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Spectroscopic properties of bacteriochlorophyll *c* in Langmuir monolayers in the absence and presence of amphiphilic peptides

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Abstract

The spectroscopic properties of bacteriochlorophyll (BChl) *c* alone, and together with synthesized, amphiphilic peptides, were measured in a Langmuir film at the air–water interface. Analysis was done using surface pressure–area isotherms, Brewster angle microscopy and absorption in situ. The BChl *c* alone, after spreading and compressing at low surface pressure, showed the typical absorption pattern for the monomeric form. The pigment aggregates that absorbed at 731 nm were formed on the water subphase when the isotherm of BChl *c* reached the kink point around 13 mN/m and the average area occupied by pigment molecule was smaller than the area of a porphyrin ring. The effect of the amphiphilic peptides on BChl *c* Langmuir film was also examined by injection of peptides beneath the pigment monolayer compressed at a certain pressure. The mixed aggregated pigment–peptide complex prepared at 20 mN/m, showed optical properties similar to those of chlorosomes with the Q_y absorption band at 739 nm. A possible structure of this complex is discussed based on the above results. In this report, we show that the results obtained by applying the spectroscopic methods in situ provide a new approach to study the chlorosome-like structure from the in vitro two-dimensional model preparation. © 2000 Elsevier Science S.A. All rights reserved.

Keywords: Amphiphilic peptide; Bacteriochlorophyll *c*; Brewster angle microscopy; Langmuir monolayer

Abbreviations: BAM, Brewster angle microscopy; BChl, Bacteriochlorophyll; Chl, Chlorophyll; E, Glutamic acid; H, Histidine; K, Lysine; L, Leucine; TLC, Thin-layer chromatography; W, Tryptophan

1. Introduction

Green bacteria are characterized by a special light-harvesting structure called a chlorosome, which is essential for the efficient energy transfer of absorbed light energy to the photochemical reaction center [1]. Chlorosomes of green sulfur bacteria, elongated bag-like structures, are mostly made up of bacteriochlorophyll (BChl) *c*, but lipid, protein, carotenoid and quinone are also present [1,2]. The far-red absorption spectrum of BChl *c* in the chlorosome is red-shifted with respect to that of the monomeric form in organic solvents. The strong orientational ordering of the BChl *c* Q_y transition dipoles along the long axis of chlorosome in situ suggests that an elementary BChl aggregate has the form of at least quasi-linear chain [3]. To study this pigment aggregation state, several model systems have been employed, for example, aggregates of BChl *c* in non-polar solvents [4–7] and aqueous solution [8,9], in a liquid crystal [10], polyacrylamide gel [8], polyvinyl alcohol film [11], at low temperatures [12], and in the multilayers of BChl *c* on solid support [13].

Two hypotheses exist about the association of the BChl *c* and protein in the intra-chlorosomal structures. One hypothesis proposes that the BChl *c* molecules can form functional aggregates by themselves without the participation of any proteins, after which proteins may play a role in stabilizing the chlorosome structure [4,6,14,15]. The other hypothesis favors the idea that the BChl *c* molecules are directly binding to the specific protein, which determines the organization and possibly causes the in vivo red shift of the absorption band [16,17].

The purpose of the present study was to gain a better understanding of the molecular self-association of BChl *c* in situ in the Langmuir monolayer at the air–water interface. Using the combination of spectroscopic and microscopic techniques, it was found that BChl *c* has an intrinsic nature to make two-dimensional organized assemblies at the

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interface. In this study, we reconstituted pigment aggregates consisting of several BChl *c* homologs, presented in vivo in *Prostecochloris* (*P.*) *aestuarii*, in the absence, and presence, of amphiphilic peptides which were chosen to mimic the chlorosomal proteins. Because the monolayers are ordered two-dimensional systems which are, to some extent, similar to the layer of BChl *c* which forms the rod surfaces, this study might represent another step towards reconstituting the chlorosome from component parts. It was shown that BChl *c* aggregates absorbed at 731 nm were formed when the area occupied by pigment was smaller than the area of a porphyrin ring and the chlorine ring is tilted with respect to surface of subphase. We found that the pigment aggregates at the interface were formed based on a specific (neither hydrophobic nor electrostatic) interaction, between functional groups of BChl *c*, being independent of the buffer saline condition. According to our results and those in the literature [9], it seems that these aggregates have a non-specific amorphous structure.

In this study, we also successfully provided a model system related to the creation of the chlorosome-like structure which could be adopted for the study of in vivo molecular organization. Using the synthesized, amphiphilic peptides (with, and without, histidine residues), the influence of the presence of these peptides on pigment aggregation was examined. It was found that the incorporation of peptides into aggregated pigment monolayer causes a red shift of BChl *c* Q_y band and results in optical properties of aggregated BChl *c*-peptide complex similar to those observed for native chlorosome. This might suggest that the amphiphilic peptides are able to stabilize the pigment aggregates by making a hydrophobic environment around them.

2. Materials and methods

BChl *c*, being a mixture of several homologs [18], was isolated from *P. aestuarii* by thin layer chromatography (TLC). Extraction, purification and identification procedures were described in Ref. [19]. Peptides, that mimic the spectral properties of α-helical photosynthetic protein, were synthesized as described earlier [20,21]. Three different amphiphilic peptides, with the hydrophobic area formed mainly by the leucine residues, were used. Two of them were 30 residue peptides: L (NH_2-EEEQQKKLLEELKKLLEELKYLLKEEQKKK–COOH) and L/H [21]. L/H, having the same sequence as peptide L, but the leucine (L) residue in the 16th position is replaced with histidine (H) [19] which is an important residue because it might be involved in vivo in binding a BChl *c* molecule at the periphery of the chlorosome core [1]. The third peptide was named peptide L/2H (NH_2-WLELHKLLLELLKHLLELLK–COOH) (see also scheme in Fig. 4) and consisted of 20 amino acids, two of them being histidines located in the hydrophobic area.

The pure pigment of the chloroform solution was spread on the water subphase from a 2 mM Tris/HCl, 2 mM $CaCl_2$, 0.5 mM sodium ascorbate and/or 500 mM NaCl (pH 8.2) buffer system. The peptides dissolved in chloroform-methanol (7:3, v:v) solution were spread on the subphase or injected beneath a pigment monolayer compressed at 10 mN/m or 20 mN/m.

A KSV-5000 Langmuir–Blodgett system (KSV, Finland) located in a soft plastic box was used for symmetrical compression and expansion at constant barrier speed. The solvents used to dissolve the pigment or peptides were allowed to evaporate for 15 min after spreading. Surface tension was measured by means of a Wilhelmy plate. All experiments were performed in dim light, with minimal difference between air temperature and the subphase temperature $(20\pm1)^\circ$C to prevent a capillary convection effect in subphase.

In situ absorbance measurements were performed by means of a single-beam spectrophotometer MCPD-1000 (Otsuka Electronics, Japan) equipped with a diode array detector. A fiber optic bundle was used to introduce the white light vertically to the water surface, whilst a small aluminum mirror was placed in the bottom of the trough to turn the light back to a receiver optic fiber. In this way the analyzing light beam travels twice through the film and the recorded absorbance was two times higher than for monolayers.

A Brewster angle microscope, type NL-EMM 633SS (Nippon Laser & Electronics Lab., Japan) based on standard microscope scheme [22,23] was used when imaging the structure of the pure pigment film. Images were recorded in an analogous way on videotape via Quality Image Improver DSV-20 (Hamamatsu, Japan) and printed, without correction of the geometrical distortion due to the oblique incident angle associated with the microscope optics, on photoprinter NC-1 (Fujix, Japan). Only reproducible Brewster angle microscopy (BAM) patterns, typical for the BChl *c* monolayer morphologies, were considered.

3. Results and discussion

3.1. Pigment Langmuir monolayer

After spreading the chloroform solution of pure pigment, no increase in the surface pressure was observed. Fig. 1 presents an example of typical compression and expansion surface pressure–area isotherms for BChl *c*. Upon film compression, the rising surface pressure could be seen for smaller area per molecule of pigment (about 3.5 nm^2) like BChl *c* rather than for other photosynthetic pigments, for example chlorophyll (Chl) *a* [24,25] and BChl *a* (results not shown). The isotherm of the pigment rises monotonically up to around 15 mN/m where the kink of the isotherms is observed. Further compression shows that the isotherm does not reach the collapse point in the investigated region of surface pressure. The average area per molecule for BChl *c*

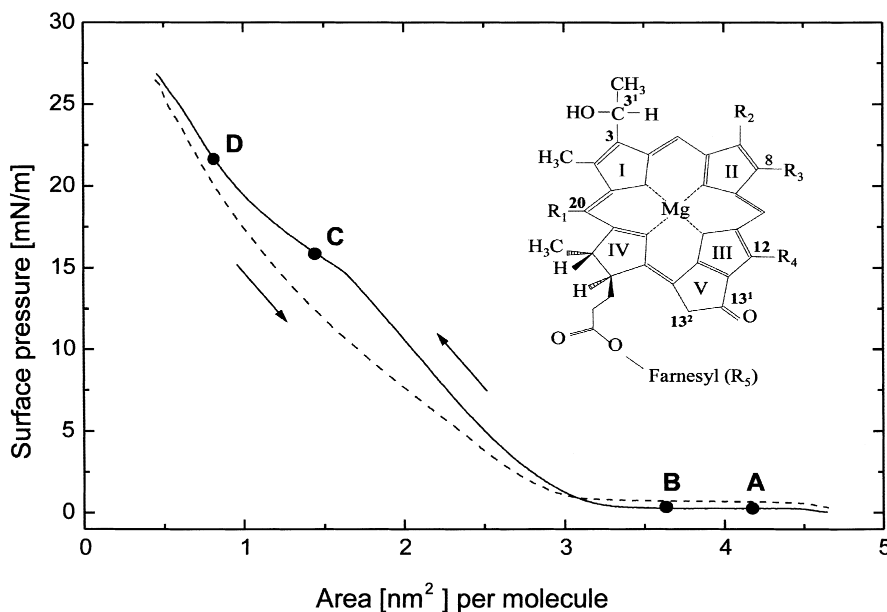

Fig. 1. Surface pressure–area isotherms of BChl *c* on the air–water interface. The points with letters designate the surface pressures (or areas) at which the BAM images were taken. Molecular structure of BChl *c* from green sulfur bacteria is also shown inside the figure (R_1=R_2=methyl, R_3=ethyl, *n*-propyl or isobutyl, R_4=ethyl or methyl, and R_5=farnesyl (91%), phytyl (3%), cetyl (3%) or tetrahydrogeranylgeranyl (2%) [1]).

at 25 mN/m (0.60 nm^2) is similar to that for R-8,12-diethyl (R[E,E] [26]) BChl *c* homolog (0.62 nm^2) [14] and also comparable with the area occupied by one Chl *a* molecule [24]. On comparison, BChl *c* and Chl *a* have similar chemical structures, but differ in compressibility (Fig. 1 and [24]), which can be seen directly from the slope of the increase of surface pressure. This indicates the difference in the intermolecular interaction between these pigment molecules on the buffer subphase. We also observed that, under the experimental conditions used, the expansion isotherm of the BChl *c* shows a hysteresis with respect to the compression isotherm (Fig. 1).

The absorbance spectra of a monolayer on the air–water interface were measured at different stages of film formation upon compression and expansion. Fig. 2A presents the representative spectra of pigment at different surface pressures. Generally, the absorption features of the film do not change on compression up to ca. 13 mN/m. At low pressures, the spectrum of BChl *c* is typical in terms of the peak position and the shape of the Soret and Q_y bands to the pigment absorption spectrum in polar organic solvents. Therefore, we could interpret the absorption spectrum at low surface pressure, in Fig. 2A, as those of the monomer. At 2 mN/m, the maxima occur at around 438 nm and at 674 nm in the B and Q regions, respectively. The absorption spectra change significantly when the BChl *c* monolayer is compressed further and the average area occupied by one molecule decreases. Starting from the point where the pressure reached 13 mN/m (1.8 nm^2), a small shoulder in the long-wave region of absorption occurred indicating some aggregate formation (Fig. 2). Compression of the monolayer caused the increase in absorption at 731 nm and also the appearance of a shoulder in the Soret region, at around 452 nm, which is characteristic for the aggregated form of the pigment. These measurements were repeated for BChl *c* on the buffer subphase containing 500 mM NaCl and the same spectral patterns seen for pigment monolayer were again observed. It indicates that the contents of salt do not change the specific interaction of a functional group of BChl *c* between pigment molecules on the interface.

According to the changes in area per molecule and considering the cross-sectional area of the porphyrin plane for BChl *c* which is similar to that for Chl *a* (equal 1.987 nm^2 [24]), we can conclude that aggregate formation starts at about 1.8 nm^2, when the average surface occupied by one pigment molecule is a little smaller than the area of a porphyrin ring and the spatial arrangement of pigment molecules is changed. It seems that at low surface pressure the molecules of BChl *c* keep a flat orientation with respect to the interface surface. With increasing surface pressure a layer of dense-packed pigment molecules is formed. A further increase of the surface pressure by reduction of the area causes BChl *c* molecules to assume a tilted position convenient for interactions with neighbor pigment molecules forming an aggregate. According to the surface pressure–area isotherm (Fig. 1), the changes of the tilt angle of the BChl *c* molecule in the Langmuir film during formation of pigment aggregate could be calculated. Taking into account the experimental molecular area occupied by BChl *c* molecule at the surface pressure between 13 and 25 mN/m and comparing it with the

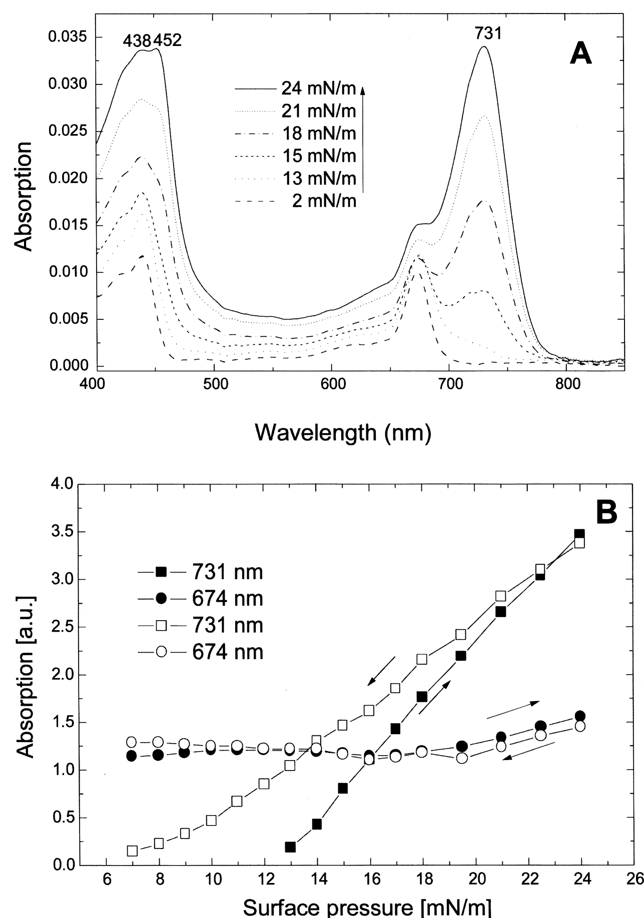

Fig. 2. (A) In situ absorption spectra of a BChl *c* monolayer on the buffer subphase measured at different surface pressures during compression. (B) The intensity of absorption vs. surface pressure during compression and expansion cycle for 674 nm and 731 nm Q_y bands.

cross-sectional area of the BChl porphirin plane, we point out that the chlorine ring is oriented at a tilted angle to the water surface at 25 and 72°, respectively.

The in situ absorption was also measured during the expansion cycle and the dependence on the absorption intensity for 674 and 731 nm bands vs. surface pressure is shown in Fig. 2B. From these results, we could see that the 731 nm band of absorption is observed at 13 mN/m for the first time, whereas it disappears during the expansion cycle at lower surface pressure at around 7 mN/m.

We can conclude that the BChl *c* in the Langmuir monolayer (in the two-dimensional system) exists in at least two different (monomeric and/or aggregate) states on the air-water interface. This is in contrast to the widely investigated Chl *a* monolayer where the pigment exists as a monomer in the Langmuir–Blodgett monolayer prepared in the air–gas condition [25]. The hysteresis of isotherms for the investigated pigment could be explained by the fact that strong pigment–pigment interactions occurred on the water surface and caused the formation of a two-dimensional BChl *c* aggregate. The process of the BChl *c* aggregation and conversion to an unaggregated form are not completely reversible and occur under different surface pressures (Fig. 2B). The pigment aggregates very easily and the strong interaction between pigment molecules could maintain such a state even at low-pressure conditions when the area occupied by pigment simply increases. Also, it is clear that the pigment aggregates are formed by means of a specific (neither hydrophobic nor electrostatic) interaction between functional groups of BChl *c* molecules that is not sensitive to the presence of salt in the subphase. It should be mentioned here that, recently, the effect of ionic strength on the aggregation of two BChl *c* homologs was reported [9]. These results indicated that a red shift of BChl *c* Q_y band was induced by a high concentration of salt (NaCl) or buffer for both R[E,E] and R[P,E] (P-propyl) homologs and that the replacement of the side chain at the C-8 position on the macrocycle induces a change in aggregation behavior. Mizoguchi et al. also proposed that different aggregates are formed from R[E,E] [27] and S[I,E] (I-isobutyl) [28] BChl *c*. The heterogeneity of BChl *c* homologs found in chlorosomes in vivo allows us to use a mixture of homologs. In our experiments, we used also low concentrated buffer with, and without, the addition of salt. On comparing our results with those in the literature [9], we could conclude about the profile of the observed BChl *c* aggregate in the Langmuir film. The absorption band of aggregated BChl *c* in Langmuir monolayer (Fig. 2A) is very broad and consists of superimposed contributions from different pigment homologs; therefore, it is difficult to observe the effects related to an individual homolog. The bands characteristic for two abundant homologs ([E,E] and [P,E]) from *P. aestuarii* overlap, giving the resultant absorption band at 731 nm being independent of the buffer saline condition. Although from the spectroscopic data it is rather difficult to point out about the features of the BChl *c* aggregates in the Langmuir film, but based on literature [9], we could say that their structure is probably non-specific and essentially identical, for at least R[E,E] and R[P,E] BChl *c*. From the results described by Ishii et al. [9], it seems that the Mg atom of BChl *c* is five-coordinated and the 13^1 keto group is involved in a linkage between pigment molecules, whereas the 3^1 OH group might be free or involved in the aggregation.

In order to observe the changes in morphology of the BChl *c* monolayer at various pressures BAM was used. Each BAM image of the BChl *c* monolayer (Fig. 3) is labeled by a letter which corresponds to the points on the compression isotherms as indicated in Fig. 1. For zero surface pressure at the open trough area, a uniform background was observed. After spreading BChl *c*, the BAM image (Fig. 3A) shows an almost regular geometrical domain organized in islands at 0 mN/m. When the area per molecule decreases and a liquid phase is in coexistence with gas phase, the packed hexagon pattern is followed by big liquid islands (Fig. 3B). Before the surface pressure starts to rise monotonically at around 3.5 nm^2 (Fig. 1), uniform films almost cover the buffer surface. Further compression makes the film completely

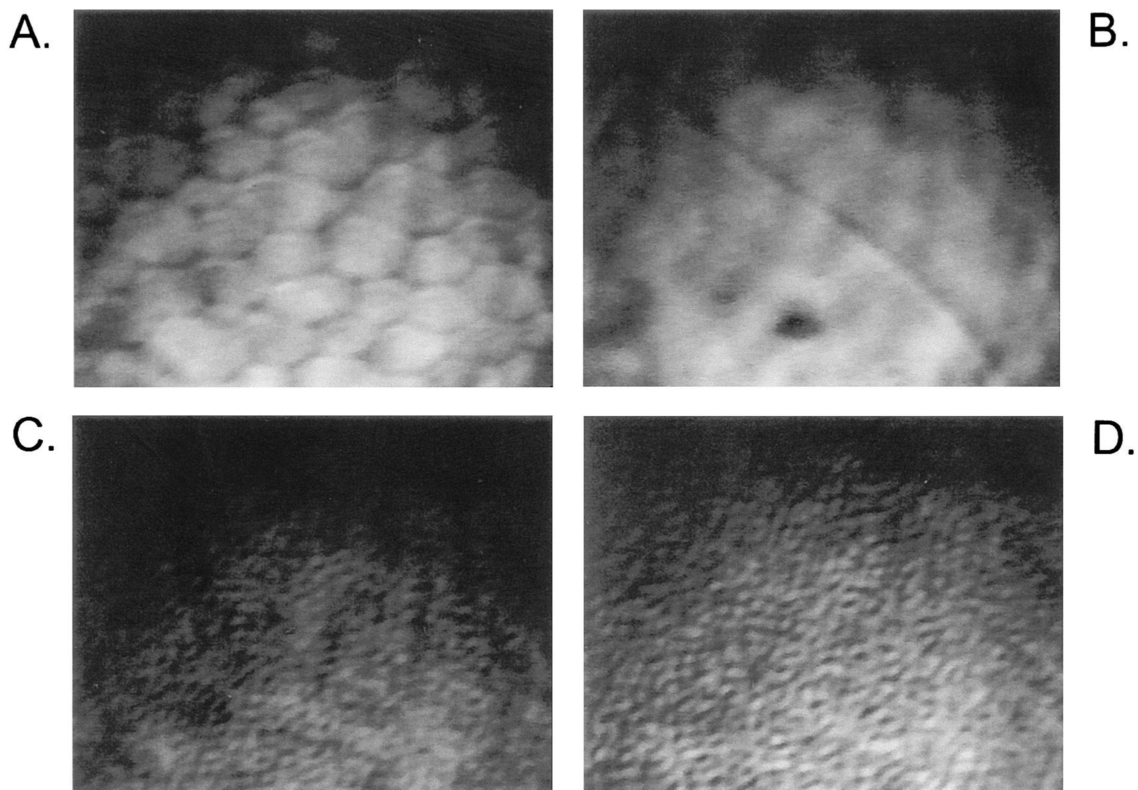

Fig. 3. BAM images of a BChl *c* monolayer on the buffer surface at various molecular areas. A 320 μm×550 μm area of the film on the trough was imaged through lenses and an extension tube set attached to the CCD camera.

homogenous and brighter because of the increase of molecular packing that causes an increase in the effective refractive index of the film. Just after passing the kink of the isotherm (around 15 mN/m (Fig. 1)), the new form of film texture appears (Fig. 3C). On increasing the pressure, the smaller clear structures appear on the buffer subphase as shown in Fig. 3D.

The BAM images clearly show the transition of the molecular structure of the monolayer. It could be interpreted as the aggregate formation, which is in agreement with the information obtained from the surface pressure–area curve and absorption spectra. This suggests that the BAM observation provides a valuable approach for easy evaluation of the state of photosynthetic pigments in situ based on changes in the refractive index of the monolayer surface.

3.2. Pigment-peptide Langmuir monolayer

For the purpose of constructing the artificial chlorosomal-like structure on the water subphase, the interaction of BChl *c* with three different amphiphilic peptides was examined. The examples of surface pressure–area isotherms of pure peptide L/2H (with two histidines that might serve as a possible binding side for BChl) and of pigment–peptide L/2H mixture are shown in Fig. 4. When spread at the air–water interface, all examined peptides formed stable monolayers. The shapes of isotherms of the peptides were similar to each other, while only the collapse point occurred at different pressures depending on the peptide hydrophobicity. Peptide L/2H, being the most hydrophobic one, shows the collapse point above 45 mN/m. It is necessary to mention here that the isotherms of the peptides are reliable only up to 30 mN/m,

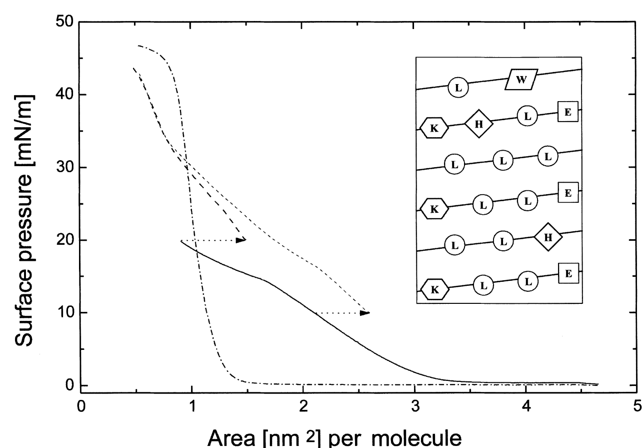

Fig. 4. Surface pressure–area isotherms of pure peptide L/2H (dot-and-dash line) and mixed BChl *c*–peptide L/2H film. The peptides were injected into the subphase underneath a BChl *c* monolayer compressed at 10 or 20 mN/m (solid lines). During 1 h, the film was expanded (dotted line-arrows), then recompressed (dashed lines). The structure of peptide L/2H is also shown inside the figure (E, glutamic acid; H, histidine; K, lysine; L, leucine; and W, tryptophan).

because above this pressure, due to the high layer viscosity, the Wilhelmy plate system appears no longer functional [29].

During the incorporation of peptides into the pigment monolayer the surface pressure was kept constant (isobaric process). This was performed by injection of the peptides into the subphase, beneath the BChl *c* monolayer compressed at 10 or 20 mN/m, and as the film expanded the area occupied by the monolayer increased (Fig. 4). The recompression isotherms measured 1 h later, followed completely new surface pressure–area curves and reached a higher surface pressure than observed for BChl *c* alone (Fig. 1).

The amphiphilic peptides were first incorporated at low surface pressures (at 10 mN/m) under the monomeric (unaggregated) pigment monolayer (Fig. 4). In such cases, the absorption at 674 nm decreased due to the dilution effect but no change in the position of BChl *c* Q_y absorption band after 1 h was observed. The mixed monolayer was compressed and the absorption spectra of pigment, measured at a higher pressure (30 mN/m), showed maxima at 674 and 731 nm (Fig. 5, dot-and-dash line). These positions of absorption maxima are similar to those observed for pure BChl *c* at pressures higher than 13 mN/m (see Fig. 2). There are two possible explanations for this. The first is that the histidine residues of peptide L/H and peptide L/2H could not interact, for some reason, with a Mg atom of monomeric BChl *c*. This may be due to the spatial conformation and/or distance between an Mg atom and histidine residue on the buffer surface which are not suitable for interactions, or the water molecule is ligated to an Mg atom similar, as in a case of Chl *a*, in mono- and multilayers [30]. The second possibility is that the monomeric pigment–peptide complex formed by hydrophobic interaction only, at 10 mN/m, is not stable and strong enough; therefore, the competition between the pigment–pigment and pigment–peptide interactions favor the former, especially when the pressure increases and the distance between BChl molecules decreases. Hence, finally at 30 mN/m only the absorption spectra similar to those for pure pigment aggregates were observed.

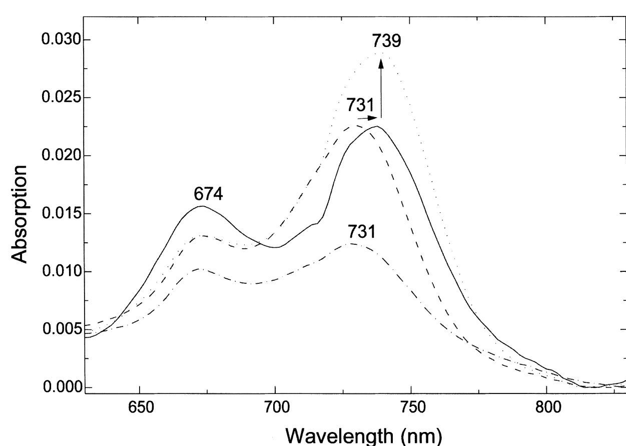

Fig. 5. The absorption spectra of pure BChl *c* at 20 mN/m (dashed line) and mixed BChl *c*–peptide L/2H film. The mixed BChl *c*–peptide L/2H film was obtained by injecting the peptides at 10 mN/m beneath the monomeric (unaggregated) BChl *c* film and compressed after 1 h up to 30 mN/m (dot-and-dash line). The solid line (multiplied by 2) shows the mixed pigment–peptide monolayers measured at 20 mN/m, 1 h after injecting peptide L/2H under aggregated pigment film, and the film was then compressed and observed at 30 mN/m (dotted line).

A different situation occurred when the peptides were applied under a monolayer of aggregated BChl *c* at 20 mN/m. The example of absorption spectra of aggregated BChl *c* taken in situ at 20 mN/m, before and 1 h after applying the peptides are shown in Fig. 5 (dashed and solid lines, respectively). The absorption of the long-wavelength BChl *c* Q_y region, in the presence of peptides at 20 mN/m, decreased by half compared with the spectrum of pure pigment at the same surface pressure, and showed the red-shift (about 7–8 nm). The absorption ratio, measured before, and 1 h after peptide injection, for the 674 nm band and long-wave band also changed (dashed and solid lines in Fig. 5), indicating that the presence of peptides strongly affected the absorption related to the aggregated pigment. Further compression of such mixed pigment–peptide monolayers caused only an increase in absorption, while the wavelength of the new band remained unchanged (Fig. 5, dotted line). Independently of the peptides used (amphiphilic without, and with one or two, histidine residues), the same effects were observed in the presence of peptides in mixed aggregated pigment–peptide monolayers. To exclude the possibility of the hydrophobic and/or electrostatic interaction between the pigment aggregates and peptides, the same experiments were performed on the buffer subphase containing 500 mM NaCl (results not shown). The absorption spectra measured 1 h after peptides injection showed the red-shift from 731 nm for pigment alone to 734 nm for pigment–peptide mixtures monolayers on the saline buffer interface, at 20 mN/m.

The experiments with, and without, salt contents suggest that in the first case the main force causing the formation of the aggregated pigment–peptide complex on the buffer subphase is probably hydrophobic and/or electrostatic interactions. Such interactions are due to the amphiphilicity of peptides rather than the presence of histidine and make the 7–8 nm red-shift of Q_y absorption of BChl *c* (Fig. 5). It indicates that the peptides which mark the spectral properties of chlorosomal proteins, are able to stabilize the aggregated pigment probably by the formation of a hydrophobic environment, similar to that found in cytoplasm in vivo or which was made by monogalactosyl diglyceride lipids in vitro [31]. This aggregated BChl *c*-peptide complex reconstituted at the interface possesses optical properties similar to those observed for chlorosome [1,32].

It was reported [1] that the antibodies prepared against the 5.7 kDa proteins, which appeared to be the only proteins that might be directly involved with the organization of chlorosomal pigments, react with the chlorosome surface, and a little is known about the function of the other chlorosome proteins. It was also described [32] that the absorption of the chlorosome after thermolysin treatment

showed the blue-shift around 6–8 nm and the protein composition of chlorosome changed. In this study, at 20 mN/m the opposite effect was observed caused by the interaction of amphiphilic peptides with the aggregated BChl *c* in the Langmuir monolayer. When the ability of peptides to interact with aggregated pigment is lost, due to the salt content in buffer solution, the peptides are possibly covered by a hydrated shell, and no significant changes in the absorption spectra are observed. Based on the above results, we assume that peptides might surround the pigment aggregates on the subphase, thus forming a layer similar to the lipid monolayer in native chlorosomes. The environmental changes around pigment molecules caused by interaction with the peptides are clearly seen from the spectral data (Fig. 5). The main force is probably hydrophobic interaction between peptides and the periphery of the pigment aggregates. It seems that our results might support the idea that the protein in vivo stabilized the shape and structure of chlorosome rather than direct binding to the BChl *c* molecules as predicted by the model of Feick and Fuller [17].

The present results have demonstrated that aggregates of BChl *c* can be formed in a two-dimensional system at the air–water interface. The surface pressure–-area isotherms showed that the monolayer organization varied as a function of surface pressure (or area), and the in situ absorption patterns of BChl *c* differ due to the spatial organization and to the physical state of pigment molecules which can exist as a monomers (up to 13 mN/m) and/or aggregates (above this pressure). It seems that the 731 nm Q_y BChl *c* band reflects the spectral properties of an amorphous non-specific pigment aggregate formed in the Langmuir film at the interface. The amphiphilic peptides stabilized such an aggregated form, most probably by the formation of a hydrophobic environment and made a chlorosomal-like structure.

The BAM technique visualized the BChl *c* monomolecular films in situ without a fluorescence probe showing that the refracting properties of pigment changed depending on the molecular aggregation of the pigment (monomer or aggregate) which could be easily recognized from BAM images.

The unique properties of BChl *c* and its interaction with amphiphilic peptides on the air–water interface enable us to mimic the chlorosomal-like structure. The applied Langmuir technique allows us to create the two-dimensional ordered model system for further studies of the relationship between structure and function in chlorosome.

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