The phase behavior of membrane proteins stems from a complex synergy with the amphiphilic molecules required for their solubilization. We show that ionization of a pH-sensitive surfactant, LDAO, bound to a bacterial photosynthetic protein, the Reaction Center (RC), leads in a narrow pH range to protein liquid-liquid phase separation in surprisingly stable 'droplets', forerunning reversible aggregation at lower pH. Phase segregation is promoted by increasing temperature and hindered by adding salt. RC light-absorption and photoinduced electron cycle are moreover strongly affected by phase segregation.

PACS numbers: 87.15.Nn ; 64.75.+g ; 87.14.Ee; 82.70.Uv
Keywords: membrane proteins, surfactants, phase separation

Recent studies of protein solutions shed new light on soft-matter physics by blowing up the conceivable panorama of complex-fluid interactions and phase behavior [1]. So far, general attention has been mainly turned to soluble proteins, performing basic enzymatic or transport tasks. The very basic purpose of cell membranes, creating compartments for life, would however be pointless without transmembrane proteins carrying on all primary exchange functions as specific ion channels, receptors for extracellular signals, 'weavers' of the membrane texture driving cell recognition, and linkers in cell adhesion [2]. Our knowledge of membrane proteins is unfortunately rather poor, even at the structural level. Obtaining membrane protein crystals is indeed extremely hard, and crystallization protocols have so far been successful only for a very limited number of proteins [3]. The hampering fact is that membrane proteins, displaying large exposed hydrophobic regions often associated with lipids, are essentially insoluble in water. Stable solutions can only be obtained by exploiting the solubilizing properties of specific detergents used for extracting them from the supporting cell membrane. Surfactants are therefore necessary 'chaperons' in order to bring membrane protein in solutions, and self-assembly phenomena unavoidably coexist with, and strongly influence, membrane-protein solution behavior. The phase behavior of membrane proteins stems therefore from a complex synergy between surfactant supramolecular aggregation and protein-surfactant specific interactions. So far, however, studies of the solution properties of membrane proteins are totally lacking.

The Reaction Center (RC) is a 100 kDa bacterial pigment-protein complex spanning the intracytoplasmic membrane and accomplishing the primary events of energy transduction by promoting light-induced charge separation across the membrane [4]. RC, which has been the first membrane protein to be crystallized, can be extracted from bacterial membranes by using lauryldimethylamino-N-oxide (LDAO), a surfactant acting as a very efficient solubiliser due to the good matching between its spontaneous curvature and the packing constraints imposed by RC structure. In this Letter we show that the interplay between the ionization state of LDAO, tuned by the solution pH, and the packing requirements for efficient solubilization of the Reaction Center induces complex association effects, leading in a narrow pH range to segregation of the protein-surfactant complexes into mesoscopic 'droplets', with a typical size of the order of few µm and a relatively narrow size distribution. This micro-phase segregation shows many features resembling a liquid-liquid phase separation. Furthermore, at variance with what is found for most spontaneous aggregation phenomena in complex fluids, phase segregation of RC/LDAO complexes is fully hindered by screening of the electrostatic interactions with the addition of salt. Finally, confinement into droplets has noticeable effects on RC photochemical reactivity for what concerns both protein absorption spectrum and dynamics of ground-state recovery after excitation. Besides describing an uncommon spontaneous re-organization of a complex fluid involving formation of structures far larger than the basic constituents, these findings, stressing the primary influence of the surrounding environment on RC biochemical activity, may therefore have impact on our understand-
Reaction Center was isolated and purified from the purple bacterium *Rhodobacter sphaeroides* R-26 according to Gray et al. We first discuss the qualitative phase behavior of RC/LDAO solutions observed in moderate acid conditions, at room temperature, and in absence of added salt. From the original stock solution, samples were prepared at RC concentration $c = 4.4 \, \mu$M in presence of 0.87 mM LDAO (value below the surfactant critical micellar concentration $c_{m.c} \approx 2$ mM), and the solution pH tuned in the range $5 < p\text{H} < 8$ by adding HCl to 10 mM imidazole buffers. For $p\text{H} > 7$ solutions look optically transparent and scatter light very weakly. By increasing acidity, a progressive growth of turbidity associated to light scattered at small angles is observed, until at $p\text{H} < 6$ fast aggregation of the solute, which precipitates as an amorphous powder, takes place. Aggregation is however almost fully reversible, as confirmed by the rapid dissolution of most of the aggregates observed by titrating the solution back to $p\text{H} = 8$. For $p\text{H} \approx 5$ precipitation kinetics becomes slower, but aggregates are harder to break up. Solution behavior around $p\text{H}=6.5$ is however very peculiar. In this region, samples persist in a strongly turbid state for days (with typical extinction coefficients of the order of $3 \sim 4 \, \text{cm}^{-1}$), with little solute precipitation or sedimentation. Severe problems to obtain homogeneous RC/LDAO solutions around pH = 6.5 have indeed been previously reported, but no detailed scrutiny of the effect has so far been performed. RC absorbance, which is strong in the blue-violet and near-infrared regions, is low within a moderately large visible spectral range, allowing for dynamic light scattering (DLS) measurements. By progressively changing the pH of dilute RC/LDAO solutions from 8 to 6.5, the scattered intensity not only grows by order of magnitudes, but also becomes strongly forward-peaked. DLS measurements concurrently show a dramatic slowing down of translational diffusion. Time-correlation functions at pH = 8 (Fig. main body) give a hydrodynamic radius $R_H \approx 5 \, \text{nm}$ for the RC/LDAO complex (yet, we unexpectedly detected the presence of a very small quantity of objects with a size around 20-50 nm, persisting even after extensive close-loop filtering). Conversely, DLS measurements at pH=6.5 suggest the presence of much larger objects, with a typical size more than two order of magnitude larger. The calculated particle size distribution shown in the upper right inset, gives an average diameter $d \approx 1.4 \, \mu$m with a standard deviation of about 40%. Direct visualization, made by sealing samples into rectangular capillaries and using phase-contrast microscopy, show that strong scattering is due to a relatively large number of liquid-like droplets (Fig.1, lower left inset), with a typical diameter around 1 – 2 $\mu$m. By counting the number of droplets within the depth of field of a 60X, 0.7 NA objective, we estimate that samples at pH = 6.5 with 1$\mu$M RC, 0.87 mM LDAO, approximately contain a volume fraction $\Phi = 2 \times 10^{-3}$ of dispersed droplets. When filtered through a 0.2 $\mu$m low protein-binding membrane, samples where observed to clarify considerably, meaning that most of the droplets are blocked by the filter. Indeed, DLS measurements of filtered samples essentially coincides with those obtained at pH=8. By comparing absorption spectra of filtered samples at pH=6.5 and pH=8, it is possible to conclude that at least 95% of the original protein amount is confined within the droplets. On the basis of the previous estimate of $\Phi$, this means that RC concentration within the droplets may reach up to 2 mM. LDAO concentration within the separated phase could not be easily measured, but since in this pH range LDAO associates to RC in a 300/400 molar ratio, it should range between 15 to 20 %. Such a high value is consistent with the observation that, by letting samples dry at very slow rate under crossed polarizers, droplets start to display, after limited volume shrinkage, liquid crystal textures observed for pure LDAO at $c \gtrsim 40\%$.

We have checked whether the observed behavior depends on temperature. Fig. 2 shows that the scattering extinction coefficient $\alpha$, measured at pH values close to the onset of the ‘anomalous’ region, strongly increases with T. For instance, 4.4 $\mu$M RC solutions at pH=7, looking almost transparent at room temperature, show an extinction coefficient $\alpha \approx 1 \text{cm}^{-1}$ at T=30$^\circ$ C. Therefore, droplet formation could be possibly associated to...
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phospholipid, which is nonionic at neutral and basic pH and
vanishes by increasing the ionic strength up to 1 M. Inset B of Fig. 2 shows indeed that strong 
turbidity enhancement at pH = 6.5 is confined to mod-
by increasing the solution ionic strength with the addi-
concentration, droplet density is found to be around
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LDAO is indeed lighter than water (α ≈ 0.35 g/l), so
that, by using the previous estimates for RC and LDAO concentration, droplet density is found to be around 1.03 g/l. A final and very peculiar aspect of RC/LDAO liquid-liquid phase separation is that it is fully hindered by increasing the solution ionic strength with the addition of KCl. Inset B of Fig. 2 shows indeed that strong turbidity enhancement at pH = 6.5 is confined to moderately low electrolyte concentration, and progressively vanishes by increasing the ionic strength up to 1 M.

The observed phase behavior for RC/LDAO solutions is strongly correlated with the ionization properties of the surfactant. LDAO is indeed a pH-sensitive amphiphile, which is nonionic at neutral and basic pH and becomes increasingly protonated (cationic) in acid conditions (reported pK ≈ 5.0). Charging of LDAO may be expected to have strong effects on RC/LDAO association for two concurrent reasons. First of all, since RC net charge in this pH range is negative, due to excess of acidic aminoacids in the protein hydrophilic caps, mutual neutralization of the protein and surfactant opposite charges may be expected to deplete the ‘surfac-
tant belt’ around the protein hydrophobic region and decrease complex solubility. The charge-neutrality point of RC/LDAO complex has indeed been reported to be pI ≈ 6.1, quite close to the phase-separation region. At the same time, LDAO spontaneous curvature is larger when the surfactant head group is charged, leading to worse structural matching. We point out that similar effects due to surfactant protonation have been clearly detected by Mel’nikova and Lindman studying DNA condensation in presence of LDAO. As we have seen, RC/LDAO liquid-liquid phase separation takes place by increasing temperature, at variance with simple binary mixtures, but similarly, for instance, to what happens (at much higher protein concentration) for normal and sickle-cell hemoglobin (Hb). It is interesting to notice that, also for sickle-cell Hb, liquid-liquid separation ‘foreruns’ much more extensive protein association, in form of Hb polymerization. Inverted miscibility gaps necessary call for temperature-dependent interactions, but is generally hard to single out and quantify specific temperature effects on interparticle forces. The situation may be simpler for RC/LDAO, since temperature changes definitely influence hydrogen-ion activity of the buffer, modifying pH and therefore surfactant ionization (for imidazole buffer d(pK)/dT ≈ −0.017 °C−1). Inset A in Fig. 2 shows indeed that the α(T) for samples with different pH at T = 25 °C collapse on a single curve when plotted versus the actual pH(T), directly measured with temperature-compensated electrodes. Our hypothesis of a mutual LDAO/RC charge-neutralization process, driving reduced RC solvation and eventually leading to phase-separation, is further supported by the ionic strength dependence of the effect, since salt addition would screen RC/LDAO opposite charge attraction.

We discuss now the effect of phase segregation on RC photochemistry, giving a rough picture of RC electron-transfer cycle. At variance with green plants or algae, energy fruition by photosynthetic bacteria is not based on direct water splitting, but rather depends on simultaneous reduction of quinones, small hydrophobic molecules fully mobile within the cell membrane. Within the RC, a bacteriochlorophyll dimer (D) acts as primary electron donor. Following photon absorption, it delivers an electron in sequence to a couple of quinones (QA and QB), generating a D+QB state. In vivo, D+ is reduced by a small soluble protein, Citochrome C2 (Cyt). Further double electron-transfer leads to formation of QBH2, which freely diffuses to another membrane protein complex (bc1) where it is finally set back to the initial charge state. This cyclic electron-transfer chain creates the proton concentration gradient across the membrane needed to drive ATP synthesis. In vitro, the light-induced D+Qb state undergoes slow charge recombination, which can be probed by exciting the state using a short light-flash, and following the time evolution of specific spectral signatures of the charge separated state. In presence of exogenous Cyt, however, which is a very efficient electron donor for D+, D+Qb recombination is quenched,
phase-separation is driven by increasing hydrophobicity

and spectral evolution becomes a straight probe of cytochrome reduction. We point out that Cyt interacts primarily with the hydrophilic region of the RC, testing therefore the local surrounding of this specific moiety.

A very schematic recollection of our findings is the following. By decreasing the pH below 7, a relevant frequency shift (more than 10 nm) and intensity decrease of the spectral band at 870 nm, due to a specific absorption by the bacteriochlorophyll dimer D, is observed. Band intensity becomes negligible for pH ≤ 6.5 (Fig. 3, inset). Band shift takes place regardless of the solubilizing buffer (imidazole, MES, or Tris), and is not therefore due to specific buffer effects. Moreover, Cyt oxidation, which at pH > 7 is extremely fast, proceeds at a slower pace at pH = 6.5, reaching full completion only after few tens of ms. The RC-Cyt redox reaction is extremely fast when the two proteins are bound in a pre-existing complex (which is therefore the case for pH > 7), while it is collision-limited, and therefore much slower, if Cyt is free. Overall kinetics at pH = 6.5 can be quantitatively described by assuming that about half of the RCs undergo reduction by collisional reaction with Cyt. Although we must defer detailed discussion of phase-separation effects on RC photochemistry to a future publication, we regard these findings as consistent with the general picture we have suggested. Quite similar blue-shift and intensity decrease of the 870 nm band is indeed observed when RC solutions are prepared at LDAO concentration lower than what needed for full RC ‘coating’, suggesting that phase-separation is driven by increasing hydrophobicity due to surfactant desorption from RC non-polar regions. Cyt oxidation kinetics becomes to a large extent collisional because of competitive binding of LDAO to RC polar caps, leading to charge-neutralization, and limiting at the same time the occurrence of RC/Cyt pre-formed complexes. Due to the presumably high viscosity of the surrounding concentrated LDAO solution, diffusion-limited Cyt oxidation is moreover particularly slow. We finally observed that, at pH = 6.5, charge recombination of the D+Q_B state markedly slows down, possibly reflecting an altered stability of Q_B in the phase-separated state. Consistently with turbidity, DLS, and direct visualization results, all these ‘anomalous’ kinetic and spectral effects vanish in presence of a sufficient amount of added KCl.

In conclusion, we have seen that RC/LDAO solutions may undergo micro-phase segregation processes which are totally absent for pure LDAO solutions, take place at very low protein concentration, strongly resemble an inverted liquid-liquid phase separation, forerun extensive (but reversible) aggregation, and disappear by increasing electrostatic screening. The origin of such complex cooperative effects may be likely traced down to RC/LDAO mutual charge neutralization effects, leading to less efficient screening of protein hydrophobic regions and to effective attractive interparticle interactions. Furthermore, phase-segregation effects on RC photochemical kinetics support the proposed mechanism, and suggest that full intelligence of membrane protein operation has to make allowance for protein specific association state.

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