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REGULAR PAPER

Effects of the measuring light on the photochemistry of the bacterial photosynthetic reaction center from *Rhodobacter sphaeroides*

Ivan Husu · Mauro Giustini · Giuseppe Colafemmina · Gerardo Palazzo · Antonia Mallardi

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Abstract The bacterial reaction center (RC) has become a reference model in the study of the diverse interactions of quinones with electron transfer complexes. In these studies, the RC functionality was probed through flash-induced absorption changes where the state of the primary donor is probed by means of a continuous measuring beam and the electron transfer is triggered by a short intense light pulse. The single-beam set-up implies the use as reference of the transmittance measured before the light pulse. Implicit in the analysis of these data is the assumption that the measuring beam does not elicit the protein photochemistry. At variance, measuring beam is actinic in nature at almost all the suitable wavelengths. In this contribution, the analytical modelling of the time evolution of neutral and charge-separated RCs has been performed. The ability of measuring light to elicit RC photochemistry induces a first order growth of the charge-separated state up to a steady state that depends on the light intensity and on the occupation of the secondary quinone (Q_B) site. Then the laser pulse pumps all the RCs in the charge-separated state. The following charge recombination is still affected by the measuring beam. Actually, the kinetics of charge recombination measured in RC preparation with the Q_B site partially occupied are two-exponential. The rate constant of both fast and slow phases depends linearly on the intensity of the measuring beam while their relative weights depend not only on the fractions of RC with the Q_B site occupied but also on the measuring light intensity itself.

Keywords *Rhodobacter sphaeroides* · Reaction center · Electron transfer · Light intensity

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Introduction

Reaction centers (RC) of photosynthetic bacteria are integral pigment-protein complexes spanning the intracytoplasmic membrane that efficiently convert photon energy into redox energy. The RCs from purple bacteria have gained an outstanding role as subject of scientific investigations in many research areas. They have been the first membrane proteins to be crystallized and are still playing the role of archetype in the field of structural biology of membrane proteins (Fyfe and Jones 2000; Nogi and Miki 2001; Jones 2007). In recent years, the RC has gained a central role also in studies dealing with chemical–physical aspects of the membrane proteins such as the interaction with surfactants (Piazza et al. 2003; Palazzo et al. 2004, 2010) and the study of protein energy landscape (McMahon et al. 1998, Goushcha et al. 2003; Francia et al. 2004, 2008). On the physiological side, the RC functionality has been extensively studied, yielding to a wealth of information on the energetics of the electron transfer and protonation

events involving the quinone acceptor complex (Okamura et al. 2000) so that the RC has become a reference model in the study of the diverse interactions of quinones with electron transfer complexes (Cramer and Knaff 1990).

A very convenient way of studying the functional properties of the RC is through flash-induced absorption changes (hereafter flash photolysis) experiments where the activity of the protein is triggered by an intense light (usually laser) pulse and its functionality is probed spectrophotometrically by means of a low intensity measuring light beam. Almost all the wavelengths suitable to probe RC functionality are actinic in nature, therefore contributing to the RC photoactivity.

The practice of RC flash-photolysis was improved over decades and the designs of experiment carefully minimize the background excitation of the sample by the measuring beam. Accordingly, the formal description of the actinic effects of the measuring light beam has been disregarded and only its influence on the observed rate of charge recombination from the secondary quinone was detailed by Mancino et al. (1984) and Paddock et al. (2006). In recent years, the investigation of RC light-induced conformational transitions has elicited experimental protocols involving non-negligible intensity measuring beams (Goushcha et al. 2003) or the use of additional high intensity continuous light (Francia et al. 2004). The use of an actinic measuring beam has been also proposed in the case of optical herbicide biosensors based on RC (Mallardi et al. 2007). In all these cases, it is important to properly model the effect of the background excitation. In the present contribution, we model the actinic effect of measuring light on the absorbance changes of RC solutions and on the features of the dark charge recombination kinetics following the firing of the intense laser pulse. It turned out that the kinetics of charge recombination can be dramatically influenced by the intensity of the measuring beam. All the model predictions have been validated experimentally.

Materials and methods

N,*N*-dimethyldodecylamine-*N*-oxide was purchased from Fluka as 30% aqueous solution. Ubiquinone-10, *o*-phenanthroline, atrazine and terbutryn were from Sigma-Aldrich. RCs were isolated from *Rhodobacter* (*Rb.*) *sphaeroides* and purified according to Gray et al. (1990). In all the preparations, the ratio of the absorption at 280 and at 800 nm was between 1.2 and 1.3. The quinone was removed from the Q_B site of RCs according to the method of Okamura et al. (1975). Light-induced redox changes of the primary donor of the RC were monitored by a home-built kinetic single-beam spectrophotometer realized with orthogonal geometry between measuring and exciting beams (see scheme in Fig. 1) described in Giustini et al. (2005). The measuring beam was provided by a laser diode (Roithner Lasertechnik; $\lambda = 870$ nm); different light intensities were obtained by changing the laser diode driving current (the photodiode monitoring current was suitably changed to optimize the response). The measuring beam irradiance was measured by a digital response photodiode (PD-300-UV Ophir optronics Ltd., Israel) providing the radiating energy power passing through the 1 cm^2 aperture. The exciting light pulse was provided by a frequency doubled Nd:YAG laser (Quanta System, Handy 710-$\lambda = 532$ nm; 7 ns pulse width; 300 mJ/pulse). In order to avoid any interference from the exciting light, the measuring beam passed through a red glass filter (Corning, no. 2-64) placed in front of the 800 μm entry slit of a grating monochromator (Oriel, mod. 7240) between the sample and the detector.

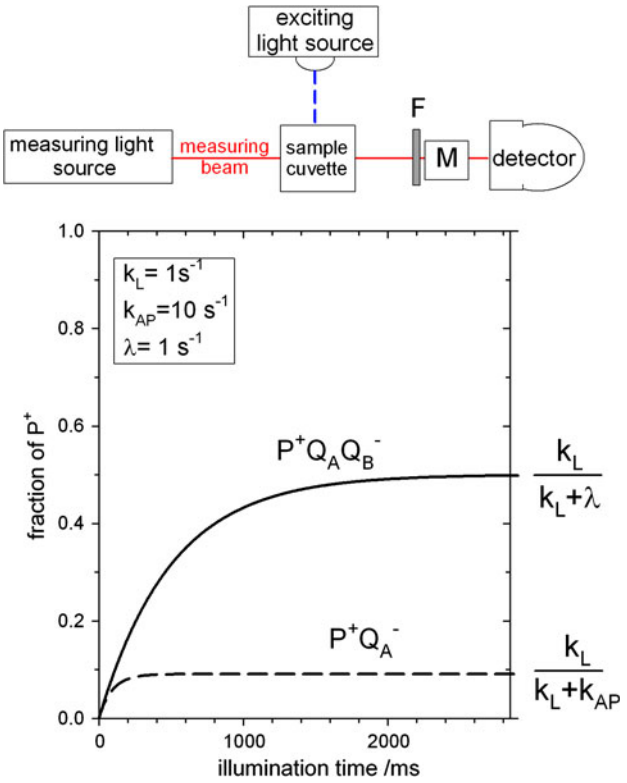

Fig. 1 *Top* scheme of a typical single-beam flash-photolysis apparatus. *F* and *M* denote the filter and the monochromator, respectively, used to protect the detector from the exciting light. *Bottom* calculated time evolution of the oxidised primary donor P^+ upon exposure to continuous actinic light. *Dashed line* denotes the prediction for RC without quinone at the Q_B site (Eq. 6); *solid line* denotes the prediction for RC with the Q_B site fully occupied (Eq. 8). The numerical values of the charge recombination rate constants used in the simulation are $k_{AP} = 10\ s^{-1}$ and $\lambda = 1\ s^{-1}$ (see Feher et al. 1989); the k_L value ($1\ s^{-1}$) corresponds to an irradiance of about 50 μW/cm^2 (see Fig. 4a)

The numerical fitting procedures and the corresponding error estimation of the best-fit parameters were performed as previously described (Mallardi et al. 1998).

Results and discussion

Basic kinetic schemes

The bacterial RC promotes the primary events of photosynthetic energy transduction by catalyzing a light-induced charge separation across the protein dielectric through a sequence of electron transfer steps, involving cofactors held at fixed distances by the polypeptide scaffolding (Feher et al. 1989). Upon photon absorption, a bacteriochlorophyll special pair, facing the periplasmic side of the membrane, acts as the primary electron donor (P) by delivering an electron to the primary acceptor, Q_A, an ubiquinone molecule bound at a site close to the opposite, cytoplasmic side of the complex. This primary charge separation, accomplished in about 200 ps, is stabilized by a subsequent electron transfer from Q_A^- to a second ubiquinone molecule, bound at the Q_B site of the RC, which is located symmetrically to the Q_A site on the same cytoplasmic side of the membrane (Feher et al. 1989; Okamura et al. 2000). In vivo, the photoxidized donor, P^+, is rapidly re-reduced by a c-type cytochrome, so that a second photo-induced charge separation can take place across the RC, leading to the double reduction and protonation of Q_B, which leaves the RC in its quinol state, QH_2. In this reaction, two protons are taken up from the cytoplasmic side of the membrane so that the RC catalyzes the first step in the generation of the transmembrane electrochemical potential for protons which is the driving force for ATP synthesis (Ort and Melandri 1982). In vitro, no physiological or artificial electron donor is available to re-reduce flash generated P^+, and the electron on Q_B can recombine with the hole on the primary donor, as shown by the following reaction which describes the photo-induced charge separation and the dark charge recombination:

$$PQ_AQ_B \underset{k_{AP}}{\overset{k_L(h\nu)}{\rightleftarrows}} P^+Q_A^-Q_B \underset{k_{BA}}{\overset{k_{AB}}{\rightleftarrows}} P^+Q_AQ_B^- \quad (1)$$

where k_L denotes the (light dependent) rate constant for the light-induced primary charge separation (it is given by the product: light intensity × absorption cross-section × quantum yield). Under physiological conditions, the $P^+Q_A^-$ charge recombination is much slower than the interquinones electron transfer steps ($k_{AP} \approx 10\ s^{-1}$, $k_{AB} \approx 6 \times 10^3\ s^{-1}$ and $k_{BA} \approx 4.5 \times 10^2\ s^{-1}$; Kleinfeld et al. 1984). Accordingly, the charge recombination of the $P^+Q_AQ_B^-$ state occurs essentially by thermal repopulation of the $P^+Q_A^-Q_B$ state and the quasi-equilibrium (between $P^+Q_A^-Q_B$ and $P^+Q_AQ_B^-$) approximation holds. Hence, the reaction 1 can be reduced to the following one

$$PQ_B \underset{\lambda}{\overset{k_L(h\nu)}{\rightleftarrows}} P^+Q_B^- \quad (2)$$

where the observable overall rate constant λ is determined by the values of k_{AP}, k_{AB} and k_{BA} and by the degree of occupation of the Q_B site (for reviews see Shinkarev and Wraight 1993; Wraight and Gunner 2008). This rate constant reaches its maximum value $\lambda = k_{AP}/(1 + k_{AB}/k_{BA}) \approx 1\ s^{-1}$ only for RC fully reconstituted at the Q_B site (a situation difficult to achieve in purified proteins).

In the absence of the secondary acceptor Q_B, or in the presence of competitive inhibitors displacing the quinone from the Q_B site, the charge-separated state is limited to the $P^+Q_A^-$ and the relevant reaction for the formation and decay of P^+ is:

$$PQ_A \underset{k_{AP}}{\overset{k_L(h\nu)}{\rightleftarrows}} P^+Q_A^- \quad (3)$$

Since the reduced and oxidised forms of P have different extinction coefficients in the visible-NIR spectral range, the transient flash-induced formation of P^+ and its subsequent decay by charge recombination can be monitored spectrophotometrically at the appropriate wavelengths and time resolution.

Exposure to actinic measuring light

The reduced primary donor P absorbs light at almost all the wavelengths suitable to probe the $P \rightarrow P^+$ oxidation (a notable exception is a band in the NIR around 1200 nm) thus the measuring beam inevitably promotes the photo-oxidation of a fraction of RCs. The time evolution under exposure to a continuous actinic measuring beam in the simple case of RCs without quinone at the Q_B site can be easily retrieved from reaction 3:

$$\frac{d[P^+Q_A^-]}{dt} = k_L[PQ_A] - k_{AP}[P^+Q_A^-] \quad (4)$$

in this case the concentrations of neutral and charge-separated proteins ($[PQ_A]$ and $[P^+Q_A^-]$, respectively) are related to the total RC concentration ($[RC]$) by

$$[RC] = [PQ_A] + [P^+Q_A^-] \quad (5)$$

and Eq. 4 can be integrated assuming that at time $t = 0$ all the RCs are in the PQ_A state, giving

$$[P^+Q_A^-] = \frac{[RC]k_L}{k_L + k_{AP}}[1 - e^{-(k_L + k_{AP})t}] \quad (6)$$

At the steady state ($t \rightarrow \infty$) the PQ_A and $P^+Q_A^-$ concentrations will be:

$$[P^+Q_A^-]_{SS} = \frac{[RC]k_L}{k_L + k_{AP}}; \quad [PQ_A]_{SS} = \frac{[RC]k_{AP}}{k_L + k_{AP}} \tag{7}$$

Equations 4–7 foretell that the exposure of the sample to the light beam will induces a first order growth of the charge-separated state $P^+Q_A^-$ up to a steady state in which only the fraction $k_L/(k_L + k_{AP})$ of the total protein is in the $P^+Q_A^-$ state. In the case of RC fully saturated at the Q_B state, Eq. 2 holds and the time evolution of the charge-separated state $P^+Q_B^-$ is

$$[P^+Q_B^-] = \frac{[RC]k_L}{k_L + \lambda}[1 - e^{-(k_L+\lambda)t}] \tag{8}$$

that is the analogous of Eq. 6.

At the steady state ($t \to \infty$) the PQ_B and $P^+Q_B^-$ concentrations will be:

$$[P^+Q_B^-]_{SS} = \frac{[RC]k_L}{k_L + \lambda}; \quad [PQ_B]_{SS} = \frac{[RC]\lambda}{k_L + \lambda} \tag{9}$$

The kinetics of P^+ formation in the two cases, simulated according to the above equations, are shown in Fig. 1 for reasonable estimates of the relevant rate constants. Since k_{AP} is at least one order of magnitude larger than λ, the steady-state fraction of P^+ formed in the case of RC deprived of quinone at the Q_B will be very small compared with the value attained in the case of RC functional at the Q_B site.

In the case, the sample is composed by RCs either with or without quinone at the Q_B site, the corresponding kinetics of P^+ formation will be the population averaged superimposition of Eqs. 6 and 8:

$$\frac{[P^+Q_A^-] + [P^+Q_B^-]}{[RC]} = (1 - f_B)\frac{k_L}{k_L + k_{AP}}[1 - e^{(k_L+k_{AP})t}] + f_B\frac{k_L}{k_L + \lambda}[1 - e^{(k_L+\lambda)t}] \tag{10}$$

where f_B is the fraction of RCs with quinone bound to the Q_B site. Note that in this case the steady-state fraction of photoexcited RCs depends linearly on f_B:

$$\frac{([P^+Q_A^-] + [P^+Q_B^-])_{SS}}{[RC]} = \frac{k_L}{k_L + k_{AP}} + \left(\frac{k_L}{k_L + \lambda} - \frac{k_L}{k_L + k_{AP}}\right)f_B \tag{11}$$

and the same holds for the steady state in the absorbance change. Thus, the absorbance change (ΔAbs) upon continuous illumination (at suitable wavelength) represents an easy-to-use method to probe the functionality of the Q_B site, and has been proposed as an analytical procedure for the quantification of some herbicides (Mallardi et al. 2007).

An example is presented in Fig. 2a where are compared the absorbance changes (bleaching at 870 nm) measured at

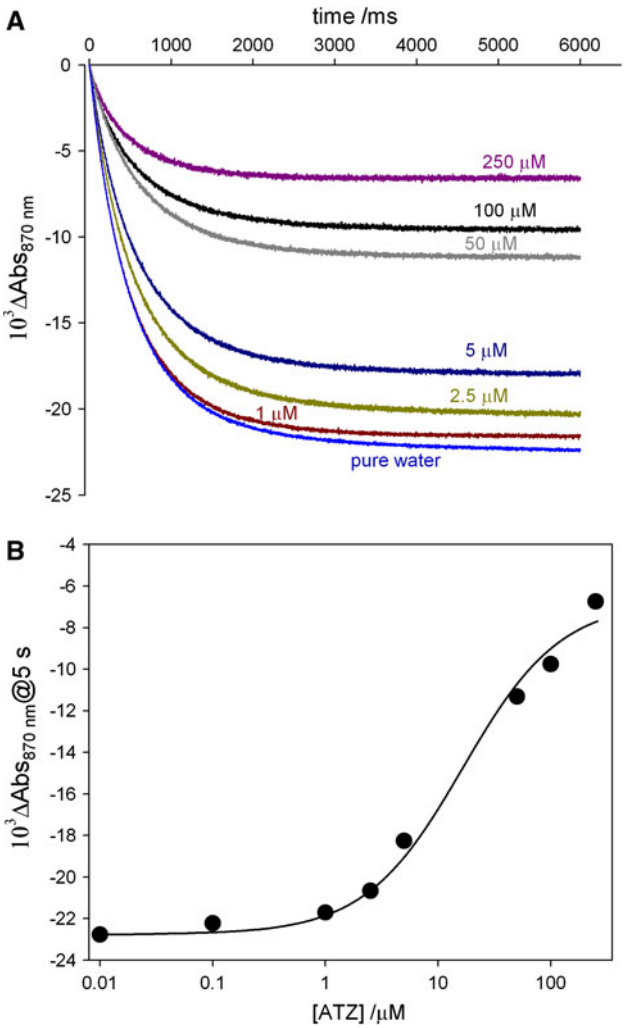

Fig. 2 Influence the herbicide atrazine (ATZ) on the measuring light (870 nm) induced bleaching. **a** Absorbance kinetics for the same RC solution (2.3 μM) in presence of different atrazine concentrations. The reference transmittance was that at time = 0 (ΔAbs($t = 0$) = 0). **b** Absorbance change at the steady state (after 5 s of illumination) as a function of the atrazine concentrations. The atrazine molecule displaces the quinone reducing the fraction of RC reconstituted at the Q_B site (f_B) and this, according to Eq. 11, reduces the extent of bleaching. The dependence of the steady-state bleaching on the herbicide concentration reflects the binding isotherm of atrazine to the RC

constant irradiance and different loading with an herbicide (atrazine) that displaces the quinone from the Q_B site (Draber et al. 1991). In the absence of herbicide most of the RCs retain the quinone bound to the Q_B site and the steady-state (after 5 s) bleaching is $\Delta Abs = -22 \times 10^{-3}$. Upon herbicide loading, the atrazine displaces more and more quinones from their Q_B sites and this, according to Eqs. 10 and 11, results in a drop in the maximum attainable bleaching ($\Delta Abs = -6 \times 10^{-3}$). Since the ΔAbs at the steady state is proportional to the fraction of RCs with quinone bound to the Q_B site, it depends on the herbicide

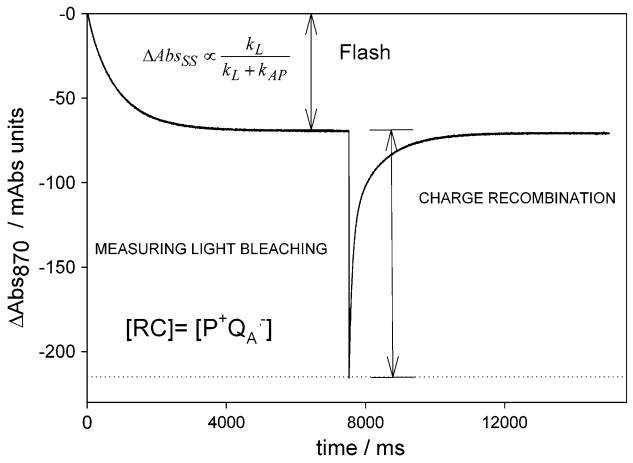

Fig. 3 Overall time course of the absorbance (at 870 nm) during a flash-photolysis experiment. The RC (2.3 μM) was fully deprived of quinone at the Q_B site (only charge recombination from $P^+Q_A^-$ is allowed). The reference transmittance was that immediately after the measuring beam was turned on ($t = 0$). After about 4 s the ΔAbs reaches a steady-state value (ΔAbs_{SS}); the exciting laser pulse (532 nm) is fired at $t = 7.5$ s driving suddenly the absorbance of the sample to the maximum value $\Delta Abs_{SS} + \Delta Abs^{\circ}_{app}$. For longer times, the absorbance relaxes back to the steady-state value

concentration according to a binding isotherm as shown in Fig. 2b.

Effect of a saturating light pulse

In biophysical studies, it is customary to probe the functionality of the RC through flash-photolysis experiments. In these experiments the state of the primary donor is probed by means of a continuous measuring beam while the electron transfer is triggered by a short intense light pulse. The experimental set-up has invariably a single-beam geometry and the signal is an absorbance change taken using as reference the transmittance measured before the light pulse. Implicit in the analysis of the data collected in these experiments, is the assumption that the measuring beam does not elicit the protein photochemistry. Since, as we have discussed above, the measuring beam is actinic in nature it is of some interest to describe analytically what happens in such flash-photolysis experiments.

Let us discuss first the case of RCs with only the Q_A site occupied (reaction 3); a real experiment is shown in Fig. 3. In the pre-flash region ($0 < t < 7.5$ s) it is evident the first order growth of P^+ due to the measuring light and discussed in the previous section. At $t = 7.5$ s, a saturating laser pulse is fired, resulting in an instantaneous (on the experimental time resolution) pumping of all the RCs in the $P^+Q_A^-$ state. The relaxation from this state is still described by the rate Eq. 4 (the sample is still illuminated by the measuring beam) but under the constraints that at the time of flash $[P^+Q_A^-] = [RC]$ and $[PQ_A] = 0$.

The differential Eq. 4 can be integrated under these constraints providing the time evolution of $[P^+Q_A^-]$ that undergoes to charge recombination:

$$\frac{[P^+Q_A^-]}{[RC]} = \frac{k_L}{k_L + k_{AP}} + \frac{k_{AP}}{k_L + k_{AP}} e^{-(k_L + k_{AP})t'} = \frac{k_L}{k_L + k_{AP}} + \frac{k_{AP}}{k_L + k_{AP}} e^{-k_{fast}t'} \quad (12)$$

where t' denotes the time elapsed after the laser pulse.

According to the above equation the relaxation from the $P^+Q_A^-$ state is mono exponential with an apparent rate constant $k_{fast} = k_L + k_{AP}$. This was confirmed experimentally by the data shown in Fig. 4a where the experimental rate of charge recombination k_{fast}, determined in RCs without quinone at the Q_B site, is reported as a function of the measuring light intensity (strictly irradiance). The rate of photo-induced charge separation (k_L) is expected to be proportional to the photon flux, thus the linear dependence of the charge recombination rate k_{fast} on the light intensity shown in Fig. 4a is in agreement with the prediction of Eq. 12. Accordingly, the extrapolation at null light intensity gives the k_{AP} value ($\approx 8.8\ s^{-1}$). A peculiar aspect of flash-photolysis experiments is that, if the change in absorbance is evaluated with respect to the pre-flash transmittance (as it is customary) it depends on the actinic power of the measuring beam. In particular, comparison of Eqs. 12 and 6 indicates that the maximum apparent variation in absorbance (at the time of the light pulse) depends on the ratio between the rate constant for the charge (photo-induced) separation k_L and rate constant for the (dark) recombination.

$$\Delta Abs^{\circ}_{app} \propto \frac{k_{AP}}{k_L + k_{AP}} \quad (13)$$

The validity of the above treatment can be also confirmed by comparing the rate of charge recombination with the values of absorbance changes measured under steady-state illumination (ΔAbs_{SS}) by the measuring beam and immediately after the light flash (ΔAbs°_{app}). According to the Eqs. 7 and 13, the ratio $r = \Delta Abs_{SS}/\Delta Abs^{\circ}_{app}$ equals the ratio k_L/k_{AP}. On the other hand, the rate of charge recombination can be written as (see Eq. 12)

$$k_{fast} = k_{AP} + k_{AP}\, r \quad (14)$$

The above equation foretells that a plot of k_{fast} (obtained by exponential regression of the charge recombination decay) as function of the ratio r between two absorbance values (see Fig. 3 for a graphical description of these parameters) is a straight line with equal intercept and slope.

Such a plot is shown in Fig. 4b where the absorbance time courses have been collected at different light intensities, for RCs deprived of quinone at Q_B, and have been analyzed extracting for each light intensity pairs of k_{fast} and

r values. It is clear that data in Fig. 4b obey to Eq. 14: k_{fast} values are a linear function of r and the intercept and slope have reasonably close numerical values (9 and 13 s^{-1}, respectively) in agreement with the estimate of k_{AP} found in Fig. 4a.

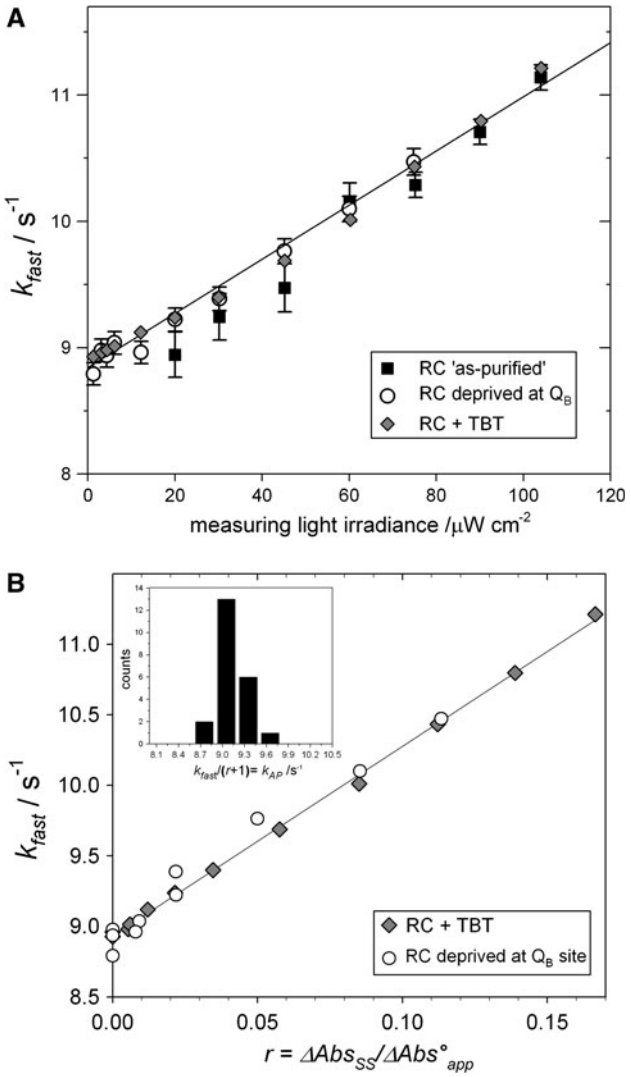

Fig. 4 **a** Dependence of the rate of $P^+Q_A^-$ charge recombination on the irradiance of the measuring light. *Grey diamonds* refer to experiments in presence of excess concentration of terbutryn (the uncertainties in this case are not shown, for the sake of readability); *hollow circles* refer to experiments on RC that have been deprived of the quinone at the Q_B site using the procedure of Okamura et al. (1975). The *straight line* represent the fit of the data to the equation $k_{fast} = k_{AP} + \alpha I$ according to Eqs. 12 and 19; best-fit parameters are $k_{AP} = 8.8 \pm 0.1$ s^{-1} and $\alpha = 0.021 \pm 0.001$ cm^2 μW^{-1} s^{-1}. *Filled squares* refer to the fast phase of the P^+ decay measured in RC 'as-purified' and thus containing a fraction of protein lacking the quinone at the Q_B site (see "Flash photolysis in case of partial occupation of Q_B site" section for details). **b** Rate of the $P^+Q_A^-$ charge recombination as a function of the ratio of the absorbance changes measured after long illumination with the measuring beam and immediately after the exciting laser pulse (see Fig. 4 for details). *Symbols* are as in **a**. *Inset* distribution of the k_{AP} values obtained from the 22 pairs of k_{fast} and r values (see text for details)

In these experiments, the unavailability of quinone bound to the Q_B site was achieved by means of two different strategies. In the first case (hollow circles) the RCs have been truly deprived of the quinones at Q_B (and of part of the quinone at Q_A as well) using a well assessed biochemical procedure (Okamura et al. 1975), thus in this case the Q_B site is empty. In the second case (grey diamonds), a competitive inhibitor of the electron transfer to Q_B (terbutrine) has been added to an otherwise classical RC preparation. The terbutrine efficiently displaces the quinone from the Q_B site according to a mechanism shared by some herbicides (as the previously cited atrazine); note that in this case the Q_B site is occupied by the terbutrine molecule. Since the two procedures give the very same results, we conclude that the occupation of the Q_B does not influence the rate of $P^+Q_A^-$ charge recombination. Eq. 14 suggests a way to extract from a single experimental trace the real $k_{AP} = k_{fast}/(1 + r)$ value avoiding the extrapolation at null measuring light intensity. This calculation has been applied to all the 22 (k_{fast},r) pairs of Fig. 4b and the distribution of the resulting k_{AP} values is shown in the inset of Fig. 4b. The whole set of result is peaked in a short range (0.8 s^{-1}) around the mean value 9.1 s^{-1} (86% of the k_{AP} values are within a 0.3 s^{-1} interval).

A completely analogous treatment holds for RCs fully reconstituted at the Q_B site. In this case, the rate Eq. 8 can be described under the constraints that at the time of flash $[P^+Q_B^-] = [RC]$ and $[PQ_B] = 0$ giving

$$\frac{[P^+Q_B^-]}{[RC]} = \frac{k_L}{k_L+\lambda} + \frac{\lambda}{k_L+\lambda} e^{-(k_L+\lambda)t'} = \frac{k_L}{k_L+\lambda} + \frac{\lambda}{k_L+\lambda} e^{-k_{slow}t'} \quad (15)$$

According to the above equation, the relaxation from the $P^+Q_B^-$ state is mono exponential with an apparent rate constant $k_{slow} = k_L + \lambda$ that depends on the light intensity of the measuring beam. This result was already obtained by Mancino et al. (1984) in the only (to our best knowledge) previous paper discussing some aspects of the intensity of the measuring beam.

Flash photolysis in case of partial occupation of Q_B site

As shown in the previous sections, the actinic effect of the measuring light has relatively simple consequences on the kinetics of dark charge recombination when the sample is an ensemble of RCs with the quinone binding sites uniformly populated. However, the purification procedure removes the quinone from the Q_B site from a significant fraction of RCs, and the complete reconstitution is difficult to achieve because the native ubiquinone is virtually water

insoluble. In this case, the situation becomes more complicated as illustrated by the experiments of Fig. 5. In this case, the same solution of RC (2.3 μM) has been subjected to flash-photolysis experiments using measuring beams (wavelength = 870 nm) with different light intensities. The ordinate axis represents the absorbance change calculated using as reference the transmittance taken immediately after turning-on the measuring beam. The exposure to the measuring light induces an absorbance change (bleaching) associated to the formation of the P^+. According to Eq. 11, the fraction of photoexcited RC at the steady state (and thus the extent of the bleaching) increases upon increasing the intensity of the measuring beam.

After 15 s of illumination, a laser pulse is fired exciting all the RCs. The total bleaching at this time (about −185 mAbs) is independent on the intensity of the measuring beam because the laser is saturating. However, since the pre-flash steady-state bleaching depends on this parameter, the drop in absorbance (bleaching) due to the laser pulse (ΔAbs°_{app}) becomes smaller upon rising the measuring light intensity. Furthermore, the relaxation kinetics of the absorbance back to the pre-flash value depends markedly on the intensity of the measuring light. For low intensity measuring beam, the full relaxation requires a couple of seconds and is markedly biphasic. At variance, the absorbance relaxes mono-exponentially in 0.1–0.2 s for high intensity measuring beam.

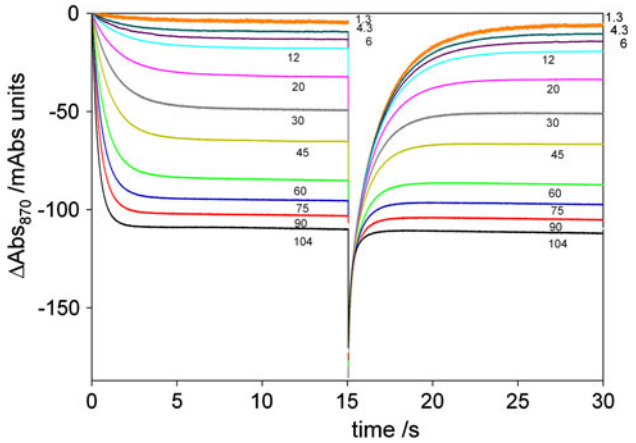

Fig. 5 Overall time course of the absorbance (at 870 nm) during a flash-photolysis experiment performed using measuring beam of different intensities (*numbers* closing each trace are the irradiance in μW/cm^2). The very same RC (2.3 μM) solution was probed with measuring light of different intensities (the measured irradiance are listed close to each trace). The RC was used 'as-purified' and this means that it is only partially reconstituted at the Q_B site. The reference transmittance was that immediately after the measuring beam was turned on ($t = 0$). After about 5 s, the ΔAbs reaches the steady-state value that depends on the measuring beam intensity; the same exciting laser pulse (532 nm) is fired at $t = 15$ s driving suddenly the absorbance of the sample to the maximum value (−185 mAbs). For longer times, the absorbance relaxes back to the relevant steady-state value

The rationale for this behaviour can be traced out to the non-uniform occupancy of the Q_B site. During the purification steps, a fraction of the RCs has lost the quinone at the Q_B site. Therefore, the overall response of the sample is due to the superimposition of both the responses of the RC with the Q_B site occupied and vacant. The lapse of time before the laser pulse is described by Eq. 10 above and the steady-state P^+ fraction depends on the true fraction of RCs deprived of quinone at the Q_B site (through f_B) and on the light intensity (through k_L).

After the laser pulse, the kinetics of P^+ relaxation will be the population averaged superimposition of the charge recombination of the $P^+Q_A^-$ and $P^+Q_B^-$ states (Eqs. 12 and 15, respectively) according to:

$$\frac{[P^+Q_A^-]+[P^+Q_B^-]}{[RC]} = \frac{(1-f_B)}{k_L+k_{AP}}\left(k_L + k_{AP}e^{-(k_L+k_{AP})t'}\right) + \frac{f_B}{k_L+\lambda}\left(k_L+\lambda e^{-(k_L+\lambda)t'}\right) \quad (16)$$

The change in absorbance evaluated with respect to the pre-flash transmittance (ΔAbs_{app}, the experimental observable in flash-photolysis experiments) will be proportional (via the Lambert–Beer law) to the difference between Eq. 16 and the pre-flash fraction of P^+ (given by Eq. 11). Consequently, ΔAbs_{app} can be written as

$$\Delta Abs_{app} \propto (1-F_B^{app})e^{-(k_L+k_{AP})t'} + F_B^{app}e^{-(k_L+\lambda)t'} = (1-F_B^{app})e^{-k_{fast}t'} + F_B^{app}e^{-k_{slow}t'} \quad (17)$$

$$F_B{}^{app} = \left(1+\frac{(1-f_B)}{f_B}\frac{k_{AP}}{\lambda}\frac{k_{slow}}{k_{fast}}\right)^{-1} \quad (18)$$

According to the above equation, the kinetics of absorbance relaxation measured after firing a saturating flash is two-exponential with the rate constant of both the fast and slow components that increases with the light intensity of the measuring beam. Actually, the kinetics of charge recombination measured in RC preparation with the Q_B site partially occupied (e.g. the traces of Fig. 5) closely follow the predictions of Eq. 17. The dependence of rate constant of the fast phase (k_{fast}) on the measuring light intensity is compared with the values obtained in RC lacking the quinone at the Q_B site in Fig. 4 (black dots). It is clear that the k_{fast} observed in RCs partially lacking the Q_B are linearly dependent on the illumination intensity in the same way does the rate of charge recombination measured in fully Q_B-deprived RCs. Also the decay constant of the slow phase depends linearly on the intensity of the measuring beam as shown in Fig. 6a.

It should be stressed that the relative effect of the measuring light intensity on the rate constants of the slow and of the fast phases is very different. This is because the

rate constants of the charge recombination kinetics from $P^+Q_A^-$ (k_{AP}) and from $P^+Q_B^-$ (λ) are very different. The relatively high k_{AP} value (9 s^{-1}) make the sum $k_{fast} = k_L + k_{AP}$ not very sensitive to the illumination; the maximum variation is around 20% for the irradiance range of the experiments of Figs. 4a and 5. On the contrary, being λ one order of magnitude smaller, the illumination considerably speeds-up the charge recombination from $P^+Q_B^-$; in Fig. 6a there is an increase in k_{slow} of about 400% upon increasing the irradiance from 1.3 to 100 μW/cm^2.

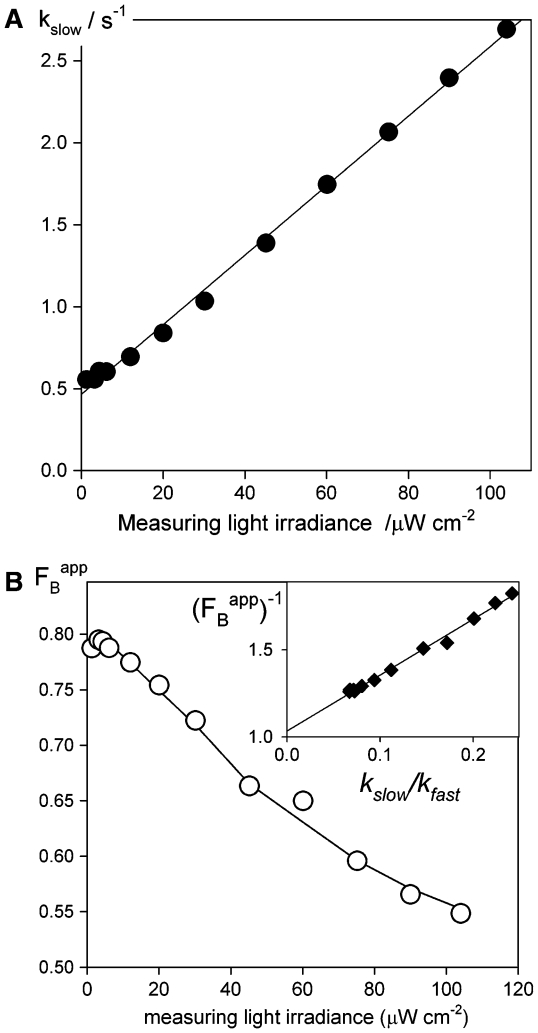

Fig. 6 **a** Rate of the $P^+Q_B^-$ charge recombination as a function of the measuring light irradiance. The data have been obtained from the deconvolution according to Eq. 17 of the experimental kinetics of absorbance relaxation shown in Fig. 6. The *straight line* represent the fit of the data to the equation $k_{slow} = \lambda + \alpha I$ according to Eqs. 15 and 19; best-fit parameters are $\lambda = 0.47 \pm 0.01$ s^{-1} and $\alpha = 0.021 \pm 0.001$ cm^2 μW^{-1} s^{-1}. **b** Influence of the measuring light intensity on the weight of the slow phase (F_B^{app}) obtained from the deconvolution (according to Eq. 17) of the after-pulse absorbance relaxation of the traces in Fig. 6. *Inset* the reciprocal of F_B^{app} as a function of the ratio r between the rate constants of the slow and fast phases of relaxation (see Eq. 17); according to Eq. 18, $(F_B^{app})^{-1}$ increases linearly with r with intercept $\approx$ 1. The *continuous line* in the main panel corresponds to the reciprocal of the least-square linear regression shown in the *inset*

A striking feature of Eqs. 17 and 18 is that the weight of the fast and slow phases of the observed kinetics cannot be identified with the fractions of RC with the quinone unbound and bound to the Q_B site of the RC. Instead, the weight of the slow phase depends also on the measuring light intensity through the term k_L in Eq. 17. The effect is dramatic as shown in Fig. 6b where the weight of the slow phase of the P^+ decay is plotted as a function of the measuring light irradiance for the same sample. At high light intensity, the slow phase accounts only for 55% of the overall P^+ decay but this percentage increases steadily upon decreasing the irradiance of the measuring beam reaching the 80% at very low irradiance value. It should be stressed that the experiments have been performed on the very same solution and that the results are highly reproducible. The data of Fig. 6b fully obey to the predictions of Eqs. 17 and 18. Indeed, the reciprocal weight of the slow phase depends linearly on the ratio between the rates of the slow and the fast phases with an intercept very close to one as dictated by Eq. 18 (see the inset of Fig. 6b). The strong linear dependence of $(F_B^{app})^{-1}$ on k_{slow}/k_{fast} fully reproduces the dependence of F_B^{app} on the irradiance of the measuring beam (solid line in the main panel of Fig. 6b). Note that, since k_{slow} and k_{fast} reduce to λ and k_{AP} for measuring light weak enough, the weight of the slow phase actually equals the fraction of RCs with the quinone bound to the Q_B site at vanishing light intensity (see Eq. 18). Accordingly, the extrapolation of F_B^{app} to null measuring light irradiance in Fig. 6b furnishes a value (0.8) in very good agreement with the estimates of occupancy of the Q_B site for purified RCs.

Concluding remarks

The analytical modelling of the time evolution of the concentrations of neutral and charge-separated RCs, during a flash-photolysis experiment has been performed taking into account the actinic nature of the measuring beam.

The ability of measuring light to elicit RC photochemistry induces a first order growth of the charge-separated state up to a steady state that depends on the intensity of the measuring light and on the occupation of the Q_B site. In customary flash-photolysis experiments this plateau corresponds to the transmittance taken as reference (pre-flash value). The instantaneous (on the experimental time-scale) laser pulse pumps all the RC in the charge-separated state. The relaxation from this state (charge recombination) is affected yet by the measuring beam.

As recalled in the introduction, customary experimental protocols minimize the actinic effect of measuring beam so

that the approximation $k_L \cong 0$ holds in the above equations.

On the other hand, the formal analysis here presented can be profitably used to tailor experiments using a high intensity measuring beam. Two examples are described below.

(i) The rate of photoexcitation k_L depends linearly on the measured values of light irradiance I

$$k_L = \alpha I \tag{19}$$

The parameter α, having units of (unit area)/(power × time), is of interest in the analysis of experimental results of RC equilibration kinetics under various illumination conditions (Goushcha et al. 2003). Accordingly, Manzo et al. (2009) have recently proposed a method to measure α based on the use of an additional orthogonal light beam. Alternatively, Eq. 16 suggests how the α-value can be evaluated using the classical set-up of Fig. 1 performing flash-photolysis experiments at different intensity of the measuring beam. The kinetics of charge recombination measured in RC preparation with the Q_B site partially occupied are two-exponential. The rate constant of both the fast and slow phases depends linearly on the intensity of the measuring beam (Eq. 16). The proportionality constant, α, relating the beam irradiance I and the rate constant k_L can be easily determined by linear regression of the plots k_{fast} versus I (Fig. 4a) and k_{slow} versus I (Fig. 6a). These analyses of the I-dependence of the k_{fast} and k_{slow} provide the same α-value (see captions in Figs. 4a, 6a) as expected.

(ii) Equation 16 shows how the relative weights of the fast and slow phases of the observed kinetics are tuned by the measuring light intensity. Actually, one can emphasize the contribution of the RCs lacking the secondary quinone to the P^+ decay simply by increasing the intensity of the measuring beam. In the case of the as-purified RC solution of Fig. 6b, the fast phase accounts for about 20% of the decay at small irradiance while its weight reaches 45% of the decay using an intense measuring beam ($I = 100$ μW/cm^2). Having comparable amounts of fast and slow phases allows a more accurate deconvolution resulting in reliable values of the rate constant for fast recombination. This enables to probe the effect of the presence of some herbicides in Q_B pocket on the rate of $P^+Q_A^-$ recombination avoiding to use of quinone removal procedures. Evidence of these effects has been proposed for RC from some purple bacteria (Baciou et al. 1990; Fufezan et al. 2005). In Fig. 4a, the k_{fast} values obtained on pristine RC solution and upon addition (on the very same sample) of terbutryne are compared. At high irradiance levels, the k_{fast} values obtained from as-purified RCs are reliable and confirm that the binding of terbutrine to the Q_B site of RCs from *Rb. sphaeroides* does not affect the recombination kinetic from Q_A^-. Decreasing the intensity of I decreases the weight of the fast phase resulting in an increase in the associated uncertainty and in a systematic bias towards lower k_{fast} values.

Finally, the analytical model proposed in this contribution is based on equations (Eqs. 1–3) that describe the RC photochemistry only in terms of the distribution of charges (conformational substates are not contemplated). Accordingly the phenomenology related to such a model can be thought as the reference behaviour when studying the coupling of RC photochemistry to its conformational dynamics.

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