spectroscopy journal

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Effect of Dehydration on Light-Adapted States of Bacterial Reaction Centers Studied by Time-Resolved Rapid-Scan FTIR Difference Spectroscopy

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Abstract: Dehydration is known to affect the rate of electron transfer backreaction from the light-induced charge separation state $P^+Q_A^-$ to the neutral ground state PQ_A in photosynthetic bacterial Reaction Centers. On the other hand, a 20 s continuous illumination period has been demonstrated to induce (at 297 K) formation of one or more light-adapted states at different levels of dehydration; these light-adapted states are believed to be related to peculiar response(s) from the protein. In this work, we applied time-resolved rapid-scan FTIR difference spectroscopy to investigate the protein response under dehydrated conditions (RH = 11%) at 281 K both after a flash and under prolonged continuous illumination. Time-resolved FTIR difference spectra recorded after a laser flash show a protein recovery almost synchronous to the electron transfer backreaction $P^+Q_A^- \rightarrow PQ_A$. Time-resolved FTIR difference spectra recorded after 20.5 s of continuous illumination (RH = 11%, T = 281 K) surprisingly show almost the same kinetics of electron transfer back reaction compared to spectra recorded after a laser flash. This means that the mechanism of formation of a light-adapted stabilized state is less effective compared to the same hydration level at 297 K and to the RH = 76% hydration level (both at 281 K and 297 K). Time-resolved FTIR difference spectra after continuous illumination also suggest that the 1666 cm^{-1} protein backbone band decays faster than marker bands for the electron transfer back reaction $P^+Q_A^- \rightarrow PQ_A$. Finally, FTIR double-difference spectra (FTIR difference spectrum recorded after 18.4 s illumination minus flash-induced FTIR difference spectrum) suggest that at RH = 11%, a light-adapted state different from the one observed at RH = 76% is formed. A possible interpretation is that at RH = 11%, the protein response is modified by the fact that only protons can move easily, differently from water molecules, as instead observed for RH = 76%. This probably makes the formation of a real light-adapted $P^+Q_A^-$ stabilized state at RH = 11% unfeasible.

Keywords: Reaction Center; rapid-scan FTIR; time-resolved infrared; electron transfer; photosynthesis; conformational change; protein response

Academic Editors: Rui Fausto and Turrell Sylvia

Received: 11 October 2024
Revised: 23 December 2024
Accepted: 28 December 2024
Published: 1 February 2025

Citation: Mezzetti, A.; Malferrari, M.; Francia, F.; Venturoli, G. Effect of Dehydration on Light-Adapted States of Bacterial Reaction Centers Studied by Time-Resolved Rapid-Scan FTIR Difference Spectroscopy. *Spectrosc. J.* **2025**, *3*, 5. https://doi.org/10.3390/spectroscj3010005

1. Introduction

The reaction center (RC) from the purple bacterium *Rba sphaeroides* is a membrane protein complex where the initial photochemistry of photosynthesis takes place. The pigments

and cofactors involved in these photochemical reactions are inserted in the polypeptide scaffold. When absorbing a photon, a so-called special pair of bacteriochlorophyll-*a* molecules (called P), located nearby the periplasmic side of the RC, is excited to P* and is then photo-oxidized, giving an electron, through an intermediate bacteriopheophytin pigment, to a ubiquinone named Q_A placed on the cytoplasmic side of the protein complex. Following this light-induced charge separation $P\ Q_A + h\nu \rightarrow P^+Q_A^-$ (which takes place in a time scale of roughly 200 ps), the electron moves from Q_A to a second ubiquinone Q_B (timescale ~ 100 µs) [1]. When no Q_B is present in the RC (e.g., because it is displaced by a specific inhibitor), Q_A^- can recombine with P^+ (reaction rate: $k \sim 10\ s^{-1}$) [1].

Several articles have studied the events associated with the photochemical reactions $P\ Q_A + h\nu \rightarrow P^+Q_A^-$ inside the RC. In particular, several studies show a peculiar protein conformational change (see [2–8] as well as the references therein). A pioneering paper [2] demonstrated that after a nanosecond photoexcitation at 298 K, the RC relaxes from a so-called dark-adapted conformation to a so-called light-adapted conformation. This light-adapted conformation leads to a stabilization of the $P^+Q_A^-$ state. Interestingly, the two protein conformations (dark-adapted and light-adapted) can be trapped when the RC is cooled to very low (cryogenic) temperatures either in darkness or under illumination [2].

It was deduced that at room temperature, the proteins rapidly "adapt" to the altered electric charge distribution generated by the photoinduced charge separation reaction and strongly slow down the electron recombination between P^+ and Q_A^-. At 298 K, this dielectric relaxation from the dark-adapted to the light-adapted conformation is believed to take place on a very short timescale, definitely shorter than the $P^+Q_A^- \rightarrow P\ Q_A$ charge recombination (around 100 ms).

Several published articles also suggest that, under continuous light, the RC can have further slower conformational changes, on a scale from seconds to minutes [5,6,8–10]. In particular, the lifetime of the $P^+Q_A^-$ state increases with the duration of the continuous light illumination period. This suggests that the persistence of the charge-separated state $P^+Q_A^-$ can induce further conformational changes inside the RC. A similar effect (formation of a long-lived "light-adapted" stabilized state) was reported for Photosystem II core complexes from *T. vulcanus* after 20 saturating ns laser flashes [11], suggesting that the possibility of forming "light-adapted" states is common to all Type II RCs [12]. This is a topic that has been recently debated in the literature [13,14].

Time-resolved light-induced difference FTIR spectroscopy is a powerful technique that makes it possible to follow the time evolution of specific molecular chemical moieties inside a protein using specific "marker" IR bands; it is widely used to investigate the photochemistry of photosynthetic RCs [15].

A key feature in time-resolved light-induced FTIR difference spectroscopy is the detection limit, which depends both on the intrinsic signal size and on the noise level, which depends on the optics, optoelectronics (source and detector), and electronics of the used instrument. Absorbance noise levels at the 10^{-6} scale are common in time-resolved FTIR difference spectra recorded on most FTIR instruments (especially commercially available ones). As a consequence, time-resolved FTIR difference spectra reporting signals with $\Delta A \leq 10^{-5}$ are very difficult to obtain with a high signal-to-noise ratio [16–18]. The situation is even more critical with irreversible reactions [19], or with rapidly photo-degrading or photo-ageing samples [20].

Recently, we used rapid-scan FTIR difference spectroscopy in order to understand more in details the stabilization process in RCs under moderately dehydrated conditions, i.e., relative humidity (RH) = 76% [20].

On the other hand, previous results [8,21] have shown that the hydration of the RCs deeply influences the $P^+Q_A^-$ → P Q_A recombination rates (both after a saturating flash and after a continuous illumination period).

In this manuscript, we extended our study to RCs under strongly dehydrated conditions, finding striking differences compared to what happens at higher hydration. Time-resolved rapid-scan FTIR difference spectra were recorded after one saturating laser flash and during/after 20.5 s of continuous illumination. Whereas there are indications suggesting formation of a light-adapted state, FTIR spectroscopy suggests that the protein conformational changes taking place under continuous illumination are different from those observed at higher hydration (RH = 76%).

2. Materials and Methods

2.1. Purification of RCs

RCs were purified from *R. sphaeroides* 2.4.1 strain following the protocol described by Baciou and Michel [22]; lauryldimethylamine N-oxide (LDAO) was used as a detergent. The Q_B site occupancy was estimated to ~57%, using the relative amplitude of the "slow" kinetic phase ($\tau \approx 1.8$ s) of P^+ decay after a laser pulse (following the approach by Kleinfeld et al. [23]), measured by time-resolved visible absorption spectroscopy, and probed at 422 nm.

2.2. Preparation of RC Films in Dehydrated Conditions

CaF_2 windows were used as support to prepare RC-LDAO films as described in Malferrari et al. [8]. In short, 50 µL of an aqueous solution containing 60 µM RC, 0.025% LDAO, 10 mM o-phenanthroline (o-Phe) as a Q_B site inhibitor, and 10 mM TRIS HCl as a buffer (pH = 8.0) were used and deposited on the CaF_2 window.

O-Phe at this concentration guarantees almost complete occupancy of the Q_B site in all RCs, so that upon illumination, no $P^+Q_B^-$ state is formed (see Figure 1). The absence of the $P^+Q_B^-$ state was confirmed by the kinetic analysis of P^+ decay (probed at 422 nm) after a 7 ns laser pulse. However, at very high concentrations, o-Phe could in principle also lead to partial displacement of Q_A from its site, thereby making the P Q_A + hν → $P^+Q_A^-$ reaction impossible in a fraction of RCs [24]. Therefore, the functionality of the Q_A site was also assessed by adding 10 mM o-Phe; this addition did not change the extent of light-induced charge separation, $P^+Q_A^-$.

A vacuum grease-sealed gastight sample compartment (≈1 mL) was built putting a second CaF_2 disk over the first one carrying the RC–LDAO film in a clipping holder, using a ≈1.5 mm rubber spacer (an O-ring covered with vacuum grease, placed between the CaF_2 disks). The relative humidity RH inside the sample compartment was set to 11% by adding ~5 µL of a saturated LiCl solution (see Figure 2). Under these conditions, hydration equilibrium of the RC-LDAO films according to the specific thermodynamic conditions is reached in a few hours [26]. RC hydration level at equilibrium was checked recording absorption spectra between 6000 and 1000 cm^{-1} using the area of the ($\nu_2 + \nu_3$) combination band of water (broad, ~5180 cm^{-1}) and the area of the amide II band of the RC (at ~1550 cm^{-1}) as an internal standard [21]. These calculations gave a (H_2O/RC) molar ratio of 935 in RC films, in excellent agreement with the literature results at the same RH level [21].

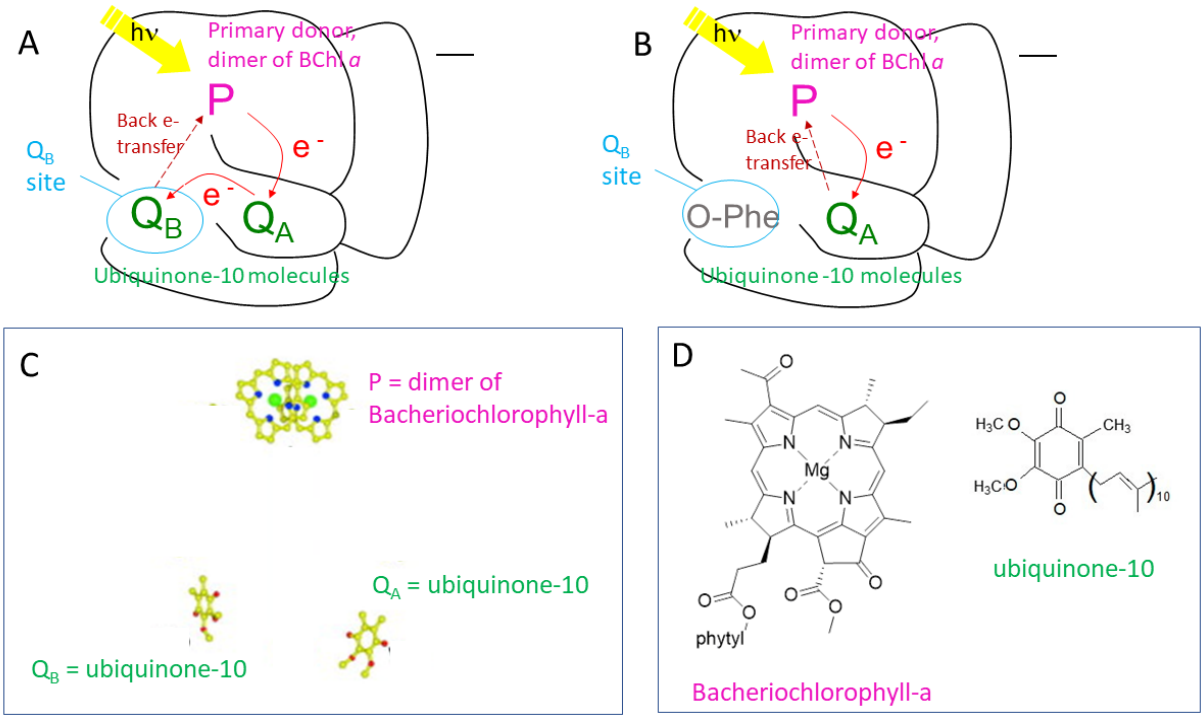

Figure 1. Scheme of the reaction center (RC) from the purple bacterium *Rba. sphaeroides*. (**A**) Untreated RC: the Q_B site is occupied by a ubiquinone-10 molecule. A laser flash entails formation of a $P^+Q_B^-$ charge separate state that recombines to PQ_B in ~1 s. (**B**) O-phenanthroline (O-Phe)-treated RC: the Q_B site is occupied by O-Phe, unable to accept electrons, so that a laser flash induces formation of a P^+Q_A state, which recombines to PQ_A in ~100 ms. (**C**) Arrangement of cofactors P, Q_A, and Q_B inside the RC. (**D**) Chemical structure of BChl a (P is a dimer of BChl a molecules) and of ubiquinone-10 (therefore, of both Q_A and Q_B). (**C**) reprinted (in form) from [25] Copyright (2001) with permission from Elsevier.

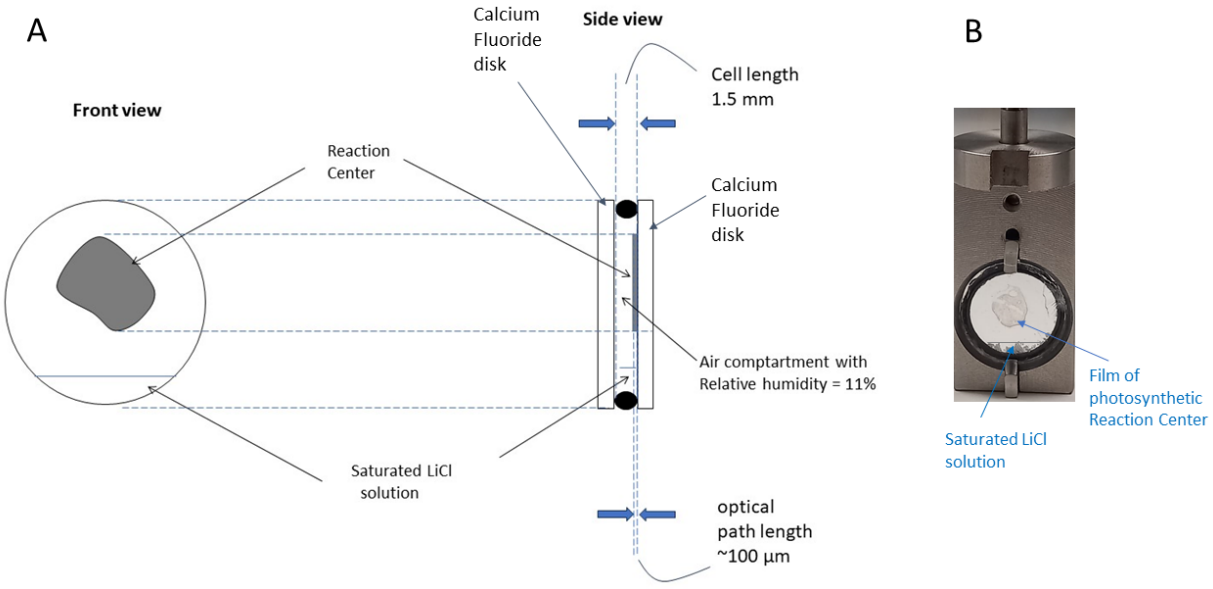

Figure 2. (**A**) Scheme of the sample compartment for FTIR experiments. (**B**) Picture of the sample compartment. See text for further details.

2.3. Static and Time-Resolved FTIR Spectroscopy

Static FTIR and time-resolved rapid-scan FTIR difference spectra were recorded on a Bruker IFS 88 spectrometer, equipped with the rapid-scan module, and with MCT-A

(N_2-cooled) and DTGS detectors. Opus software 2.0 (running on OS2) was used to manage the experiment and treat data.

A N_2 flux cryostat (Oxford instruments, Oxon, UK) was used to keep the sample at 281 ± 1 K during the experiments. FTIR absorbance spectra (6000–1000 cm^{-1}) were measured in the dark employing the DTGS detector in order to assess the level of hydration of the RC-LDAO films.

In time-resolved rapid-scan FTIR difference spectra, mirror speed was set to maximum speed (5.063 cm/s) and the single-sided, fast return option was used to record the interferogram. The MCT-A detector was employed. Resolution was set to 4 cm^{-1}.

Two different approaches were used, as described in Mezzetti et al. [27] (laser flash-induced rapid-scan FTIR difference spectroscopy experiments) and in Mezzetti et al. [28] (rapid-scan FTIR difference spectroscopy experiments during and after continuous illumination with a lamp).

In the first case, interferograms in the 1900–1000 cm^{-1} range were acquired after a 7 ns flash at 532 nm from a frequency-doubled pulsed Nd:YAG laser (Quantel, Les Ulis, France). A 2 ms delay was inserted between the flash and the beginning of interferogram recording thanks to a pulse generator; the experiment timing was checked on a digital oscilloscope (Tektronix, Beaverton, Oregon, USA). Experimental scheme is shown in Figure 3A. The results from 3000 cycles (recorded on 3 different samples) were averaged in order to obtain a good signal-to-noise ratio. These time-resolved FTIR difference spectra are named FL-TRIR difference spectra hereafter. (Time-resolved step-scan FTIR difference spectroscopy [18], which has a much higher time resolution, could not be applied due to the high number of flashes required (>20.000) and to the partial (photo-)degradation of the sample. The same phenomenon was also observed at RH = 76% [20].)

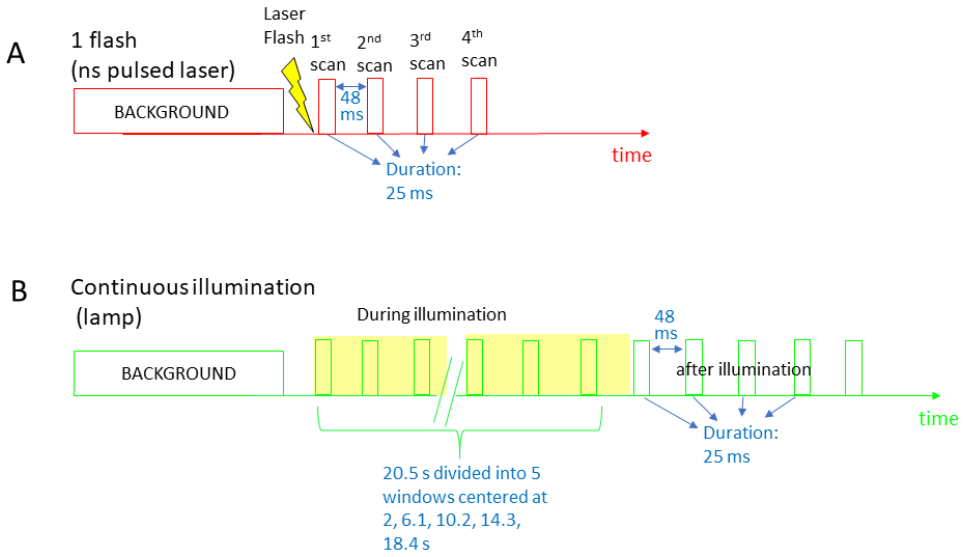

Figure 3. (**A**) Scheme of flash-induced rapid-scan FTIR difference spectroscopy experiments (giving FL-TRIR difference spectra). Each marked interval represents the time required to record an interferogram. Each interferogram is recorded in a separate memory. Fifteen seconds were introduced at the end of the measuring period as a delay time between cycles to let the system to recover completely to the initial state. (**B**) Time-resolved rapid-scan FTIR difference spectroscopy experiments during and after 20.5 s of continuous illumination (giving UI-TRIR difference spectra and AI-TRIR difference spectra, respectively). During the illumination period, interferograms are averaged on a 4.1 s time window; averages are then Fourier transformed. After the illumination period, interferograms are recorded at increasing times, with a time resolution of 73 ms. A wait-time of 1 min was introduced at the end of the measuring period to let the system to recover completely to the initial state before starting a new measurement cycle.

In the second case (see also Figure 3B), time-resolved FTIR difference spectra were recorded under continuous illumination (lasting 20.5 s; these spectra are named UI-TRIR-difference spectra hereafter) and after continuous illumination (named AI-TRIR difference spectra hereafter). As a source of continuous illumination, a 250 W tungsten halogen lamp (Oriel) was used. The light from the lamp was collimated using an optical condenser and it was filtered through an 8 cm thick water layer, through a 50% neutral density filter, and through two colored glass filters providing transmission centered at 760 nm with <1% transmittance for $\lambda < 700$ nm and $\lambda > 850$ nm. The duration of photoexcitation was controlled by a Uniblitz electro-programmable mechanical shutter system (Vincent Associates, Rochester, New York, NY, USA) that has a ~3 ms closure time.

During illumination, UI-TRIR difference spectra were recorded and averaged in time windows of 4.1 s, so that the series of 5 consecutive spectra allowed for monitoring the evolution during the 20.5 s continuous illumination. After closing the shutter (i.e., after the end of the illumination period), AI-TRIR difference spectra were recorded with a time resolution of 73 ms. The experimental scheme is shown in Figure 3B. The results from 4000 cycles (recorded on 3 different samples) were averaged in order to obtain a good signal-to-noise ratio.

In all the time-resolved rapid-scan FTIR difference spectra (FL-TRIR, UI-TRIR, and AI-TRIR), the absence of unwanted photothermal heating signals was checked.

3. Results

FL-TRIR difference spectra after a saturating laser flash are shown in Figure 4A. All bands decay completely within 200 ms, in a roughly synchronous way. Detailed kinetic analysis (global exponential fitting, 2D correlation analysis) was hampered by the limited number of time-resolved spectra and by baseline correction issues.

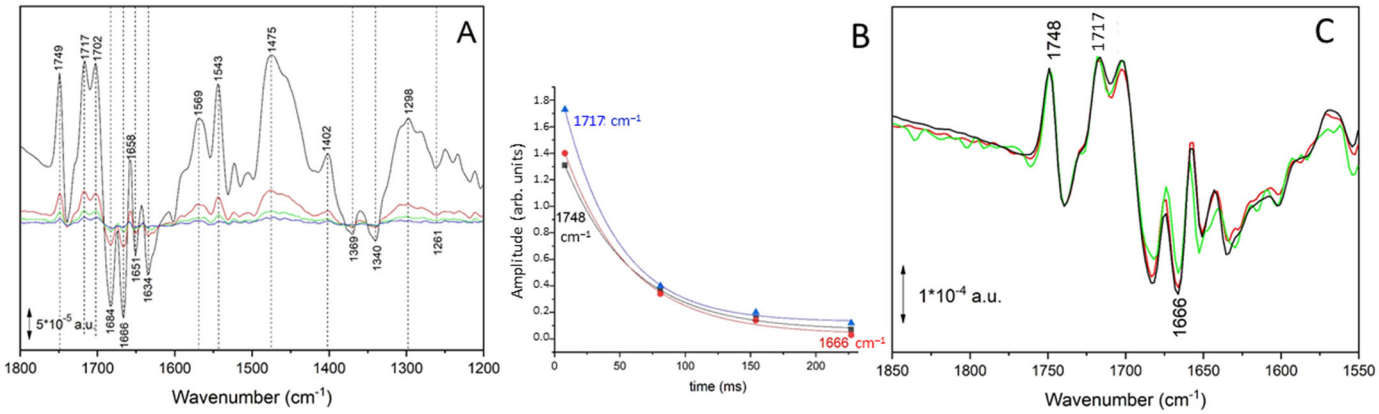

Figure 4. (**A**) FL-TRIR difference spectra recorded 8 (black trace), 81 (red trace), 154 (green trace), and 227 (blue trace) ms after a saturating laser flash. (**B**) Time evolution of specific bands for P^+ (1748, 1712 cm^{-1}) and a protein conformational change associated with the formation of a Q_A^- state (1666 cm^{-1}). Kinetics constants found with a mono-exponential fitting: τ (1748 cm^{-1}) = 51.5 ± 4.6 ms; τ (1717 cm^{-1}) = 41.7 ± 3.5 ms; τ (1666 cm^{-1}) = 50.0 ± 6.3 ms. (**C**) Superposition of spectra recorded 8, 81, and 154 ms after a saturating laser flash normalized with respect to the 1748 (+) cm^{-1} band.

It should be underlined that the fast decay in FTIR difference signals in Figure 4 made it possible to record only 4 FL-TRIR difference spectra after the flash. The accuracy of our analysis somehow suffers from the limited number of spectra.

Figure 5A shows UI-TRIR difference spectra recorded at increasing times after onset of continuous light. As can be seen, almost no time evolution is observed in shape or band intensity, meaning that a photo-accumulated state is already formed 2 s after the

beginning of the illumination period, and this state does not seem to evolve significantly in the following 16.4 s. This is confirmed by the time evolution of the marker bands at 1748 cm^{-1} (positive; attributed to the 10a ester C=O vibration [29]), 1717 cm^{-1} (positive; attributed to the 9-keto C=O vibration [29]), and 1666 cm^{-1} (negative, attributed to a protein conformational change associated with the formation of Q_A^- (The same band is placed at 1670 cm^{-1} in RCs in solution [27] and at 1668 cm^{-1} in RCs under moderately dehydrated condition (RH = 76%) [8]) (see Figure 5B).

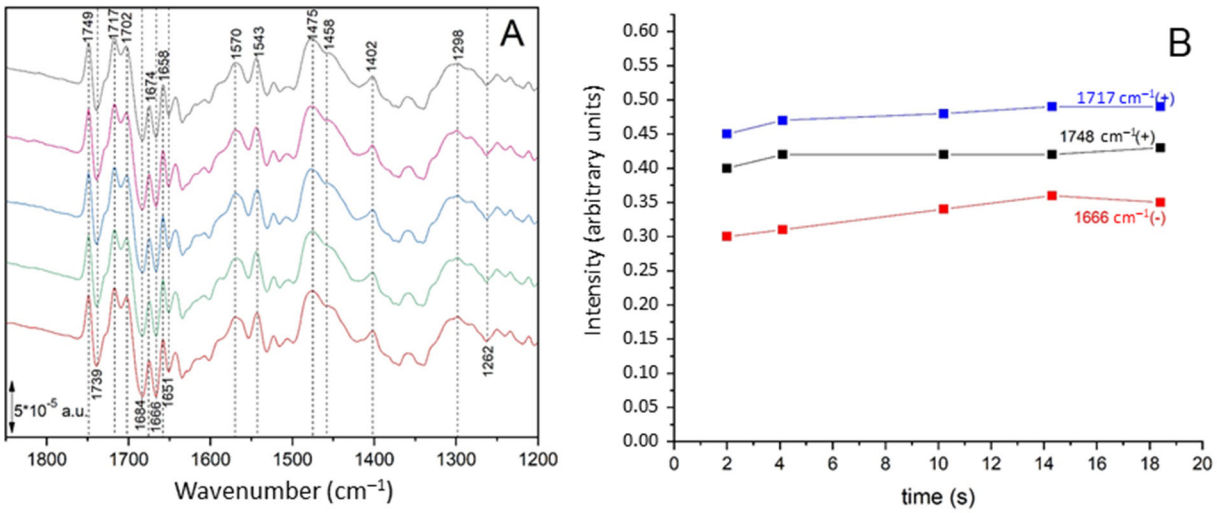

Figure 5. (**A**) UI-TRIR difference spectra recorded under continuous illumination. Spectra were recorded (from top to bottom) 2 s (grey trace), 6.1 s (magenta trace), 10.2 s (blue trace), 14.3 s (green trace), and 18.4 s (red trace) after onset of illumination. (**B**) Time evolution of specific bands for P^+ (1748, 1717 cm^{-1}) and a protein conformational change associated with the formation of a Q_A^- state (1666 cm^{-1}).

Figure 6A shows the AI-TRIR difference spectra recorded after the end of the 20.5 s continuous illumination period. Interestingly, whereas it seems that the decay of the 1748 cm^{-1} P^+ marker band is slower compared to spectra recorded after one flash (but not for the 1717 cm^{-1} band, where small contributions from the protein could exist), this does not seem to be related to a peculiar conformational change in the protein, at least not to the one responsible for the 1666 (-) cm^{-1} band, which conversely seems to decay even faster compared to the time-resolved spectra recorded after one flash (FL-TRIR difference spectra).

Here, it is however important to underline two points: First, the signal size in these spectra is very small (~10^{-5} a.u.) and, differently from spectra recorded under continuous illumination (Figure 5), no intensive averaging using large time windows is possible. On the other hand, the experiment was repeated every 100 s, so that it was not possible to achieve the same number of cycles of measurement as in FL-TRIR difference spectra (Figure 4). Therefore, the non-optimal signal-to-noise ratio reported in Figure 6 should be considered both as a good achievement, in technical terms, but also a limiting factor, in the exploitation of experimental data.

Second, the fast decay in FTIR difference signals in Figure 6 made it possible to record only three AI-TRIR difference spectra after the end of the illumination period. Taking the last spectrum recorded during the illumination period (Figure 5) as equivalent to a hypothetical spectrum at t = 0, it is possible to try to fit the decay of marker bands with four points to an exponential decay (see Figure 6B). The accuracy of these fittings will anyhow suffer from the very limited number of points, as in the case of FL-TRIR difference spectra.

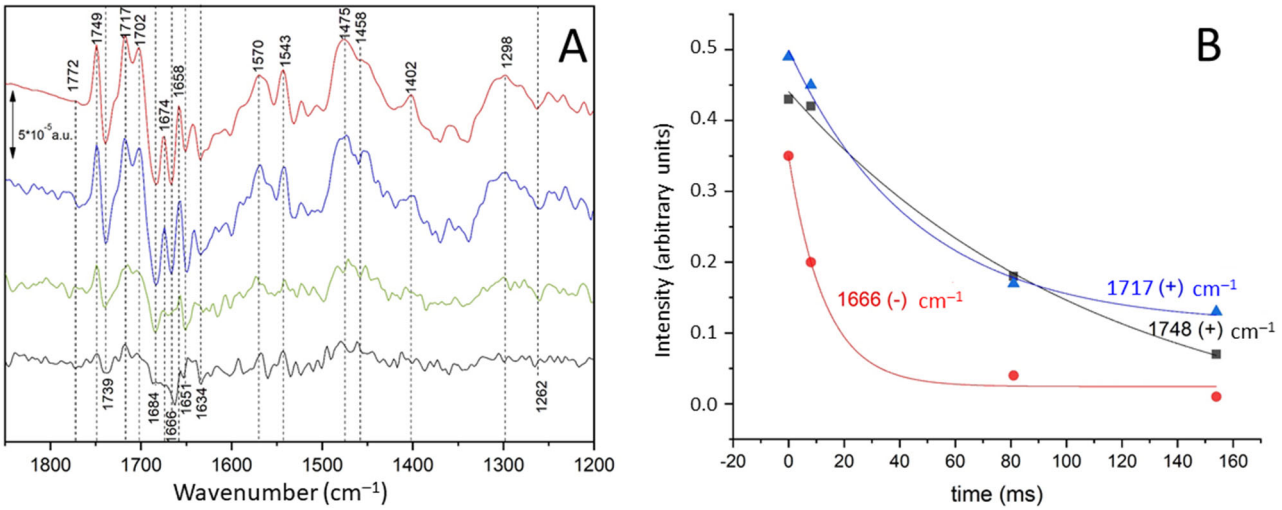

Figure 6. (**A**) AI-TRIR difference spectra recorded after 20.5 s of continuous illumination and (**B**) time evolution of specific bands for P^+ (1748, 1717 cm^{-1}) and a protein conformational change associated with the formation of a Q_A^- state (1666 cm^{-1}). Kinetics constants found with a mono-exponential fitting: τ (1748 cm^{-1}) = 112 ± 45 ms; τ (1717 cm^{-1}) = 46 ± 13 ms; τ (1666 cm^{-1}) = 13.0 ± 3.0 ms.

In Figure 7, we compare the UI-TRIR difference spectrum recorded during continuous illumination with the first spectrum FL-TRIR difference spectrum recorded after one saturating flash. This comparison should evidence the possible formation of a light-adapted state after 18.4 s of continuous illumination, compared to the state formed after a single 7 ns saturating laser pulse. As can be observed, the two difference spectra are not perfectly equivalent. The double-difference spectrum (black trace in Figure 7) highlights that the major changes concern the amide I region (1690–1610 cm^{-1}), even though clear changes can also be observed in the 1780–1690 cm^{-1} region.

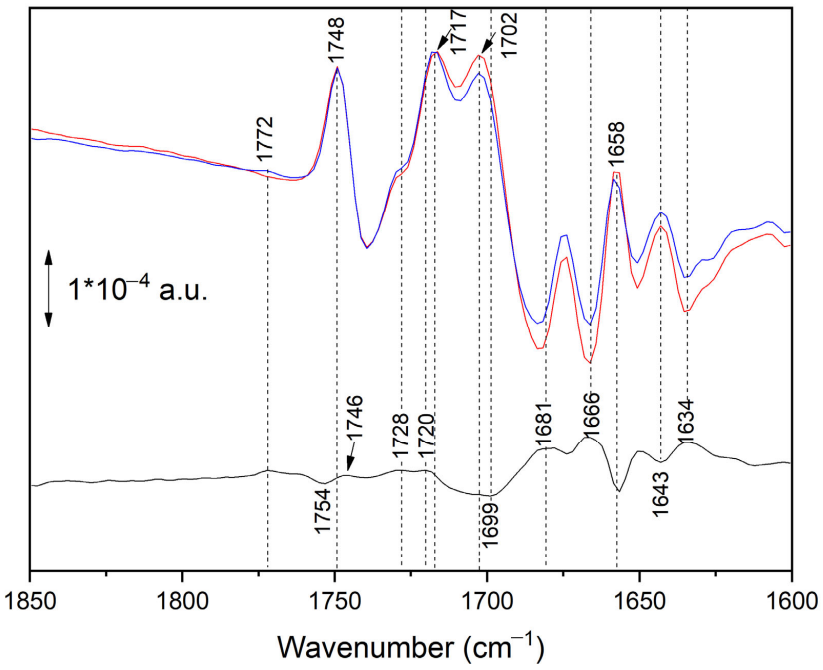

Figure 7. Comparison between FTIR difference spectra recorded after 18.4 s of continuous illumination (blue trace, last of the UI-TRIR difference spectra) and 8 ms after a saturating laser flash (red trace, first of the FL-TRIR difference spectra). In black, the double-difference spectrum obtained by subtracting the red trace from the blue trace.

4. Discussion

Formation of light-adapted states in bacterial photosynthetic RCs have been reported in several conditions. In particular, our group [8] reported that in moderately dehydrated conditions (RH = 76%) and in strongly dehydrated conditions (RH = 11%), exposure to a 20 s continuous illumination at 297 K slows down the electron transfer backreaction $P^+Q_A^- \rightarrow PQ_A$ in both cases. In the same work, relying on static light-minus-dark FTIR difference spectra recorded between 4000 and 1000 cm^{-1}, we deduced that the response of the protein under illumination at the two hydration levels was not the same [8].

Recently, we have shown that in bacterial RCs at RH = 76% and 281 K the protein backbone response is at least partially (negative band at 1668 cm^{-1} attributed to a conformational change related to the monoelectronic reduction in Q_A (corresponding to the 1666 cm^{-1} band in the present paper)) perfectly synchronous with the electron transfer backreaction, both after a 7 ns saturating flash and after a 20.5 s continuous illumination period [20].

The results presented here suggest that the situation is dramatically different in strongly dehydrated RCs (RH = 11%) in the following points:

(1) After a flash, the 1666 cm^{-1} negative band (equivalent to the 1668 cm^{-1} band at RH = 76%) decays with roughly the same kinetics of the 1748 cm^{-1} characteristic of P^+ (in other terms, the 1666 cm^{-1} conformational change relaxes almost synchronously with the back electron transfer reaction).

(2) After 20.5 s, the 1666 cm^{-1} negative band seems to decay significantly faster than the band at 1748 cm^{-1} characteristic of P^+ (this not only suggests that the 1666 cm^{-1} conformational change relaxes faster than the back electron transfer reaction, but also that this conformational change does not seem to be related to the formation of the light-adapted state). Interestingly, this band seems to decay faster than after a single saturating flash.

(3) According to the kinetic fitting of all the time-resolved FTIR difference spectra, the lifetime of the $P^+Q_A^-$ state is just slightly prolonged after 20.5 s of continuous illumination as compared to the lifetime after a laser flash. This result is at variance with the results obtained by time-resolved Vis spectroscopy by our group (where a strong stabilizing effect was observed [8]), and is likely to be related to the difference in temperature at which the experiments were carried out (281 K for time-resolved FTIR spectroscopy reported in the present manuscript, 297 K for time-resolved Vis spectroscopy).

(4) Compared to the results obtained at RH = 76%, other mechanisms seem to be responsible for the (relative, if any) stabilization of the $P^+Q_A^-$ state under continuous illumination at RH = 11%. The double-difference spectrum in Figure 7 suggests that proton movement involving protonation/deprotonation events involving side chains of Glu and Asp amino acids in the 1775–1690 cm^{-1} region might be involved. It is interesting to note that in this region, the double-difference spectra "light-adapted minus non-light adapted" obtained at RH = 76% and at RH = 11% differ most. At RH = 76%, positive peaks are observed at 1752, 1733, and 1721 cm^{-1} and negative peaks are observed at 1744 and 1694 cm^{-1} [20]; at RH = 11%, positive peaks are observed at 1772, 1746, 1728, and 1720 cm^{-1} and negative peaks are observed at 1754 and 1699 cm^{-1}. Conversely, at lower wavenumbers (in the amide I region), the pattern in the two double-difference spectra is strikingly similar, the double difference spectrum obtained at 76% showing positive peaks at 1679, 1667, and 1630 cm^{-1} and negative peaks at 1654 and 1646 cm^{-1} [20], and the double-difference spectrum obtained for RH = 11% showing positive peaks at 1681, 1666, and 1634 cm^{-1} and negative peaks at 1658 and 1643 cm^{-1}.

(5) This suggests that the light-adapted RC has a similar response of the protein backbone at the two hydration levels (RH = 76% and 11%) but a different response in term of proton movements involving Glu and Asp side chains.

(6) In Figure 6, there are bands lying in the amide I region (positive bands at 1658 and 1643 cm^{-1} and negative bands at 1651 and 1634 cm^{-1}) that seem to be roughly synchronous—in decay—to the ones reflecting progressive reduction in the primary donor (from P^+ to P). As mentioned above, peaks at these positions can also be observed in the double-difference spectrum in Figure 7. Unfortunately, no precise identification for these band exists to date. For the 1634 cm^{-1} negative band, a possible attribution to an internal water molecule can also be proposed.

On the basis of the points listed above, we can propose, as a working hypothesis, that at RH = 76% [8,20], the protein backbone response is stabilized by water molecule displacements that are absent at RH = 11% (as observed in [8]), most likely due to a shrinking effect at the most dehydrated condition and possibly to the lack of specific water molecules in key position inside the RC. This does not allow for the formation of a real light-adapted state conformation at RH = 11% (at least at 281 K). In addition, a protein response can be seen by the movement of protons responsible for the protonation/deprotonation of Glu and Asp side chains at both hydration levels. This kind of protein response seems to be not the same in the two cases.

5. Conclusions

In this work, we applied time-resolved FTIR difference spectroscopy to investigate the protein response at RH = 11% at 281 K both after a flash and during/under 20.5 s of continuous illumination. Spectra recorded after a laser flash show a protein recovery almost synchronous to the electron transfer backreaction. Conversely, spectra recorded after 20.5 s of continuous illumination basically show no difference in kinetics of electron transfer compared to spectra recorded a laser flash. This means that the mechanism of formation of a light-adapted stabilized state is less effective compared to the same hydration level at 297 K (as observed before [8]) and to the RH = 76% hydration level (both at 281 K [20] and 297 K [8]). It is interesting to notice that in spectra recorded after 20.5 s of continuous illumination, the 1666 cm^{-1} band seems to decay faster than marker bands for the electron transfer backreaction $P^+Q_A{}^- \rightarrow PQ_A$.

FTIR double-difference spectra (obtained by subtracting a flash-induced FTIR difference spectrum from an FTIR difference spectrum recorded after 20.5 s illumination) suggest that at RH = 11% and 281 K, the light-adapted state formed after 20.5 s of continuous illumination is different from the one observed at RH = 76% [8,20]. A possible interpretation is that at RH = 11% and 281 K, the protein response is modified by the fact that at this hydration level and at this temperature, only protons can move easily, and movement of water molecules is hampered compared to at RH = 76%. This makes the formation of a real light-adapted stabilized state unfeasible.

Author Contributions: A.M. and G.V. conceived the study; A.M., M.M. and F.F. collected the data; A.M. analyzed the data; A.M. wrote the draft of the manuscript; A.M., M.M., F.F. and G.V. corrected the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data can be provided upon request to the corresponding author, A.M.

Acknowledgments: The authors thank Winfried Leibl (CEA-Saclay, France) for help during FTIR measurements and helpful discussion.

Conflicts of Interest: The authors declare no conflict of interest.

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