Biochimica et Biophysica Acta, 1102 (1992) 253–259

BBABIO 43701

Sensitive analysis of the occupancy of the quinone binding site at the active branch of photosynthetic reaction centers

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(Received 19 November 1991)
(Revised manuscript received 15 May 1992)

Key words: Bacterial photosynthesis; Reaction center; Quinone

A sensitive method for determining the degree of the occupancy of the primary quinone site in reaction centers of photosynthetic bacteria is described. By measuring the transient absorbance bleaching of the primary electron donor during the time interval 1 ns to 1 ms after an exciting laser pulse, small amounts of quinone-depleted reaction centers can be detected with an accuracy of about ±3%. In the case of *Rb. sphaeroides*, standard preparations typically contain 20% reaction centers in which the quinone binding site at the active branch is not occupied.

Introduction

Heterogeneities may cause considerable obstacles in the spectroscopic characterization of complex photochemical systems, such as photosynthetic reaction centers (RCs). In RC preparations a heterogeneity caused by partial occupancy of the binding site of the primary quinone (Q_A) cannot be excluded a priori and deserves a closer investigation. Apart from possible structural effects of an empty binding site on electron transfer reactions not directly involving Q_A, the loss of Q_A will inhibit all electron transfer processes in which Q_A is directly involved. This could give rise to an apparent wavelength dependence of the 100 ps electron transfer reaction and has serious consequences for quantum yield determinations.

In Q_A-depleted RCs the forward electron transfer from the bacteriopheophytin anion (H^-) to Q_A occurring on the 100 ps timescale [1–3] is blocked, forcing the reoxidation of H to occur via recombination with the cation of the primary donor (P^+) on the 10 ns timescale [4,5]. Under certain circumstances, which have been explored recently [6], a partial loss of Q_A could lead to an apparent wavelength dependence of the measured rate $P^+H^- \rightarrow P^+Q_A^-$ when fitted monoexponentially. On the other hand, in the presence of 100% Q_A such a wavelength dependence has to be attributed to other heterogeneities in the RC preparation causing a distribution of the rate $P^+H^- \rightarrow P^+Q_A^-$ [7]. Thus, a sensitive determination of the occupancy of the Q_A site is indispensable for the interpretation of wavelength-dependent kinetics.

A partial loss of Q_A will also affect the result for the quantum yield of charge separation (QY) from $^1P^*$, when determined by the standard method of measuring the induction rate of photooxidation of P on the ms timescale [8,9]. RCs not containing Q_A will not contribute to the bleaching of P on this timescale and lead to an underestimation of QY. On the other hand, the reported value of 1.02 ± 0.04 for the QY in RCs of *Rb. sphaeroides* [8] was calculated using the difference extinction coefficient $\Delta\epsilon_{865} = 112\ mM^{-1}\ cm^{-1}$ for the bleaching of P absorbance at 865 nm, as determined in an independent measurement [10] from the incomplete bleaching observed under steady-state illumination. The incomplete bleaching could be due to contributions of the bacteriochlorophyll (BChl) monomers or the dimer cation P^+ to the absorbance at 865 nm. However, it also could be due to an incomplete occupancy of the Q_A site, since RCs depleted of Q_A re-

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Abbreviations: RC, reaction center; P, bacteriochlorophyll dimer; H, bacteriopheophytin at the active branch; Q_A, quinone at the active branch; Q_B, secondary quinone; LDAO, lauryldimethylamine oxide; BChl, bacteriochlorophyll; BPh, bacteriopheophytin; QY, quantum yield of charge separation.

cover completely on the 100 μs timescale and do not contribute significantly to the bleaching under steady-state illumination. In the latter case, the above value of $\Delta\epsilon_{865}$ would be underestimated, leading to an overestimation of QY, as discussed in Ref. 8. Indeed, a slightly higher value of $\Delta\epsilon_{865} = 118$ mM^{-1} cm^{-1} was also determined in Ref. 10 from the coupling of the bleaching at 865 nm to the oxidation of cytochrome *c* after excitation with a short flash. With $\Delta\epsilon_{865} = 118$ mM^{-1} cm^{-1} the reported value for the QY in *Rb. sphaeroides* would have to be corrected to 0.98 ± 0.04. These measurements, in principle, avoid the problem of Q_A-depleted RCs, but introduce the uncertainty of the difference extinction coefficient for the oxidation of cytochrome *c*. Only a highly accurate determination of the occupancy of the Q_A site will allow the confirmation or reliable correction of the value of $\Delta\epsilon_{865}$ and, thereby, of QY in *Rb. sphaeroides*.

Moreover, the knowledge of the occupancy of the Q_A binding site is of utmost significance for experiments on modified RCs, since the majority of mutagenic and chemical modifications have been found to reduce the Q_A binding affinity considerably. This holds for: (i) various mutations of neighboring or more remote amino acid residues in RCs of *R. viridis* [11], *Rb. sphaeroides* [12] and *Rb. capsulatus* [13] and for (ii) chemical exchange of prosthetic groups as the quinones [14,15] or of pigments, such as the bacteriochlorophyll monomer (A. Struck, personal communication). Therefore, in the light of the increased interest in modified RCs [16], a sensitive determination of the occupancy of the Q_A binding site is becoming increasingly important.

A first estimate of the Q_A content is usually achieved by measuring the bleaching of the Q_x absorption band of P around 865 nm under saturating steady-state illumination. This determination of the Q_A content rests on the knowledge of $\Delta\epsilon_{865}$, the uncertainty of which was discussed above. Additionally, data obtained at high actinic intensities are problematic, since the possibility of non-reversible or slowly reversible photochemistry cannot be rule out. Indeed, a bleaching of the ground-state absorbance of P after steady-state illumination recovering on the timescale of minutes (*Rb. sphaeroides*) or hours (*Chloroflexus aurantiacus*) has been observed [17].

The determination of the Q_A content via the observation of the oxidation of cytochrome at the beginning of continuous illumination [18] or via chemical extraction of the quinones [15] yields only approximate values. This is due to experimental limitations and the uncertainty with respect to the presence of secondary quinone.

More reliable values can be expected from transient absorption measurements at low excitation conditions. However, for measurements on the ps or ns timescales, certain features of the transient difference spectra of P^+H^- and $P^+Q_A^-$ have to be known for estimating the Q_A content. An unambiguous direct measurement of these spectra is difficult on samples which might be heterogenous with respect to the Q_A site occupation.

In principle, there are several spectral regions well suited for the determination of the Q_A content:

(1) The absorption band of H at 665 nm. Here the amount of Q_A-depleted RCs could be estimated from the amount of residual H^- absorption in the time window after $P^+H^- \rightarrow P^+Q_A^-$ in Q_A-containing RCs but before $P^+H^- \rightarrow PH$ recombination in Q_A-depleted RCs. This estimate rests on the assumption that $P^+Q_A^-$ has no contribution to the difference absorbance. $P^+Q_A^-/PQ_A$ [3,9] and P^+/P [19] difference spectra, however, show slightly negative difference absorbance in this spectral region. If this is true, a difference absorbance of zero at 665 nm for delay times over 1 ns would actually imply a certain number of RCs remaining in the state P^+H^- at these delay times. These would need to be Q_A-depleted RCs.

(2) In the Q_x absorption band of H at 545 nm. In this spectral region, the bleaching of the Q_x absorption band of H is superimposed on a broad spectral feature with positive absorption attributed to P^+. The attempt to deduce the H^- concentration by distinguishing between both spectral contributions is hampered by superimposed electrochromic shifts on the Q_x transitions of the bacteriopheophytins, as observed in RCs of *Rb. sphaeroides* [3] and *Chloroflexus aurantiacus* [20], and by uncertainties with respect to the shape of the P^+ absorption band.

(3) In the low energy Q_x transition of P at 865 nm. This spectral region is dominated by the P absorbance and is not expected to show appreciable absorbance due to one of the transient states under consideration, apart from minor contributions discussed later. The decay of the various intermediate states in Q_A-containing and Q_A-depleted RCs to the ground state P can be discerned by the different life-times of these states. The respective amplitudes of the ground-state absorbance recovery in principle allow the determination of the relative concentrations of these states.

In RCs lacking Q_A, P^+H^- recombines on the 10 ns timescale, accompanied by a recovery of the bleaching of the 865 nm band. In contrast, the bleaching persists on this timescale in Q_A-containing RCs, due to the formation of the long-lived state $P^+Q_A^-$. For the necessary delay times of several tens of nanoseconds, however, the standard technique of pump-probe-measurements with an optical delay between exciting and probing pulse suffers from the problem of changing the profile of the exciting beam and/or changing the overlap of the two beams at different delay times. Thus, experiments with the necessary precision are very difficult. A quantitative determination also must account for the fraction of Q_A-depleted RCs recombining to

the triplet state $^3P^*$ [4,5], since in those RCs the bleaching persists for microseconds [21]. Therefore, an independent measurement of the yield of $^3P^*$ formation, on RCs deliberately depleted of Q_A, is necessary.

Alternatively, one can determine the amount of RCs lacking Q_A from the amplitude of the absorption recovery on the microsecond time scale, reflecting the decay of $^3P^*$ [21]. Again, the relative yield of $^3P^*$ formation Φ_T has to be known from independent measurements on RCs deliberately depleted of both quinones for the quantitative determination of the Q_A content. With a typical value of Φ_T of 0.3 [4,5], only a fraction of the Q_A-depleted RCs is observed in a measurement on the microsecond timescale, thus reducing the accuracy of the measurement.

Thus, it seems very difficult to perform a determination of the occupancy of the Q_A site with an accuracy of a few percent employing the methods used to date. It is the goal of this paper to demonstrate that, by the simultaneous observation of the P absorbance bleaching in an expanded time window, ranging from nanoseconds to milliseconds, it is possible to measure this occupancy with an accuracy of better than 3%. The expanded time window is achieved in a pump-probe experiment using an electronic delay between the excitation and the probing laser pulses, thus also eliminating the problems caused by an optical delay. Furthermore, no independent determination of recombination parameters, especially the triplet yield Φ_T, in Q_A-depleted RCs is necessary.

Experimental procedures

Materials

Q_A-containing RCs of *Rb. sphaeroides R26* in Tris buffer (20 mM, pH 8.0) were prepared by standard methods [5], with either 0.1% Triton X-100 or 0.08% lauryldimethylamine oxide (LDAO) as detergent. The concentration of RCs was adjusted to approx. 20 μM to give an absorbance of approx. 0.5 at the probing wavelength of approx. 870 nm (pathlength 2 mm). The concentration was not well matched for the different samples described below, with the absorbances ranging from 0.5 to 0.8. For measurements at low temperatures a 60% (v/v) glycerol/buffer solution was used. For the investigation on the effect of excess quinone, ubiquinone$_{10}$ (Sigma) was dissolved in methanol (spectroscopic grade, Aldrich Chemie) to give a concentration of 10 mM, then the appropriate amount of this solution was added to the sample to yield a concentration of ubiquinone$_{10}$ of 100 μM.

Apparatus

RCs were excited at 600 nm by a Nd:YAG-laser-pumped dye laser (pulse width 1.8 ns) with an energy density of 0.5 mJ/cm^2, corresponding to approx. 20% excited RCs per pulse [1]. The P groundstate absorbance was indeed observed to bleach by approx. 20%. For this low excitation probability effects from multiple excitation can be neglected. Indeed, in measurements of the recombination dynamics in Q_A-depleted RCs of *Rb. sphaeroides*, which normally were done using the same excitation probability, no change in the characteristics was found when attenuating the excitation energy by a factor of three (Volk, M., Häberle, T., Ogrodnik, A. and Michel-Beyerle, M.E., unpublished results). The rate of excitation is chosen to ensure complete recovery of the whole sample before the next excitation pulse (e.g. approx. 1 Hz for RCs of *Rb. sphaeroides* with a lifetime of $P^+Q_A^-$ of approx. 135 ms at room temperature).

Absorption changes in the Q_y band of P around 870 nm are probed with a second dye laser (pulse width 1.5 ns) being pumped by a N_2 laser. A sequence of probing laser pulses is generated after each exciting pulse by repeated triggering of the probing laser with the maximum repetition rate of the detecting system of 16.7 Hz. The delay between the exciting and the first of the probing laser pulses can be adjusted electronically between 1 ns and 10 ms (programmable delay generator Le Croy 4222). To correct for trigger jitter and drifts of the lasers, the actual delay time between the actinic and the first probing pulse is monitored with a time-to-digital-converter (Le Croy 4202, resolution 150 ps). Thus, the absorbance changes in the interval between 1 ns and 10 ms after excitation can be monitored by varying the delay time between the exciting pulse and the first of the probing pulses, whilst the recombination of $P^+Q_A^-$ in the 100 ms range is measured with the sequence of probing pulses generated after each exciting pulse.

The probing pulse energy is measured by integrating the signal of a diode (Centronics OSD 60-3T, risetime 20 ns) during a 100 ns interval after the probing laser is triggered (integrating analog-to-digital-converter Le Croy 2249W). Excitation light (600 nm) is blocked from the diode by suitable filters (Schott RG695). Due to the small fluorescence yield in RCs [23], fluorescence from the sample (around 920 nm) can be estimated to be too small to distort the results. To experimentally rule out artifacts from scattered excitation or fluorescence light, control measurements are done at a delay time of -10 ns, that is with the first probing pulse

[1] The energy density of 0.5 mJ/cm^2 actually corresponds to 30% excited RCs in the first layer of the sample. However, due to the absorption of the exciting light in the sample the energy density decreases in the sample. A numerical simulation shows that, for a sample with an absorbance of 0.3 at the excitation wavelength, the overall probability of excitation is reduced by a factor of 1.5 compared to that in the first layer [22].

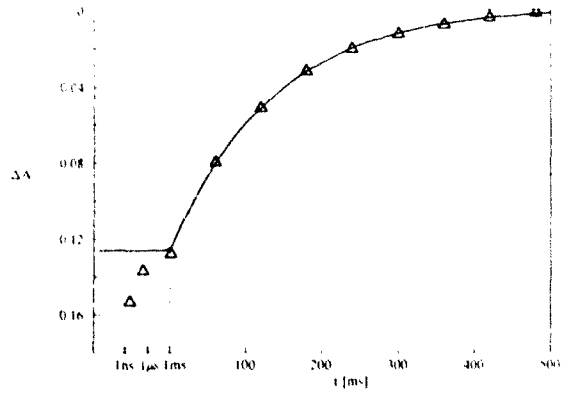

Fig. 1. Time-dependence of the bleaching ΔA of the P ground-state absorbance at 865 nm in RCs of *Rb. sphaeroides* in buffer solution (20 mM Tris (pH 8.0), 0.08% LDAO) at room temperature. The timescale is logarithmic for delay times under 1 ms and linear for longer times. The solid curve is a monoexponential fit of the data at delay times of 1 ms or longer, yielding a time constant of 135 ms.

arriving at the sample before the excitation pulse. Under these conditions no bleaching caused by the excitation light should be measured, whilst scattered excitation or fluorescence light detected by the diode still would be integrated and would contribute to the signal, thus inducing an apparent bleaching. Similar effects would arise from electronic artifacts. No such spurious 'absorbance change' was observed at -10 ns in any of the measurements described below.

By detecting the relative energy of each excitation pulse, the measured difference absorbance can be corrected for the fluctuating energy of excitation. After averaging over 100 single measurements, an accuracy of $2 \cdot 10^{-4}$ is obtained. A typical measurement, showing the complete recovery of the bleaching of P absorbance between 4 ns and 500 ms, is shown in Fig. 1.

A magnetic field of up to 700 G is applied to the sample with a pair of Helmholtz-coils.

Determination of Q_A content

The time-dependence of the P groundstate absorbance bleaching $\Delta A(t)$ at delay times t of nanoseconds or longer after excitation is given by

$$\Delta A(t) = \Delta A_0 e^{-t/\tau_1} + \Delta A_{depl}((1-\Phi_1) e^{-t/\tau_{RP}} + \Phi_1 e^{-t/\tau_3}) \quad (1)$$

Here ΔA_0 and ΔA_{depl} denote the bleaching at $t = 0$ due to Q_A-containing and Q_A-depleted RCs, respectively, τ_1 the recombination lifetime of $P^+Q_A^-$ (30–120 ms [14]) and τ_{RP} the recombination lifetime of P^+H^- in Q_A-depleted RCs (10–20 ns [4,5]). During this recombination, part of the RCs lacking Q_A form $^3P^*$ with the yield Φ_1. $^3P^*$ decays to the groundstate with a lifetime τ_3 (50–150 μs [21]). Eqn. 1 is valid as long as the forward electron transfer from H^- to Q_A is considerably faster than recombination of P^+H^-, as is the case in native RCs.

ΔA_0 can be obtained from the bleaching ΔA in the millisecond range, since at this time all Q_A-depleted RCs have returned to the ground state P, whilst recombination of $P^+Q_A^-$ has not proceeded considerably. The decay of $P^+Q_A^-$ within 1 ms can be accounted for by extrapolation to $t = 0$ with the lifetime of this state obtained in the same measurement. We have refrained from analysing the difference absorbance at delay times shorter than 4 ns in order to avoid an unreliable deconvolution in the temporal range where exciting and probing pulses overlap. Instead, ΔA_{depl} can be extrapolated from $\Delta A(4\text{ ns}) - \Delta A_0$, if the values of τ_{RP} and Φ_1 are known, since τ_3 is in the microsecond range. In case the recombination dynamics in Q_A-depleted RCs, characterized by τ_{RP} and Φ_1, have not yet been determined for the species under investigation, $\Delta A(0\text{ ns})$ has to be extrapolated from additional measurements of the time-dependence of $\Delta A(t)$ on the timescale of the P^+H^- recombination. Comparison of the initial bleaching of P absorbance of Q_A-containing (ΔA_0) and Q_A-depleted (ΔA_{depl}) RCs then allows the direct determination of the amount of Q_A depletion [2].

In Q_A-depleted RCs the yield of $^3P^*$ is appreciable ($\Phi_1 \approx 30\%$ at 290 K in *Rb. sphaeroides*) and is reduced by a factor of 2 in an external magnetic field of several hundred G [4,5]. A magnetic field-induced change of the absorption signal on the 100 ns timescale is indicative of this change in Φ_1. A magnetic field, however, should not influence the bleaching at 4 ns, when all excited RCs are observed, or 1 ms, when only Q_A-containing RCs should contribute to the signal. The quantitative consistency of the magnetic field effect on $\Delta A(100\text{ ns})$ with the magnetic field dependence of Φ_1 can be used as an additional check for the obtained content of Q_A and provides an additional test for the assumption that the observed nanosecond component of $\Delta A(t)$ is indeed due to recombination of RCs lacking Q_A.

In order to make measurements on the nanosecond and millisecond timescales comparable, rotational depolarization of the P bleaching has to be considered.

[2] Here it is assumed, that the absorbance changes around 865 nm of both states P^+H^- and $P^+Q_A^-$ are due only to the bleaching of P. However, the bacteriopheophytin (BPh) anion shows a weak absorption in this spectral region. The exact value of the extinction coefficient of H^- in the RC is not known. From the difference spectrum of BPh^-/BPh in solution [24] it can be estimated to be smaller than 15% of the extinction coefficient of P at 865 nm. Thus, the amount of Q_A-depleted RCs is underestimated by at most this factor. This leads to an overestimation of the Q_A content, which is highest (less than 5% absolute) in the case of a Q_A content of 50% and amounts to less than 2% (absolute) for a Q_A content of 10% or 90%.

Preferentially, RCs with the Q_y transition moments of P or the bacteriochlorophyll monomers in the direction of the excitation polarization are excited at 600 nm, whilst the measured bleaching of P is due to RCs with the Q_y transition moments in the direction of the probing polarization. The measured value of the difference absorbance, therefore, depends on the relative orientation of exciting and probing polarization. For the wavelengths used here, the resulting photodichroism amounts to a difference of 40% for the bleaching measured with parallel or perpendicular polarization of exciting and probing light in the case of *Rb. sphaeroides* [25,26]. Due to rotational diffusion this photodichroism decays with time constants of about 90 ns [26] and 3 μs [22] in buffer solution and in a 60% (v/v) glycerol/buffer solution, respectively, at room temperature. The difference absorbance measured before and after this rotational depolarization cannot be compared directly, except if measurements are done with the magic angle of 54.7 degrees between the polarization of the exciting and the probing light, in which case no effect of the rotational depolarization will be observed. Alternatively, measurements are done at lower temperatures, where RCs do not rotate.

The probing pulse separation of 60 ms is not enough to ensure complete recovery of Q_A-containing RCs excited by a probing pulse until the following pulse. RCs accumulated in the state P^+Q^- ($P^+Q_A^-$ or $P^+Q_B^-$ with Q_B the secondary quinone) by the probing pulses preceding an exciting pulse will not contribute to the observed difference absorbance. Their number n^*_{acc} can be calculated from the number of RCs excited by one probing pulse n^*_{pr}, the recombination lifetime τ_r of P^+Q^- and the pulse separation of 60 ms, taking into account that RCs excited at the ith preceding probing pulse had $i*60$ ms to recombine:

$$n^*_{acc} = \sum_i n^*_{pr} e^{-i*60\text{ms}/\tau_r} = n^*_{pr} \frac{1}{e^{60\text{ms}/\tau_r} - 1} \qquad (2)$$

The energy density of one probing pulse is less than 1 $\mu J/cm^2$, exciting a fraction of less than 0.1% of the RCs. For $\tau_r = 120$ ms a fraction of 0.15% of the Q_A-containing RCs will have accumulated in the state P^+Q^- before an exciting pulse. This is considerably smaller than the accuracy of the Q_A content determination described here and will be neglected. Even for $\tau_r = 500$ ms only a fraction of 0.8% of Q_A-containing RCs can be accumulated in the state $P^+Q_A^-$ which still can be neglected.

RCs in which the rate for the reaction $P^+H^- \rightarrow P^+Q_A^-$ is slow and becomes comparable to the recombination lifetime of P^+H^-, e.g., RCs depleted of the central Fe^{2+} ion [27,28], show recombination of P^+H^- even when containing Q_A. This will considerably hinder a proper determination of the Q_A content.

Results

The occupancy of the Q_A site was measured in three different samples of RCs of *Rb. sphaeroides*. The influence of excess quinone, as well as the influence of different detergents, on the Q_A binding ability was investigated.

Table I(a) gives the bleaching of the P ground state absorbance, measured at 889 nm on a RC sample with Triton X-100 as the detergent, frozen at 90 K in order to exclude rotational depolarisation. The recombination lifetime τ_r of $P^+Q_A^-$ was found to be 29 ± 5 ms. Inserting the values for τ_{RP} and Φ_f from [5] into Eqn. 1, ΔA_Q and ΔA_{depl} were determined from the measured bleaching at 4 ns and 1 ms, as described before. These values indicate that a portion of (19 ± 2)% of the RCs was depleted of Q_A. The uncertainty of this value was estimated from the uncertainties of the measured bleaching and the recombination parameters used for its deduction. The bleaching at 100 ns to 500 ns corresponds exactly to the value expected with this amount of Q_A-depleted RCs using Eqn. 1 and a value of $\Phi_f = 0.71$ [5]. Application of a magnetic field of 700 G (data not shown) had no influence on the bleaching measured at 4 ns and 1 ms, but reduced the bleaching at 100 ns to 500 ns in exact correspondence with the reduction of Φ_f by a magnetic field [5]. This confirmed the notion of Q_A-depleted RCs being responsible for

TABLE I

Bleaching of the P ground state absorbance in Rb. sphaeroides RCs

(a) 60/40 (v/v) glycerol/20 mM Tris (pH 8.0), 0.1% Triton X-100, 90 K; (b) 20 mM Tris (pH 8.0), 0.1% Triton X-100, room temperature, with 5-fold excess of ubiquinone$_{10}$ and; (c) 20 mM Tris (pH 8.0), 0.08% LDAO, room temperature. Sample conditions: $\Delta A(t)$, bleaching measured at different delay times t between excitation and probing pulses (uncertainty ± 0.0002); probing wavelength 889 nm (a) and 869 nm (b, c); τ_r, measured recombination lifetime of $P^+Q_A^-$ (± 20%); τ_{RP}, recombination lifetime of P^+H^- in Q_A-depleted RCs (± 8%), from Ref. 5; Φ_f, yield of $^1P^*$ during recombination of P^+H^- in Q_A-depleted RCs (± 0.02), from Ref. 5; ΔA_Q, ΔA_{depl}, bleaching at $t = 0$ due to Q_A-containing and Q_A-depleted RCs (± 0.002), determined as described in the text; c_{depl}, fraction of Q_A-depleted RCs, determined from ΔA_Q and ΔA_{depl} (± 3% (absolute)).

Sample	(a)	(b)	(c)
$\Delta A(t)$			
4 ns	−0.1509	−0.0907	−0.1548
100 ns	−0.1440	−0.0874	–
200 ns	−0.1437	−0.0869	−0.1385
500 ns	−0.1434	−0.0867	–
1 ms	−0.1193	−0.0848	−0.1308
τ_r (ms)	29	135	135
τ_{RP} (ns)	21.2	13.0	13.0
Φ_f	0.71	0.30	0.30
ΔA_Q	−0.124	−0.085	−0.132
ΔA_{depl}	−0.029	−0.006	−0.028
c_{depl}	19%	7%	18%

the decay on the nanosecond timescale.

To investigate the extent of Q_A reconstitution, the measurements were repeated with a sample from the same preparation, but containing excess quinone. Ubiquinone$_{10}$ was added to the RC preparation to final concentrations of 20 μM RC and 100 μM ubiquinone$_{10}$, as described above. The measurements were done at room temperature in buffer solution at a probing wavelength of 867 nm. To avoid effects from rotational depolarisation the angle between exciting and probing polarization was carefully adjusted to the magic angle of 54.7 degrees. Measurements were also done at 52.7 and 56.7 degrees (data not shown), showing the expected increase or decrease of the bleaching due to rotational depolarization on the 100 ns time scale. No effects from rotational depolarization were seen when measuring under the magic angle, confirming that it had been chosen accurately.

Table I(b) shows the results of these measurements. τ_1 was found to be approx. 135 ms, the same as for the sample of Table I(c) which did not contain excess quinone. The sample with the excess quinone, however, showed a small deviation from a monoexponential behaviour (data not shown). A minor component (approx. 20%) with a time constant of about 500 ms might reflect recombination from partially occupied Q_B sites [18]. Again ΔA_Q and ΔA_{depl} were calculated from the bleaching at 4 ns and 1 ms, Table I(b), using the values of τ_{RP} and Φ_T from [5]. Even in the presence of excess quinone, a portion of $(7 \pm 3)\%$ RCs were found to be depleted of Q_A. Besides the uncertainties of measurement and recombination parameters, the uncertainty given here includes an estimation of residual effects of rotational depolarization, deduced from the measurements at different angles between the exciting and probing polarization. Again the bleaching measured at 100 ns to 500 ns corresponds to the values expected for the determined amount of Q_A depletion.

Table I(c) and Fig. 1 show the measurement on a sample with LDAO as the detergent at room temperature, again measured with exciting and probing polarization under the magic angle, with no excess quinone added. $P^+Q_A^-$ decayed monoexponentially with $\tau_1 \approx$ 135 ms. A portion $((18 \pm 3)\%)$ of the RCs were found to have lost Q_A in this case.

In the past we had occasionally determined the occupancy of the Q_A site with a setup using an optical delay line with a maximum delay time of 92 ns, therefore giving access only to the nanosecond and 100 ms ranges [5] and yielding less precise values. In agreement with the results obtained in the improved apparatus described here, all these measurements showed that, in our standard preparations of *Rb. sphaeroides*, typically 15–25% RCs are depleted of Q_A. This holds for preparations with Triton X-100 as the detergent as well as for those with LDAO. In contrast, standard preparations of *Chloroflexus aurantiacus* [28] showed a portion of only 10% Q_A depleted RCs, if LDAO was used as the detergent, but of up to 80% in the presence of Triton X-100.

Conclusion

By measuring the total recovery dynamics of the bleaching of the Q_x ground-state absorbance band of P in the time-window from nanoseconds to milliseconds it is possible to determine the occupancy of the Q_A binding site with an accuracy of better than 3%. The high precision of this method rests on highly accurate measurements of the bleaching ($\Delta(\Delta A) = 2 \cdot 10^{-4}$) on the nanosecond and millisecond timescale without any changes in the optical setup. The extended time-window allows us to account for the appearance of $^3P^*$ in recombination processes, occurring in the fraction of RCs that lack the primary quinone.

First applications of this method indicate that preparations of RCs tend to be inhomogenous with respect to the occupancy of the primary quinone site. RCs of *Rb. sphaeroides* were found to be depleted of Q_A by typically 20%. Reconstitution of Q_A by adding excess quinone does not necessarily lead to a full occupation of the Q_A site. Therefore, transient absorption measurements should not be analysed without accounting for such losses.

Acknowledgement

Financial support from the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 143) is gratefully acknowledged.

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