Quinone Pathways in Entire Photosynthetic Chromatophores of *Rhodospirillum photometricum*

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In photosynthetic organisms, membrane pigment–protein complexes [light-harvesting complex 1 (LH1) and light-harvesting complex 2 (LH2)] harvest solar energy and convert sunlight into an electrical and redox potential gradient (reaction center) with high efficiency. Recent atomic force microscopy studies have described their organization in native membranes. However, the cytochrome (cyt) bc₁ complex remains unseen, and the important question of how reduction energy can efficiently pass from core complexes (reaction center and LH1) to distant cyt bc₁ via membrane-soluble quinones needs to be addressed. Here, we report atomic force microscopy images of entire chromatophores of *Rhodospirillum photometricum*. We found that core complexes influence their molecular environment within a critical radius of ∼250 Å. Due to the size mismatch with LH2, lipid membrane spaces favorable for quinone diffusion are found within this critical radius around cores. We show that core complexes form a network throughout entire chromatophores, providing potential quinone diffusion pathways that will considerably speed the redox energy transfer to distant cyt bc₁. These long-range quinone pathway networks result from cooperative short-range interactions of cores with their immediate environment.

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Keywords: atomic force microscopy; membrane protein; membrane structure; photosynthesis; quinone diffusion

**Introduction**

Photosynthesis is an important biological process performed by plants, algae and some bacteria using several pigment–protein complexes in specialized photosynthetic membranes. In photosynthetic purple bacteria, this process requires high connectivity between several different membrane protein complexes: the peripheral light-harvesting complex 2 (LH2), the central light-harvesting complex 1 (LH1), the photochemical reaction center (RC) and a proton-translocating cytochrome (cyt) bc₁ complex that converts energy into an electrochemical potential gradient, as well as the ATP synthase, which is able to convert energy into a phosphodiester bond of ATP. The pigment–protein complexes (including the RC and LHs) are organized to form the photosynthetic unit (PSU) and are located in the specialized intracytoplasmic membranes.

Up to now, a wealth of structural information on individual photosynthetic proteins has been acquired for LH2, the RC, the bc₁ complex and the ATP synthase. The pigment–protein complexes (including the RC and LHs) are organized to form the photosynthetic unit (PSU) and are located in the specialized intracytoplasmic membranes.

Atomic force microscopy (AFM), with high lateral resolution, high signal-to-noise ratio and the possibility of nano-manipulation, has developed into a powerful tool to directly and precisely visualize biological samples under physiological conditions. Based on the advantages of the technique, AFM has significantly advanced the elucidation of the native architecture of photosynthetic membranes. The first views of the photosynthetic apparatus at submolecular
resolution provided insights into the multicomponent energy-transducing membranes from different photosynthetic bacteria: Blastochloris viridis, Rhodospirillum photometricum, Phaeospirillum molischianum and Rhodopseudomonas palustris. Based on such data, three-dimensional models of a PSU have been elaborated. The various strategies that purple bacteria have evolved for the harvesting and utilization of light energy have been reviewed.

The photosynthetic membranes in R. photometricum are stacked disc-like invaginations of the cytoplasmic membrane. High-resolution AFM images have provided deep insights into the assembly of LH2 antennae and core complexes. Furthermore, in membranes adapted to different light intensities, the photosynthetic apparatus organized in two types of membrane domains: domains of hexagonally packed LH2 and domains containing disordered core complexes with LH2 antennae following a eutectic phase behavior with $\sim 3.5$ LH2 rings per core. Such an assembly pattern of photosynthetic protein complexes has been interpreted to be essential for excitation energy transfer in the PSU (Fassioli F. et al., submitted to Biophys. J.) and to be a prerequisite for rapid quinone/quinol (Q/QH2) diffusion.

In this work, we have imaged entire chromatophore sacs of R. photometricum using AFM. The chromatophores showed a B850/B800 ($A_{847}/A_{881}$) absorption ratio of 1.86, offering an unstudied medium-light-adapted membrane architecture, different from those with ratios of 2.6 and 2.1 previously studied. The tetraheme cytochrome (4Hcyt) subunit of RC on the periplasmic surface of the chromatophores from R. photometricum was visualized for the first time. Imaging of cytoplasmic and periplasmic surfaces of the chromatophores proved that surface adsorption does not alter the supramolecular architecture of the complexes. Detailed pair correlation function (PCF) analysis allowed understanding of short- and long-range membrane organization. Based on this and on the analysis of lipid membrane spaces around core complexes, we depict quinone diffusion pathways that imply a favorite assembly of core complexes interspaced by an LH2 ring and a critical short-range radius of only $\sim 250$ Å inside within which cores influence their molecular environment.

**Results**

**Absorption spectrum of the isolated chromatophores from R. photometricum**

The absorption spectrum of the chromatophore membranes isolated from R. photometricum and analyzed here is illustrated in Fig. 1. Most of the absorption bands originate from the bacteriochlorophyll (BChl) molecules present in the membrane: BChls have a Soret band near 371 nm; the 480-nm band is mainly due to carotenoid embedded within the pigment–protein complexes; absorption bands at 794 and 847 nm are attributed to the BChl in LH2; and the 881-nm band arises mainly from LH1. The arrows show the near-infrared absorption bands of LH2 at 794 nm (1), LH2 at 847 nm (2), and LH1 at 881 nm (3), consistent with previous results. The inset shows room temperature near-infrared absorption in the region of 800–900 nm. The normalized absorption spectra of the chromatophores under different light conditions are compared. The recorded absorption ratio of $A_{847}/A_{881}$ was 1.86 for medium-light-adapted membranes analyzed in this work (black line), in between absorption ratios of 2.6 and 1.6 for low-light-adapted (dark gray line) and high-light-adapted (light gray line) membranes, respectively.

**The distribution of core complexes in the entire chromatophores**

Overview AFM topographs of six entire vesicular chromatophores absorbed on mica are shown in Fig. 2. These chromatophores are sacs, with diameters ranging from 400 to 500 nm, and consist of two membranes stacked on top of each other. All vesicular chromatophores that we have seen shared the same overall architecture, with similar core complex distributions, with the six representative chromatophores...
shown (Fig. 2). The main protruding features (∼2 nm) observed on the tip-exposed membrane surface are attributed to the H-subunit of the photosynthetic RC, which protrudes into the cytoplasm. In contrast, a slightly opened chromatophore partially exposed the periplasmic surface inside the chromatophore sac (Fig. 2a). On the periplasmic surface, the strongly protruding structures (∼4 nm) are assigned to the 4Hcyt subunits of RCs, which were imaged in R. photometricum for the first time. This is reminiscent of the structure of RC existing in B. viridis.7,16 Similar to our previous studies on R. photometricum,18 it is worth noting that no feature attributable to the dimeric cyt bc\textsubscript{1} complex was visible on the periplasmic surface as well (see also Supplementary Material 1), given that the cyt bc\textsubscript{1} complex is expected to protrude into the periplasm.\textsuperscript{11}

A more detailed analysis on a chromatophore sac structure is shown in Fig. 3. In height (Fig. 3a) and deflection (Fig. 3b) images, we viewed the stacked double-bilayer architecture of entire chromatophore sacs. The periplasmic surface of the bottom membrane (Fig. 3b, area 1) exposing non-membranous 4Hcyt subunits and the cytoplasmic surface of the top layer (Fig. 3b, area 2) featuring the strongly protruding H-subunits of the RCs can both be seen. Cross-sectional analysis in the height AFM image revealed that the 4Hcyt subunits on the lower periplasmic membrane surface protruded by 3.7±0.5 nm (n=20), stronger than the H-subunits with a protrusion height of 1.9±0.2 nm (n=50) above the top cytoplasmic membrane (Fig. 3c). This is comparable with the X-ray crystallographic data of RC.\textsuperscript{7} With the use of feedback control in contact-mode AFM, the apparatus tries to scan a sample with a fixed cantilever deflection (i.e., constant force). However, the deviation of this prefixed deflection value is used for driving the feedback, and therefore the deflection image is actually the error signal of feedback delay, directly proportional to the surface corrugation amplitude. More protruding objects give stronger deflection signals with identical feedback speed. Generally, the deflection signal is more sensitive than the height signal. Therefore, the strong signal detectable in the deflection AFM image is used for precise complex position definition for PCF analysis (Fig. 3d). The cantilever deflection is also increased on the further protruding 4Hcyt compared with the H-subunit on the other side of the RC. PCF analyses, on the basis of the acquired AFM images, result in probability graphs reporting the core complex distribution on the periplasmic (Fig. 3e) and cytoplasmic (Fig. 3f) membrane surfaces. In both cases, short-range PCF peaks corresponding to core complexes in direct contact (∼120 Å) and to core–LH2–core assemblies (∼175 Å) were observed. The similar PCF analysis pattern of core complexes on both cytoplasmic and periplasmic surfaces provided strong evidence that no reorganization of protein components in the membrane driven by adsorption onto the mica...
support took place. This was also confirmed by high-resolution imaging (Supplementary Material 1).

Two-dimensional PCF analysis based on the AFM topographs allowed us to have an overview of the lateral distribution of the core complexes in the individual entire chromatophores (Fig. 3e and f). In order to uncover preserved structural organization, we averaged the PCF analyses of several chromatophores. The average PCF analysis showed significant peaks at distances of only up to \( \sim 250 \) Å, indicating that complexes influence their local environment in the order of magnitude of their own size but not at a longer range (Fig. 4a).

Distinct core pair distance probabilities of 1.25 at 127±8 Å (peak and full width at half-height of a Gaussian fit of the peak) and 1.7 at 179±21 Å were found, corresponding to core–core and core–LH2–core assemblies, respectively (Fig. 4b). A weak peak of 1.1 appears at 256±32 Å. The significant pair probabilities at short distances indicate that the core complexes influence their molecular environment up to a radius of \( \sim 250 \) Å and assemble in a most favorable pattern in which two cores are separated by one LH2.

**Size mismatch between LH2 and core complexes creates a lipid environment around cores**

One of the remaining enigmas of the supramolecular assembly of photosynthetic complexes is the absence of cyt \( bc_1 \) in proximity to RCs; indeed, the \( bc_1 \) complex was not observed in any purple bacterial membranes studied so far. This absence implies that Q/QH\(_2\) must travel far to find a distant \( bc_1 \) complex.
complex. We have previously reported that the membrane regions in which core complexes cluster were less rigid than the protein-dense paracrystalline antenna domains (Fig. 5a), interpreted to favor Q/QH₂ diffusion in core complex proximity and to inhibit Q/QH₂ diffusion through LH2-dense regions. Indeed, diffusion of membrane-dissolved Q/QH₂ needs a lipid environment, as found around cores (Fig. 5b).

In this study, we went further by analyzing the direct local environment of the core complex and LH2 depending on their molecular neighbors. From our PCF analysis, we know precisely the outer interaction diameters of LH2 ($D_{\text{LH2}}=75\,\text{Å}$) and the core complex ($D_{\text{CORE}}=127\,\text{Å}$). From these, we can calculate the free angular segment in any complex assembly (Supplementary Material 2). Analyses of complex architectures with different central complexes and different numbers of different kinds of neighboring complexes were performed, and the free angular segment occurring on the central complex was calculated (Table 1). We found that the free angular segment on the perimeter of a core complex is on average significantly larger than that of LH2’s. The majority of core complexes are surrounded by seven loosely packed LH2’s providing 55° of free perimeter. In contrast, an LH2 surrounded by six LH2’s in a paracrystalline packing has a saturated perimeter (0°). It is noteworthy that domains consisting of hexagonal LH2 paracrystals grow under low-light conditions. We have ordered the complex assembly patterns in Table 1 from bottom to top by increasing lipid space on the complexes’ perimeters. Given the lipophilicity of the membrane-dissolved Q/QH₂, quinones will be found preferentially in membrane regions resembling those shown in the top rows of Table 1. In other words, Q/QH₂ diffuse in the core-rich regions in close proximity to the LH1–RC core complexes where they are reduced and do not enter the LH2 areas shown at the bottom of Table 1. Since LH2-occupied areas make about 60% of the membrane surface (considering the interaction diameters of 75 and 127 Å for LH2 and the core complexes, respectively, and the LH2/core ratio of 4.8 in this sample), Q/QH₂ diffusion will be accelerated to find distant cyt $bc_1$ complexes. Therefore, the size mismatch of the ‘rare’ core complexes within the more abundant LH2’s intrinsically accumulates Q/QH₂ in close proximity to cores.

It cannot be excluded that additional aspects are needed or contribute to a favored Q/QH₂ passage. Comparing the complex arrangements depicted in Fig. 5, frequent in the native membrane are one with LH2’s in a hexagonal arrangement (Fig. 5a) and another with a central core complex surrounded by seven LH2’s in contact with the core and a gap, with an eighth LH2 plugging the gap at a larger distance (Fig. 5b). It might be argued that the eighth LH2 in proximity to the core equally hampers Q/QH₂ passage. However, in this assembly (Fig. 5b), the situation is still significantly different compared with the LH2 surrounded by six LH2’s (Fig. 5a): First, the remaining free angular space (55°; see Table 1) on a core perimeter, with seven LH2 neighbors and an eighth at a larger distance, can be distributed between any of the seven LH2’s and create, maybe dynamically, gaps at places different from where the eighth LH2 is situated—a situation that is impossible in the saturated assembly of six LH2’s around a central LH2, where all rings have the same size. Second, our analysis shows that with highest probability a second core will be close to such a core and ‘create’ itself a similar situation with a gap just beside it. Third, due to the different size of the core compared with the LH2, the angular arrangement of the LH2 neighbors around a core is also different and variable compared with the constant 60-deg assembly of LH2 in LH2-
only regions. Intuitively, such a non-ordered assembly is also more diffusible compared with the paracrystalline assembly in LH2 regions. Taken together, angular variability and gap-opening dynamics may be additional aspects favoring Q/QH2 diffusion around cores, beyond the size-mismatch-creating perimeter gaps.

**Quinone pathways in the entire chromatophores**

Above we have seen that quinones will, due to their lipophilic nature, diffuse in the lipid spaces close to core complexes. We have also shown, through PCF analysis, that core complexes reveal the same distributions not only over entire chromatophores but

<table>
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<th>Image</th>
<th>Sketch</th>
<th>Central complex</th>
<th>LH2 neighbors (n)</th>
<th>Core neighbors (n)</th>
<th>Free angular segment (°)</th>
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<td>LH2</td>
<td>5</td>
<td>1</td>
<td>—</td>
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The column marked by a dash indicates that the assembly is (and other rare assemblies are) geometrically ‘impossible’ if all complexes were in direct contact. In this case, it appears that the LH2’s are separated from one another. With increasing distance from a complex, the angle occupied on the perimeter of the central complex decreases (Supplementary Material 2).
also among the chromatophores of cells grown in the same flask. In addition, PCF analysis indicated that membrane protein interactions are only of short range; indeed, cores influence their membrane environment to a maximum extension of $\sim 250 \text{ Å}$. How can such a system ensure efficient reduction energy transfer to distant cyt $bc_1$ complexes? To investigate this, we have computed the core interactions on the basis of our chromatophore AFM images. Using the experimental coordinates of all core complexes within the chromatophores, we evaluated the connectivity of core complexes as a function of their interaction distance. Entire core interactions are found within the significant short-range distance ($\leq 250 \text{ Å}$) that has been shown to emerge from the PCF analysis as the critical distance within which a core influences its environment. Interestingly, this resulted, readily and without further treatment, in long-range networks that we depict as quinone diffusion pathways (Fig. 6; see Materials and Methods). Hence, it seems that the answer lies in the use of cooperative short-range interactions to create a long-range membrane structure. We have tested the use of shorter ($\leq 150 \text{ Å}$) and longer ($\leq 350 \text{ Å}$) interaction radii to create networks. Unconnected ‘core islands’ within the chromatophore emerge in the first case, while the entire chromatophore would be perfused by quinones in the second. We feel that this is strong evidence that nature has developed a simple mechanism to guide quinone diffusion: the assembly size mismatch of molecules ensures a more lipid environment in core proximity and the ‘crystallization’ of the excess LH2 to form impenetrable domains. Well-defined Q/QH$_2$ pathways will direct quinone diffusion to distant $bc_1$ complexes and therefore speed this bottleneck process. This will allow $bc_1$ complexes to be situated at the interface between photosynthetic and respiratory machineries, where they are best placed to function under changing environmental conditions.

Discussion

It has been shown that the organization of the photosynthetic apparatus and the relative ratio between LH2 and LH1 in the photosynthetic membrane are modulated in response to light intensity.$^{18,27}$ As a surprise, it has been found that core complexes remain in similar molecular environments whatever the growth conditions were.$^{18}$ Indeed, as shown by PCF analysis, cores influence the membrane organization in an $\sim 250$-Å-radius environment, with a most probable assembly (core–LH2–core) at an $\sim 180$-Å radius. This finding and the fact that LH2 paracrystals were rigid$^{27}$ led us to think that the core environment was an adaptation for Q/QH$_2$ diffusion, especially in light of the fact that the cyt $bc_1$ complex has so far never been observed in proximity to cores.$^{10}$

This specific core environment may have important physiological implications. First, it might play a key role in energy transfer by maximizing the capacity for excitation capture under different light conditions (Fassioli F. et al.,$^{28}$ submitted to Biophys. J.). Second, the size mismatch of the core complexes with the more abundant LH2’s breaks the dense hexagonal packing of LH2 rings and locally creates a more lipidic environment favorable for Q/QH$_2$ diffusion.

It is important to note that core complexes also form hexagonal lattices, similar to LH2’s, in species devoid of LH2, such as $B. \text{ viridis}$,$^{16}$ but also in $R. \text{ palustris}$. This would be very unfavorable for Q/QH$_2$ transfer. However, core complexes are not circular, but the LH1 assembly is elliptic following the long RC axis.$^{16}$ We have measured an LH1 ellipticity of 10% to 15% in native membranes,$^{23}$ in agreement with X-ray analysis.$^9$ Following our considerations (Supplementary Material 2), lipid gaps of about 4° (corresponding to $\sim 16$ Å) are formed on the perimeter of an elliptic hexagonally packed core complex; this would be sufficient for Q/QH$_2$ diffusion.

So far, there is still no microscopic evidence for the presence of the dimeric cyt $bc_1$ complex in the photosynthetic membranes in the various species examined.$^{16,19-25}$ This may lead to the assumption that cyt $bc_1$ complexes are not in proximity to many core complexes. However, this is in stark opposition to the generally accepted view emerging from spectroscopic analysis that showed efficient coupling between core and cyt $bc_1$ complexes, interpreted as localization proximity or even the formation of supercomplexes.$^{52}$ The long-range quinone pathways as we describe them here may reconcile the discrepancy between functional measurements—rapid
connection between RCs and cyt $bc_1$ complexes—and structural analyses by AFM—absence of cyt $bc_1$ complexes. Indeed, the quinone pathway networks will quickly furnish reduction energy from cores to distant cyt $bc_1$ complexes.

**Materials and Methods**

**Bacterial culture and membrane preparation**

*R. photometricum* (DSM 122) was grown anaerobically and photoheterotrophically on modified Hutner medium under medium-light (30 W m$^{-2}$) conditions and harvested in late-log phase. Cells were harvested and washed twice with 10 mM Tris–HCl, pH 7.0, and then broken in 20 mM Tris, pH 8.0, 0.5% ethylenediaminetetraacetic acid and 25 μg mL$^{-1}$ of DNase by a single passage through a French pressure cell. Lysates were loaded directly onto 5% about 40% sucrose and contained the different proteins of the photosynthetic apparatus. The membranes were washed with 10 mM Tris–HCl, pH 8.0, in a centrifugal concentrator (220,000 × g, 1.5 h) and kept at 4 °C for AFM analysis.

**Atomic force microscopy**

A Nanoscope-E atomic force microscope (Veeco, Santa Barbara, CA, USA) equipped with a 160-μm scanner (J-scanner) and oxide-sharpened Si$_3$N$_4$ cantilevers (length=100 μm, k=0.09 N/m; Olympus Ltd., Tokyo, Japan) was operated in contact mode at ambient temperature and pressure. For imaging, minimal loading forces of ~100 pN were applied at scan frequencies of 4–7 Hz using optimized feedback parameters. The mica supports were immerged in 40 μL of adsorption buffer (10 mM Tris–HCl, pH 7.5, 150 mM KCl and 25 mM MgCl$_2$). Subsequently, 2 μL of membrane solution was injected into the buffer drop. After ~1 h, the sample was rinsed with recording buffer (10 mM Tris–HCl, pH 7.5, and 150 mM KCl).

**Data analysis**

All data analyses were performed using self-written routines in C or Java plug-ins for the ImageJ image processing package. For two-dimensional PCF, a program into which the $x$ and $y$ coordinates of all complexes within an image delimited by a polygon are entered was written. The program calculates the separation vector, $r$, for all pairs of complexes ($AB$)—hence, the probability $p(r)$ of finding a complex ($B$) in a distance interval of $(r±dr)$ from a given complex ($A$). This is normalized with respect to the surface area included within the radial interval and the total number of complexes ($B$) within the membrane. If the distribution is random, $p(r)$ is always close to 1.

Quinone pathway maps of chromatophores were constructed from the RC coordinate lists by joining each RC to all other RCs within a test radius. Specifically, we computed the interactions between any core complex and its neighboring core complexes and then connected the core pairs by lines. We have studied the generation of core networks from a short radius ≤150 Å to a larger radius ≤350 Å. All RCs are connected in the network at a critical radius ≤250 Å in Fig. 6.

**Acknowledgements**

This study was supported by an ANR (Agence Nationale de la Recherche) PNANO-06-023 grant (to S.S. and J.S.) and by a research support grant from ‘Ville de Paris’ (to S.S.).

**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2009.07.044.

**References**


