the slower peaks could not be described satisfactorily and it is probably related to the dynamics of aggregates in solution. The aggregates are well defined entities with a relatively low polydispersity ascertained by the width of the slow peak (see Fig. 2). The two observed linear regions for the slow mode are considered to be representing two individual diffusional modes that occur when molecules (aggregates) begin to interact (see Fig. 5) [16]. On the other hand, self-organized amphiphilic systems often exhibit shape fluctuations or shape transitions where the different shaped objects may coexist, yielding an augmented effective number of components. The only real experimental value accessible from the DLS experiment is the hydrodynamic radius of the different species in the solution (see Table 2). The detection of aggregates with radii in the range of 30–110 nm indicates the better resolution of the DLS to the gel filtration.

In order to characterize the aggregates, DLS experiment were performed in the absence of the protein. The hydrody-

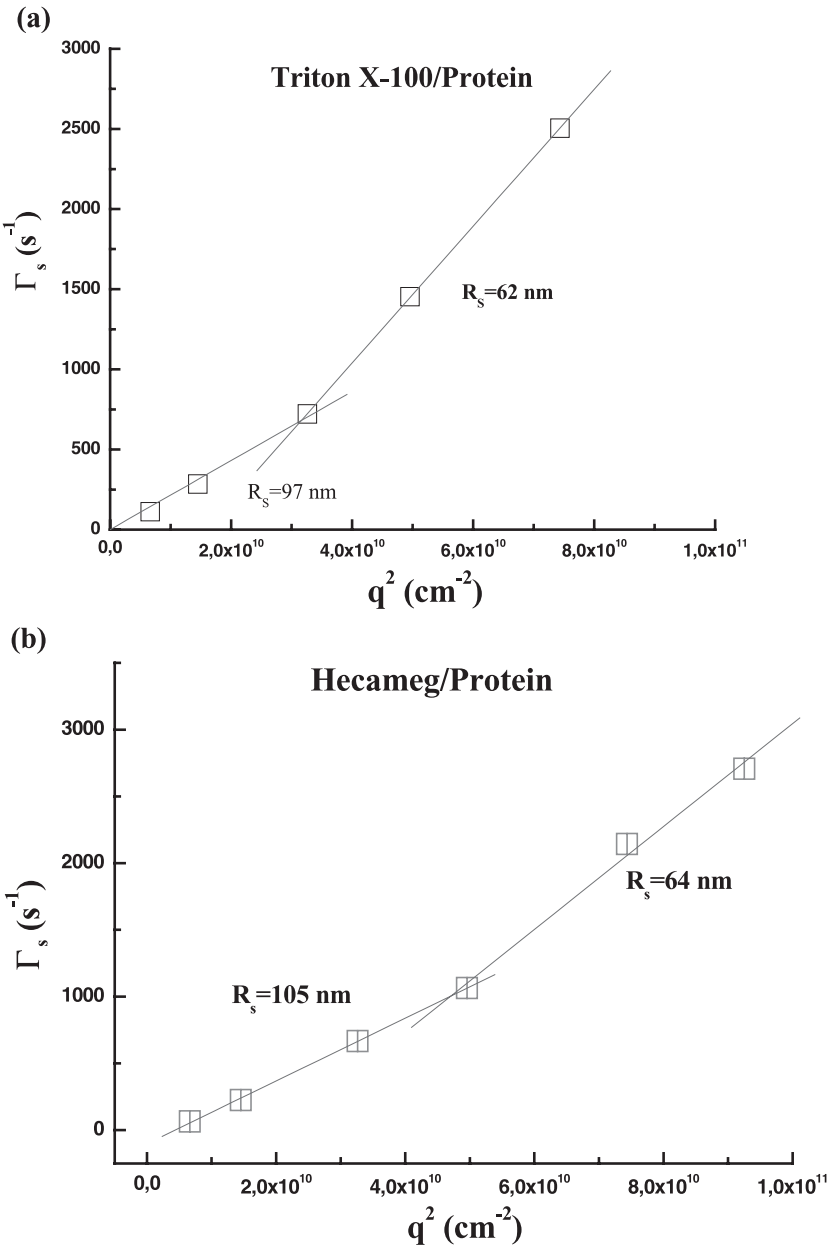

Fig. 5. The angular dependence of the slow characteristic relaxation rate Γ_s versus q^2: the two observed linear regions for the slow mode are considered to be representing two individual diffusional modes for (a) Triton X-100/protein and (b) Hecameg/protein.

namic radius of pure Triton X-100 micelles in water could not be detected in our system due to the low surfactant concentration; nevertheless, the slow relaxation peak was present. On the contrary, for the other two pure nonionic surfactants Octyl-POE and DDM micelles, the measured R_h values were 3.03 and 3.65 nm, respectively. In the presence

Table 2
Micelle radii of the different surfactants and protein/surfactant solutions at 298 K

Surfactant	R_h/nm (fast)	R_h/nm (slow 1)	R_h/nm (slow 2)	Protein/surfactant	R_h/nm (fast)	R_h/nm (intermediate)	R_h/nm (slow 1)	R_h/nm (slow 2)
Triton X-100	4.84[a]	–	105	Triton X-100	5.3	–	62	97
Octyl-POE	3.03	38	80	Octyl-POE	3.2	20	66	108
DDM	3.65	27	84	DDM	4.6	–	36	100
Hecameg	–	–	–	Hecameg	3.2	19	64	105

[a] From Ref. [11].

of the protein, those values increase to 3.2 and 4.6 nm, respectively, since they now correspond to the solubilized protein/surfactant micelles. Our results are in good agreement with the values reported in the literature with aggregation numbers between 90 and 125 for the different surfactants [17–20].

Our objective is to compare our experimental findings with those found in the literature. Our results for Triton X-100 micelles are consistent with the findings of Brown et al. [21] with respect to the appearance of both the fast and slow diffusive relaxation modes. However, there is disagreement with the results of Phillies et al. [22,23], who did not see any clear evidence for the slow modes. We must emphasize that the slow modes appear in all surfactant solutions studied both in the presence and in the absence of the membrane protein. A direct comparison between the pure surfactant and the protein/surfactant solution (see Fig. 6) for Triton X-100 reveals the presence of only one slow process in the ILT. This in turn, strengthens our claim that the fast process is due to the protein species and the slow due to aggregates of the surfactant or protein/surfactant aggregates.

Based on the shape-factor or asymmetry factor, a value of 0.75 indicates the form of a sphere for the aggregated species. This form is present in the slow process in all surfactants indicating a common structure for the different surfactants with similar radii in the range of ~ 100 nm. This is in correlation to quasielastic neutron scattering experiments which have shown an aggregation of the micelles to clusters (about 100 nm) of small spherical micelles [24]. The locally increased concentration of the mixed micelles has been proposed to assist the formation of membrane protein crystals [25]. Further, the DLS indicate the presence of smaller particles with size of about 60 nm expect the DDM with a size of 36 nm. The obtained higher R_h values indicate that the smaller aggregates act as intermediates for the formation of the higher aggregate micelles from the monomer micelles.

It is known that there is a severe loss of stability of membrane proteins when detergents with an octyl or heptyl chain are used [1]. In contrast, long alkyl chains are the mildest and preserve the membrane protein in a "native" state. The presence of particles with intermediate size (about 20 nm) as in the case of Octyl-POE and Hecameg are an indication that these detergents destabilize the form of the protein leading the protein to aggregation (Table 1).

5. Conclusions

Structure and dynamic of an outer surface protein from the photosynthetic bacterium *C. tepidum* was studied by DLS and HPLC gel filtration. For that purpose, the membrane protein was isolated and studied in four different nonionic surfactants Triton X-100, Hecameg, DDM and Octyl-POE. The structural evolution of the aggregates formed was examined by means of a combined analysis of static and DLS measurements. The simultaneous presence of different sizes is revealed in the 3–6 nm and 20–110 nm range, respectively. The overall picture emerging from this study is thus one in which the smaller size is related to the hydrodynamic radius of the individual membrane protein/detergents micelles, whereas the larger size is associated with the presence of complex structures.

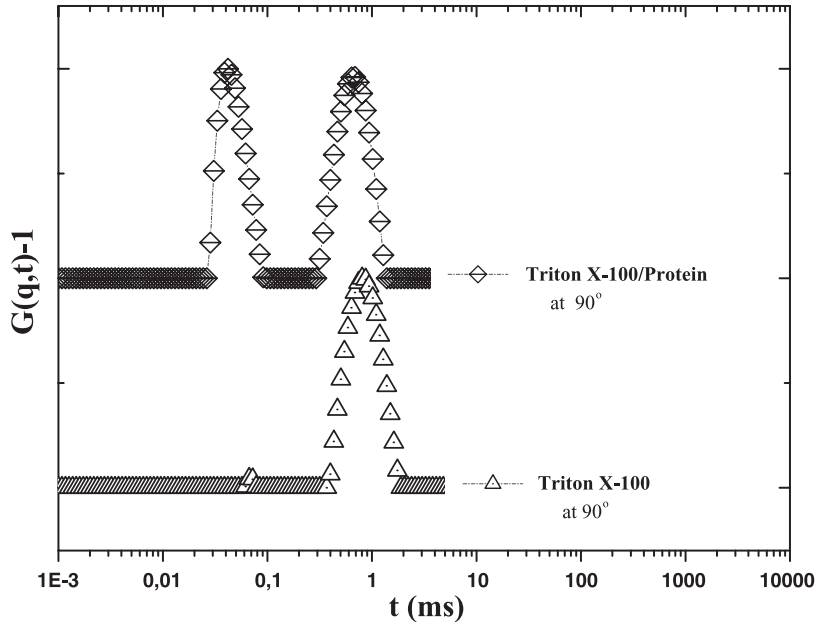

Fig. 6. A direct comparison between the pure surfactant and the protein/surfactant solution for Triton X-100 reveals the presence of only one slow process in the inverse Laplace transformation that is attributed to large Triton X-100 aggregates.

Acknowledgements

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