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ARTICLE *in* LANGMUIR · JUNE 1998

Impact Factor: 4.46 · DOI: 10.1021/la971276w

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Surface and Spectroscopic Properties of Photosystem II Core Complex at the Nitrogen/Water Interface

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Received November 20, 1997. In Final Form: March 2, 1998

We have studied surface and spectroscopic properties of Photosystem II core complex (PS II CC) for the first time in monolayers at the nitrogen/water interface. A new instrument was thus specially built to perform absorption and fluorescence spectroscopic measurements directly at the nitrogen/water interface. The effect of initial surface density, incubation time, and compression speed have been studied. When PS II CC was spread at an initial surface pressure of 5.7 mN/m and immediately compressed at a speed of 40 nm^2/molecule·min, it retained its native spectroscopic characteristics. Even though a slower speed of compression (10 nm^2/molecule·min) produced more homogeneous films, the absorption maxima suffered a blue shift, indicating denaturation of PS II CC. Compression at a speed of 80 nm^2/molecule·min produced aggregates of intact PS II CC as indicated by $\Delta V-A$ isotherms, absorption spectra, and fluorescence micrographs. We also conclude that spreading of PS II CC at an initial surface pressure of 0.6 mN/m followed by a 30 min incubation time is inadequate to maintain PS II CC surface and spectral properties. Indeed, $\pi-A$ and $\Delta V-A$ isotherms measured in that condition showed transitions which suggested that PS II CC underwent physical changes during compression. Moreover, absorption and fluorescence maxima were blue shifted, indicating that PS II CC is denatured under that condition.

Introduction

Photosystem II (PS II) is a membrane pigment−protein complex responsible for the visible light driven cleavage of water molecules which produces molecular oxygen. It is present in higher plants and algae. PS II is made of several polypeptides, two of which are inner (core) antennae (CP47 and CP43, named after their apparent molecular masses). These membrane proteins contain 15−20 chlorophylls (chl) *a*.[1,2] CP47 and CP43 are believed to have six transmembrane α-helices that span the membrane.[3] These antennae collect and funnel excitation energy to chl located in the reaction center (RC) of the complex, where photochemical charge separation takes place. The RC is composed of five α-helices containing membrane polypeptides, namely D1 (34 kDa), D2 (32 kDa), the α (9 kDa) and *β* (4.5 kDa) subunits of cytochrome (cyt) b_{559} and the small psbI gene product (4 kDa).[4] The RC also comprises the following reaction species: the photooxidable special chl *a* (P680) whose nature, monomeric vs dimeric, is still under intense debate, two intermediate pheophytins *a*, two to four accessory chl *a* and two plastoquinone electron acceptors (Q_A and Q_B) and other ionic cofactors. In thylakoid membranes, PS II is also associated with an outer antenna, the light harvesting complex (LHC), and to an undetermined number of other intermediate antennas (CP29, CP26, and CP24) all binding chl *a*, chl *b*, and various carotenoids. PS II is also associated with three extrinsic polypeptides which stabilize the tetranuclear manganese cluster facing the lumenal side of thylakoid membranes and which is responsible for the binding of water molecules and production of molecular oxygen. Those PS II-associated water soluble polypeptides protecting the oxygen evolving complex (OEC) respectively mass 33, 23 and 17 kDa.

Although tremendous efforts have been made to crystallize PS II,[5] to this day, its exact three-dimensional structure is still unknown. The putative model of PS II RC is based on our knowledge of the bacterial RC structure, which has close homology to the higher plants RC,[6] and whose atomic structure has been solved at a resolution of 2.3 Å using well-ordered three-dimensional crystals.[7] This intense research on bacterial RC structure has stimulated considerable interest in the study of its mono- and multilayer organization.[8−11] This interest toward protein monolayers was not limited to bacterial RC. In recent years, many studies have been undertaken to characterize

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S0743-7463(97)01276-6 CCC: $15.00
Published on Web 06/18/1998

protein monolayers.[12] Membrane proteins are especially interesting to study in monomolecular assemblies since their structure contains hydrophilic area that can interact with the subphase as well as hydrophobic domains which can interact either with each other or with detergent and lipids. Working with such proteins directly at the air/water interface could become a valuable approach for their two-dimensional crystallization.[13] Indeed, membrane proteins form mainly two-dimensional crystals.[14] Up to now, only two-dimensional crystals of PS II CC have been produced.[5] Since monolayers offer a two-dimensional organization of proteins at the interface, the monolayer methodology could be used to induce crystallization of PS II CC if excess detergent can be removed to optimize protein−protein interactions. Also, the recent development of in situ X-ray diffraction of protein crystal at the air/water interface[15] may open the way to new research avenues that were previously unexplored.

In the present work, we have used the smallest complex able to evolve molecular oxygen called PS II core complex (PS II CC). It contains CP47, CP43, D1, D2, α, and β subunits of cyt b_{559}, psbI gene product, and the extrinsic 33 kDa polypeptide, as well as all functional cofactors located in those polypeptides which are essential to photosynthesis. However, since protein properties can be drastically altered when spread at the interface,[16] especially labile proteins such as PS II CC, finding the appropriate set of experimental conditions to maintain the native properties of this complex is of major importance. As an example, one must find a way to elude the effect of water surface tension which can denature protein structure upon spreading. We investigated the effect of two different spreading conditions and three compression speeds on the physical, microscopic, and spectroscopic characteristics of PS II CC monolayers. These are crucial factors influencing the homogeneity and intactness of proteins at the interface. We used surface pressure−area ($\pi-A$) and surface potential-area ($\Delta V-A$) isotherms to study the complex behavior at the nitrogen/water interface. We also developed a new in situ analytical instrument to further demonstrate under which conditions the protein−pigment complex spread at the interface retains its native absorption and fluorescence characteristics. Indeed, the study of these spectroscopic properties of chl *a* bound to PS II CC allows the characterization of the complex integrity. To investigate the homogeneity of the film, we also observed the microscopic distribution of chl *a* fluorescence in PS II CC at the interface using in situ fluorescence microscopy.

Materials and Methods

Photosystem II Core Complex Isolation and Purification. PS II CC was extracted and purified from fresh spinach leaves using a previously published method.[17] All manipulations were performed in darkness or under dim green light, and samples were always kept on ice. The method of van Leeuwen et al.[17] was modified as following: filtration of the crude extract was done with 10 layers of cheesecloth, and isolation of PS II CC from LHC by FPLC was performed at 4 °C with a HiLoad 16/10 Q Sepharose high performance column (Pharmacia Biotech Inc.). The column was previously equilibrated with BTS400 (20 mM of (Bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane (Bis-Tris, Sigma Chemical Co.) at pH 6.5, 20 mM $MgCl_2$ (Fisher Scientific Co.), 5 mM $CaCl_2$ (Omega Chemical Compagny Inc.), 10 mM $MgSO_4$ (Sigma Chemical Co.), 400 mM sucrose (ACP Chemicals Inc.) and 0.03% (w/v) *n*-dodecyl-β-D-maltoside (DM, Calbiochem). Approximately 20 mL of the extract containing PS II CC and LHC was loaded onto the ion exchange column. This volume corresponds to a total of 35 mg of chl. The column gel was then entirely green and the maximum absorption of the eluate at 280 nm (A_{280}) reached 0.9 au. LHC was eluted from the column until the eluate became nearly colorless and the A_{280} was lower than 0.02 au, which took 30 min at a flow rate of 3 mL/min. After complete elution of LHC, PS II CC was still anchored as a deep green band on the first few centimeters of the gel. Its elution was performed by increasing the $MgSO_4$ concentration to 75 mM. Total procedure, from leaves to pure PS II CC fractions took approximatly 6 h. Samples were then aliquoted, rapidly frozen in liquid nitrogen, and stored at −80 °C until use.

Photosystem II Core Complex Characterization. Chl concentration of PS II CC was measured with a double beam UV−vis spectrophotometer (Model 553, Perkin-Elmer Ltd) using a buffered 80% aqueous acetone pigment extraction as described by Porra et al.[18]

Functional integrity of PS II CC was checked by oxygen evolution measurements. A Clark-type electrode cell (Hansatech Ltd) contained PS II CC at a chl concentration of 5 μM and 1 mM of 2,5-dichloro-*p*-benzoquinone (Pfaltz & Bauer, Inc.) as electron acceptor. The solution was kept at 20 °C during measurements. We used a saturating red light centered at 660 nm to induce oxygen evolution.

To evaluate the molar concentration of PS II CC, we have determined its cyt b_{559} content. Most authors now agree that there is only one cyt b_{559} (divided into two subunits) per PS II CC.[19] We used the method of De Las Rivas et al.[20] which consists of measuring the difference spectrum between the reduced (dithionite) and oxidized (ferricyanide) form of cyt b_{559} using an extinction coefficient of 17.5 $mM^{-1}\,cm^{-1}$. The sample was diluted with BTS400 (without detergent) to a chl concentration of 0.1 mM. Difference spectra were recorded on a Hewlett-Packard single beam diode array spectrophotometer (Model 8452A).

The analysis of the polypeptide composition was carried out on a Bio-Rad Mini-protean II electrophoresis cell, with experimental conditions as published by Haag et al.[21] Gels were Coomassie stained and polypeptide identification was performed using low range molecular weight standards (Bio-Rad Laboratories). PS II CC were diluted five times into sample buffer and a volume of 10 μL was deposited on top of the stacking gel. Gels were run at 200 V for 50 min.

UV−visible absorption spectra of PS II CC were recorded with a Hewlett-Packard single beam diode array spectrophotometer (Model 8452A). In order to obtain optical densities lower than 1 au, samples were diluted with BTS400 to obtain a PS II CC concentration of 0.17 μM.

Fluorescence emission spectra were measured using a Spex Fluorolog II spectrometer. Excitation was set at 440 nm. Excitation and emission slits were fixed at 0.5 mm. PS II CC concentration was adjusted to 0.17 μM by dilution with BTS400. All measurements were recorded at room temperature.

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Isotherm Measurements of Photosystem II Core Complex. The Langmuir trough, built in our laboratory, is made of aluminum covered with a sheet of adhesive precoated Teflon (Johnston Industrial Plastics). The width of the trough is 121 mm, and the monolayer can be compressed from a length of 767.0 mm down to 19.9 mm which gives a compression factor of 38.5. This is particularly important to measure the entire isothem of PS II CC. The trough's depth is 6 mm. Surface pressure (π) was detected with a Wilhelmy plate system using a filter paper.[22] Surface potential (ΔV) was measured with an ^{241}Am radioactive electrode (Nuclear Radiation Development) coupled with a platinum reference electrode immersed into the subphase.[23] All trough operations were controlled by computer. Monolayers were always kept under nitrogen to prevent hazardous contact of molecular oxygen with our sample.

We used the same subphase conditions for PS II CC as those reported for monolayers of bacterial RC.[9] Hence, the subphase contained 2 mM ascorbic acid (Fisher Scientific Co.) and 10 mM tris(hydroxymethyl)aminomethane buffer (Tris, Boehringer Mannheim) at pH 8. However, our conditions were different from this publication with regards to the nature of the salt and its concentration. Indeed, preliminary results (not shown) indicated that using a salt concentration as low as 2 mM $CdCl_2$ led to dissolution of PS II CC into the subphase. Other reports showed a similar behavior for bacterial RC from *Rhodobacter sphaeroides* and *Rhodopseudomonas viridis*.[11c] It was shown that using a dense salt solution could minimize leakage of protein material into the subphase.[24] However, potentially hazardous effects of $CdCl_2$ on PS II CC[25] compelled us to use a less damaging salt. We therefore measured our isothems with 500 mM NaCl (Ultrapure, J. T. Baker Inc.). This salt concentration does not solubilize the extrinsic 33 kDa polypeptide since a 1.5 M NaCl wash will not.[26] Tris and NaCl were purified with chloroform (Megasolv-HPLC 99.98%, Omega Chemical Company Inc.).[23] Ultrapure water was obtained from a four cartridge demineralizing system (NANOpure, SYBRON|Barnstead). Its specific resistivity and surface tension were greater than $18 \times 10^6\ \Omega$·cm and 70 mN/m, respectively. The Trurnit method was used to spread PS II CC at the nitrogen/water interface.[27] To study the influence of protein surface density on the integrity of PS II CC at the interface, two initial surface pressures of 0.6 ± 0.1 and 5.7 ± 0.2 mN/m were investigated by spreading 20 and 40 μL of a 12.7 ± 0.3 μM PS II CC solution, respectively. Also, we were interested to follow the effect of compression speed on the integrity and packing of the protein complex. Therefore, three compression speeds, 10, 40, and 80 nm^2/molecule·min, were investigated. When PS II CC was spread at 0.6 mN/m, the monolayer was incubated for 30 min prior to compression. A molecular mass of 240 kDa was used for PS II CC.[28]

Design of the Instrument for *in Situ* Spectroscopic Measurements at the Air/Water Interface. We collected all monolayer spectra on a new instrument specially built for in situ measurements at the air/water interface. Its design, which is shown in Figure 1, allows to measure absorption and fluorescence spectra as well as $\pi-A$ isotherms of monolayers of PS II CC. All components of the instrument (trough, spectrofluorometer, and spectrophotometer) are computer controlled. The homemade Langmuir trough is made of aluminum covered with adhesive precoated Teflon and equipped with a Langmuir film balance having a typical precision of ± 0.5 mN/m. Translation of the

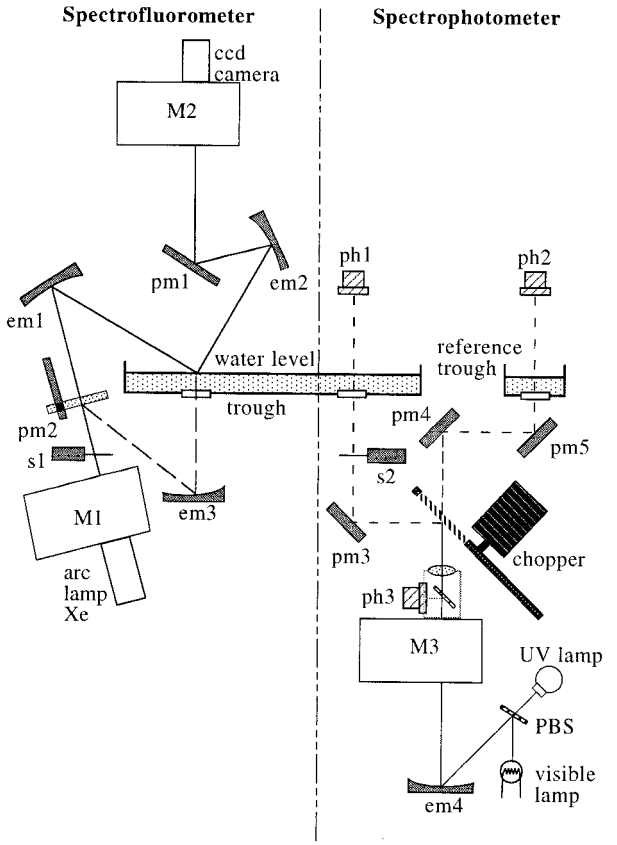

Figure 1. Schematic representation of the spectrophotometer–spectrofluorometer designed for in situ measurements at the air/water interface. Key: ph, photodiodes; pm, plan mirror; em, ellipsoidal mirror; M, monochromator; PBS, Polka beam splitter.

monolayer permits consecutive measurements of absorption or fluorescence spectra with the same sample. A slow flow of nitrogen can be distributed at the air/water interface with a pierced tube fixed on the side of the trough. The left part of Figure 1 shows the spectrofluorometer, which is assembled from parts of a Spex Fluorolog II (Spex Industries Inc.). Optics are fixed on two positions permitting excitation from below (dashed line) or from above (solid line) the trough. In fact, the excitation path is selected by the position of the plan mirror (pm2). In the present work, excitation was done from above the monolayer only. The excitation light comes from a 150 W xenon lamp. The beam passes through a monochromator (M1) and is then focused at the air/water interface by an elliptical mirror (em1 or em3). A shutter (s1) is placed in the light path to protect the sample from photobleaching. The fluorescence is collected with a Photometrics extented UV CCD camera (model CH 250, Optikon Corp. Ltd) via a second monochromator (M2) whose optics were modified to select an appropriate 200 nm detection range. The sensitivity of the instrument is high enough to measure the spectrum of a chl *a*/phospholipid (1:100) monolayer with a signal-to-noise ratio of 20 for one second acquisition time. The acquisition time can be varied between several msec to minutes. The right part of Figure 1 shows the spectrophotometer, a UV–visible dual beam instrument designed for the measurement of low absorbance values. To increase the signal stability, all optical components, except the two detectors, were placed under the trough to minimize vapor condensation. Deuterium and tungsten lamps are used as light sources for UV and visible regions, respectively. They can be used either simultaneously or independently. The two light beams are combined with a Polka beam splitter (model 38105, Oriel Corp.) and this radiation is directed toward the monochromator M3. Subsequently, approximately 4% of the beam is redirected to a reference photodiode (ph3) to adjust the light intensity at all wavelengths by a feedback loop. The light reaches a chopper which is made from mirrors especially cut so that all light is directed either to the monolayer or to the

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reference trough. When no sample is spread at the air/water inferface, a shutter (s2) can block the signal to photodiode 1 (ph1). The reference signal reaching the reference photodiode 2 (ph2), I_0', is then recorded as a function of wavelength. The shutter is then opened and a baseline is recorded in absence of monolayer at the air/water interface. As both light paths are identical, the signal obtained when the two detectors ph1 and ph2 are used is almost a flat line and is amplified by a lock-in (model SR830 DSP, Stanford Research Systems). Spreading of material at the interface decreases the signal to ph1 thus increasing the combined signal producing a square wave $I_0 - I$, which is sent to the lock-in. Absorption is calculated when this square wave signal is divided by the recorded reference signal I_0' using eq 1. The baseline is calculated in the same way and

$$A = -\log\left[1 - \left(\frac{I_0 - I}{I_0'}\right)\right] \quad (1)$$

is then subtracted to give the true absorbance. Compared to conventional instruments, this design has a superior precision for low values of absorbance. The output to the lock-in being the square wave $I_0 - I$ (or when the sample beam is blocked, I_0'), 0.5% noise on both measurements introduces, for a 1×10^{-3} au full scale absorption measurement, an uncertainty of 1×10^{-5} au. In conventional designs, when I_0 and I are measured using $A = -\log I/I_0$ with a precision of 0.5%, the uncertainty of the same measurement becomes 5×10^{-3} au.

Fluorescence Microscopy. Epifluorescence microscopy (Nikon, model UM-2) was used to further investigate the state of PS II CC monolayers. For that purpose, the trough (see "Isotherm Measurements of PS II CC") was installed directly under the objective lens of the microscope. Excitation light was produced with a mercury short arc lamp (HBO 100 W/2, Osram). The light beam was sent through a neutral density as well as a blue filter (430−490 nm). The filter set used to correctly observe chl *a* fluorescence was a combination of a blue excitation filter (Nikon, M420-490, B-3A filter combination), a dichroic mirror (Nikon, DM580, G-2A Filter Combination) and a barrier filter (Nikon, M590, G-2A filter combination). A 20× objective (Nikon, MPlan 20) was used to focus the light beam on the monolayer interface. Emission light was collected with a low light level video camera (Hamamatsu, C2400−09), recorded with a video-cassette recorder (Sony, VO-9800) and printed using a video graphic printer (Sony, UP-850).

Results and Discussion

Characterization of the Photosystem II Core Complex in Solution. On the basis of cyt b_{559} measurements, we estimated the PS II CC concentration of the samples used in this work to be 12.7 ± 0.3 μM. Similar values were always consistently obtained with our PS II CC preparations. The chl *a*/chl *b* ratio of PS II CC samples was 11 which is comparable to other published data.[29] Samples showing a chl *a*/chl *b* ratio lower than 6 were discarded since the presence of higher amounts of chl *b* is known to indicate that one of the minor outer antenna is contaminating the samples.[21] This was confirmed by electrophoretic analysis, which showed the presence of CP29 in those samples. Gel electrophoresis of our PS II CC preparations indicated the presence of CP47, CP43, D1, D2, cyt b_{559}, and the extrinsic 33 kDa. There was no trace of PS I nor LHC. We calculated that the samples used in this work contained 42 ± 2 chls per PS II RC which is similar to other published results.[5b,19c] Oxygen evolution was 1030 ± 90 μmol of O_2/mg of chl·h which is comparable to published values for similar complexes[17,19b] although we regularly obtained average values of up to 1300 μmol of O_2/mg of chl·h. All preparations exhibiting oxygen evolution rates lower than 1000 μmol of O_2/mg of chl·h were discarded.

We measured the absorption spectrum of PS II CC in solution. The positions of the two main maxima located at 437 and 676 nm, which depend on different pigments present in the complex, are in agreement with published data.[19a,21] It is generally agreed that denaturation of PS II has strong effects on its spectroscopic characteristics. The following examples from different authors[30] illustrate this tendency. They observed that the Q_y red absorption band, located at 676 nm in intact PS II RC suffered a blue shift down to 670 nm under progressively harsher conditions. In fact, on the basis of fluorescence lifetime measurements, Booth et al.[30a] qualified as *inactive* any sample showing a red absorption maximum at wavelengths lower than 673 nm. Seibert et al.[30f] stated that this red band is mainly due to chl absorption. This shift was therefore due to changes in the chl/protein matrix. In our experiments, we observed that PS II CC also showed blue shifts of the red absorption band of up to 7 nm in denaturating conditions. Therefore, this parameter was used to characterize the structural integrity of PS II CC in monolayers. The fluorescence spectrum of PS II CC was also measured in solution. We observed the typical bands at 683 and 735 nm.[31] PS II RC fluorescence also shows a blue shift with denaturation.[30b−e] Hence, the results obtained in solution were used as basis of comparison for spectra measured at the nitrogen/water interface.

Conditions That Maintain Native Characteristics of PS II CC in Monolayers. *Effect of Compression Speed on Surface Pressure Isotherms of PS II CC.* $\pi-A$ and $\Delta V-A$ isotherms of PS II CC were measured at two initial surface pressures of spreading (or surface densities) and three different compression speeds. We wanted first to verify the effect of the spreading density on the integrity of PS II CC when spread in monolayers at the air/water interface and second to choose the appropriate speed of compression in regards to equilibrium vs denaturation. Figure 2A shows $\pi-A$ isotherms of PS II CC measured at three different compression speeds (curves a, b, and c). In those experiments, PS II CC was spread at an initial surface pressure of 5.7 mN/m which corresponded to a surface density of 300 nm^2/molecule, and compression was started immediately after spreading. We observed an increase in surface pressure with compression speed between approximately 300 and 30 nm^2/molecule. This indicates that more material is being compressed at the interface when the speed is greater. Indeed, at the two lowest compression speeds (curves b and c, Figure 2A), the compression time needed to reach any molecular area is two and eight times longer (respectively) than at the fastest compression speed (curve a, Figure 2A). Therefore, at lower compression speeds, the water soluble DM (dodecyl maltoside) molecules, which are in large excess at the interface, have more time to leave the interface, thus lowering the measured surface pressure. All three curves merge at 33 mN/m (30 nm^2/molecule), which is presumed to correspond to the surface pressure where excess detergent molecules have been ejected into the subphase.

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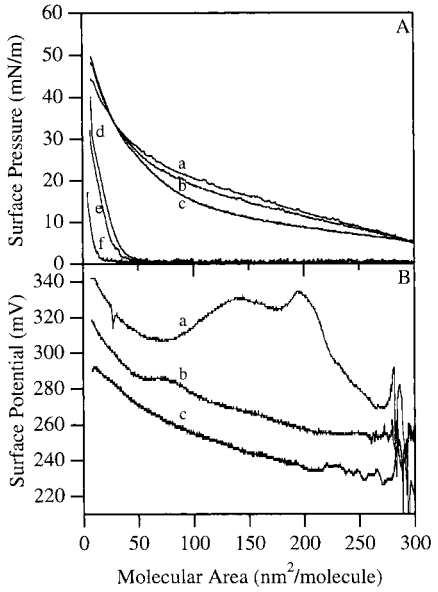

Figure 2. Effect of different compression speeds on $\pi-A$ (A) and $\Delta V-A$ (B) isotherms of PS II CC (curves a−c) and of DM (curves d−f) spread at an initial surface pressure of 5.7 mN/m. Key: curves a and d, 80 nm^2/molecule·min; curves b and e, 40 nm^2/molecule·min; curves c and f, 10 nm^2/molecule·min. All compressions were started immediately after spreading. Subphase buffer contained 10 mM tris(hydroxymethyl)aminomethane buffer at pH 8, 2 mM ascorbic acid, and 500 mM NaCl. To improve clarity, $\Delta V-A$ isotherms b and c were moved downward to lower surface potentials by 30 and 40 mV from the original values, respectively.

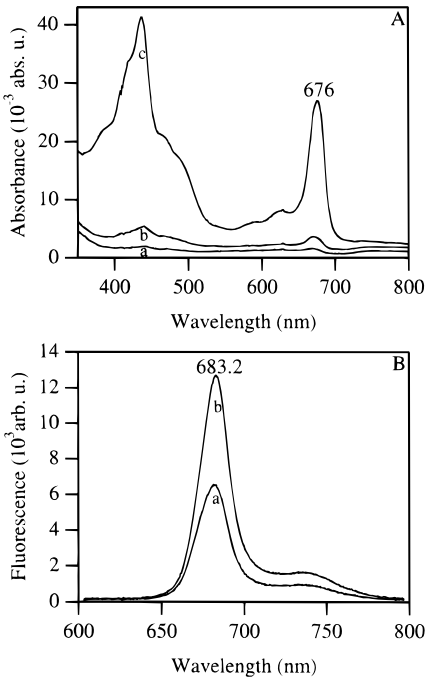

Figure 3. In situ absorption (A) and fluorescence (B) spectra at the nitrogen/water interface of PS II CC spread at an initial surface pressure of 5.7 mN/m and compressed immediatly at 80 nm^2/molecule·min. In situ fluorescence measurements were made at $\lambda_{exc} = 440$ nm. Key: curves a, 10 mN/m; curves b, 20 mN/m; curve c, 30 mN/m.

Effect of Compression Speed on Surface Pressure Isotherms of DM. To verify to which extent the presence of DM could influence the shape of PS II CC $\pi-A$ isotherms, we spread at the interface 40 μL of the DM containing buffer that is used to solubilize PS II CC. Results are shown in Figure 2A (curves d, e, and f). For comparison purposes, all parameters were set as if it actually was PS II CC being compressed at the interface. Therefore, molecular area, compression speeds, and so on all refer to PS II CC. It can be seen that no matter the compression speed, an isotherm of DM was measured only at molecular areas lower than 50 nm^2/molecule. Hence, we believe that most of the features present in isotherms a, b, and c (Figure 2A) are attributable to PS II CC although modulated by its interaction with detergent molecules. Contrary to what is expected for a lipid, the compression speed had a drastic effect on DM isotherms. Indeed, isotherms of DM show that at any given molecular area, higher compression speeds give higher surface pressures. As mentionned above, this phenomenon is most likely due to the solubility of DM. Lower compression speeds probably gave more time to the detergent to form micelles that were solubilized into the subphase thus lowering the surface pressure at all molecular areas (curve f, Figure 2A). Therefore, the compression speed dependence of DM $\pi-A$ isotherms (curves d, e, and f) followed what was observed with PS II CC (curves a, b, and c). This hypothesis is also supported by the fact that incubating the DM monolayer at zero surface pressure for 30 min before compression (as performed in Figure 5) did not allow one to measure $\pi-A$ isotherms of DM, the surface pressure being constantly at 0 mN/m (at all compression speeds).

Other authors discussed the influence of lauryldimethylamineoxide (LDAO) on isotherms of bacterial RC.[11c] When they spread bacterial RC at the interface, they observed a slope change at a surface pressure of 31−32 mN/m which was attributed to two different states of RC at the interface. They reported that at low surface pressure the detergent played a major role in the monolayer structure by covering the hydrophobic surface of protein subunits. This state was designated as a liquid-expanded state (state II). At surface pressures above the transition of 31−32 mN/m, detergent molecules were believed to be ejected from the monolayer and RC could then form a more strongly interacting lattice which they identified as a condensed state I. Our results favor this global interpretation.

Effect of Compression Speed on PS II CC Surface Potential Isotherms. Figure 2B shows $\Delta V-A$ isotherms of PS II CC. The general shape of the curves shows that surface potential rises with compression. This is due to the increase in PS II CC surface concentration at the nitrogen/water interface upon compresion. Typical surface potentials obtained immediately after spreading were ranging from 220 to 250 mV and rose to values from 330 to 350 mV at the end of compression. At a compression speed of 80 nm^2/molecule·min (curve a), intense unreproducible fluctuations were observed in the isotherms that are probably attributable to patches of aggregated PS II CC being randomly detected by the radioactive electrode. Additional evidences from absorption spectroscopy and fluorescence microscopy (Figures 3A and 4, respectively) also indicate the presence of aggregates of intact PS II CC when the film is compressed at this highest compression speed (see discussion below). Such fluctuations largely decreased at 40 nm^2/molecule·min (curve b) and are almost undetectable at 10 nm^2/molecule·min (curve c) thus revealing a homogeneous film leading to reproducible $\Delta V-A$ isotherms especially in the last part of the isotherm at molecular areas lower than 200 nm^2/molecule.

Spectroscopic Characteristics of Intact PS II CC at the Interface. In situ absorption and fluorescence spectral

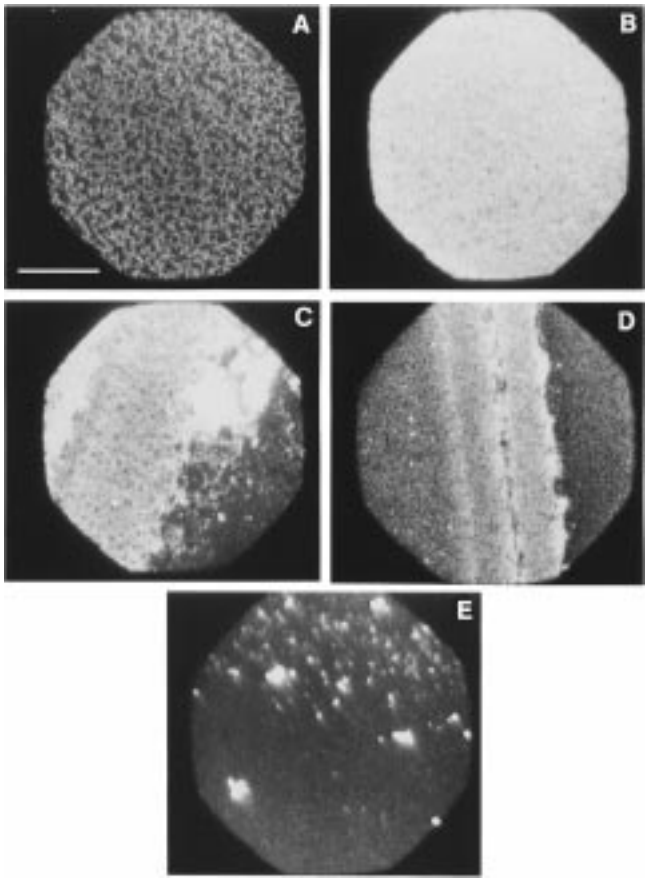

Figure 4. In situ fluorescence microscopy of PS II CC spread at the nitrogen/water interface. A−C: Initial surface pressure of speading (π_i) = 5.7 mN/m, immediate compression, speed = 80 nm^2/molecule·min. Key: A, π = 5.9 mN/m; B, π = 25.0 mN/m; C, π = 30.0 mN/m. D−E: π_i = 5.7 mN/m, immediate compression, speed = 10 nm^2/molecule·min. Key: D, π = 30.0 mN/m; E, π = 7.7 mN/m. Scale bar on micrograph A = 100 μm.

characteristics of PS II CC spread in monolayers at the nitrogen/water interface are presented in Table 1. As mentioned above, the absorption and fluorescence maxima are important indicators of the state of PS II CC at the interface. A typical in situ absorption spectrum whose characteristics are close to those measured in solution is shown in Figure 3A. In this case, PS II CC was spread at an initial surface pressure of 5.7 mN/m followed by an immediate compression of the film at a speed of 80 nm^2/molecule·min. Similar results were obtained at 40 nm^2/molecule·min (see Table 1). In both cases, the red absorption band position was located at 676 nm which is identical to what was obtained for intact PS II CC in solution (see above). These results thus suggest that those experimental conditions lead to the formation of PS II CC films that are not denatured. A large increase in absorption values can be seen when PS II CC is compressed to 30 mN/m at a speed of 80 nm^2/molecule·min (curve c, Figure 3A and Table 1). The intensity of this sudden rise was highly variable. This observation is an additional indication for the presence of aggregates of intact PS II CC at the interface which were randomly detected by absorption as observed in surface potential. This is further supported by the experiments in fluorescence microscopy (see below). When the film was compressed at 10 nm^2/molecule·min, the red maximum was blue shifted to 673.5 nm which indicates that the complex suffered some denaturation (see Table 1).

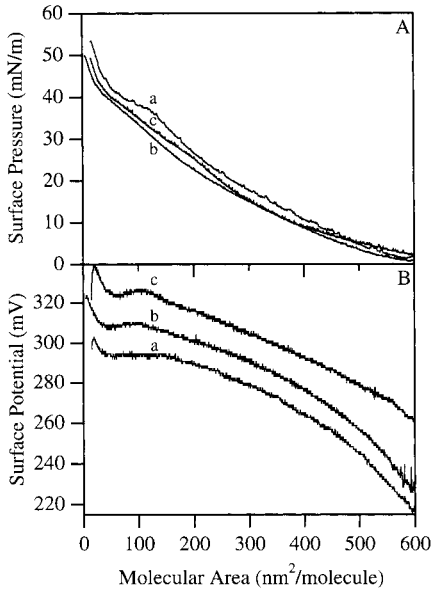

Figure 5. Effect of different compression speeds on $\pi-A$ (A) and $\Delta V-A$ (B) isotherms of PS II CC spread at an initial surface pressure of 0.6 mN/m. Key: curves a, 80 nm^2/molecule·min; curves b, 40 nm^2/molecule·min; curves c, 10 nm^2/molecule·min. All compressions started after a 30 min incubation time. To improve clarity, the $\Delta V-A$ isotherm b was moved downward to lower surface potentials by subtracting 13 mV from the original values.

Table 1. In Situ Absorption and Fluorescence Characteristics of PS II CC Monolayers[a]

π_i, mN/m	compression speed, nm^2/molecule·min	π, mN/m	λ_A, ± 0.2 nm	I_A, au	λ_F, ± 0.5 nm	I_F, au
5.7	10	10	673.5	0.0026	682.9	4053
		20	674.5	0.0078	684.2	14888
		30	676.0	0.0144	684.2	11065
5.7	40	10	676.0	0.0057	682.5	5842
		20	676.0	0.0147	683.9	25056
		25			684.2	56811
5.7	80	10	676.0	0.0005	682.2	6543
		20	676.0	0.0037	683.2	12688
		30	676.0	0.0270		
0.6	10	0	675.0	0.0009	681.5	2712
		10	669.0	0.0015	681.8	1962
		20	670.5	0.0020	681.8	1690
		30	671.0	0.0030	683.5	2583
0.6	40	10	672.0	0.0013	678.8	1217
		20	672.0	0.0019	682.9	1746
		30	672.0	0.0042	683.9	2928
0.6	80	10	672.0	0.0010	680.5	2007
		20	672.5	0.0015	682.9	2161
		30	672.5	0.0023	683.9	5218

[a] π_i is the initial spreading surface pressure and λ_A and λ_F are respectively the absorption red band and fluorescence maxima whereas I_A and I_F are the corresponding intensities of those maxima.

Figure 3B shows typical in situ fluorescence spectra of PS II CC at the nitrogen/water interface in the same experimental conditions as in Figure 3A. It is well-known that the emission observed from PS II CC comes from antenna chl *a* molecules.[31a] In intact PS II CC, all other photoactive molecules such as carotenoids and chl *b* transfer their excitation energy to a neighboring chl *a*.[31c] Although steady-state measurements are not the most powerful fluorescence technique to study PS II CC, we detected interesting features which can be used as indicators of PS II CC integrity at the interface. Figure 3B shows the fluorescence characteristics of PS II CC which was spread at an initial surface pressure of 5.7

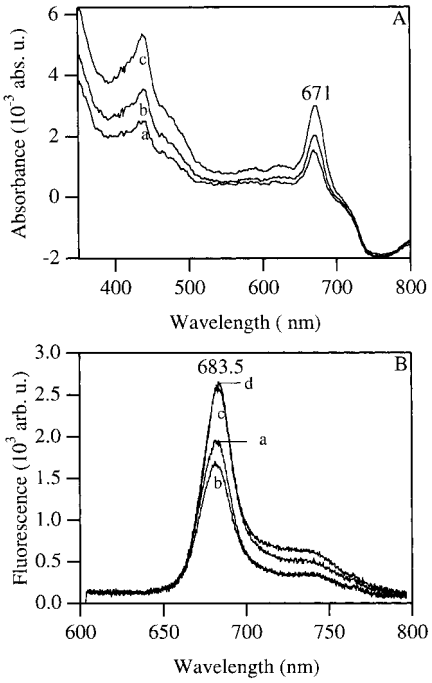

Figure 6. In situ absorption (A) and fluorescence (B) spectra at the nitrogen/water interface of PS II CC spread at an initial surface pressure of 0.6 mN/m followed by a 30 min incubation time prior to compression at 10 nm^2/molecule·min. In situ fluorescence measurements were made at $\lambda_{exc} = 440$ nm. Key: curves a, 10 mN/m; curves b, 20 mN/m; curves c, 30 mN/m; curve d, 40 mN/m.

mN/m followed by an immediate compression at a speed of 80 nm^2/molecule·min. Comparable results were obtained at 40 nm^2/molecule·min. In those two cases, the fluorescence maximum measured at 10 mN/m is centered at 682 nm and increasing surface pressure shifts this value to the red up to 683−684 nm (see Table 1). Comparing these values to the ones obtained with PS II CC in solution which showed a maximum located at 683 nm, these values indicate that PS II CC is intact in these films, even at lower surface pressure. At 25 mN/m, the emission intensity reached 56 811 counts for a compression speed of 40 nm^2/molecule. Such a strong fluorescence suggests that the pigments in PS II CC are not damaged. Spreading PS II CC at an initial surface pressure of 5.7 mN/m and compressing the film at a speed of 10 nm^2/molecule·min also gives similar fluorescence characteristics although with a much lower fluorescence intensity (Table 1). Indeed, maximum fluorescence emission is red shifted from 682.9 (10 mN/m) to 684.2 nm (30 mN/m) and its intensity reaches 11 065 counts (Table 1). However, we noticed the presence of a small shoulder at 765 nm, which was observed under denaturating conditions (see Figure 6B). Hence, even though PS II CC was spread at 5.7 mN/m, it may not all be in its native state when using such a low compression speed of 10 nm^2/molecule·min. Indeed, the emission maximum was blue shifted to 680.2 nm at the early stages of compression (at pressures lower than 10 mN/m, results not shown). We have demonstrated by polarization-modulated infrared reflection absorption spectroscopy (PM-IRRAS) that the secondary structure of the polypeptide backbone of PS II CC is not denatured in these conditions.[32] Therefore, only protein−pigment interactions can be affected by use of such compression speeds. In fact, chl *a* may be disrupted from PS II CC and thus more susceptible to damage. However, if damage does not occur, the red shift observed at higher surface pressures (see Table 1) may be explained by the reduction of the distance between individual chl molecules. Indeed, knowing that diluted chl *a* molecules in lipid monolayers have a maximum fluorescence band located at 678 nm and that a red shift is detected in chromophores whenever the distance between them is reduced to a point at which energy transfer becomes possible, one could think that compression leads to such changes in spectra where chl *a* molecules are detached from PS II CC. Increasing the surface pressure could thus shift the spectral bands due to the reduction of intermolecular spacing as chl *a* molecules get closer together. In conclusion, both absorption and fluorescence spectra of PS II CC demonstrated that compression at 10 nm^2/molecule·min is inadequate to preserve the native characteristics of PS II whereas compression at 80 nm^2/molecule·min leads to the formation of an inhomogeneous film. Finally, the best spectral characteristics were obtained when PS II CC was compressed at 40 nm^2/molecule·min.

Fluorescence Microscopy of Intact PS II CC Monolayers. Figure 4 shows micrographs of PS II CC films using in situ fluorescence microscopy. During the first few minutes after spreading, whatever the compression speed, rapid movements in the film made the collection of images so difficult that compression had to be stopped in order to acquire images. This phenomenon was observed until the film reached a surface pressure of 10 mN/m. Whenever PS II CC was spread at an initial surface pressure of 5.7 mN/m followed by an immediate compression, the film had an inhomogeneous texture as shown in Figure 4 (part A). The darker areas in Figure 4 (part A) are probably made of excess detergent domains which were gradually eliminated at higher surface pressures. Indeed, when the surface pressure reached 25 mN/m, the film became very homogeneous as shown in Figure 4 (part B). These images showed an intensely bright film that was bleached in a matter of seconds. However, all films showed areas of tremendous heterogeneity, their occurrence being proportional to the compression speed. Examples of those remarkable features are shown in parts C and D of Figure 4. These structures were always oriented perpendicular to the direction of compression. Although those kinds of heterogeneities were observed most frequently at pressures higher that 30 mN/m, they were occasionally observed at lower surface pressures, but especially with the high compression speed of 80 nm^2/molecule·min. Those structures are most likely formed by aggregated domains of intact PS II CC being stacked together by the movement of the mobile barrier. These microscopic observations thus support our previous explanation for the measurement of intense unreproducible fluctuations in $\Delta V-A$ isotherms (curve a, Figure 2B) and the large increase in absorption values at 30 mN/m (curve c, Figure 3A and Table 1) observed at this high compression speed. Nevertheless, the most frequently observed type of heterogeneity noticed at low surface pressure can be viewed in Figure 4E. Those small and rapidly moving features were so intensely fluorescent that, in order to record adequate images, sensitivity had to be substantially reduced. Therefore, the dark background in Figure 4 (part E) is highly fluorescent.

Molecular Area of PS II CC in Monolayers. Molecular area of PS II CC at the nitrogen/water interface was calculated using isotherms obtained when PS II CC was spread at an initial surface pressure of 5.7 mN/m followed by an immediate compression (Figure 2A). Since the

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isotherms shown in Figure 2A all merge at 33 mN/m, where excess detergent molecules should have left the interface (see curves d, e, and f, Figure 2A), we used the corresponding value for the molecular area of 31 nm^2/molecule as a basis of comparison with the published data. Our calculations supposed that all PS II CC remained at the interface. Although some proteins can be solubilized into the subphase upon spreading at the interface, several indications strongly suggest that this loss was insignificant. Indeed in situ absorption and fluorescence measurements after succion of PS II CC monolayer from the interface have shown that there was no detectable spectroscopic signal emerging from possibly solubilized PS II CC, even after the spreading and removal of three consecutive monolayers. Moreover, the very high reproducibility of the isotherms shown in Figure 2A also strongly suggests that the loss of proteins into the subphase was minimal. Our calculation of the molecular area of PS II CC also supposed that PS II CC formed a monomolecular array which was confirmed by X-ray reflectivity measurements.[32] Our value of 31 nm^2/molecule for the molecular area of PS II CC can be compared with literature data obtained by electron microscopy. Hence, Boekema et al.[28] estimated the top view of PS II CC dimer to measure 17.2 nm × 9.7 nm. Considering that PS II CC have an ellipsoidal shape, we calculated from these measurements that a single PS II CC would occupy approximately 65 nm^2. Santini et al.[33] calculated the volume of PS II CC to be 640 nm^3. Dividing this value by their estimated thickness of 13.5 nm, which is large compared to other published values,[28] we obtained a molecular area of 47 nm^2. These values are 1.5−2 times larger than our value of 31 nm^2/molecule. Since the lateral pressure in membranes is still unknown, although there is a large consensus for values ranging between 25 and 35 mN/m,[34] the molecular area of PS II CC was also calculated using values obtained at 25 mN/m. It that case, the molecular area of PS II CC varies from 54 to 67 nm^2, depending on the compression speed (see Figure 2A). These values are in good agreement with the previously published data mentioned above.[28,33]

Conditions That Alter PS II CC Structure in Monolayers. *Effect of Compression Speed on Surface Pressure and Surface Potential Isotherms of PS II CC.* To evaluate the effect of low surface density on PS II CC monolayers, a second set of experimental conditions was studied where PS II CC was spread at an initial surface pressure of 0.6 mN/m and incubated for 30 min prior to compression. The compression speeds used were the same as described above. Figure 5A shows $\pi-A$ isotherms obtained under these conditions. PS II CC was spread at an initial surface pressure of 0.6 ± 0.1 mN/m which rose to 1.3 ± 0.6 mN/m at constant molecular area after the 30 min incubation time, thus suggesting that PS II CC spreads out and unfolds at the interface. This interpretation is supported by our PM-IRRAS measurements showing that a large proportion of the α-helices of PS II CC is transformed to β-sheets in these conditions.[32] Transitions in the $\pi-A$ isotherms can be seen at all compression speeds, the most obvious one being observed at approximately 37 mN/m at a compression speed of 80 nm^2/molecule·min (curve a). In all isotherms, no matter the compression speed, these transitions were observed at surface pressures between 30 and 40 mN/m. Figure 5B shows $\Delta V-A$ isotherms that also displayed corresponding transitions as a plateau where the most striking one is observed at 80 nm^2/molecule·min (curve a). These transitions observed in both surface pressure and surface potential isotherms indicate that a physical process is taking place at the interface which affects the structure of PS II CC. Indeed, PM-IRRAS measurements have shown that the secondary structure of PS II CC undergoes important structural changes during those transitions, from unfolded β-sheets to the formation of new α-helices.[32] Also, at molecular areas lower than 50 nm^2/molecule all isotherms indicated the presence of more compact films characterized by the rapid increase in surface pressure upon further compression. Furthermore, when PS II CC was spread and incubated at a low initial surface pressure, we observed that the compression resulted, at all compression speeds, in the formation of a rigid film which could be lifted from the interface with a syringe. This phenomenon was observed by other groups and was explained by nonspecific interactions between denatured side chains of the protein.[35] Under these conditions, no randomly occurring fluctuations in surface potential were observed in the $\Delta V-A$ isotherms that could be attributable to aggregates of intact PS II CC in contrast to isotherms obtained when spreading is performed at 5.7 mN/m (compare Figures 5B and 2B). The incubation at low surface pressure could have allowed the destruction of all intact protein particles which were further transformed as incubation proceeded into superaggregates of interacting unfolded polypeptide chains at the interface. This hypothesis is also supported by absorption spectroscopy (Figure 6A, see below) and PM-IRRAS measurements.[32]

Spectroscopic Characteristics PS II CC at the Interface. When PS II CC was given time to relax at a surface pressure of 0.6 mN/m during 30 min prior to compression, the red absorption band at 10 mN/m was blue shifted as low as 672−669 nm at all compression speeds (see Figure 6A and Table 1). We also noted a large drift in the baseline that was stronger in the UV region when samples were incubated at low surface pressures as illustrated in Figure 6A in contrast to spreading at 5.7 mN/m (Figure 3A). This effect was more pronounced at lower compression speeds. This observation was interpreted by the presence of superaggregates which scatter light. Further compression allows one to observe these superaggregates by naked eye as intense green strips that can be removed from the interface as dark green threads. Finally, an important decrease in the absorption intensities was observed in all spectra taken after the 30 min incubation time (see Table 1). This phenomenon may partly be due to the lower surface density of PS II CC at the interface in these conditions. Indeed, under these conditions, molecular areas between 230 and 270 nm^2/molecule were obtained at 20 mN/m depending on the compression speed (see Figure 5A), whereas molecular areas between 70 and 110 nm^2/molecule were obtained at the same surface pressure when PS II CC was spread at 5.7 mN/m (see Figure 2A). However, since the position of the red absorption band clearly shows denaturation, which is supported by our PM-IRRAS measurements under the same conditions,[32] the release of individual pigments from PS II CC which are randomly floating at the interface may also be responsible for this observation.

In these experimental conditions, the intensity of fluorescence is reduced by 2−10-fold (compare Figures

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3B and 6B and see Table 1). This is interpreted as an indication of denaturation which is also supported by our PM-IRRAS data.[32] As an example, in the case of monolayers compressed at 80 nm^2/molecule·min, the fluorescence maximum was shifted from 680.5 to 683.9 nm with increasing pressure but the fluorescence intensity is not restored to values obtained with samples which were not incubated at low surface pressure (compare Figures 3B and 6B and see Table 1). To differentiate between the effect of spreading at low surface pressure vs incubation time on PS II CC integrity, the fluorescence of PS II CC was immediatly measured after spreading at low surface pressure. Evidences of denaturation could be observed even though the spectrum was taken within less than 1 min after spreading (see 0 mN/m spectrum in Table 1). Indeed, the fluorescence maximum was blue shifted to 681.5 nm and the intensity of fluorescence was already as low as 2712 counts. Increasing the surface pressure by compression did not significantly change the fluorescence intensity, although a red shift to 683.5 nm was observed. In fact, we think that after the incubation at low surface pressure, PS II CC is completely denatured, leaving chl *a* molecules floating randomly at the interface surrounded by a loose protein matrix. As explained before, reduction of the surface area induced by compression could bring chl *a* molecules closer together. A red shift in the fluorescence emission would consequently be observed. This shift would increase as the distance between chls get smaller, thus permitting stronger dipole−dipole interactions. This would also explain the low fluorescence intensity which is observed. If chl *a* aggregates were formed, the quantum yield of fluorescence will decrease according to the intermediate strength exciton coupling model. Therefore, we do not think that the red shift observed in those cases indicate a renaturation of the complex at the interface, since neither the intensity of fluorescence nor the absorption characteristics are restored. We also observed a relative increase in the 735 nm band which may also be an additional indication of denaturation. Indeed, it is well-known that chl−water interaction in hydrated chl *a* aggregates will lead to bands in the region between 725 and 735 nm.[36] These results thus further support our interpretation that chls are released from PS II CC in these conditions. Figure 6B also shows new features in its red most region, where chl *a* fluorescence vibrational satellites bands are located, particularly at approximately 715 and 765 nm. Since those bands are not present in intact PS II CC fluorescence spectrum in solution or under conditions where PS II CC is believed to be intact (Figure 3B), we conclude that they result from denaturation of PS II CC which leads to the formation of hydrated chl *a* oligomers.[36]

Since PS II CC contains more chls than PS II RC, we believe that the protein environment of CP47 and CP43 as well as that of the RC is greatly affected by the incubation at low surface pressure. There may be a partial or complete disruption of pigments from PS II CC, thus indicating that its structure is drastically altered as shown for the polypeptide backbone of PS II CC by our PM-IRRAS measurements.[32] This disruption could either be reversible or irreversible, depending on the experimental conditions. This damage could affect only outer chls which may not need to be located in an exact position for the complex to be functional. In that case, the damage could be reversed by an increase in surface pressure. In contrast, the damage could involve specific pigments whose exact location are essential to the function of PS II CC. Damage involving displacement of those pigments could be irreversible.

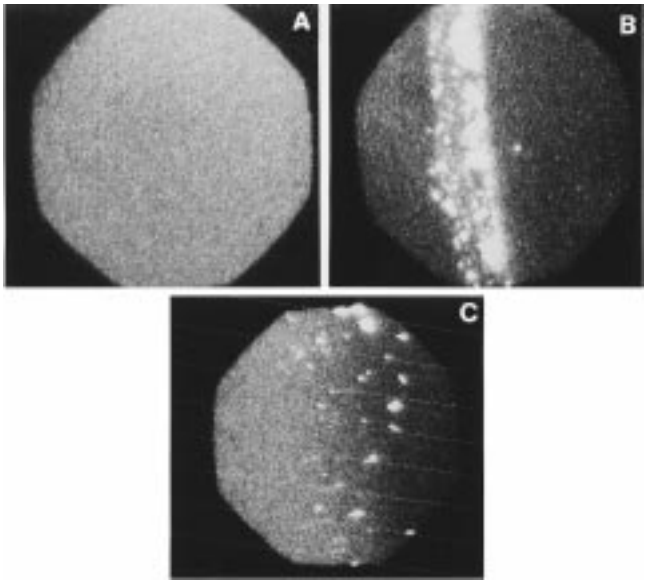

Figure 7. In situ fluorescence microscopy of PS II CC spread at the nitrogen/water interface, incubation time = 30 min, π_i = 0.6 mN/m. Key: A, compression speed = 80 nm^2/molecule·min, π = 5.0 mN/m; B, compression speed = 40 nm^2/molecule·min, π = 41.0 mN/m; C, compression speed = 10 nm^2/molecule·min, π = 11.0 mN/m. See Figure 4 for the scale bar.

Fluorescence Microscopy of PS II CC at the Interface. When PS II CC was spread at 0.6 mN/m and incubated for 30 min, images showed very homogeneous monolayers, as shown in Figure 7 (part A). We noticed that the intensity of fluorescence was greatly reduced compared to what was observed when PS II CC was spread at 5.7 mN/m (see Figure 4B) although the intensity was still proportional to the surface pressure. The occurrence of heterogeneities was also greatly reduced. In fact, at surface pressures lower than 30 mN/m, intense fluorescent aggregates as shown in Figure 7 (part B) were almost never observed. Indeed, mainly small fluorescent domains could seldom be observed (Figure 7 (part C)). We noted that the faster the compression speed, the greater was the probability of observing those heterogeneities.

Conclusion

From the measurement of both surface and in situ spectroscopic properties of PS II CC in monolayers, we have identified experimental conditions that should be avoided to prepare monolayers of intact PS II CC. Indeed, whenever PS II CC was spread and incubated at low surface pressure, both the absorption and fluorescence characteristics showed signs of degradation which is consistent with our PM-IRRAS data.[32] Indeed, the red absorption band of PS II CC suffered an irreversible blue shift that is correlated to a significant denaturation. Also, the fluorescence studies have illustrated that at the very beginning of the 30 min incubation time, i.e., immediately after spreading, the low surface pressure was already affecting the spectral properties of PS II CC, since the fluorescence maximum was blue shifted to 681.5 nm. The $\pi-A$ and $\Delta V-A$ isotherms measured under these same experimental conditions showed transitions which indicated that PS II CC underwent physical changes during compression. This suggested that the protein may be unfolded at lower surface pressures and that its secondary structure was changing as surface pressure increased as

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clearly demonstrated by our PM-IRRAS data.[32] Fluorescence microscopy revealed that PS II CC films that had been subject to low surface pressure incubation were very homogeneous but showed low intensity, thus indicating that the complex is denatured. Heterogeneities were observed at surface pressures higher than 30 mN/m and were correlated with the transition noted in the $\pi-A$ and $\Delta V-A$ isotherms.

Our results also showed that when PS II CC was spread at an initial surface pressure of 5.7 mN/m and immediately compressed, the compression speed is an important parameter affecting its integrity. When PS II CC was compressed at 10 nm^2/molecule·min, the absorption spectra showed a slight indication of degradation that seemed reversible. In fact, the red absorption band measured at 10 mN/m was blue shifted down to 673.5 nm, but this value returned to 676.0 nm with compression. This observation was also detected by fluorescence, although less evidently. However, $\pi-A$ and $\Delta V-A$ isotherms, absorption spectra and fluorescence microscopy indicated that when the film was compressed too rapidly, at 80 nm^2/molecule·min, PSII CC formed superaggregates at the interface. Considering our ultimate aim to prepare ordered two-dimensional crystals of PS II CC, the formation of such aggregates must be avoided. Therefore, we can conclude that the best experimental condition allowing to maintain the native PS II CC spectral characteristics and where reduced aggregation was obtained is by spreading PS II CC at an initial surface pressure of 5.7 mN/m followed by an immediate compression at 40 nm^2/molecule·min.

Acknowledgment. The authors are indebted to Natural Sciences and Engineering Research Council of Canada, the Fonds FCAR and the FRSQ for financial support. J.G. was also supported by Le Syndicat des Chargé(e)s de Cours de l'Université du Québec à Trois-Rivières and the Fondation du CEU. Authors also thank Prof. Robert Carpentier for the use of the Hansatech system for oxygen evolution measurements.

LA971276W