Photochemical & Photobiological Sciences

Accepted Manuscript

This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/pps

Dehydration affects the electronic structure of the primary electron donor in bacterial photosynthetic reaction centers: evidence from visible-NIR and light-induced difference FTIR spectroscopy

Marco Malferrari[1], Paola Turina[2], Francesco Francia[1], Alberto Mezzetti[3,4], Winfried Leibl[4], Giovanni Venturoli[1,5,*]

[1]Dipartimento di Farmacia e Biotecnologie, FaBiT, Università di Bologna, Bologna, Italy

[2]Dipartimento di Scienze Biologiche, Geologiche e Ambientali, BiGeA, Università di Bologna, Bologna, Italy

[3] LASIR, UMR 8516, Université Lille 1, Cité Scientifique, 59655 Villeneuve d'Ascq, France

[4] Service de Bioénergétique, Biologie Structurale et Mécanismes UMR 8221, IBITeC-S, Bat 532, CEA-Saclay, 91191 Gif-sur-Yvette, France

[5] Consorzio Nazionale Interuniversitario per le Scienze Fisiche della Materia (CNISM), c/o Dipartimento di Fisica e Astronomia, DIFA, Università di Bologna, Bologna, Italy.

[*]Corresponding author:

e-mail: giovanni.venturoli@unibo.it; Tel. +39 051-2091288; Fax: +39 051 242576

Abbreviations: BChl, bacteriochlorophyll; Bphe, bacteriopheophytin; CTAB, cetyl-trimethyl-ammonium bromide; ENDOR, electron nuclear double resonance; EPR, electron paramagnetic resonance; FTIR, Fourier transform infrared; HOMO, highest occupied molecular orbital; LDAO, lauryldimethylamine N-oxide; MO, molecular orbital; NIR, near infrared; P, primary donor; $Q_{A,B}$, quinones in the RC; Q_y, optical transition in BChls; *Rb.*, *Rhodobacter*; RC, reaction center;; TRIPLE, electron-nuclear-nuclear triple resonance.

Abstract

The photosynthetic reaction center (RC) is a membrane pigment-protein complex that catalyzes the initial charge separation reactions of photosynthesis. Following photoexcitation, the RC undergoes conformational relaxations which stabilize the charge-separated state. Dehydration of the complex inhibits its conformational dynamics, providing a useful tool to get insights into the relaxational processes. We analyzed the effects of dehydration on the electronic structure of the primary electron donor P, as probed by visible-NIR and light-induced FTIR difference spectroscopy, in RC films equilibrated at different relative humidities r. Previous FTIR and ENDOR spectroscopic studies revealed that P, an excitonically coupled dimer of bacteriochlorophylls, can be switched between two conformations, P_{866} and P_{850}, which differ in the extent of delocalization of the unpaired electron between the two bacteriochlorophyll moieties (P_L and P_M) of the photoxidized radical P^+. We found that dehydration (at r = 11%) shifts the optical Q_y band of P from 866 to 850-845 nm, a large part of the effect occurring already at r = 76%. Such a dehydration weakens light-induced difference FTIR marker bands, which probe the delocalization of charge distribution within the P^+ dimer (the electronic band of P^+ at 2700 cm^{-1}, and the associated *phase-phonon* vibrational modes at around 1300, 1480, and 1550 cm^{-1}). From the analysis of the P^+ keto C=O bands at 1703 and 1713-15 cm^{-1}, we inferred that dehydration induces a stronger localization of the unpaired electron on P_L^+. The observed charge redistribution is discussed in relation with the dielectric relaxation of the photoexcited RC on a long (10^2 s) time scale.

1. Introduction

The reaction center (RC) of purple photosynthetic bacteria has become a model system in the study of dynamics/function relationships in proteins which catalyze light-induced electron transfer processes. This integral membrane pigment-protein complex promotes the early photochemical events in the conversion of solar excitation energy into chemical free energy[1]. Two protein subunits, L and M, spanning the photosynthetic membrane and related by an approximate C_2 symmetry, form the scaffold to bind the cofactors, which, in the RC from *Rhodobacter* (*Rb.*) *sphaeroides*, studied in the present work, are: four bacteriochlorophylls (BChl) a, two bacteriopheophytins (BPhe) a, two quinones (Q_A and Q_B), a carotenoid, and a non-heme iron. Although also the BChl, BPhe and quinone pigments are arranged in two pseudo-symmetrical branches, associated to the L and M subunits and sharing their approximate C_2 symmetry axis, the initial electron transfer proceeds along the L-branch only. Following photoexcitation, a dimer P of BChl a molecules (called P_L and P_M according to their coordination by a histidine residue of either the L or M subunits), acts as the primary donor by delivering an electron, via an intermediate BPhe cofactor, to the primary quinone acceptor Q_A. Since P and Q_A are located close to the periplasmic and cytoplasmic sides of the complex, respectively, this sequential electron transfer leads (within ~ 200 ps) to electric charge separation across the protein dielectric. Formation of the primary charge separated state $P^+Q_A^-$ is followed by electron transfer to the secondary quinone acceptor Q_B on the 100-μs time scale[1-3].

During the last three decades, a large body of experimental studies, based on biochemical[4], crystallographic[5, 6] and spectroscopic approaches[7-15], has demonstrated that light-induced electron transfer processes are associated to RC conformational rearrangements, which affect the stability of the charge separated state. These conformational relaxations from dark- to light-adapted RC states involve most likely different processes, which influence selectively specific electron transfer steps and encompass a large time scale (from 10^{-6} to 10^2 s), as indicated by studies in which the RC was photoexcited by short laser pulses[8, 10] or by continuous prolonged illumination[11-16].

Despite extensive efforts, a clear, complete picture of the structural changes associated to the photo-activity of the RC is still lacking. Different conformational events, localized in different protein domains, have been proposed to participate in the RC response to charge separation: (a) small- and large-scale protein structural changes on the quinone acceptor side of the RC[5, 9]; (b) dielectric relaxation of water molecules weakly bound to the RC in proximity of Q_A[5, 16, 17]; (c) proton uptake and fast protonation of aspartate or glutamate residues on the cytoplasmic RC side[18]; (d) rearrangements involving groups of the primary donor P[16], as well as amino acid residues and bound water molecules near P[12, 13, 19]; (e) redistribution of the electric charge density, within the P^+ dimer, between the two BChl molecules (P_L and P_M) which form the primary electron donor[19, 20].

In a series of papers[21-29], we have shown that a useful approach in the study of the conformational protein dynamics associated with RC photo-activity consists in varying the hydration state of the RC. In fact, dehydration of the RC, both in the absence[29] and in the presence of a sugar[21, 22, 24-26, 28] or polymeric[23] solid matrix, inhibits progressively and selectively the RC dynamics, and affects specific electron transfer steps. It came out that several effects of inhibition of protein dynamics on electron transfer, observed at cryogenic temperatures in hydrated RCs[8, 10], are mimicked in dehydrated RCs at room temperature[21-29].

In an attempt to better characterize the structural rearrangements which stabilize the charge-separated $P^+Q_A^-$ state, this dehydration approach has been combined with light-minus-dark difference FTIR spectrroscopy[16], a method which proved to be very powerful in identifying light-induced structural changes involving cofactors, protein residues, and solvent molecules interacting with the RC[9, 16, 17, 30-34]. In this previous study[16] we focused on the comparison between moderately and strongly dehydrated RC-films, equilibrated at relative humidity r = 76% or r = 11%. Several changes, induced by RC dehydration in the light-minus-dark difference FTIR spectra, could be identified, and attributed to water molecules hydrogen-bonded to the RC in the vicinity of Q_A, to NH or OH stretching modes of amino acid residues, as well as to the 9-keto C=O stretching modes of the two BChls of P[16]. The latter finding pointed to a possible role of structural rearrangements occurring on P (and in its local environment) in the stabilization of the charge separated $P^+Q_A^-$ state upon prolonged illumination.

Dehydration-induced reorganizations in the dimeric structure of P are also suggested by the blue shift observed upon RC dehydration[21, 23] in the position of the absorption band peaking at 866 nm in RC suspensions at pH = 8. This NIR band of the RC is identified as the low energy exciton component of the Q_y transition of the BChls of the P dimer[35-37]. Upon dehydration of RC incorporated into polyvinyl alcohol (PVA) films[23], or embedded into glassy trehalose matrices[21], the band maximum shifts from 866 nm to 850 nm. Such an effect has been observed as a result of a number of different perturbations of the P environment, including e.g. changes in the detergent type and concentration[38-40], as well as single point mutations in the proximity of P[41]. Investigations on the electronic structure of the radical cation P^+ by electron paramagnetic resonance spectroscopy and related multiple resonance techniques (ENDOR/TRIPLE) showed that the ~16 nm blue shift of the Q_y band of P is systematically associated with a significant change in the spin density distribution of P^+ within the dimer, which becomes more asymmetric when the Q_y band is blue shifted[20, 40, 42, 43]. It has been inferred that two distinct conformations of the primary electron donor exist, called P_{866} and P_{850} from the position of the Q_y band, and differing in the extent of delocalization of the unpaired electron over the P_L and P_M halves of the P^+ dimer [20].

The symmetry/asymmetry of the charge distribution within the P^+ dimer is consistently probed by the intensity of a broad absorption band of the light-minus-dark P^+Q^-/PQ FTIR difference spectrum, appearing in the mid-IR near 2700 cm^{-1} in *Rb. sphaeroides* RCs[30]. This band has been shown to correspond to an electronic transition of the photoxidized primary donor and to reflect the magnitude of the resonance interaction between the two BChls P_L and P_M of the P^+ dimer[30, 44]. Additionally, three difference IR marker bands, observed at ≈ 1550, 1480, and 1300 cm^{-1}, have been proved to be mostly related to the BChl dimeric nature of P^+ [31, 44]. The presence and intensity of these so called *phase-phonon*[45, 46] bands appear to be systematically correlated with those of the 2700 cm^{-1} transitions[19, 47-50], i.e. with the delocalization of the charge on the P^+ dimer.

In the present paper, we have studied the effect of RC dehydration on the electronic structure of the P dimer, by examining RC-films equilibrated at different relative humidities. In order to get information on charge localization within the Bchl P^+ dimer, we have analyzed, as a function of the relative humidity, the shift of the Q_y band of P and selected regions of the light-minus-dark difference FTIR spectrum, which include the electronic band of P^+ at 2700 cm^{-1} and the three *phase-phonon* bands in the 1600-1200 cm^{-1} range. We have found that dehydration results in a more localized charge distribution as compared to RC in solution, and that the transition from the P_{866} to the P_{850} conformation occurs over a large part of the RC population already under moderately dehydrated conditions (at r = 76%). From the response to dehydration of the spectral contributions attributed to the 9-keto C=O modes of P_L and P_M, we concluded that dehydration leads to an increased charge density on the P_L BChl moiety of P^+.

2. Experimental

2.1 RC purification and sample preparations

RCs were isolated from *Rb. sphaeroides wt* 2.4.1 strain and purified as described previously[51], using lauryldimethylamine-N-oxide (LDAO) as a detergent. The ratio of the absorption at 280 and 800 nm was 1.3. RC-LDAO films were prepared on CaF_2 windows following essentially the procedure described in detail in ref.[29]. Briefly, 40-60 μL (in the case of RC films for light-induced difference FTIR measurements) or 160 μL drops (for visible-NIR measurements) of a solution containing 60 μM RC, 10 mM TRIS HCl buffer at pH = 8.0, 0.025% LDAO and 10 mM o-phenanthroline were laid on the CaF_2 window and dried for about 4 hours under N_2 flow at room temperature. The inhibitor o-phenanthroline was used to block light-induced electron transfer from the primary photoreduced Q_A^- to the secondary Q_B quinone acceptor, in order to obtain pure $P^+Q_A^-$ /PQ_A light-induced FTIR spectra. Visible-NIR measurements were also performed in RC-films prepared in the absence of o-phenanthroline.

The control of the hydration state of the RC-LDAO films was achieved by an isopiestic method[29], which consists in equilibrating the sample with hydrating saturated salt solutions, providing defined values of relative humidity r. Equilibration was obtained by placing the window on which the RC film had been formed into a sample holder equipped with a second CaF_2 window, to form a small volume, gas-tight cavity, which also contained the hydrating solution. Visible-NIR and difference FTIR measurements have been performed at temperatures of 295K and 281K, respectively. This difference in temperature has a small effect (see values in brackets below) on the relative humidity corresponding to a given saturated hydrating solution[52]. The following solutions have been used in visible-NIR measurements to obtain the desired r values (indicated in brackets, for T = 295K): KNO_3 (94%), KCl (84%), NaCl (75%), NH_4NO_3 (63%), $Mg(NO_3)_2$ (54%), K_2CO_3 (43%), $MgCl_2$ (33%), LiCl (11%). Light-minus-dark FTIR measurements have been performed at T = 281K in fully hydrated samples (solution RCs), in the presence of KNO_3 (r = 96%), of NaCl (r = 76%), and of LiCl (r = 11%) saturated solutions. By this method, when the relative humidity inside the holders was varied over the explored range (11% ≤ r ≤ 96%), a steady hydration level could be reached in a few hours, as shown by previous measurements[29], in which the content of residual water was monitored by following the evolution of the NIR water combination band around 5155 cm^{-1}. RC-LDAO films, equilibrated at r = 96% and r = 76% with D_2O, were obtained following a sequence of dehydration/re-hydration equilibria, as described in detail by Malferrari and coworkers[53], which yields an efficiency of D_2O replacement larger than 95%.

2.2 Visible-NIR and difference FTIR spectroscopic measurements

Visible-NIR absorption spectra of solution RCs and RC films were recorded on a Jasco V-550 spectrophotometer. Light-minus-dark FTIR difference spectra were recorded essentially as described by Breton and coworkers[54], using a MCT-A detector. The sample was placed in a nitrogen cryostat (Oxford Instruments) to keep the temperature stable within ± 1 °C during the measurements. The mirror speed was set to 2,531 cm s^{-1}, and the interferograms were collected in the double-sided forward-backward mode. Interferograms in the 4000-1000 cm^{-1} range were acquired at 281K, before and during continuous illumination (lasting 20 s). Continuous photoexcitation was provided by a 250 W tungsten halogen lamp collimated by an optical condenser, and filtered by a 8 cm thick layer of thermostated water, a 50 % transmittance neutral density filter, and two coloured glass filters, resulting in a transmitted band centered at 760 nm, with 0.01 transmittance for λ<700 nm and λ>850 nm. The duration of the photoexcitation cycles was controlled by a Uniblitz electro-programmable shutter system (Vincent Associates, Rochester, NY) with opening/closing times ~ 3 ms. Photoexcitation cycles were separated by appropriate dark

periods (80 s or 140 s for RC equilibrated at r = 11% or r = 76% and 96%) to allow a complete recovery of the RC neutral state (PQ_A) from the charge-separated $P^+Q_A^-$ state induced by the 20 s period of continuous illumination. $P^+Q_A^-/PQ_A$ FTIR difference spectra in H_2O were obtained by averaging 2.280.000, 1.300.000, 118.800, and 108.000 interferograms at r = 11%, r = 76%, r = 96% and in fully hydrated conditions, respectively. Spectra in D_2O were obtained by averaging 396.000 at r = 76 %, and 120.000 interferograms at r = 96%.

3. Results

3.1 Effects of dehydration on the Q_y absorption band of the primary donor P

Figure 1 shows the NIR absorption spectra recorded between 650 and 900 nm in solution RC and in a RC film equilibrated at different values of relative humidity r by using the isopiestic method described in the Experimental section. Samples were prepared in the absence of o-phenanthroline. The bands centered at around 760 nm and 800 nm are principally assigned to the Q_y transitions of the two BPhes and of the two monomeric BChls, respectively, while the band at 866 nm in solution RC, as mentioned in the Introduction, is ascribed to the low energy exciton component of the Q_y transition of the BChl dimer P, which acts as the primary electron donor. The assignment of the fourth small band, centered at ~ 670 nm, is uncertain[37]. The main spectral change induced by dehydration is a blue shift of the Q_y band of the P dimer. A small shift is already detectable in the largely hydrated RC films, equilibrated at r = 94%. Further dehydration results in a progressive shift down to 856 nm at r = 75% and to 851 nm at r = 11%. The shift reverts totally upon re-suspension of the most dehydrated (r = 11%) RC film (not shown). A direct comparison of the spectra recorded in the RC film (without normalization to the amplitude of the 804 nm band) does not reveal any significant decrease in the oscillator strength of the 804 nm band (not shown). Also the intensity of the Q_y band of the dimer is not significantly altered upon dehydration (see Figure 1), except for a small decrease when reducing the hydration level from r = 76% to r = 11%. The amplitude of the band around 750 nm is somewhat increased upon formation of the RC film (independently of the relative humidity), possibly reflecting a very limited release of BChls from the RC[37].

The dependence of λ_{max} for the Q_y transition of the dimer upon the relative humidity r is presented in Figure 2 for two RC films, prepared in the absence and in the presence of o-phenanthroline, respectively. The effect of dehydration on the position of the Q_y band of P has been studied also in the presence of o-phenanthroline to allow a comparison with light-minus-dark difference FTIR spectra, which have been recorded in the presence of the inhibitor (see Experimental and section 3.2 and 3.3), in order to block electron transfer from the photoreduced

primary acceptor Q_A^- to the secondary acceptor Q_B. In this way, upon photo-excitation, pure $P^+Q_A^-/PQ_A$ difference FTIR spectra have been obtained, free of any spectral contributions of the $P^+Q_B^-$ state, which, in the absence of the inhibitor, could come from the RC sub-population retaining the secondary quinone acceptor after purification. We have chosen to examine pure $P^+Q_A^-/PQ_A$ difference spectra because most of the FTIR studies of the primary donor available in the literature have been performed at low temperature (100 K), i.e. under conditions in which the electron is no longer transferred from Q_A^- to Q_B (see Discussion). Furthermore, the effects of dehydration on $P^+Q_A^-/PQ_A$ spectra can be discussed in relation with the dielectric relaxation of the photo-excited RC, which has also been studied in the $P^+Q_A^-$ state in the presence of o-phenanthroline[16].

Both in the absence and in the presence of the inhibitor, the large part of the blue shift of λ_{max} for the Q_y transition of the dimer is observed following a decrease in the RC hydration level from solution to r ~ 60%. A smaller blue shift smoothly occurs when RC films are further dehydrated down to r = 11%. The blue shift appears to be systematically larger in the samples supplemented with o-phenanthroline. The additional blue shift in λ_{max} measured in the presence of the inhibitor (3 nm at r=94%) seems to increase with dehydration (6 nm at r = 11%). The blue shift observed under both conditions points to an effect of dehydration on the geometry and electronic structure of the P BChl dimer, indicating that a transition from the P_{866} to the P_{850} RC conformation takes place[20], and suggesting that partial dehydration of RC promotes a redistribution of the electric charge within the BChl dimer of the photoxidized primary donor, reducing the charge delocalization over the two P_L and P_M moieties (see Introduction).

3.2. The response to dehydration of the electronic P^+ band at 2700 cm^{-1} and of the phase-phonon bands in the 1600-1200 cm^{-1} region of the light-minus-dark difference FTIR spectrum

In view of the results presented in the previous section, we have examined the response to dehydration of light-induced difference FTIR bands which provide information on the charge delocalization and the extent of coupling within the two BChl molecules constituting the oxidized primary electron donor. These bands include the broad band, centered around 2700 cm^{-1}, due to an electronic transition of P^+ [30], which dominates the 4000-1000 cm^{-1} region, as well as three bands, centered at ~ 1550, ~1480, and ~1300 cm^{-1} (see Figure 3). The latter bands have been attributed to *phase-phonon* vibrational modes that, being formally forbidden in monomeric BChl molecules, are allowed by coupling to the electronic transition of P^+ [45, 46].

In a previous study[16], by comparing difference FTIR spectra measured in moderately and strongly dehydrated RC-films (at r = 76% and r = 11%, respectively), it was observed that the amplitude of the electronic band of P^+ was decreased in the more dehydrated sample. The smaller

amplitude of the light-induced band, observed at the lower water content, reflected mostly the decreased extent of P steadily oxidized by continuous photoexcitation, as shown by time resolved optical assessment of P photoxidation under the same illumination regime used in the difference FTIR measurments[16]. As a consequence, to compare correctly the light-minus-dark FTIR difference spectra measured at different hydration levels, we have normalized them to the extent of photo-induced P^+, estimated from the amplitude of the differential bands at 1749/1739 cm^{-1}, which have been attributed to the 10a-ester C=O mode of the BChls of the P dimer. The adequacy of this procedure is discussed extensively by Malferrari and coworkers[16].

Figure 3 (panel A) compares the normalized light-minus-dark FTIR difference spectra in the 4000-1000 cm^{-1} range recorded at 281 K in solution RCs and in RC films at different hydration levels, i.e. equilibrated at r = 96%, 76%, and 11%. We focused on this range of hydration levels, because a large part of the blue shift observed in the position of the Q_y optical band of P occurs when the relative humidity at which the RC film is incubated is decreased to r = 76%, with a more limited additional shift observed at lower r values, down to r = 11% (see Figure 2).

As shown in Figure 3A, the amplitude of the normalized electronic band of P^+ in RC films equilibrated at r = 96% is significantly lower than that recorded in solution RCs. Upon further dehydration, the band amplitude is further reduced. Most of the effect occurs however between full hydration (solution) and r = 76%. Only a slight additional weakening of the band takes place when r is decreased from 76% to 11%, in agreement with previous observations[16].

The large noise between 3600 and 3200 cm^{-1} in the spectrum recorded in solution RCs and in hydrated (r = 96%) RC films is due to the background absorption of water in this spectral region (cf. e.g. ref.[19]). The noise is consistently much lower in the spectra recorded in the two dehydrated samples (r = 76%, r = 11% in Figure 3A) and following D_2O substitution even at r = 96% (Figure 3B), see below. The possibility that the stretching band of water, positioned between 3600 and 3200 cm^{-1}, can distort the difference electronic band of P^+ around 2700 cm^{-1}, affecting in particular its amplitude, is rather remote. However, to exclude such an eventuality, we measured, as a control, light-minus-dark difference spectra of RC films after D_2O substitution at r = 96% and r = 76% (Figure 3 B). The attenuation caused by D_2O replacement at both hydration levels on the bands on the top and on the lower wavenumber ridge of the electronic band in the 2700-2200 cm^{-1}, has been already observed (at r = 76 %) and discussed in detail by Malferrari and coworkers[16]. Apart from these changes, the spectra in Figure 3B show that, also in D_2O replaced RC films, dehydration results in a significant decrease of the P^+ electronic band.

Therefore, from the results presented in Figure 3 we infer that dehydration affects the electric charge distribution within the photoinduced cation radical P^+, since the attenuation of the P^+

electronic band is diagnostic of an increased localization of the spin density on one of the two BChl moieties of P^+ (either P_L^+ or P_M^+)[19, 30, 31, 43, 44, 47, 49, 50].

This conclusion is fully supported by the response to dehydration of the three positive *phase-phonon* bands observed in the light-minus-dark spectrum between 1600 and 1200 cm^{-1} (see Figure 4). As mentioned above, the presence of these bands highly correlates with the electronic transition of P^+ at 2700 cm^{-1}, and their intensity is considered a sensitive probe of resonance interaction within the P^+ dimer[19, 44, 47, 49, 50]. Figure 4 shows that the bands centered around 1300, 1480 and 1550 cm^{-1} are progressively weakened upon dehydration of the RC. The intensity of all the three *phase-phonon* bands recorded in RC films at r = 96% appears to be significantly decreased when compared to solution RCs. An additional reduction is observed upon dehydration to r = 76%, while a very limited attenuation occurs when the hydration level is further decreased to r = 11 %, particularly in the bands at ~1300 and 1480 cm^{-1}. The attenuation of the *phase-phonon* bands occurs therefore in parallel with the decrease in intensity of the 2700 cm^{-1} electronic band of P^+.

Following dehydration, also the intensity of the two positive bands centered at 1250 and 1234 cm^{-1} is decreased. These bands are absent, together with the three *phase-phonon* bands, in the spectra of the two heterodimer mutants in which His M200 or His L173 is replaced by Leu[47], and their intensity is affected similarly to the intensity of the 1300, 1480 and 1550 cm^{-1} bands by mutations which introduce hydrogen bonds to the keto carbonyls of P[48]. This strongly suggests that also the bands at 1250 and 1234 cm^{-1} are related to the dimeric nature of P^+. Similar considerations apply to the band centered at ~1570 cm^{-1}, which is progressively weakened by dehydration, in parallel with the band at 1550 cm^{-1}.

Besides the overall attenuation of the bands described above, significant changes occur in the profile of the *phase-phonon* band at ~ 1300 cm^{-1}. When the hydration level is decreased from solution RCs to RC films at r = 76 %, the peak of the *phase-phonon* band shifts from 1297 cm^{-1} to 1309 cm^{-1} (or the shoulder around 1309 cm^{-1}, already observable at r = 96%, increases becoming the band maximum at r = 76%, as suggested by the band profile at an intermediate hydration value, r = 96%). It is noteworthy that a comparable shift of the 1295 cm^{-1} band to 1309 cm^{-1} has been observed in the H-bond mutant LH(M160)[48], which was shown to be characterized by a much larger localization of the spin density on the L-half of the P^+ dimer, as compared to the wild type.[55] Extensive drying (from r = 76% to r = 11%) appears to further affect the profile of the bands at ~1300 and ~1480 cm^{-1}, as previously observed[16]. Also the large band on the high-energy side of the *phase-phonon* region at ~1570 cm^{-1}, weakened by dehydration, shifts to higher wavenumbers, from 1566 cm^{-1} in solution to 1572 cm^{-1} at r = 76%.

The changes observed at strong dehydration in several spectral features of the attenuated bands in the 1600-1200 cm^{-1} region suggest modifications in the structure of the P dimer, affecting most likely C-C and/or C-N and/or C-H porphyrin ring modes[48, 56].

3.3. Effects of dehydration on the light-minus-dark difference spectrum in the 1800-1600 cm^{-1} region

The results presented so far clearly indicate that dehydration perturbs the electronic structure of the BChl P dimer, favouring a markedly asymmetric charge distribution within the two BChls of the P^+ dimer. Some of the alterations induced by dehydration in the light-minus-dark FTIR spectrum are similar to changes produced by single-site mutations which shift the charge density of P^+ to the L-half of the dimer. However, it has to be noticed that the most prominent spectral effects of RC dehydration, i.e. the shift of the Q_y transition of P from 866 nm in solution to 850-845 nm in RC films at r = 11%, as well as the attenuation of the electronic band of P^+ at ~2700 cm^{-1} and of the associated *phase-phonon* bands, are compatible with a large localization either on the L- or on the M-half of the P^+ dimer. For instance, the electronic band of P^+ is absent in both the heterodimer mutants HL(M202) and HL(L173) of *Rb. sphaeroides*[30], for which the unpaired electron is localized on the BChl moiety, i.e. on P_L^+ and P_M^+ respectively[43].

The analysis of the difference spectrum in the 1800-1600 cm^{-1} region can in principle help to discriminate between asymmetric charge re-distribution in favour of P_L^+ or P_M^+. This region includes in fact two positive peaks, positioned at 1713-1715 and 1703 cm^{-1}, which are due to the 9-keto C=O stretching modes of the P_L and P_M BChl molecules, respectively.[31, 48] A re-distribution of the spin density leading to a higher localization of the unpaired electron on either the P_L^+ or the P_M^+ BChl moiety is therefore expected to result in a change of the relative intensity of the two sub-bands.

Figure 5 compares light-induced $P^+Q_A^-/PQ_A$ spectra recorded over this region in solution RCs and in RC films equilibrated at r = 96%, r = 76%, and r = 11%. In solution RCs, the sub-band at 1703 cm^{-1} is more intense than the one centered at 1714 cm^{-1}, in agreement with previous observations[19, 31] in hydrated films of *Rb. sphaeroides* RCs at 100K (which, when cooled in the dark[19], exhibit sub-band peaks at 1713 and 1703 cm^{-1}). Upon dehydration, in RC films at r = 96%, the amplitude of the lower-energy peak decreases, and the intensity of the two sub-bands becomes essentially equal. When RC films are equilibrated at a lower relative humidity, r = 76%, a further reduction of the sub-band attributed to P_M is observed, so that the amplitude of the higher-energy sub-band is now prevailing. Further dehydration does not cause additional changes in the relative intensity of the sub-bands, but only a very small decrease in the amplitude of both of them.

We take the above described reversion in the relative amplitude of the 9-keto C=O sub-bands as a strong evidence that dehydration leads to a substantial increase in the charge density on the P_L half of the P^+ dimer . The dehydration-induced reversion of the relative amplitude of the two sub-bands is accompanied by a slight but significant increase in their splitting. The sub-band attributed to P_M is in fact shifted to 1702 and 1701 cm^{-1} at r = 96% and r = 76%. Conversely, the P_L peak (at 1714 cm^{-1} in solution RCs) moves to higher wavenumbers, i.e. 1716 cm^{-1} at r = 76%. Under extensively dehydrated conditions (r = 11%), the position of the P_L peak remains essentially unchanged (1716-1717 cm^{-1}), while the P_M peak appears to revert towards 1703 cm^{-1}. We notice that a similar increase in the splitting of the 9-keto C=O sub-bands, paralleled by a relative strengthening of the P_L sub-band, has been observed in a mutant of *Rb. sphaeroides*, RE(L135), specifically designed to stabilize, through long-range electrostatic interactions, P_L^{+}[19]. Indeed, ^1H special TRIPLE spectra showed that the ratio of the net spin densities on P_L and P_M , ρ_L/ρ_M, increases from 2.1 in wild-type RC to 3.1 in the RE(L135) mutant[19].

Besides the changes in the 9-keto C=O modes described above, the only other significant alterations in response to RC dehydration over the 1800-1600 cm^{-1} spectral range occur in the amide I region. In this region the amplitudes of the negative signal at ~1669 cm^{-1} and of its positive counterpart at ~ 1658 cm^{-1} are similar in solution RCs and in RC films at r = 96%, but undergo a significant reduction at r = 76 %. This band has been tentatively attributed to a peptide C=O from a transmembrane helix in the environment of the P dimer[31]. Under extensive dehydration (at r = 11%), the negative signal shifts from 1669 cm^{-1} to 1666 cm^{-1}, and increases in amplitude, while the positive signal is further weakened. The sensitivity of the 1669(-)/1658(+) signals to the RC hydration level is also consistent with the notion that the hydration state of the RC affects the local environment of the primary donor P.

4. Discussion

4.1 Effects of dehydration on the electronic structure of the primary donor P

In the present study we investigated the effects of RC dehydration on the electronic structure of the primary electron donor P, as probed by: i) the position of the Q_y optical transition of the BChl dimer P; ii) the light-minus-dark FTIR difference band around 2700 cm^{-1} and the related *phonon-like* bands between 1200 and 1600 cm^{-1} ; iii) the light-induced difference bands attributed to 9-keto C=O stretching modes of P_L and P_M**.** These markers of the dimeric nature of P showed that the RC hydration influences the coupling between P_L and P_M and the charge localization within the BChl dimer of the photoinduced cation radical P^+.

RC dehydration induced a progressive blue shift in the peak of the Q_y band of P, from 866 nm in solution RCs down to 851 nm and 845 nm, in the absence and presence of o-phenanthroline, respectively, in agreement with previous non-systematic data obtained upon dehydration of RCs incorporated into PVA films[23] or into glassy trehalose matrices[21]. Such an effect has been previously observed under a variety of circumstances which perturb the P environment. Positively charged detergents such as cetyl-trimethyl-ammonium bromide (CTAB) induce a blue shift of the Q_y band in LDAO solubilized RC from *Rb. sphaeroides*, which is reverted on the subsequent addition of the anionic surfactant deoxycholate.[20, 38] When RC is solubilized by zwitterionic surfactants (sulfobetaine), the position of the Q_y band is tuned by the detergent/RC ratio[20, 40]. A comparative study in RC purified from different species of purple bacteria, related by a strong structural and photochemical similarity, showed the existence of two classes of RC, characterized by Q_y transitions at 865 and 850 nm, respectively[38]. Furthermore, in the two mutants of *Rb. sphaeroides*, in which the histidine residues coordinating the central Mg^{2+} ions of the two BChls of P (His-L173 and His-M202) have been changed to leucine, obtaining BChl/Bphe heterodimers, the Q_y band is shifted to 850 nm.[40, 41]

For all cases mentioned above, analysis of the electronic structure of P^+ by ENDOR/TRIPLE spectroscopy showed that the shift of the Q_y transition of P was systematically associated with an increased asymmetry of the spin density distribution within the P^+ dimer [20, 42, 43]. This body of results led to the notion, originally proposed by Müh and coworkers[20], of two distinct conformations of the primary electron donor, corresponding to the two values of λ_{max} of the Q_y transition (λ_1=866 nm, λ_2=850 nm), which differ in the extent of delocalization of the unpaired spin density on P^+. Noteworthy, Müh and coworkers[20] showed that there exist only two conformations of P^+ and that the perturbations responsible for the shift of the spin density in favour of P_L^+ or P_M^+ affect the distribution of the RC population between the two conformers. This means that the observed progressive shift in λ_{max} of the Q_y transition between λ_1 and λ_2 reflects a change in the relative amplitude of two large, not-resolvable, optical bands positioned at λ_1 and λ_2. Transitions between the two P^+ conformations are also induced in site-directed mutants with altered H-bond interactions[55] or by replacement of ionizable residues near P, only capable of long-range electrostatic interactions with the dimer[19].

In the light of the data summarized above, we interpret the shift in λ_{max} of the Q_y band observed in response to dehydration as reflecting the transition from the native conformation, characterized by a moderately asymmetric spin density distribution ($\rho_L/\rho_M \approx 2$ both in LDAO RC solutions and in intact membranes[42]), to a conformation in which the charge on P^+ is strongly localized on one of the two BChl moieties of the dimer.

The shift in the spin density between the dimer halves P_L and P_M has been rationalized on the basis of MO-models, which consider the interaction of the frontier orbitals of the two BChl moieties of the dimer, and allow to trace spin density asymmetries back to structural and/or environmental parameters.[44, 57] Essentially, these models assume a difference $\Delta\alpha$ between the Coulomb energies of the highest occupied molecular orbitals (HOMOs) of the two dimer halves P_L and P_M, due to different interactions with the protein environment. The interaction of the two HOMOs, described by the resonance integral β, gives rise to two supermolecular orbitals, separated in energy by

$$\Delta E = \sqrt{\Delta\alpha^2 + 4\beta^2} \qquad (1)$$

In the cation radical P^+, the unpaired electron resides in the upper orbital of the dimer. Hückel MO calculations, performed on a simplified model of the P dimer with four π centers (ethylene dimer), show that, for $\Delta\alpha>0$, i.e. for a higher Coulomb energy of the P_L HOMO, the ratio of the spin density ρ_L/ρ_M is a monotonically increasing function of $(\Delta\alpha/\beta)$[57]. An increased localization of the spin density on P_L can therefore result from an increase in $\Delta\alpha$ and/or a decrease in β. The Coulomb energies, and therefore $\Delta\alpha$, can be altered by several factors, including : (i) the H-bonding pattern with the 2-acetyl and 9-keto groups of the BChl molecules; (ii) a rotation of the 2-acetyl group with respect to the macrocycle plane[57, 58]; (iii) the introduction of electric charges in the vicinity of the macrocycle[59]. A change in the distance and/or in the angle between the two macrocycles can affect the resonance integral β. This simple model accounts for the shifts in the spin density of the dimer observed in response to the many different perturbations summarized above. In particular, two-orbital models, extended to include electron-phonon coupling[46, 60], provided a reasonable interpretation of ρ_L/ρ_M changes induced by site-directed mutations near P[61].

By adopting the described MO model, it is conceivable that RC dehydration induces the transition from the P_{866} to the P_{850} conformation by altering $\Delta\alpha$ and/or β through different mechanisms. Dehydration might lead to structural rearrangements of the RC-detergent complex which result in reorientation of polar side chains, interacting asymmetrically with the dimer, and/or of the protein backbone, thus affecting $\Delta\alpha$. This parameter could also be changed due to dehydration-induced alterations of long-range electrostatic interactions with amino acid residues belonging to the hydrophilic periplasmic side of the RC that act differently on P_L and P_M. Changes in the dimer geometry (distance and angle between the two macrocycles), affecting β, cannot however be excluded.

We do not have a specific interpretation for the slightly more pronounced shift observed in the presence of o-phenanthroline, particularly at low hydration levels. The binding of o-phenanthroline at the Q_B site could in principle distort the structure of the cofactor binding pocket, and it cannot be

excluded that such a structural perturbation propagates over a long distance, affecting the protein environment of the primary donor. Unfortunately no crystallographic structure of the RC is available with o-phenanthroline bound at the Q_B site. However, a comparison of the structures of the RC with the Q_B site occupied by ubiquinone-10 (PDB accession code 2GNU) or by terbutryn (PDB accession code 2BNP) does not reveal significant structural alterations in either the Q_B or P binding pockets. Since terbutryn and o-phenanthroline bind at the same site, as reported by crystallographic data in the RC from the related species *Rps. viridis*[62], it seems reasonable to assume that also the binding of o-phenanthroline does not cause structural perturbations at the resolution of crystallographic studies. An alternative possibility to explain the small difference induced by o-phenanthroline in the dehydration-induced shift of the Q_y band is that this hydrophobic molecule, present at a concentration comparable to that of the detergent, perturbs the structure of the LDAO belt surrounding the RC, and/or its interaction with the protein, in turn affecting to some extent the configuration of the P dimer.

It has to be mentioned that a shift of the Q_y band of P from 866 to 846 nm has been also observed upon detergent depletion from a RC-LDAO suspension, causing RC aggregation[39]. This suggests that also protein-protein interactions could play a role in tuning the λ_{max} value of the transition. A shift of λ_{max} from 866 to 850 nm also occurs following ionization of the LDAO detergent at pH < 7.0, which causes a reversible emulsification of the RC-detergent complex[63, 64]. In the latter case, however, the RC conformational change probed by the λ_{max} shift was shown to always precede droplet formation[64], excluding that protein-protein interactions determine the spectral shift, and rather indicating that the P environment perturbation is due to the electrostatic interaction between the RC and the LDAO, cationic at acidic pHs. Since dehydration leads to an amorphous matrix in which RC-LDAO complexes are packed closely together, it could be argued that direct interactions between RC-detergent complexes might be responsible for the transition from the P_{866} to the P_{850} conformation. However this possibility can be reasonably discarded, because a comparable shift of the Q_y band has been also observed upon dehydration of RC in PVA[23] and trehalose[21] amorphous matrices, in which, due to the large volume excess of the embedding medium and homogeneity[65] of the matrix, contacts between RC-detergent complexes are unlikely. We rather believe that the observed effects are mainly due to a reduction in the complement of water molecules interacting with the RC-LDAO complex.

The light-minus-dark FTIR difference spectrum of bacterial RCs offers marker bands of the dimeric character of the primary electron donor P, highly sensitive to alterations in the charge distribution between P_L^+ and P_M^+ within the dimer. They include primarily the broad absorption band in the mid-IR at ~ 2700 cm^{-1} in *Rb. sphaeroides* and the associated *phase-phonon* bands in the

1200-1600 cm^{-1} region. The band at ~ 2700 cm^{-1} was interpreted by Breton and coworkers[30] as an electronic transition of P^+, in terms of a MO-model of the dimer with energetically different BChl halves, i.e. involving the two supermolecular orbitals, separated in energy by ΔE (see eq.1 above). Therefore the band can be used to probe the degree of delocalization of the unpaired electron within the P^+ dimer. This is firmly established by a large number of experimental studies, in which the electronic band of P^+ was examined in parallel with ENDOR and TRIPLE resonance spectra of the photo-induced radical P^+, allowing to determine the ratio (ρ_L/ρ_M) of the spin densities on P_L and P_M[19, 30, 43, 47, 48, 55]. In all these studies, the intensity of the electronic band of P^+ was always found proportional to the extent of charge delocalization within P^+, decreasing under conditions which induced a larger localization on the P_L^+ or P_M^+ halves of the dimer. Variations in the amplitude of the three *phase-phonon* bands (at ~1560, ~1480, and ~1295 cm^{-1}), reflecting porphyrin modes enhanced by the resonance between the two macrocycles of the oxidized dimer[45, 46], are highly correlated with changes in the amplitude of the 2700 cm^{-1} band under a variety of conditions affecting the delocalization of the charge density on P^+[19, 44, 47, 49, 50].

The indication that RC dehydration results in a charge distribution more localized on one of the two BChls of the P^+ dimer is supported by the response of these more specific probes of charge delocalization. Following dehydration, in fact, the amplitudes of the 2700 cm^{-1} band (Figure 3) and of the *phonon-like* bands (Figure 4) decrease, being halved in RC films equilibrated at r = 76% as compared to RC in solution. Artifacts due to interference of the 2700 cm^{-1} band with the stretching band of water between 3600 and 3200 cm^{-1}, are excluded by D_2O replacement (Figure 3B).

Among the three *phase-phonon* bands, the one at ~1300 cm^{-1} better reports on the dimeric character of P^+, since the other two might also contain minor contributions from the BChl cation C-C modes (the band at ~ 1550 cm^{-1}) and the quinone anion C-O and C-C modes (the band at ~1480 cm^{-1})[31]. The peak of this lowest energy *phase-phonon* band appears to be shifted from 1297 cm^{-1} in solution RCs (and at r = 96%) to 1309 cm^{-1} at r = 76%. A similar shift (of larger extent) has been reported in LH(M160) mutant chromatophores in parallel with an attenuation of the 1295 cm^{-1} band[48]. In this mutant, the introduction of a hydrogen bond to the 9-keto carbonyl of P_M induces a strong localization of the P^+ spin density on P_L (ρ_L/ρ_M = 4.94 in purified RCs isolated from LH(M160) as compared to ρ_L/ρ_M = 2.09 in wild type)[55], suggesting a similar dehydration-induced charge displacement toward P_L.

The latter suggestion is confirmed by the spectral alterations caused by dehydration in the differential band at 1715-1703(+)/1683(-) cm^{-1}, ascribed to light-induced changes in the 9-keto C=O stretching modes of the P dimer[31]. The positive component is resolved in two sub-bands, exhibiting maxima at 1715-1713 and 1703 cm^{-1}, attributed to the contribution of P_L and P_M respectively[31, 48].

The relative amplitude of the two sub-bands is modulated by the hydration level: the sub-band at 1703, which is more intense than the one at 1714 cm^{-1} in solution RCs, is weakened following dehydration, so that the sub-band at 1714 cm^{-1}, corresponding to the P_L^+ contribution, prevails in RC films at r = 76%. Such a reversion in the relative amplitude of the P_L and P_M bands indicates that dehydration increases significantly the spin density asymmetry already present within the P^+ dimer of hydrated RCs ($\rho_L/\rho_M = 2.1$[19, 20]), leading to a strong charge localization on P_L. This conclusion is also consistent with the slight increase in the sub-bands splitting induced by dehydration. A similar increase in the splitting has been observed in fact in a mutant, RE(L135), in which P_L^+ is more stabilized relative to P_M^+, as compared to wild type RCs.

An evaluation of the extent by which dehydration increases the charge localization on the P_L^+ moiety of P^+ can be attempted by comparing the effects induced by dehydration with those observed in the RE(L135) mutant. The dehydration-induced alterations in the relative amplitudes of the P_L and P_M sub-bands are significantly larger than those observed in the mutant: in the latter the attenuation of the 1700 cm^{-1} band results in equal amplitudes of the two P_L and P_M sub-bands, while dehydration at r = 76% causes a reversion in the relative amplitude of the two spectral components, suggesting a larger displacement of the charge on the P_L moiety of the P^+ dimer. This is consistent with the weakening of the P^+ electronic band and of the *phase-phonon* bands detected in the mutant relative to wild type RCs, as compared to the more prominent decrease induced by dehydration. Notably, a closer comparison of the profiles of the 1300 cm^{-1} bands recorded in the wild type and in the RE(L135) mutant by Johnson and coworkers[19] (see their Figure 3, upper panel) reveals an impressive similarity between the changes induced by mutation and by dehydration. The weak shoulder present at ~1309 cm^{-1} in the wild type band increases, becoming a resolvable peak in the mutant. The resulting band profile is very similar to that recorded by us in moderately dehydrated RCs, at r = 96% (see Figure 4). Further dehydration induces a further increase of this component, which at r = 76% becomes the dominating peak of the band. This again suggests that dehydration causes a larger shift of the charge distribution towards P_L, as compared to that observed in the RE(L135) mutant. Since a spin density ratio $\rho_L/\rho_M = 3.1 \pm 0.1$ has been determined in the RE(L135) mutant, as compared to $\rho_L/\rho_M = 2.1 \pm 0.1$[19], this can be taken as a lower limit for the increase in P_L^+ charge localization caused by dehydration.

Most of the large attenuation of the IR marker bands of the P^+ dimer discussed above occurs upon dehydration of RC films at r = 76%, further dehydration causing only a very limited weakening. This behaviour has significant implications in the light of a previous work, in which the residual water content of RC films equilibrated over the same range of relative humidity was determined, obtaining water sorption isotherms for the RC-detergent system[29]. Two main

populations of water molecules adsorbed to the RC-detergent complex could be characterized: one tightly bound at primary hydration sites and one weakly bound to the complex. When r is decreased from 94% to 76% the complement of residual water in the RC films decreases from ~$30 \cdot 10^3$ H_2O molecules per RC to ~$5 \cdot 10^3$ H_2O/RC. A further decrease to r = 11% reduces the hydration by a lesser extent (~$1 \cdot 10^3$ H_2O/RC). Notably at r ≥ 76% the tightly bound water molecules are still present and start to be partially removed only when r is decreased below about 40%. Therefore it is clear that the transition from the P_{866} to the P_{850} RC conformation, observed in the present work, is associated with depletion of weakly bound water molecules. It has been shown[29] that, over this range of dehydration, only modest alterations are observed in the intramolecular combination (around 5155 cm^{-1}) and intermolecular association (around 2130 cm^{-1}) water bands. At variance, relevant changes in the structure of the combination and association bands occur at r < 40%, i.e. when the population of water molecules tightly bound to the RC-detergent complex starts to be depleted (see ref.[29] for more details). This evolution of the system appears consistent with the observation that dehydration from r = 76 % to r =11 % leads to clear alterations in the structure of the two *phase-phonon* bands at ~ 1480 and ~1300 cm^{-1}, with, however, only a negligible additional attenuation of the bands. The latter observation is in agreement with the very limited additional weakening of the P^+ electronic band at 2700 cm^{-1}, as well as with the negligible changes in the relative amplitude of the 9-keto C=O bands of P_L and P_M. It appears therefore that, if structural changes take place in the dimer as very dehydrated conditions (r = 11%) are approached, they do not further affect the distribution of charge within the P^+ dimer, which has been already altered under less dehydrated conditions.

On the basis of the MO-model of the dimer summarized above, the shift of the spin density towards P_L can be plausibly accounted for by different structural and/or energetic perturbations of the dimer. Since the effect takes place upon the removal of weakly bound water molecules, most of which interacts with the detergent belt[29], dehydration of the micelle surrounding the RC could affect the steric constraints imposed on the RC by the detergent, influencing the relative stability of the P_{866} and P_{850} conformations. Changes in the dimer environment could be equally caused by the removal of water molecules weakly bound to the protein at the water exposed periplasmic surface of the RC. The high resolution crystallographic structure determined by Koepke and coworkers[66] reveals a large number of water molecules, at the RC-water interface, in the proximity of the dimer. Although these crystallographic water molecules likely belong to the tightly bound population, the removal of more disordered water molecules weakly bound to this primary hydration shell could well affect the dimer environment: dielectric data in the terahertz frequency domain suggest that water-protein interactions extend to a distance of approximately 10-20 Å into the bulk[67, 68]. The RC

structure also reveals a few cavities, not far from the binding pocket of the dimer, which could accommodate weakly bound water molecules. Depletion of weakly bound water molecules could affect the asymmetry of the P environment by producing a rearrangement of polar side chains or, more indirectly, by tuning their electrostatic interaction with the dimer due to changes in the local dielectric constant.

4.2 Implications for the stability of the primary charge separated state

The question has been posed whether the spatial distribution of the unpaired electron on P^+ affects the stability of the primary charge separated state, i.e. the lifetime τ_{AP} of $P^+Q_A^-$ recombination following a short photoexcitation[20, 38, 40, 42, 55]. By considering the whole body of studies in which the charge distribution on P^+ was perturbed by different factors *in vitro* and *in vivo* no unambiguous correlation emerges between τ_{AP} and the spin density ratio ρ_L/ρ_M. For instance, τ_{AP} is decreased in both heterodimer mutants, in spite of the fact that the electron is confined to P_L or to P_M respectively in the two mutants[41, 43]. Also, site-directed mutations which increase ρ_L/ρ_M decrease τ_{AP}, but the opposite effect is observed upon increasing the concentration of zwitterionic detergents, which also increases ρ_L/ρ_M[40]. From these and other observations it has been concluded that the charge distribution within the P^+ dimer does not play a significant role in the stability of $P^+Q_A^-$ [40]. This conclusion is corroborated by the results of the present paper, when considered in conjunction with those of a previous study[29], in which the $P^+Q_A^-$ recombination kinetics in RC films have been examined systematically over a large range of relative humidities (3% < r < 94%). No effect was observed on the kinetic parameters of $P^+Q_A^-$ after a short (ns) laser pulse when the hydration of the RC film was reduced by decreasing r from 94% down to 40%. Since the transition from the P_{866} to the P_{850} conformation occurs essentially over this range of relative humidities, we infer that the increased charge localization on P_L^+ does not influence the stability of the $P^+Q_A^-$ state generated by a short photoexcitation pulse. At lower hydration levels (when r is decreased from ~40% to ~9%), the kinetics accelerate progressively becoming strongly distributed. This effect has been interpreted[29] as due to a dehydration-induced inhibition of the conformational RC relaxation from the dark-adapted to the light-adapted conformation[8, 10].

The conclusion that the kinetics of $P^+Q_A^-$ recombination after a short (ns) light pulse are not affected by the asymmetry of charge distribution within the P^+ dimer does not exclude, however, that this parameter may influence the conformational RC rearrangements occurring upon continuous illumination on the second to minute time scale, and resulting in a dramatic stabilization of the charge separated state of the RC[11, 14, 15, 69, 70]. We have recently observed[16] that the response to dehydration of the $P^+Q_A^-$ recombination kinetics after a prolonged illumination (20 s) is markedly

different from that observed after a short photoexcitation[16, 29]. In particular, we found that in RC films dehydrated at r = 76%, continuous photoexcitation resulted in the appearance of an extremely slow phase of $P^+Q_A^-$ recombination, with a lifetime $\tau_{AP} \approx 8$ s, accounting for about 50% of the decay[16]. This kinetic component is absent in solution RCs, where, following a 20 s continuous photoexcitation, $P^+Q_A^-$ recombines with a lifetime $\tau_{AP} \approx$ 85-125 ms, i.e. by the same kinetics observed after a pulsed photoexcitation (M. Malferrari, F. Francia, G. Venturoli, unpublished results, and ref.[24]). The slow phase, reflecting a strong stabilization of the charge separated state, appears at the same dehydration levels which induce the redistribution of the P^+ dimer charge in favour of P_L^+. In the more dehydrated system (r = 11%), $P^+Q_A^-$ recombination after continuous photoexcitation is again accelerated ($\tau_{AP} \approx 100$ ms), probably reflecting the impairment of the overall RC dynamics (both on short and long time scales)[16]. It is important to notice that the light-minus-dark difference FTIR spectra, which probe the shift of the charge density to P_L^+, have been measured in the present work using the same photoexcitation regime (20 s continuous illumination) employed in the $P^+Q_A^-$ recombination measurements. In view of the above considerations it is tempting to speculate that the dehydration-induced RC conformational changes associated with redistribution of the charge on the P^+ dimer are related to the structural rearrangements which occur upon illumination on the longer time scale and give rise to a strong stabilization of the primary charge separated state in moderately dehydrated RC (r = 76%). In this respect, it may be relevant that the comparison between difference IR marker bands of the dimeric nature of P^+, recorded in RCs cooled at 100K in the dark and in the light, has suggested that the relaxed, light-adapted state is associated with an increased stabilization of the charge on P_L^+ relative to P_M^+[19]. The degree of delocalization of the electron hole on P^+ has been already proposed, among other structural factors, as playing a role in the stabilization of $P^+Q_A^-$ following photoexcitation in the minute time scale[70]. The notion that reorganization of the protein around P^+ plays a role in stabilizing charge separation on the long time scale is also in line with recent studies performed in mutant RCs, characterized by different H-bonding patterns of the P dimer. Interestingly, evidence of a light-induced change of the local dielectric constant in the vicinity of the P dimer has been provided[12], and proton release by one or more amino acid residues, mainly involving water molecules near P, has been proposed as the relaxation event which stabilizes the charge separated state on a long time scale[13].

5. Conclusions

The reported results show that partial dehydration of the RC affects the electronic structure of the primary electron donor P, stabilizing the unpaired electron of the photoinduced radical on the P_L^+ half of the dimer. This charge redistribution is consistently indicated by the response to

dehydration of different spectroscopic markers. The transitions between the two conformations differing in P^+ charge localization is caused by the removal of weakly bound water molecules, pointing to subtle structural rearrangement in the P environment and/or changes of the local dielectric constant around the dimer.

The dehydration-induced displacement of the charge density in favour of the L-moiety of P^+ does not correspond to any alteration of the stability of the primary charge separated state ($P^+Q_A^-$) induced by short (ns) photoexcitation pulses, suggesting that delocalization of the unpaired electron within the P^+ dimer does not play a role in the fast RC relaxation which follows charge separation. However, the changes induced by dehydration on the electronic structure of P take place at dehydration levels which strongly affect the stability of $P^+Q_A^-$ following a prolonged (tens of seconds) continuous illumination. We propose that the extent of charge delocalization may be related to the dielectric relaxations responsible for the stabilization of charge separation under continuous illumination.

Acknowledgements

This work was supported by MIUR of Italy and by the French Infrastructure for Integrated Structural Biology (FRISBI)ANR-10-INSB-05-01. M.M. gratefully acknowledges a short-term EMBO Fellowship (ASTF 256-2013).

References

1. G. Feher, J. P. Allen, M. Y. Okamura and D. C. Rees, Structure and function of bacterial photosynthetic reaction centers, *Nature*, 1989, **339**, 111-116.
2. A. J. Hoff and J. Deisenhofer, Photophysics of photosynthesis. Structure and spectroscopy of reaction centers of purple bacteria, *Phys. Rep.*, 1997, **287**, 2-247.
3. C. A. Wraight, Proton and electron transfer in the acceptor quinone complex of photosynthetic reaction centers from *Rhodobacter sphaeroides*, *Frontiers in Bioscience (Landmark)*, 2004, **9**, 309-337.
4. P. Brzezinski and L. E. Andréasson, Trypsin treatment of reaction centers from *Rhodobacter sphaeroides* in the dark and under illumination: protein structural changes follow charge separation, *Biochemistry*, 1995, **34**, 7498-7506.
5. G. Katona, A. Snijder, P. Gourdon, U. Andréasson, O. Hansson, L. E. Andréasson and R. Neutze, Conformational regulation of charge recombination reactions in a photosynthetic bacterial reaction center, *Nat. Struct. Mol. Biol.*, 2005, **12**, 630-631.
6. A. B. Wöhri, G. Katona, L. C. Johansson, E. Fritz, E. Malmerberg, M. Andersson, J. Vincent, M. Eklund, M. Cammarata, M. Wulff, J. Davidsson, G. Groenhof and R. Neutze, Light-induced structural changes in a photosynthetic reaction center caught by Laue diffraction, *Science*, 2010, **328**, 630-633.
7. H. Arata and W. W. Parson, Enthalpy and volume changes accompanying electron transfer from P-870 to quinones in *Rhodopseudomonas sphaeroides* reaction centers, *Biochim. Biophys. Acta*, 1981, **636**, 70-81.
8. D. Kleinfeld, M. Y. Okamura and G. Feher, Electron-transfer kinetics in photosynthetic reaction centers cooled to cryogenic temperatures in the charge-separated state: evidence for light-induced structural changes, *Biochemistry*, 1984, **23**, 5780-5786.
9. E. Nabedryk, K. A. Bagley, D. L. Thibodeau, M. Bauscher, W. Mäntele and J. Breton, A protein conformational change associated with the photoreduction of the primary and secondary quinones in the bacterial reaction center, *FEBS Lett.*, 1990, **266**, 59-62.
10. B. H. McMahon, J. D. Müller, C. A. Wraight and G. U. Nienhaus, Electron transfer and protein dynamics in the photosynthetic reaction center, *Biophys. J.*, 1998, **74**, 2567-2587.
11. A. J. Manzo, A. O. Goushcha, N. M. Berezetska, V. N. Kharkyanen and G. W. Scott, Charge recombination time distributions in photosynthetic reaction centers exposed to alternating intervals of photoexcitation and dark relaxation, *J. Phys. Chem. B*, 2011, **115**, 8534-8544.
12. S. S. Deshmukh, J. C. Williams, J. P. Allen and L. Kalman, Light-induced conformational changes in photosynthetic reaction centers: dielectric relaxation in the vicinity of the dimer, *Biochemistry*, 2011, **50**, 340-348.
13. S. S. Deshmukh, J. C. Williams, J. P. Allen and L. Kalman, Light-induced conformational changes in photosynthetic reaction centers: redox-regulated proton pathway near the dimer, *Biochemistry*, 2011, **50**, 3321-3331.
14. L. Kalman and P. Maroti, Conformation-activated protonation in reaction centers of the photosynthetic bacterium *Rhodobacter sphaeroides*, *Biochemistry*, 1997, **36**, 15269-15276.
15. F. van Mourik, M. Reus and A. R. Holzwarth, Long-lived charge-separated states in bacterial reaction centers isolated from *Rhodobacter sphaeroides*, *Biochim. Biophys. Acta, Bioenerg.*, 2001, **1504**, 311-318.
16. M. Malferrari, A. Mezzetti, F. Francia and G. Venturoli, Effects of dehydration on light-induced conformational changes in bacterial photosynthetic reaction centers probed by optical and differential FTIR spectroscopy, *Biochim. Biophys. Acta, Bioenerg.*, 2013, **1827**, 328-339.

17. T. Iwata, M. L. Paddock, M. Y. Okamura and H. Kandori, Identification of FTIR bands due to internal water molecules around the quinone binding sites in the reaction center from *Rhodobacter sphaeroides*, *Biochemistry*, 2009, **48**, 1220-1229.
18. A. Remy and K. Gerwert, Coupling of light-induced electron transfer to proton uptake in photosynthesis, *Nat. Struct. Biol.*, 2003, **10**, 637-644.
19. E. T. Johnson, F. Müh, E. Nabedryk, J. C. Williams, J. P. Allen, W. Lubitz, J. Breton and W. W. Parson, Electronic and vibronic coupling of the special pair of bacteriochlorophylls in photosynthetic reaction centers from wild-type and mutant strains of *Rhodobacter sphaeroides*, *J. Phys. Chem. B*, 2002, **106**, 11859-11869.
20. F. Müh, J. Rautter and W. Lubitz, Two distinct conformations of the primary electron donor in reaction centers from *Rhodobacter sphaeroides* revealed by ENDOR/TRIPLE-spectroscopy, *Biochemistry*, 1997, **36**, 4155-4162.
21. G. Palazzo, A. Mallardi, A. Hochkoeppler, L. Cordone and G. Venturoli, Electron transfer kinetics in photosynthetic reaction centers embedded in trehalose glasses: trapping of conformational substates at room temperature, *Biophys. J.*, 2002, **82**, 558-568.
22. F. Francia, G. Palazzo, A. Mallardi, L. Cordone and G. Venturoli, Residual water modulates Q_A^- -to-Q_B electron transfer in bacterial reaction centers embedded in trehalose amorphous matrices, *Biophys. J.*, 2003, **85**, 2760-2775.
23. F. Francia, L. Giachini, G. Palazzo, A. Mallardi, F. Boscherini and G. Venturoli, Electron transfer kinetics in photosynthetic reaction centers embedded in polyvinyl alcohol films, *Bioelectrochemistry*, 2004, **63**, 73-77.
24. F. Francia, G. Palazzo, A. Mallardi, L. Cordone and G. Venturoli, Probing light-induced conformational transitions in bacterial photosynthetic reaction centers embedded in trehalose-water amorphous matrices, *Biochim. Biophys. Acta*, 2004, **1658**, 50-57.
25. F. Francia, M. Dezi, A. Mallardi, G. Palazzo, L. Cordone and G. Venturoli, Protein-matrix coupling/uncoupling in "dry" systems of photosynthetic reaction center embedded in trehalose/sucrose: the origin of trehalose peculiarity, *J. Am. Chem. Soc.*, 2008, **130**, 10240-10246.
26. F. Francia, M. Malferrari, S. Sacquin-Mora and G. Venturoli, Charge recombination kinetics and protein dynamics in wild type and carotenoid-less bacterial reaction centers: studies in trehalose glasses, *J. Phys. Chem. B*, 2009, **113**, 10389-10398.
27. L. Cordone, G. Cottone, S. Giuffrida, G. Palazzo, G. Venturoli and C. Viappiani, Internal dynamics and protein-matrix coupling in trehalose-coated proteins, *Biochim. Biophys. Acta, Proteins and Proteomics*, 2005, **1749**, 252-281.
28. A. Savitsky, M. Malferrari, F. Francia, G. Venturoli and K. Möbius, Bacterial photosynthetic reaction centers in trehalose glasses: coupling between protein conformational dynamics and electron-transfer kinetics as studied by laser-flash and high-field EPR spectroscopies, *J. Phys. Chem. B*, 2010, **114**, 12729-12743.
29. M. Malferrari, F. Francia and G. Venturoli, Coupling between electron transfer and protein-solvent dynamics: FTIR and laser-flash spectroscopy studies in photosynthetic reaction center films at different hydration levels, *J. Phys. Chem. B*, 2011, **115**, 14732-14750.
30. J. Breton, E. Nabedryk and W. W. Parson, A new infrared electronic transition of the oxidized primary electron donor in bacterial reaction centers: a way to assess resonance interactions between the bacteriochlorophylls, *Biochemistry*, 1992, **31**, 7503-7510.
31. E. Nabedryk, Light-induced Fourier transform infrared difference spectroscopy of the primary electron donor in photosynthetic reaction centers, in *Infrared spectroscopy of biomolecules*, ed. H.H. Mantsch and D. Chapman, Wiley-Liss, New York, 1996, pp. 39-82.
32. A. Mezzetti and W. Leibl, Investigation of ubiquinol formation in isolated photosynthetic reaction centers by rapid-scan Fourier transform IR spectroscopy, *Eur. Biophys. J.*, 2005, **34**, 921-936.

33. E. Nabedryk and J. Breton, Coupling of electron transfer to proton uptake at the Q_B site of the bacterial reaction center: A perspective from FTIR difference spectroscopy, *Biochim. Biophys. Acta, Bioenerg.*, 2008, **1777**, 1229-1248.
34. S. Hermes, J. M. Stachnik, D. Onidas, A. Remy, E. Hofmann and K. Gerwert, Proton uptake in the reaction center mutant L210DN from *Rhodobacter sphaeroides* via protonated water molecules, *Biochemistry*, 2006, **45**, 13741-13749.
35. D. W. Reed and G. A. Peters, Characterization of the pigments in reaction center preparations from *Rhodopseudomonas spheroides*, *J. Biol. Chem.*, 1972, **247**, 7148-7152.
36. I. K. Mikhailyuk, P. P. Knox, V. Z. Paschenko, A. P. Razjivin and H. Lokstein, Analysis of absorption spectra of purple bacterial reaction centers in the near infrared region by higher order derivative spectroscopy, *Biophy. Chem.*, 2006, **122**, 16-26.
37. A. V. Hughes, P. Rees, P. Heathcote and M. R. Jones, Kinetic analysis of the thermal stability of the photosynthetic reaction center from *Rhodobacter sphaeroides*, *Biophys. J.*, 2006, **90**, 4155-4166.
38. S. Wang, S. Lin, X. Lin, N. W. Woodbury and J. P. Allen, Comparative study of reaction centers from purple photosynthetic bacteria: isolation and optical spectroscopy, *Photosynth. Res.*, 1994, **42**, 203-215.
39. P. Gast, P. W. Hemelrijk, H. J. VanGorkom and A. J. Hoff, The association of different detergents with the photosynthetic reaction center protein of *Rhodobacter sphaeroides* R26 and the effects on its photochemistry, *Eur. J. Biochem.*, 1996, **239**, 805-809.
40. F. Müh, C. Schulz, E. Schlodder, M. R. Jones, J. Rautter, M. Kuhn and W. Lubitz, Effects of zwitterionic detergents on the electronic structure of the primary donor and the charge recombination kinetics of $P^+Q_A^-$ in native and mutant reaction centers from *Rhodobacter sphaeroides*, *Photosynth. Res.*, 1998, **55**, 199-205.
41. L. M. McDowell, D. Gaul, C. Kirmaier, D. Holten and C. C. Schenck, Investigation into the source of electron transfer asymmetry in bacterial reaction centers, *Biochemistry*, 1991, **30**, 8315-8322.
42. J. Rautter, F. Lendzian, W. Lubitz, S. Wang and J. P. Allen, Comparative study of reaction centers from photosynthetic purple bacteria: Electron paramagnetic resonance and electron nuclear double resonance spectroscopy, *Biochemistry*, 1994, **33**, 12077-12084.
43. M. Huber, R. A. Isaacson, E. C. Abresch, D. Gaul, C. C. Schenck and G. Feher, Electronic structure of the oxidized primary electron donor of the HL (M202) and HL (L173) heterodimer mutants of the photosynthetic bacterium *Rhodobacter sphaeroides*: ENDOR on single crystals of reaction centers, *Biochim. Biophys. Acta, Bioenerg.*, 1996, **1273**, 108-128.
44. W. W. Parson, E. Nabedryk and J. Breton, Mid- and near-IR electronic transitions of P^+: New probes of resonance interactions and structural asymmetry in reaction centers, in *Photosynthetic Bacterial Reaction Center II: Structure, Spectroscopy, and Dynamics*, ed. J. Breton and A. Vermeglio, Plenum Press, New York, 1992, pp. 79-88.
45. J. R. Reimers and N. S. Hush, Nature of the ground and first excited states of the radical cations pf photosynthetic bacterial reaction centers, *Chem. Phys.*, 1995, **197**, 323-332.
46. J. R. Reimers and N. S. Hush, The effects of couplings to symmetric and antisymmetric modes and minor asymmetry on the spectral properties of mixed-valence and related charge-transfer systems, *Chem. Phys.*, 1996, **208**, 177-193.
47. E. Nabedryk, S. J. Robles, E. Goldman, D. C. Youvan and J. Breton, Probing the primary donor environment in the Histidine^{M200}-Leucine and Histidine^{L173}-Leucine mutants of *Rhodobacter capsulatus* by light-induced Fourier transform infrared difference spectroscopy, *Biochemistry*, 1992, **31**, 10852-10858.
48. E. Nabedryk, J. P. Allen, A. K. W. Taguchi, J. C. Williams, N. W. Woodbury and J. Breton, Fourier transform infrared study of the primary electron donor in chromatophores of *Rhodobacter sphaeroides* with reaction centers genetically modified at residues M160 and L131, *Biochemistry*, 1993, **32**, 13879-13885.

49. E. Nabedryk, W. Leibl and J. Breton, FTIR spectroscopy of primary donor photooxidation in Photosystem I, *Heliobacillus mobilis*, and *Chlorobium limicola*. Comparison with purple bacteria, *Photosynth. Res.*, 1996, **48**, 301-308.
50. E. Nabedryk, C. Schulz, F. Müh, W. Lubitz and J. Breton, Heterodimeric versus homodimeric structure of the primary electron donor in *Rhodobacter sphaeroides* reaction centers genetically modified at position M202, *Photochem. Photobiol.*, 2000, **71**, 582-588.
51. L. Baciou and H. Michel, Interruption of the water chain in the reaction center from *Rhodobacter sphaeroides* reduces the rates of the proton uptake and of the 2nd electron transfer to Q_B, *Biochemistry*, 1995, **34**, 7967-7972.
52. L. Greenspan, Humidity fixed points of binary saturated aqueous solutions, *J. Res. Natl. Bur. Stand. A.*, 1977, **814**, 89-96.
53. M. Malferrari, G. Venturoli, F. Francia and A. Mezzetti, A new method for D_2O/H_2O exchange in infrared spectroscopy of proteins, *Spectroscopy: An International Journal*, 2012, **27**, 337-342.
54. J. Breton, D. L. Thibodeau, C. Berthomieu, W. Mäntele, A. Vermeglio and E. Nabedryk, Probing the primary quinone environment in photosynthetic reaction centers by light-induced FTIR difference spectroscopy, *FEBS Lett.*, 1991, **278**, 257-260.
55. J. Rautter, F. Lendzian, C. Schulz, A. Fetsch, M. Kuhn, X. Lin, J. C. Williams, J. P. Allen and W. Lubitz, ENDOR studies of the primary donor cation radical in mutant reaction centers of *Rhodobacter sphaeroides* with altered hydrogen-bond interactions, *Biochemistry*, 1995, **34**, 8130-8143.
56. E. T. Shimomura, M. A. Phillippi, H. M. Goff, W. F. Scholz and C. A. Reed, Infrared spectroscopy of oxidized metalloporphyrins: detection of a band diagnostic of porphyrin-centered oxidation, *J. Am. Chem. Soc.*, 1981, **103**, 6778-6780.
57. M. Plato, F. Lendzian, W. Lubitz and K. Möbius, Molecular orbital study of the electronic asymmetry in primary donors of bacterial reaction centers, in *Photosynthetic Bacterial Reaction Center II: Structure, Spectroscopy, and Dynamics*, ed. J. Breton and A. Vermeglio, Plenum Press, New York, 1992, pp. 109-118.
58. M. Plato, E. Trankle, W. Lubitz, F. Lendzian and K. Möbius, Molecular orbital investigation of dimer formations of bacteriochlorophyll a. Model configurations for the primary donor of photosynthesis *Chem. Phys.*, 1986, **107**, 185-196.
59. M. Plato, W. Lubitz, F. Lendzian and K. Möbius, Magnetic resonance and molecular orbital studies of the primary donor cation radical $P960^+$ in the photosynthetic bacterium *Rhodopseudomonas viridis, Isr. J. Chem.*, 1988, **28**, 109-119.
60. Z. Gasyna and P. N. Schatz, Analysis of the intervalence band in the oxidized photosynthetic bacterial reaction center, *J.Phys. Chem.*, 1996, **100**, 1445-1448.
61. F. Müh, F. Lendzian, M. Roy, J. C. Williams, J. P. Allen and W. Lubitz, Pigment-protein interactions in bacterial reaction centers and their influence on oxidation potential and spin density distribution of the primary donor, *J. Phys. Chem. B*, 2002, **106**, 3226-3236.
62. H. Michel, O. Epp and J. Deisenhofer, Pigment protein interactions in the photosynthetic reaction center from *Rhodopseudomonas viridis, EMBO J.*, 1986, **5**, 2445-2451.
63. R. Piazza, M. Pierno, E. Vignati, G. Venturoli, F. Francia, A. Mallardi and G. Palazzo, Liquid-liquid phase separation of a surfactant-solubilized membrane protein, *Phys. Rev. Lett.*, 2003, **90** 208101.
64. G. Palazzo, A. Mallardi, F. Francia, M. Dezi, G. Venturoli, M. Pierno, E. Vignati and R. Piazza, Spontaneous emulsification of detergent solubilized reaction center: protein conformational changes precede droplet growth, *Phys. Chem. Chem. Phys.*, 2004, **6**, 1439-1445.
65. M. Malferrari, A. Nalepa, G. Venturoli, F. Francia, W. Lubitz, K. Möbius and A. Savitsky, Structural and dynamical characteristics of trehalose and sucrose matrices at different

hydration levels as probed by FTIR and high-field EPR, *Phys. Chem. Chem. Phys.*, 2014, **16**, 9831-9848.

66. J. Koepke, E. M. Krammer, A. R. Klingen, P. Sebban, G. M. Ullmann and G. Fritzsch, pH modulates the quinone position in the photosynthetic reaction center from *Rhodobacter sphaeroides* in the neutral and charge separated states, *J. Mol. Biol.*, 2007, **371**, 396-409.
67. S. Ebbinghaus, S. J. Kim, M. Heyden, X. Yu, U. Heugen, M. Gruebele, D. M. Leitner and M. Havenith, An extended dynamical hydration shell around proteins, *Proc. Natl. Acad. Sci. U.S.A.*, 2007, **104**, 20749-20752.
68. B. Born, S. J. Kim, S. Ebbinghaus, M. Gruebele and M. Havenith, The terahertz dance of water with the proteins: the effect of protein flexibility on the dynamical hydration shell of ubiquitin, *Faraday Discuss.*, 2009, **141**, 161-173.
69. U. Andréasson and L. E. Andréasson, Characterization of a semi-stable, charge-separated state in reaction centers from *Rhodobacter sphaeroides*, *Photosynth. Res.*, 2003, **75**, 223-233.
70. S. S. Deshmukh, H. Akhavein, J. C. Williams, J. P. Allen and L. Kalman, Light-induced conformational changes in photosynthetic reaction centers: impact of detergents and lipids on the electronic structure of the primary electron donor, *Biochemistry*, 2011, **50**, 5249-5262.

Figure captions

Figure 1.

Visible-NIR absorption spectra recorded at 295K in the Q_y absorption region of bacteriochlorins for RC in solution (black) and in a RC-film equilibrated at different values of relative humidity: r = 94% (green), r = 75% (red), r = 11% (blue). The inhibitor o-phenanthroline was not added to the samples. Spectra have been normalized to the amplitude of the bacteriochlorophyll peak at 804 nm in solution.

Figure 2.

The dependence of the position (λ_{max}) of the Q_y transition of the P dimer upon the hydration level of RC-films equilibrated at different relative humidities. Data have been acquired in two RC-films, prepared in the absence (open circles) and in the presence (filled circles) of o-phenanthroline, respectively. The dashed horizontal line corresponds to the λ_{max} value measured in solution, both in the absence and in the presence of the inhibitor. A 1 nm experimental error is estimated in λ_{max} determinations.

Figure 3.

Light-induced FTIR difference spectra at 281 K in the 4000-1000 cm^{-1} range of RCs at different hydration levels. Vertical bars mark the position of the three *phase-phonon* bands. Samples have been prepared in the presence of o-phenanthroline. Panel A compares the spectra recorded in solution RCs (black) and in RC films hydrated with H_2O at relative humidity r = 96% (green), 76%, (red), and 11% (blue). Panel B shows the effect of D_2O substitution in RC films equilibrated at r = 96 % (green) and r= 76 % (red). The spectra have been normalized on the basis of the differential bands at 1749/1739 cm^{-1} attributed to the 10a-ester C=O mode of P (see text for further details). The increment between major ticks of the y-axis corresponds to 2.6 x 10^{-4} absorbance units.

Figure 4.

Light-induced $P^+Q_A^-/PQ_A$ FTIR difference spectra in the 1600-1200 cm^{-1} range, dominated by the *phase-phonon* bands, acquired in solution RCs (black) and in RC films hydrated with H_2O at relative humidity r = 96% (green), 76%, (red), and 11% (blue). Samples have been prepared in the presence of o-phenanthroline. The spectra have been normalized to the differential bands at 1749/1739 cm^{-1} attributed to the 10a-ester C=O mode of P. The increment between major ticks of the y-axis corresponds to 1.3 x 10^{-4} absorbance units.

Figure 5.

Light-induced FTIR difference spectra in the 1800-1600 cm^{-1} range of solution RCs (black) and of RC films hydrated with H_2O at relative humidity r = 96% (green), 76%, (red), and 11% (blue). Samples have been prepared in the presence of o-phenanthroline.The spectra have been normalized to the differential bands at 1749/1739 cm^{-1} attributed to the 10a-ester C=O mode of P. The increment between major ticks of the y-axis corresponds to 2.6 x 10^{-4} absorbance units.

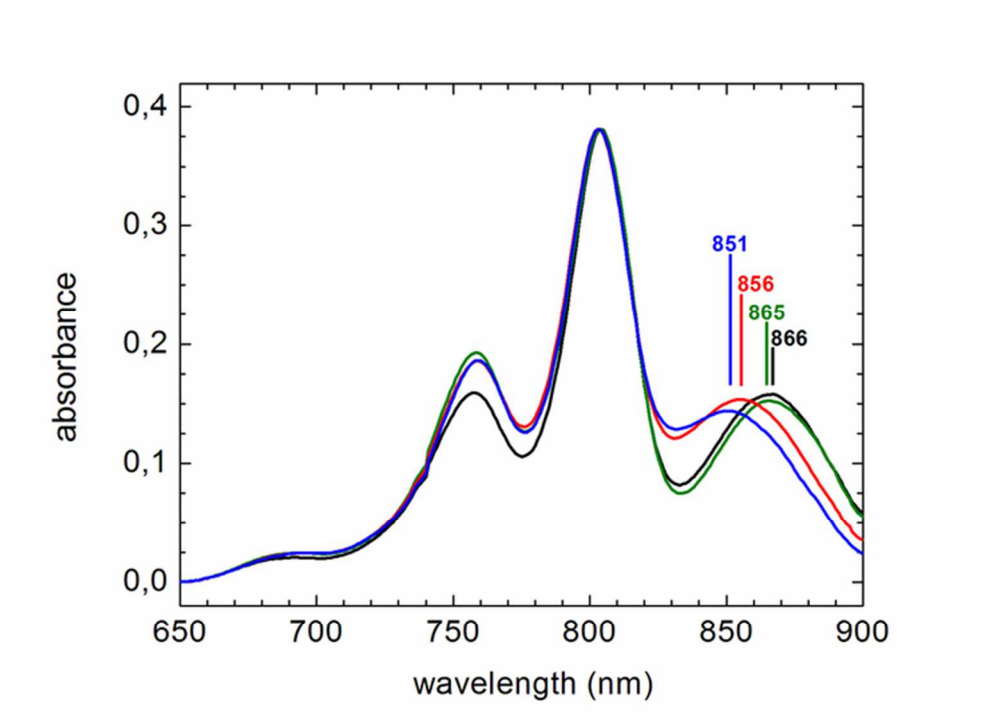


Figure 1
63x46mm (300 x 300 DPI)

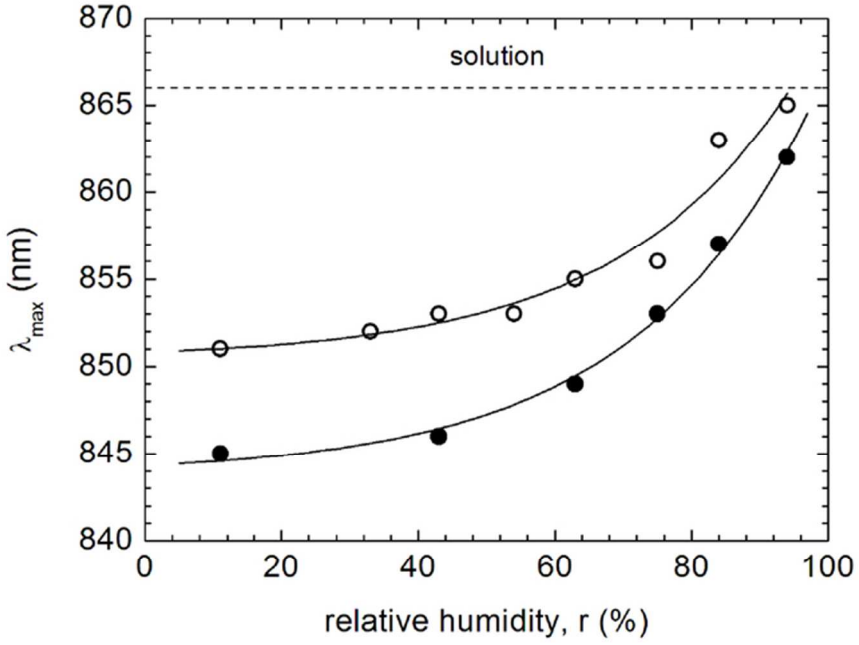


Figure 2
63x46mm (300 x 300 DPI)

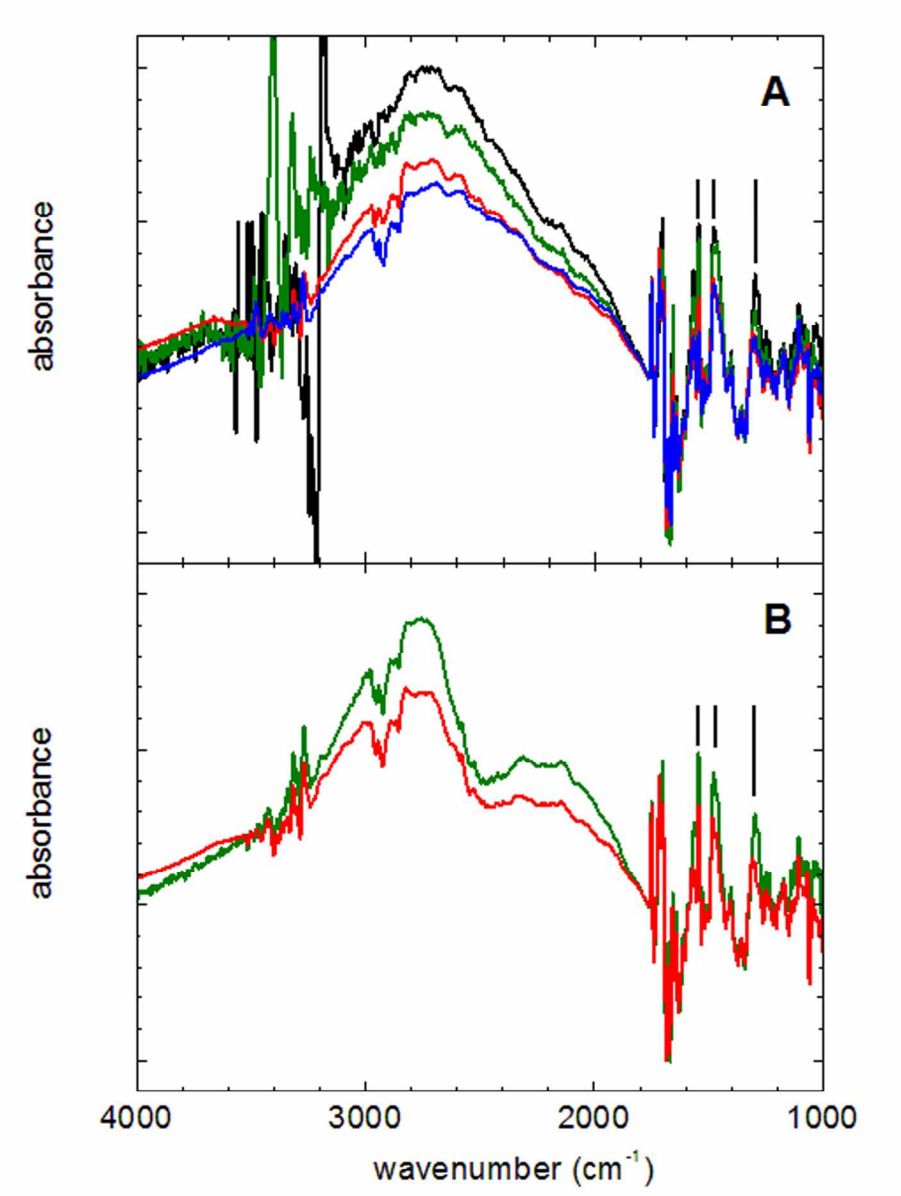


Figure 3
115x154mm (300 x 300 DPI)

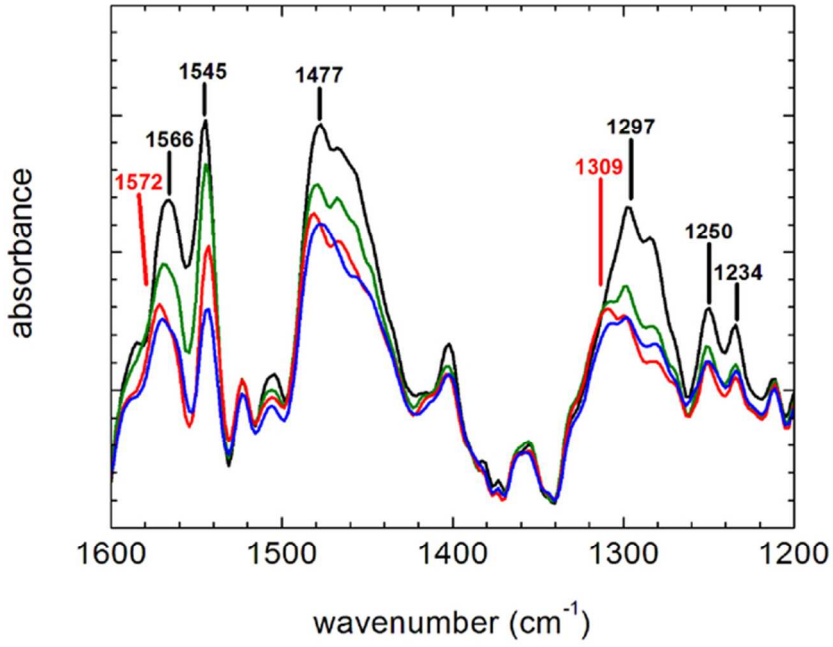


Figure 4
63x46mm (300 x 300 DPI)

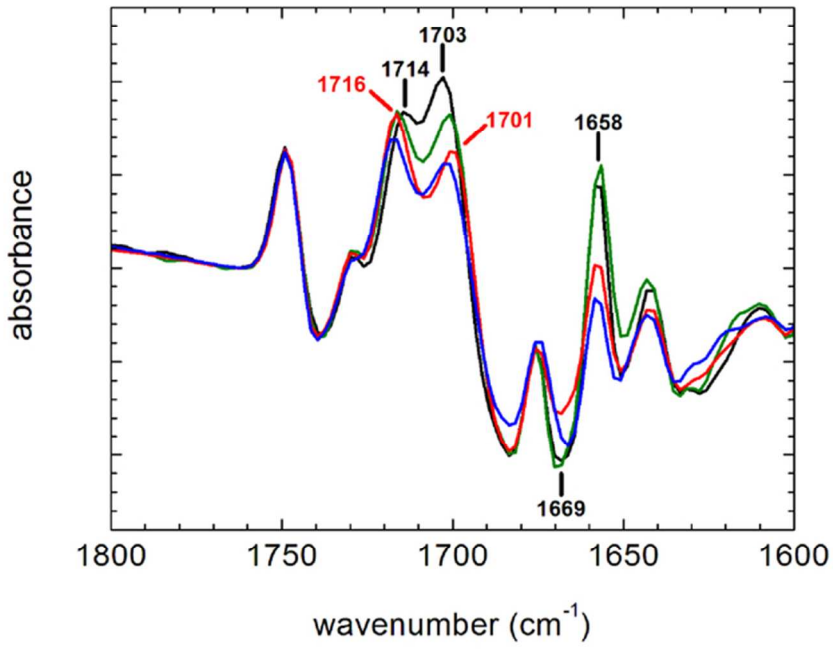
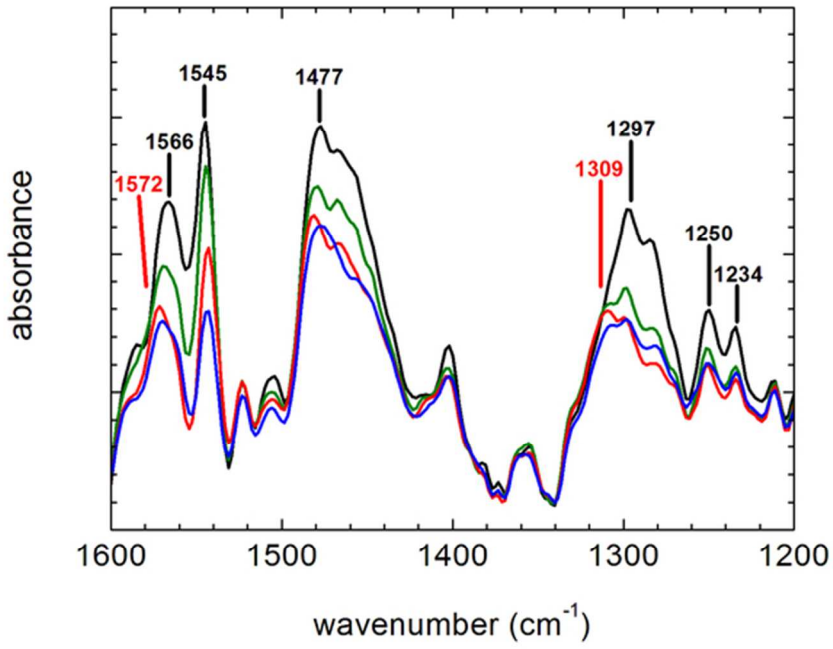
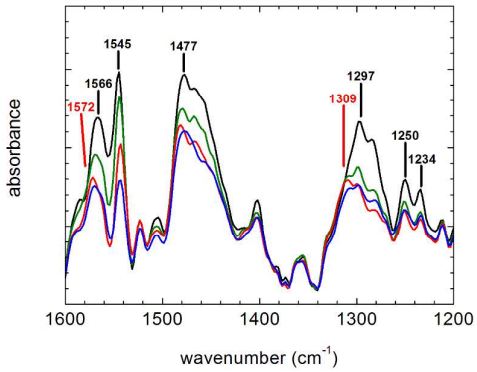


Figure 5
63x46mm (300 x 300 DPI)



visual abstract
58x42mm (300 x 300 DPI)

Graphical abstract



Visible-NIR and light-induced difference FTIR spectroscopy provide evidence that dehydration of bacterial photosynthetic reaction centers alters the electronic structure of the primary electron donor. Implications for photocatalytic activity are discussed.