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Biochimica et Biophysica Acta 1504 (2001) 311–318

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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Long-lived charge-separated states in bacterial reaction centers isolated from *Rhodobacter sphaeroides*

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Received 3 May 2000; received in revised form 27 September 2000; accepted 26 October 2000

Abstract

We studied the accumulation of long-lived charge-separated states in reaction centers isolated from *Rhodobacter sphaeroides*, using continuous illumination, or trains of single-turnover flashes. We found that under both conditions a long-lived state was produced with a quantum yield of about 1%. This long-lived species resembles the normal P^+Q^- state in all respects, but has a lifetime of several minutes. Under continuous illumination the long-lived state can be accumulated, leading to close to full conversion of the reaction centers into this state. The lifetime of this accumulated state varies from a few minutes up to more than 20 min, and depends on the illumination history. Surprisingly, the lifetime and quantum yield do not depend on the presence of the secondary quinone, Q_B. Under oxygen-free conditions the accumulation was reversible, no changes in the normal recombination times were observed due to the intense illumination. The long-lived state is responsible for most of the dark adaptation and hysteresis effects observed in room temperature experiments. A simple method for quinone extraction and reconstitution was developed.

Keywords: Reaction center; Protein quake; Charge recombination; Protein conformation; Charge separation

1. Introduction

The isolated reaction center (RC) of the photosynthetic purple bacteria has been available for over 30 years now [1]. Especially after its crystal structure was solved [2] it has been the most popular subject for the study of light-driven electron transfer in biology [3]. A chain of consecutive electron transfer reactions results in a charge-separated state that is stable for about 1 s when the secondary acceptor Q_B is present. Especially the primary steps which occur on a picosecond time scale have been the center of attention of many ultrafast spectroscopy studies [3] and many theoretical works [4]. The detergent-isolated RC is very stable and can stand quite a bit of interrogation by optical spectroscopy. However, especially among the researchers who are interested in the secondary electron transfer processes [5], it is well known that experiments should be performed on dark-adapted RCs. The reason for this is that long-lived states tend to accumulate with prolonged illumination. These long-lived states have not been characterized properly and are often somehow ascribed to a 'bad fraction' of the sample. Here we measured

Abbreviations: LDAO, N,N-dimethyl-dodecylamino-N-oxide; PMT, photomultiplier tube

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0005-2728/01/$ – see front matter
PII: S0005-2728(00)00259-0

the difference spectra and lifetimes of these 'anomalous states' using both continuous illumination and trains of single-turnover flashes. Under all conditions the quantum yield of the long-lived state is approximately 1–2%, and we show that it is *not* a small fraction of the RCs that is responsible for this, but rather that every turnover has a finite probability ($\sim$1–2%) to lead to this anomalous state. Under anoxygenic conditions the accumulation is fully reversible. Rather surprisingly, the formation of the long-lived state does not depend on the presence of the secondary quinone (Q_B). As we will show, the absorption difference spectrum of the long-lived state is very similar to that of the normal $P^+Q_A^-$ or $P^+Q_B^-$ difference spectra, in both the Q_y and quinone absorption regions. The results point to a large conformational change, i.e. a 'reversible catastrophic collapse', of the protein induced by the electric field of the charge-separated state.

2. Materials and methods

RCs from *Rhodobacter sphaeroides* were prepared as in [6], with minor changes as described below. Chromatophores were diluted to an absorbance of 50 at 850 nm, and incubated with 1% LDAO. Centrifugation steps were carried out as described. The supernatant was subjected to a DEAE-Sepharose-FF column (Pharmacia), equilibrated with 20 mM Tris–HCl, pH 8.0, 0.3% LDAO. Free pigments were removed by washing with 20 mM Tris–HCl, pH 8.0, 0.3% LDAO, 100 mM NaCl. The RCs were eluted with 145 mM NaCl in a gradient from 100 to 200 mM NaCl in 20 mM Tris–HCl, pH 8.0, 0.3% LDAO. Fractions containing RCs were combined, diluted two-fold in 20 mM Tris–HCl, pH 8.0 and were loaded on a HR 5/5 column filled with Source 15Q (Pharmacia), and equilibrated with 20 mM Tris–HCl, pH 8.0, 0.025% LDAO. RCs were purified and eluted using a linear salt gradient with 20 mM Tris–HCl, pH 8.0, 0.025% LDAO, (0–500 mM NaCl), at a flow rate of 2 ml/min in 30 min. Fractions with RCs having a 280/800 nm ratio below 1.3 were pooled and desalted in an Amicon cell with membrane YM-30. This isolation procedure produced RCs with a Q_B content/activity of about 60%; experiments were performed on these samples and on samples in which Q_B was either reconstituted or extracted. Because we wanted to minimize the duration and harshness of the isolation and reconstitution procedures we developed some simple reconstitution and extraction procedures which could be integrated within the normal isolation and purification scheme. The extraction of Q_B was attained by an extended washing procedure. RCs were prepared as described above and again subjected to a HR 5/5 column filled with Source 15Q. The column was washed for 2 h with 20 mM Tris–HCl, pH 8.0, 0.3% LDAO. RCs were eluted with a linear pH gradient in 30 min (down to pH 6.5). The front part of the main RC peak contains pure RCs with a Q_B content/activity of less than 5%. After the elution the buffer was exchanged in an Amicon cell, membrane YM-30.

Reconstitution of Q_B activity was achieved by adding ubiquinone (UQ_{10} from Sigma) to the buffers used for the second purification step in the normal procedure. The problem of the low solubility of the ubiquinone in water was solved by dissolving the quinone in the stock solution of 30% LDAO used for making the buffers. This way no external (methanol-dissolved) quinone additions were needed, and the micellar phase of the buffer had a relatively high quinone concentration. After elution from the column the samples contained essentially no unbound quinones. The Q_B activity of the reconstituted samples was typically higher than 95%, as determined from flash recombination experiments. In a similar way also different quinones such as menaquinone could be reconstituted (results not shown) after extraction of the native quinones.

Experiments were performed at room temperature, in 10 mM Tris–HCl pH 8.0, containing 0.025–0.05% LDAO and 150 mM NaCl (optional). In buffers with a low salt concentration the LDAO–RC complexes had a blue-shifted and hypochromic P-band, peaking at 855 nm, as was observed before for RCs isolated using zwitterionic detergents [7,8]. This effect was, however, not reported for LDAO, so it is observed here as a result of the prolonged washing on the column, and is probably due to the replacement of some lipid molecules by detergent. The recombination rates from both $P^+Q_A^-$ and $P^+Q_B^-$ were both slowed down by approximately 30% in the blue-shifted form. The blue shift of the P-band has no effect on the accumulation effects studied here, how-

ever, we found that the addition of 150 mM NaCl (equivalent to a physiological salt solution) gives rise to a close to complete reversal of the blue shift to ~864 nm, therefore most experiments were performed in this buffer. Anoxygenic conditions were obtained by using the oxygen dissipating system of glucose, glucose oxidase and catalase [10]. We found that excess glucose resulted in aggregation of the RCs, probably due to digestion of the detergent (LDAO) by the glucose oxidase, therefore only minimal excess glucose was added and a detergent concentration of 0.035% LDAO was used, slightly above the critical micellar concentration of LDAO.

Accumulation experiments with continuous actinic light were performed in a Unicam UV/Vis spectrophotometer. Spectra were recorded before and after illumination. The cuvette was homogeneously illuminated through an RG 830 filter using a light-guide system (~1–5 mW/cm^2). The time-dependent difference spectra were analyzed using global analysis [9]. Transient absorption measurements using trains of single-turnover flashes were performed on a home-built single-beam spectrophotometer. Actinic light was from a 10 μs xenon flash through an RG780 filter (>80% saturating), absorption changes were measured at 605 nm, the PMT was protected by a complementary BG39 filter, measuring light at the sample was 50–500 nW/cm^2.

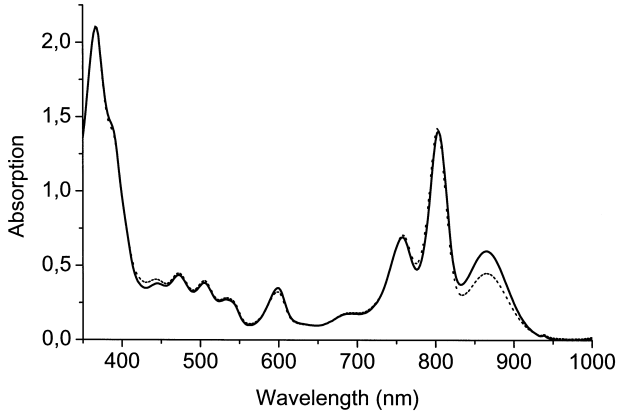

Fig. 1. Absorption spectra of RCs before (solid curve) and ~30 s after (dashed curve) continuous illumination with ~2 mW/cm^2 for 10 min. This sample had a Q_B content of 70%.

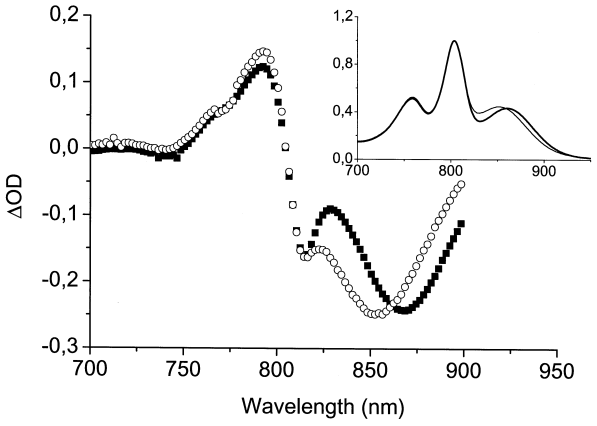

Fig. 2. Decay-associated spectra of the recovery from continuous illumination as in Fig. 1. Sample 1 (■) had >95% Q_B activity and sample 2 (○) had ~5% Q_B activity. The inset shows the effect of adding 150 mM NaCl to the Q_B extracted sample.

3. Results

Fig. 1 shows two typical absorption spectra taken before and ~30 s after saturating continuous illumination. Prominent differences between the two spectra are a bleaching of the P-band in both the Q_y and Q_x regions, a band shift of the B-band, and induced absorption in the quinone region around 450 nm. The spectral features resemble those of the P^+Q^- [11], however this species should not be present after more than a few seconds. To follow the recovery of the sample to the dark-adapted spectrum, spectra were recorded every 30 s. The decay-associated difference spectra of the recovery, obtained by global analysis of these spectra, are shown in Fig. 2 for two different samples, one in which the Q_B activity was reconstituted and one in which Q_B had been extracted. The illumination time was 10 min of close to saturating light (~2 mW/cm^2), and spectra were recorded every 30 s starting ~10 s after switching off the actinic light. The spectral difference (blue shift of the special pair band in the Q_B-extracted sample) between the two samples is due to the difference in the isolation procedure of the two samples [7,12]. The inset of Fig. 2 shows the effect of adding salt to the Q_B-extracted sample, most of the blue shift of the spectral pair band, which is a result of extended washing, can be reversed by adding salt. The difference in lifetime between the slow Q_A and Q_B recovery observed here was not observed as a systematic difference. The fits required two more components

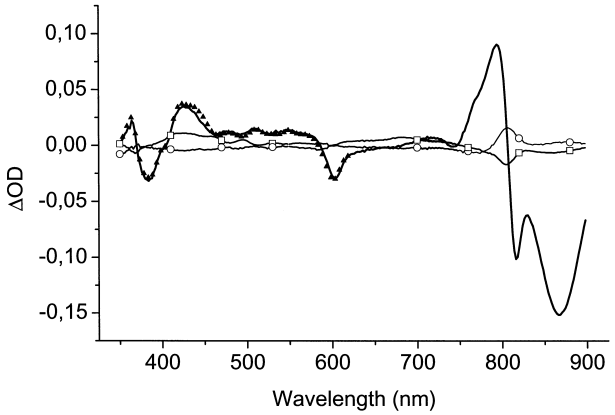

Fig. 3. Decay-associated spectra after 15 min of continuous illumination, together with the flash-induced (10 μs xenon flash through an RG780 filter) difference spectrum (▲) of a dark-adapted sample. The solid line (without symbols) corresponds with the decay-associated difference spectrum of the 5 min component, the traces marked with ○ and □ correspond to lifetimes of 40 and 1000 min (fixed parameter in the fit). The flash-induced difference spectrum was normalized to the accumulated signal at 605 nm.

with longer lifetimes (not shown, but see Fig. 3), which were of a compensating nature and were due to the non-exponentiality of the recovery (see Section 4). The light-induced changes were close to fully reversible ($>90\%$) under anoxygenic conditions. In the presence of oxygen permanent damage occurs to the RCs under these intense and prolonged illumination conditions. However, also in the presence of oxygen the accumulation effects can be observed, and can be distinguished from the irreversible processes by global analysis of the recombination process (results not shown). Fig. 3 shows the difference spectrum over a wider spectral range, in combination with the spectrum of the normal recombination component as measured with single-turnover flashes on an identical dark-adapted sample. Clearly the differences between the flash-induced P^+Q^- (sum of amplitudes of the 0.1 and 1 s decay components of the traces) and the accumulated spectrum (5 min component) are very small. As in Fig. 2, besides the major 5 min component longer-lived components, with spectra that are of compensating nature over the whole wavelength range, occur which result from the non-exponential nature of the decay. The spectrum of the accumulated species appears to be that of ubisemiquinone [13,14]. The difference around 430 nm is too small to be taken as indicative of a change in protonation [14], moreover the sign of the difference would actually indicate a lower degree of protonation in the accumulated species.

All recovery experiments showed highly non-exponential decays (see e.g. the recovery from continuous illumination in Fig. 5), therefore the global analysis gives only some average of the lifetime. For an analysis with a lifetime distribution function more time points would be required. For the point that we want to make here the spectral information is more important. Note also that when both time and spectral information are pursued at some point the measuring light will cause accumulation and thus perturb the recovery kinetics (see below).

Fig. 4 shows the effect of a train of flashes, every 12 s a flash is given and the points in the graph represent the amplitudes of the different recombination lifetimes for each individual trace/flash. The lower curve represents the absorption before each flash relative to the absorption before the first flash (with an arbitrary offset, this signal of course equals zero before the first flash) and thus shows the effects of accumulation due to previous flashes. The pulse train clearly causes the build-up of some long-lived state, and some steady-state population of this state is reached within about 100 flashes. The upper two curves represent the decay parameters of each trace. This particular sample had a Q_B activity of $\sim 60\%$,

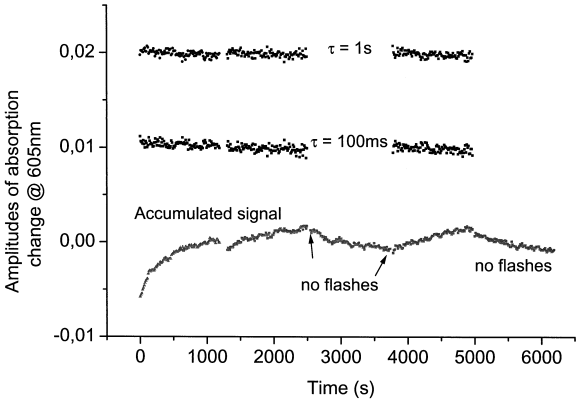

Fig. 4. Transient absorption measurements (detection at 605 nm) with excitation by trains of xenon flashes. Time between the flashes was 12 s, bunch size was 100 shots. Two periods without actinic flashes are indicated. The upper two sets of points correspond to the amplitudes of the bi-exponential fit of the individual traces (lifetimes were fitted globally). The lower set of points corresponds with the absorption of the sample before the flash (shifted arbitrarily for the figure).

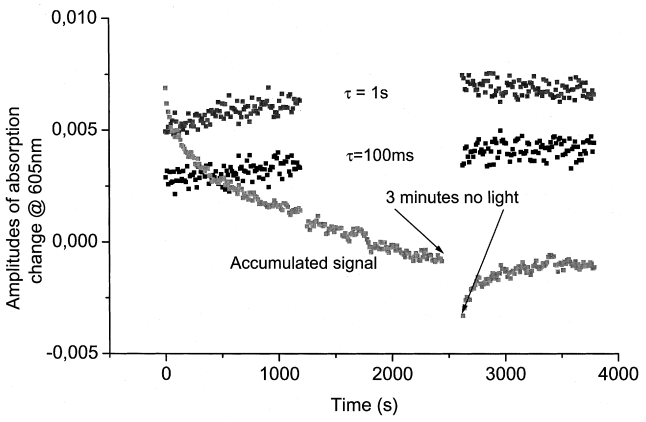

Fig. 5. Transient absorption measurements as in Fig. 4 but with 15 min continuous pre-illumination, ~2 mW/cm^2. $OD_{870} = 0.2$. Measuring light was 250 nW/cm^2.

but experiments on samples with lower or higher Q_B activity gave the same results. In the interval between the flash trains the decay of the accumulated population can be seen. From the initial slope of the offset parameter (ratio between the change in the offset parameter and the sum of the amplitudes of the normal recombination processes) a quantum yield of about 1% can be estimated. All decays were fitted with the same decay times, in this case the traces were analyzed globally in groups of 100 traces. However, also fits of the individual traces showed no effect of the pre-illumination on the normal recombination times. Note that the sum of the three components is not constant, the build-up of the accumulated state does not lead to a proportional decrease of the amplitudes of recombination components, this is due to the fact that the experiment was not done at a concentration where the Q_y transmission change is linear with the number of absorbed quanta.

In Fig. 5 this experiment is repeated but this time with continuous pre-illumination. Due to the pre-illumination higher initial populations of the accumulated state can be reached. The continuous illumination is switched off just before the start of the first pulse train. As can be seen, the recovery from the continuous illumination competes with the pumping by the flash train, the decay is faster when the flashes are off. This experiment was performed with a four times higher intensity of the measuring light. The effect of the measuring light can be clearly appreciated from the effect of the extended dark period between the last two bunches of measurements. During the dark period the concentration of the accumulated state drops much faster than in the preceding period where the measuring light was on. Therefore the measuring light (in this case ~200 nW/cm^2 at 605 nm) pumps a significant fraction of the sample into the accumulated state. In these experiments there is no effect of the pre-illumination on the recombination lifetimes obtained from the individual decay traces. Since in this experiment a significant fraction of the RCs were accumulated in the long-lived state prior to the first pulse, it is clear that after recovery from the long-lived state the RC behaves as before, i.e. the accumulation is reversible and no indications of long-term memory effects are found when looking at the flash recovery kinetics. Note that the phenomena described here make it a priori difficult or impossible to measure light adaptation effects (other than the effects described here) in isolated RCs. The measurement that one would like to make to study the changes in the electron transfer rates would be to probe the ground state that is produced after the long-lived state has recombined, however this is obviously impossible due to the slow recombination from this state.

4. Discussion

Accumulated species in isolated RCs have been described before [15–19], however these were always produced under reducing conditions and contained reduced bacteriopheophytin. The spectrum of the accumulated species observed here is very similar to that of the normal P^+Q^- radical pairs.

As to the nature of the long-lived state we can disqualify many of the options. The fact that the quantum yield for the production of the long-lived state was the same for continuous and single-turnover flash illumination indicates that the effect is not due to double excitation of the RCs. The accumulation effect does not depend on the presence of Q_B which is the best possible proof that these effects are not caused by double reduction of Q_B or the release of Q_B^- from the RC. A possible explanation would be the exchange of an electron between a PQ RC and a P^+Q^- RC resulting in a P^+Q and a PQ^- RC, which could have a similar overall difference spectrum and would have a diffusion-limited recombination time.

However, the effects do not disappear at higher detergent concentrations (no differences were observed from 0.025 to 0.1% LDAO) which makes it less likely that the exchange occurs between RCs that share a detergent micelle. Therefore, what we are left with as the most likely candidate is a large conformational change of the protein. We suggest that changes in the protein structure directly induced by the radical pair play a decisive role here. Light-induced structural changes [20–25] and structural heterogeneity [26,27] have indeed been observed in the bacterial RC both in the pheophytin and in the Q_B region. These changes can easily give rise to changes in transfer rates by several orders of magnitude [23,29]. We recently found a strong link between the protein dynamics and the accumulated state. The dephasing dynamics of the amide-I band of the protein, as measured using mid-IR photon echo spectroscopy, are changed in the accumulated state, in a way that points to a reduction of the motional freedom of the protein in this state (F. van Mourik, G.H.M. Knippels, A.F.G. van der Meer, A.R. Holzwarth, J. Phys. Chem., in preparation). The long lifetime of the accumulated state suggests a major conformational change. We would like to describe this as a 'catastrophic event/collapse' of the protein which occurs in a small fraction of the protein quakes [28] that take place in the protein as a result of repeated charge separation cycles. We have not looked so far at these effects in membrane-bound RCs. Possibly the membrane can stabilize the RC and prevent these conformational changes. However, we think that the observed effects are quite natural considering the stress invoked on the RC by the radical pair. An electron and a hole at a distance of 2.5 nm exert an attractive force of $\sim$30 pN/ε_r. To appreciate the magnitude of this one could imagine this force working on a surface of 1 nm^2 which would correspond to a (unidirectional) pressure of 30/ε_r kbar. For small values of the dielectric constant this corresponds to a pressure sufficient to denature most proteins [30,31]. This calculation is obviously wrong, since it does not include the dielectric properties of the solvent and most importantly dielectric relaxation of the solvent and the protein [32–34]. However, it demonstrates the need for the protein to provide for shielding of the charges by means of dielectric relaxation, and indeed most model calculations for the relative dielectric constant use values of $\sim$20. However, it should be realized that this value is time/frequency-dependent and at very short times a value of $n^2 = \sim$2–3 would be more appropriate. The dielectric response of the RC is furthermore not homogeneous, molecular dynamics studies show that most of the response occurs in the vicinity of Q_B^- [35], which could actually explain the observation here that the yield of the accumulated state does not depend on the presence of Q_B. In the state $P^+Q_B^-$ the charges are effectively shielded ($\varepsilon_r \sim 20$) so that the driving force for the conformational change is effectively diminished. Note that although in Q_B-containing RCs the charge-separated state lives about 10 times longer than in RCs with only Q_A, the total time spent in the state $P^+Q_A^-$ during a cycle is the same in both cases (because recombination from $P^+Q_B^-$ goes via $P^+Q_A^-$). Photo-acoustic measurements on RCs have pointed to significant (negative) changes in the volume of the RC on a time scale faster than about 20 ns [36–38], which do not depend on the presence of Q_B and probably occur in the state $P^+Q_A^-$. The photo-acoustic signal was taken as an indication that electrostriction effects play a role in the RC [38]. However, the high dielectric constant derived from this effect seems to be in conflict with the low degree of dielectric relaxation of $P^+Q_A^-$ as obtained from molecular dynamics studies [35]. Therefore it is quite possible that the long-lived state reported on here has a disproportional contribution in these photo-acoustic experiments and could possibly (despite its low quantum yield) be responsible for a significant fraction of the signal.

The actual lifetime of the long-lived state is variable and depends on the duration of the illumination. This could be due in a trivial way to an inherent distribution of these lifetimes which is then weighted/distorted by the accumulation time. Equilibration between the ground state and the accumulated state goes with the sum of the pumping and the decay rate and therefore shorter-lived states reach their equilibrium population faster. However, under illumination conditions where each RC is excited about 20 times/min in combination with a quantum yield of about 1% (resulting in a pumping rate of 0.2/min) this effect should not play a role for illumination times longer than $\sim$5 min. Of course this argument does not hold if the quantum yield is not con-

stant over the distribution. However, by fitting the slow decays with a distribution of lifetimes (results not shown), we found indications that longer illumination times shift the distribution of lifetimes to longer times and result in narrower lifetime distributions. Also, when performing these experiments at lower temperatures (277 K) the effects become more pronounced, the long lifetime becomes even longer and there is a stronger dependence on the illumination time (M. Kapustina et al., private communication). Therefore we tend to believe that there is some conformational memory effect in the reaction center that causes this. However, even after long saturating illumination we found that the decay times of $P^+Q_B^-$ and $P^+Q_A^-$ (as measured with single-turnover flashes) are the same as in dark-adapted RCs. The effect on the long-lived kinetics indicates that there is some memory of having been in the long-lived state that pertains after recovery to the ground state. However, the conformational change of the protein responsible for this memory effect does not seem to affect the normal recombination kinetics (but see below). This qualifies the conformational changes associated with this possible memory effect as protein modes normal to the (dielectric) reorganization processes that occur during the electron transfer processes in the reaction center.

Because we do not see any change in the normal recombination rates we have to conclude that previous experimental papers on light adaptation effects [39–42] in isolated RCs at room temperature were a misinterpretation due to the effects described here [43]. Under the experimental conditions of these papers accumulation effects are unavoidable and are a probable explanation for the observed non-linear effects. This does not mean that the non-linear models proposed in these works are not important for the electron transfer reactions in RCs, however we found no evidence for memory effects between consecutive cycles (i.e. memory extending over at least a few seconds) in isolated RCs at room temperature. Note that single molecule spectroscopy has recently given some very clear examples of memory effects in proteins at room temperature on a timescale of tens of seconds [44].

Unfortunately it is not possible to optimize the experimental conditions so as to produce an ensemble of ground-state reaction centers that have a significant population that has 'recently' recombined from the long-lived state, as would be necessary for investigating the 'ground-state memory'. Therefore it is also not clear if the conformational change gates the recombination process or the other way around, i.e. if the recombination process in the long-lived state requires a reversal of the conformational change or if it is the electron–hole pair that keeps the protein locked in this conformation.

As discussed above, we consider the long-lived states to be the result of some conformational change of the protein giving rise to a dramatic slowing down of the electron transfer rate by up to three orders of magnitude. Since it is possible to accumulate this state it can be studied in detail and elucidation of its nature could give some clues about the normal electron processes that occur within the bacterial RC. We are currently performing EPR and FTIR experiments on the accumulated state for further characterization.

Acknowledgements

This work was supported by the Volkswagen Stiftung. Schwerpunkt 'Electron transfer processes in Biological and Chemical Systems'.

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