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ORIGINAL PAPER

Turnover of ubiquinone-0 at the acceptor side of photosynthetic reaction center

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Abstract The steady-state operation of photosynthetic reaction center from *Rhodobacter sphaeroides* was investigated by measuring the rate of cytochrome photooxidation under intensive continuous illumination (808 nm, 5 W cm^{-2}). The native quinone UQ_{10} in Q_B binding site of the reaction center was substituted by tailless UQ_0 and the binding parameters and the turnover rate of the UQ_0 was studied to test the recently discovered light-intensity dependent acceptor side effect (Gerencsér and Maróti 2006). The binding parameters of UQ_0 ($k_{on} = 2.1 \times 10^5$ M^{-1} s^{-1} and $k_{off} = 100$ s^{-1}) were characteristic to the RC exposed to high light-intensity. The dissociation constant ($K_D = 480$ μM) determined under high light intensity is 2–3 times larger than that determined from flash-experiments. The light-intensity dependent acceleration of cytochrome turnover measured on reaction center of inhibited proton binding was independent of the type of the quinone and was sensitive only to the size ("pressure") of the quinone pool. The dissociation constants of different types of semiquinones show similarly high (several orders of magnitude) increase in the modified conformation of the Q_B binding pocket due to high intensity of illumination. This result indicates the exclusive role of the quinone headgroup in the binding of semiquinone to different conformations of the protein.

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Abbreviations

DEAE	Diethylaminoethyl
DMBQ	2,5-Dimethyl-1,4-benzoquinone
EDTA	Ethylenediaminetetraacetic acid
LDAO	*N*,*N*′-Dimethyl dodecylamine *N*-oxide
NHE	Normal hydrogen electrode
P (P^+)	Reduced (oxidized) bacteriochlorophyll dimer
Q_A and Q_B	Primary and secondary quinone, respectively
RC	Reaction center protein
SOD	Superoxide dismutase
TMPD	*N*,*N*,*N*′,*N*′-Tetramethyl-1,4-phenylenediamine
Triton X-100	Polyoxyethylene(10) isooctylphenyl ether
UQ_n	2,3-Dimethoxy-5-methyl-1,4-benzoquinone with *n* isoprenoid units in the tail

Introduction

The reaction center (RC) of photosynthetic bacteria is a membrane-bound pigment-protein complex where the light-energy is converted to proton electrochemical potential by coupling light-induced electron- and transmembrane proton transfer (Paddock et al. 2003; Wraight 2005). The RC from the purple bacterium *Rhodobacter sphaeroides* consists of three polypeptide subunits (L, M and H) and several pigment molecules bound to the protein scaffold (four bacteriochlorophylls, two bacteriopheophytins, one non-heme

Fe^{2+} and two ubiquinone-50 molecules) (Stowell et al. 1997). The energy of the excited state of bacteriochlorophyll dimer (P^*) is utilized in the consecutive electron transfer from P^* via series of electron acceptor molecules [bacteriochlorophyll monomer, bacteriopheophytin, primary quinone (Q_A)] to the secondary quinone (Q_B). The two quinones form a functional entity, the acceptor quinone complex. The different protein environments around the quinones modify diversely the physico-chemical properties of UQ_{10} in solution. The Q_A works as one-electron gate and is never protonated, while Q_B performs full reduction with two electrons and two protons: $Q_B + 2e^- + 2H^+ \rightarrow Q_BH_2$ (Fig. 1). The sequence of the electron and proton transfer steps to Q_B was determined by driving-force assay (Graige et al. 1996). The two-electron gate mechanism of Q_B is assured by the much larger binding affinity of semiquinone than quinone or quinol to the RC (Wraight 1982; Diner et al. 1984). The second reduction and protonation decreases the binding affinity of the quinone at the Q_B site, therefore the quinol is replaced by an oxidized quinone from the pool. The cyclic electron transfer and the generation of transmembrane proton transport are supplied by the membrane-bound cytochrome bc_1 complex via oxidation of quinol and reduction of cytochrome c_2, which serves as electron donor to the P^+.

The isolated RC solubilized in detergent maintains all relevant functions of RCs embedded in bacterium membrane. The functions of electron donor cytochrome c_2 on the periplasmic surface and electron acceptor UQ_{10} in the membrane can be redeemed by the externally added mitochondrial cytochrome c and ubiquinone-n, respectively. Upon steady-state operation of the RC, the pools of either the electron donor or the acceptor will be depleted finally and the turnover will be terminated. The cytochrome turnover is one of the most efficient methods to monitor the steady-state operation of the RC, as the absorption change of redox transition of cytochrome is much larger than that of any other reactions accompanying the function of RC. Since the continuous illumination mimicks the physiological conditions, it will be preferred instead of multiple flash excitations in this study.

According to the quinone reduction cycle, the coupling between the electron transfer and the proton uptake is strong and the electrons and protons leave the RC in the form of quinol. This is expressed by the simple correlation between the rate of the electron input, cytochrome photo-oxidation (k_{cyt}) and the rate of RC (quinone) turnover (k_{RC}): $k_{cyt} = 2k_{RC}$. However, the strong coupling can be lost under appropriate conditions of high light excitation (Gerencsér and Maróti 2006). The rate of the photocycle depends on the rates of the individual steps on a complex way but cannot be larger than that of the slowest one. If step i is the rate-limiting reaction ($k_i << k_j$, $i \neq j$) in the photocycle then the observed rate would be approximately equal to the rate of the rate-limiting step. As the average light intensity on the surface of the Earth is rather low, the corresponding photochemical reaction ($PQ_A \rightarrow P^+Q_A^-$) becomes usually the rate-limiting step in the photocycle. However, the photosynthetic bacteria have efficient light harvesting apparatus in their membrane, which may increase the efficiency of light utilization by about two orders of magnitude. By taking the exciting light intensity above 1 W cm^{-2}, different donor and acceptor side reactions become rate-limiting in the photocycle (Osváth and Maróti 1997; Gerencsér et al. 1999). The proton-activated electron transfer ($k_{AB}^{(2)}$) becomes the rate-limiting step of the photocycle in the following cases. (1) The dominating internal proton delivery pathway is severely blocked by mutations of protonatable amino acids in key positions, e.g. L213DN single (Takahashi and Wraight 1990) and L210DN/M17DN double (Paddock et al. 2001) mutants. (2) The pH is increased above nine to limit the availability of H^+ ions

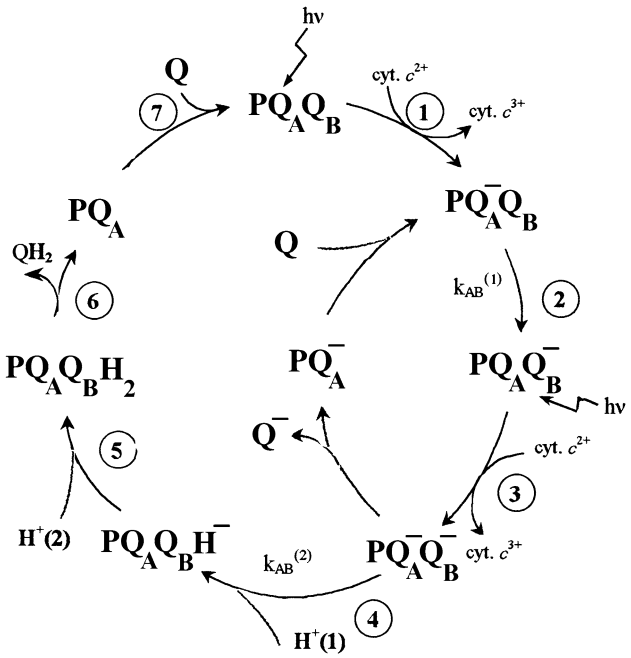

Fig. 1 Light-induced redox changes of cofactors forming photocycle in bacterial RC. Short living states, fast reactions and uptake of Bohr protons are omitted. In the conventional route of redox transitions the quinone in the Q_B site accepts two electrons and two protons that results in the release of the quinol (the chain of reactions is showed by *circle*). The electrons and protons are supplied by reduced cytochromes and by bulk phase or internal protonatable groups, respectively. The strong coupling of electron and proton transfer can be quantified by 1:1 proton/electron stoichiometry. Under high light intensity, when proton binding is retarded, release of semiquinone can be observed (see the banching of the main route after reaction 3). As no proton uptake occurs in the shunt of the electron transfer, the strong coupling between electron and proton transfers is lost

from the solution to reduce Q_B to Q_BH_2 in wild type RC. (3) The proton uptake of the wild type RC is inhibited by divalent transition metal ions (Paddock et al. 1999; Gerencsér and Maróti 2001), which are coordinated by histidines (HisH126 and HisH128) and by aspartic acid, either AspH124 (in case of Cd^{2+}) or AspM17 (Ni^{2+}) at the entry point of the proton delivery channel of the RC (Axelrod et al. 2000). The inhibition of the dominating proton pathway does not result in complete inhibition of the proton transfer, because the protons may be delivered to Q_B on alternative pathways by 2–3 orders of magnitude smaller rates. In accordance with the decreased rate of proton uptake in these cases, the rate of cytochrome turnover of the RC will become also small but shows unexpected light intensity dependence (Gerencsér and Maróti 2006). The observed increase of rate ("acceleration") was interpreted by release of semiquinone from the RC of altered conformation state induced by high light intensity in the presence of large quinone pool (Fig. 1). The probability of replacement of semiquinone by oxidized quinone in the Q_B binding site was significantly enhanced by the increase of the lifetime of biradical semiquinone due to the slow proton binding. One of the consequences of the unbinding of semiquinone is the loss of strong coupling between electron and proton transfer (Gerencsér and Maróti 2006).

As the observed acceleration of the cytochrome turnover depends on the binding properties of ubiquinone at the Q_B binding site, comparative studies will be performed in RCs where the native and hydrophobic UQ_{10} is replaced by its hydrophilic analogue UQ_0. Due to the same headgroups, the electron transfer remains fast (McComb et al. 1990; Warncke et al. 1994) but the weaker binding to Q_B site and different solubility of UQ_0 in detergent solution slow down the cytochrome turnover resulting in similar deceleration as experienced in native RC of inhibited proton uptake. The slow turnover of the quinone at Q_B site will favor the establishment of long-lived semiquinone in the Q_A site. The UQ_0 substitution will open the stage to understand the light-dependent acceleration of cytochrome turnover observed in RC with and without proton binding inhibition.

Materials and methods

The isolation of RCs from carotenoidless mutant (strain R26) of photosynthetic bacterium *Rhodobacter sphaeroides* was performed as previously described (Maróti and Wraight 1988b). The zwitterionic detergent (LDAO) was removed by overnight dialysis of the RC at 4°C against 1 mM Tris buffer (pH 8) and non-ionic detergent (0.03% Triton X-100). The concentration of the RC was determined on the basis of bacteriochlorophyll content, either from the steady-state absorption of bacteriochlorophyll monomer at 802 nm ($\varepsilon = 288\ mM^{-1}\ cm^{-1}$) or from light-induced absorption change at 430 nm ($\Delta\varepsilon = 26\ mM^{-1}\ cm^{-1}$) due to the oxidation of bacteriochlorophyll dimer (Gerencsér et al. 1999). During preparation, the native UQ_{10} content in the Q_B site of the RC dropped to 10% after the LDAO treatment. It was further reduced below 5% by washing the RC on the DEAE sephacell column with solution containing 1% LDAO, 1 mM EDTA and 10 mM Tris, according to the procedure described by (Okamura et al. 1975). Ubiquinone UQ_6 and UQ_0 (Sigma) dissolved in ethanol were externally added to restore the Q_B activity of the RC. Cytochrome *c* from horse heart (type VI, Sigma) was reduced (>95%) by bubbling H_2 gas through its aqueous solution in the presence of palladium black (Debus et al. 1985). The concentration of reduced cytochrome *c* was calculated using extinction coefficient of $\varepsilon_{550}(\text{reduced}) = 27.6\ mM^{-1}\ cm^{-1}$ (Rosen et al. 1980).

The steady-state absorption spectra were recorded by dual-beam spectrophotometer (Unicam UV4). Kinetic absorption measurements were carried out by a single-beam spectrophotometer of local design (Maróti and Wraight 1988a; Osváth and Maróti 1997). Continuous excitation was provided by laser diode illumination according to (Gerencsér and Maróti 2006). The rate of the photochemical reaction (k_p) was determined from the kinetics of P^+ generation under continuous illumination in the absence of external electron donor (cytochrome c^{2+}). The performance and analysis of measurement of cytochrome photo-oxidation during continuous illumination was previously described (Gerencsér and Maróti 2006). The fast cytochrome turnover was measured routinely with shorter sidechain quinones (UQ_6, UQ_0), as the measured kinetics are influenced by the slow intermicellar quinone movement of longer sidechain quinone (UQ_{10}).

Redox potentiometry was carried out as described in (Wraight 1981). Addition of two redox mediators (25 μM DMBQ and 20 μM TMPD) was sufficient to establish fast redox equilibrium between the platinum electrode and the redox active molecules in solution in the investigated E_h range. The E_h was adjusted by Na-ascorbate to reductive and by H_2O_2 to oxidative direction. The ferri-/ferrocyanide redox couple could not be used as it associates with transient metal ions with high affinity. The sample was prepared in a 1 × 1 cm redox cuvette where all relevant physical-chemical parameters (pH, E_h) could be controlled. On each occasion, 200 μl solutions were taken from the redox cuvette and put into a 3 × 3 mm rectangular cuvette filled by N_2 gas previously to excite by a single laser flash. The redox potential was referred always to NHE otherwise the reference electrode was indicated.

The kinetic traces recorded by a digital oscilloscope (Hitachi VC-6025) were transferred via RS232 to the IBM computer system of local design. The steady-state

absorption spectra were converted to ASCII file using the software Vision32 and the ASCII files were elaborated by Origin 6.0. Model calculations and fits were carried out by Mathcad 4.0.

Results

The rate of cytochrome turnover (photo-oxidized cytochrome molecules per time per RC) was measured under continuous illumination, where the native UQ_{10} content was decreased to 1 UQ_{10}/RC and the Q_B activity was restored by UQ_0. After a fast transient period, the amount of photo-oxidized cytochrome (cyt c^{3+}) increased in proportion with the time of illumination (Fig. 2a). Larger amount of added UQ_0 evoked larger rate of the cytochrome turnover (see the slope of the traces). Linear increase of the rate of cytochrome turnover was observed as a function of quinone concentration up to 1 mM that was followed by tendency of saturation (Fig. 2b). Similar behavior was described earlier by (Paddock et al. 1988; Gerencsér and Maróti 2004). Here, however, the observed saturation rate was higher ($k_{cyt} = 1{,}600\ s^{-1}$) and depended surprisingly on the concentration of the RC. By decrease of the RC concentration from 1 μM to 35 nM, the saturation value diminished to $k_{cyt} = 260\ s^{-1}$ (Fig. 3, open square) as obtained by (Paddock et al. 1988). More unexpectedly, the saturation rate increased in a similar way by increase of the concentration of the cytochrome. Additional increase can be observed by setting the ionic strength to 30 mM, where the turnover rate of cytochrome is at maximum (Gerencsér et al. 1999). The change of the cytochrome concentration modified not only the amplitude, but the shape of quinone concentration dependence, as well: the half-rise quinone concentration value, where the cytochrome turnover rate is equal to half of the saturation rate, shifted to 800 μM. To our further surprise, the saturation rate showed remarkable light-intensity dependence, which relationship deviated clearly from linearity (Fig. 4). Here k_{cyt} was plotted versus k_p, the photochemical rate constant that differs from the light intensity by a scaling factor only. The measured rate showed steep initial slope that gradually decreased with increasing light intensity. The turnover rate, however, did not saturate even at the highest light intensity used in our experiments.

By addition of transition metal ions to the RC solution, the proton uptake is inhibited and becomes the rate-limiting reaction in the photocycle, thus decelerated cytochrome turnover can be measured at saturating quinone concentration (Fig. 5). The shape of the kinetics of cytochrome photo-oxidation measured in presence of large UQ_0 pool deviates clearly from that of UQ_6 pool experienced earlier (Gerencsér and Maróti 2006). At the onset of the excitation, 1 [cyt c^{3+}]/[RC] appeared immediately and additional

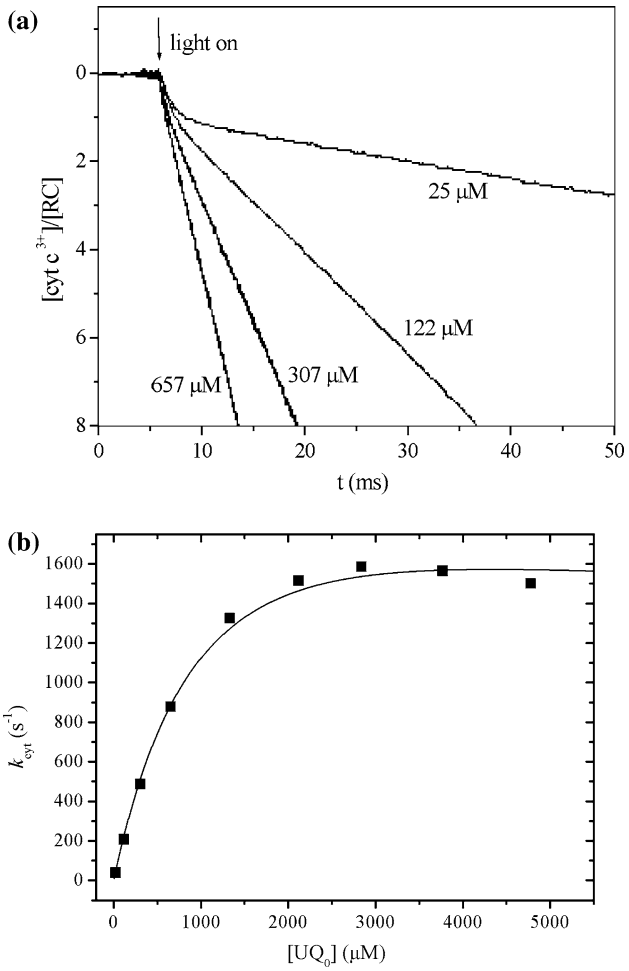

Fig. 2 The turnover of quinone acceptor complex of isolated RC with externally added UQ_0 monitored by photo-oxidation of cytochrome under continuous excitation. The steady-state rate of cytochrome turnover that follows the fast transient at the onset of illumination shows remarkable quinone concentration dependence (**a**). The observed rate increases linearly at small quinone concentration and saturates in the millimolar concentration range (**b**). The measured points were fitted by curve calculated from model in Fig. 8 using parameters of $k_{Qon} = 6.3 \times 10^5\ M^{-1}\ s^{-1}$ and $k_{Qoff} = 300\ s^{-1}$ with constrain of $K_D(Q) = 480$ μM (Fig. 3). The rate of cytochrome donation ($k_C = 2{,}500\ s^{-1}$) was obtained from the fit of light-intensity dependence of cytochrome turnover (Fig. 4). Conditions: 1 μM RC, 80 μM cyt c^{2+}, 0.03% Triton X-100, 5 mM Mes, 30 mM NaCl, pH 6.0 and 100% light intensity

oxidized cytochromes formed with gradually decreased rate. After switching off the light, fast dark reaction can be seen, which is responsible for re-reduction of photo-oxidized cytochrome produced during the light phase. Two different dark (fast and slow) phases can be distinguished. The rate of re-reduction is so remarkable, that it modifies the kinetics measured under illumination. The bimolecular character of the dark reaction can be easily recognized on the kinetics: the reaction was accelerated by accumulation of oxidized cytochrome. The dark reaction modifies the

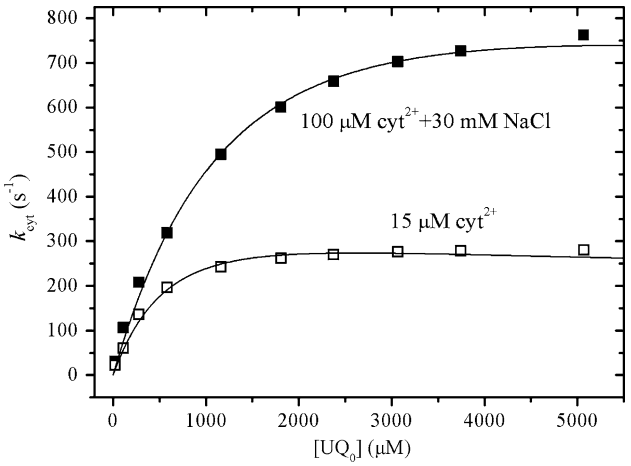

Fig. 3 The rate of cytochrome turnover as a function of UQ_0 concentration. Higher saturation rates can be obtained by increase of the concentrations of the RC and the reduced cytochrome and by setting the ionic strength to 30 mM. The measured data were fitted by curves calculated from the model in Fig. 8 with parameters of $k_{Qon} = 2.1 \times 10^5\ M^{-1}\ s^{-1}$, $k_{Qoff} = 100\ s^{-1}$ and $k_{QH2} \sim 8 \times 10^3\ s^{-1} \pm 4 \times 10^3$. The donation rate of cytochrome (k_C) changed from 250 s^{-1} (*open square*) to 820 s^{-1} (*filled square*) by modification of cytochrome concentration and ionic strength of the solution. The dissociation constant of UQ_0 was calculated from the fitting parameters ($K_D(Q) = k_{Qoff}/k_{Qon} = 480\ \mu M$). Conditions: 35 nM RC, 0.03% Triton X-100, 5 mM Mops, pH 6.6 and 15 μM cyt c^{2+} (*open square*) or 100 μM cyt c^{2+} +30 mM NaCl (*filled square*)

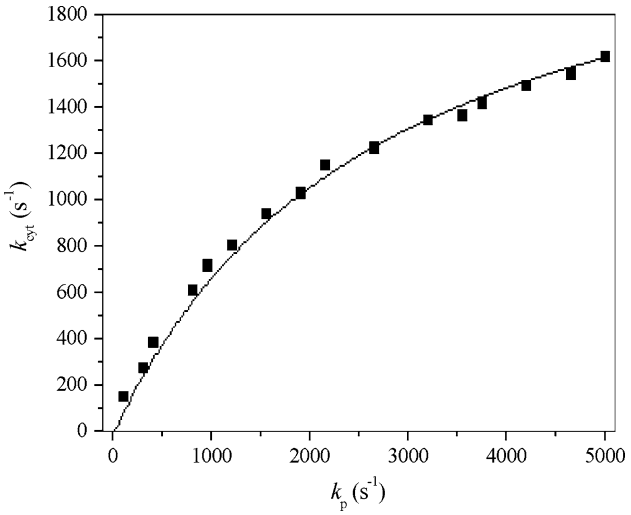

Fig. 4 Non-linear light intensity dependence of the rate of cytochrome turnover in the saturating range of quinone concentration. The light intensity is expressed as the rate of photochemical reaction (k_p). The data were fitted by a curve derived from model of simplified regular photocycle (Fig. 9) with $k_C = 2{,}500\ s^{-1}$. Conditions were the same as in Fig. 2b, but 3 mM UQ_0

kinetics of cytochrome turnover so drastically that no steady-state phase can be identified. In our previous work (in case of UQ_6 pool), we showed that the dark reaction was related to the unbinding of the semiquinone and to the

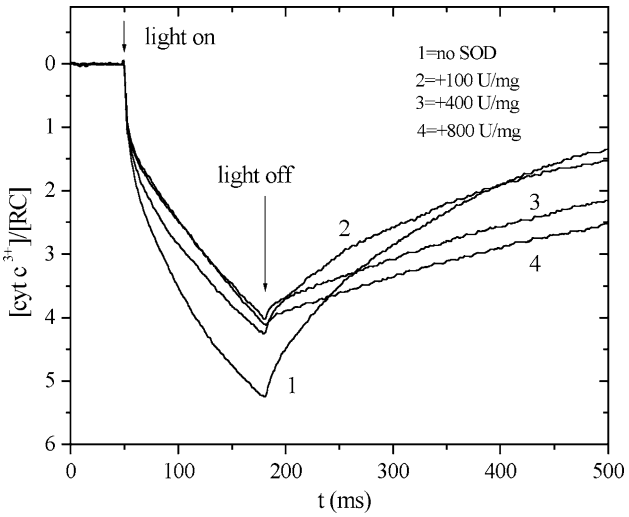

Fig. 5 Kinetic traces of oxidation and re-reduction of cytochrome in RC whose proton uptake is inhibited by addition of bivalent transition metal ions (trace 1). Notice the gradually decreasing slope of the trace and the lack of well-defined steady-state phase following the fast transient at onset of the illumination. The dark reaction (re-reduction of cytochrome) became more competitive by accumulation of oxidized cytochrome under illumination and can be visualized after switching off the light. Large UQ_0 pool is necessary for the fast dark reaction, which could be hardly detected in case of UQ_6 pool. Addition of SOD (traces 2–4) reduces the efficiency of the dark reaction: The kinetics recorded under illumination converges to straight line and the amplitude of the faster component in the dark reaction decreases drastically. Conditions: 1 μM RC, 0.03% Triton X-100, 40 mM NaCl, 60 μM cyt c^{2+}, 500 μM $NiCl_2$, 3 mM UQ_0, pH 8.4 and 100% light intensity. The amounts of added SOD are indicated on the graph

production of super oxide radical under semi aerobic conditions (Gerencsér and Maróti 2006). If the super oxide radical formed during the illumination is involved in the dark reaction measured in case of large UQ_0 pool, then addition of super oxide dismutase (SOD) would diminish the dark reaction. Indeed, increasing amount of SOD made the dark reaction slower and modified the kinetics measured in light, as well. The amplitude of the faster dark reaction dropped significantly.

In contrast to the behavior of untreated RC (Fig. 4), linear light-intensity dependence of cytochrome turnover was measured on RC of inhibited proton transfer in presence of large UQ_0 pool (Fig. 6). The enhancement of the rate of cytochrome turnover was mild (Fig. 6a) and much smaller than observed with UQ_6 pool (Fig. 6b). While 40 μM UQ_6 contributed to 10 times increase of the measured rate, 3 mM UQ_0 caused only four times acceleration of the cytochrome turnover. As the enhancement depends on the size of the quinone pool (Gerencsér and Maróti 2006), the actual effect should be even smaller.

By lowering the redox potential, the dark reaction was accelerated and below certain E_h value (+100 mV) became

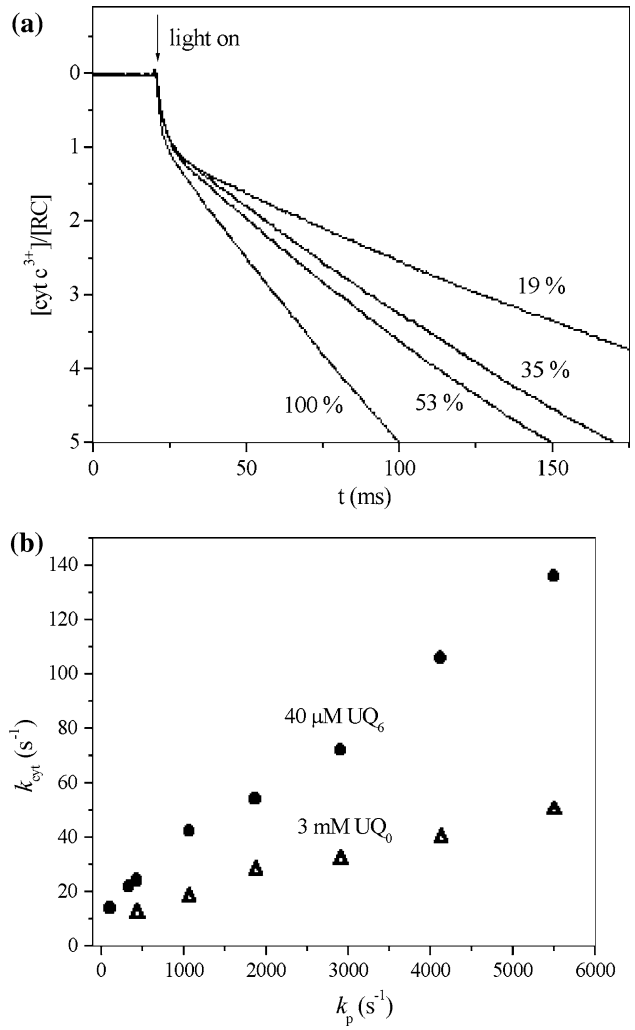

Fig. 6 Light-intensity dependence of cytochrome turnover of RC of inhibited proton binding and substituted UQ_6 pool by UQ_0. The addition of SOD diminishes the dark reaction and makes possible the determination of the steady-state rate of cytochrome photo-oxidation even in the presence of large UQ_0 pool (see Fig. 5). Kinetic traces of cytochrome turnover at different light-intensities and at high UQ_0 concentrations (**a**) and the light-intensity dependence of the rates together with RC of UQ_6 pool (**b**). Notice the two orders of magnitude larger UQ_0 pool that makes the light-intensity dependent acceleration even weaker in the case of UQ_0. Conditions: 1 μM RC, 60 μM cyt c^{2+}, 0.03% Triton X-100, pH 8.4, 100–500 μM $NiCl_2$ and 60 mM NaCl and 40 μM UQ_6 (*filled circle*) and 40 mM NaCl and 3 mM UQ_0 (*open circle*)

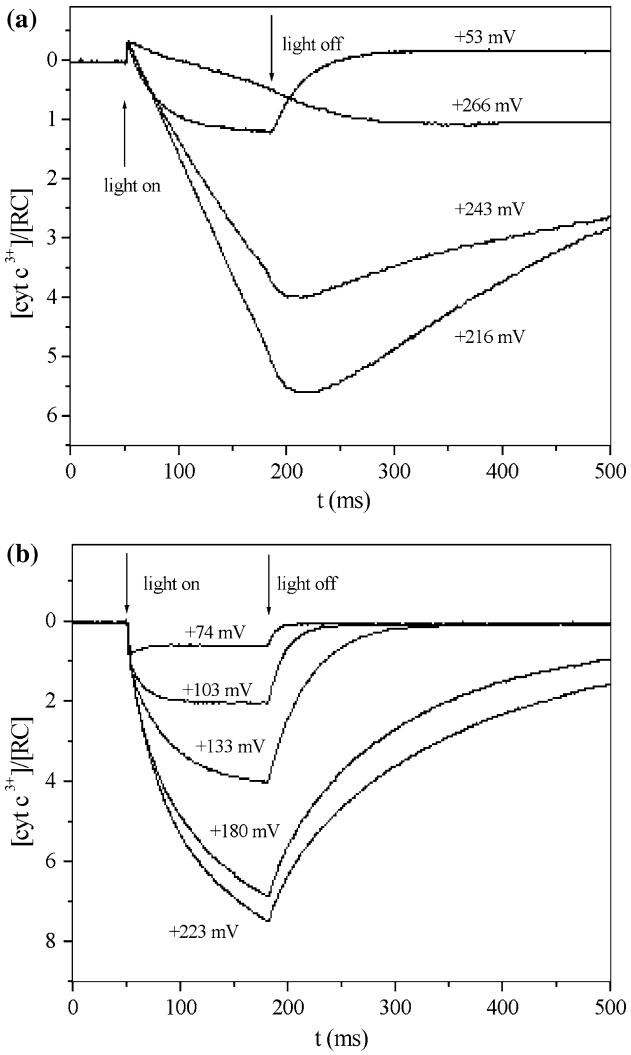

Fig. 7 Oxidation and re-reduction of cytochrome under different actual redox potentials. The turnover of the cytochrome was slowed down by inhibition of either donor (**a**) or acceptor (**b**) side of the RC. At high (200 mM) ionic strength, the bimolecular reaction of cytochrome and RC becomes rate-limiting step in the photocycle (**a**). Notice the transient peak (P^+ signal) immediately after switching on the excitation. The rate of re-reduction of oxidized cytochrome in the dark increased by lowering the E_h. The acceptor side of the RC was slowed down by inhibition of proton binding (**b**). The dark reaction was more efficient and became competitive with the photo-oxidation of cytochrome during illumination. Similarly to the former case, its rate depends strongly on the prevailing E_h. Conditions: 1 μM RC, 60 μM cyt c^{2+}, 0.03% Triton X-100, 200 mM NaCl, 3 mM UQ_0, 20 μM TMPD, 25 μM DMBQ and pH 8.4 (**a**). The condition was the same as in the case of (**a**), but the ionic strength was decreased to 40 mM and additionally 500 μM $NiCl_2$ was added (**b**)

so efficient that only stochiometric amount of oxidized cytochrome could be detected under illumination (Fig. 7). The effect of E_h on the fast (proton transfer was not inhibited) cytochrome turnover was investigated as a control experiment to make distinction between the reduction of cytochrome by redox mediator available in the solution and redox agent produced by RC turnover (Fig. 7a). The fast cytochrome turnover was adjusted to the rate of inhibited proton binding by setting the ionic strength to 200 mM. At this ionic strength, the bimolecular reaction of reduced cytochrome and RC is severely hindered (Gerencsér et al. 1999). After the onset of the excitation, small absorption increase due to the formation of P^+ was detected, as the rate of binding of reduced cytochrome became very slow. The kinetics during the illumination could be

well approximated by straight line. After turning off the excitation, additional cytochrome oxidation was observed (see curves detected at +243 and +216 mV) followed by moderate reduction of oxidized cytochrome in the dark. The cytochrome oxidation under illumination and cytochrome reduction in dark were accelerated by lowering the E_h. Although the covered E_h ranges did not match in the two experiments, we can clearly notice the difference between the kinetics measured at $E_h = 220$ mV: the dark reaction was much faster in the case of inhibited proton transfer.

Discussion

The present study was undertaken to characterize the photocycle of bacterial RC from *Rba. sphaeroides* under intense illumination if the Q_B binding site was occupied by UQ_0, a water soluble UQ_{10} analogue. The discussion will focus on two basic aspects of these experiments, (1) the binding properties of UQ_0 and (2) the enhancement of the rate of inhibited cytochrome turnover under high light intensity.

Saturation of the rate of cytochrome turnover as a function of UQ_0 concentration

If few UQ_0 molecules are in solution, the docking of quinone to the Q_B site will be slow and will become the rate-limiting step of the photocycle. The RC will be trapped mostly in the PQ_A^- form. The observed rate of the cytochrome turnover increased upon increase of the concentration of the quinone, confirming the bimolecular character of reaction of UQ_0 and RC and saturated at high quinone concentration (Figs. 2, 3, 4). The saturation rate and the half-saturating UQ_0 concentration were considered to be directly related to the electron transfer to Q_B and to quinone binding affinity, respectively (Paddock et al. 1988). Our experiments, however, did not support the simple connection since the measured rate depended remarkably on the cytochrome donation rate (Fig. 3). Not the concentration itself, but the donation rate of cytochrome was relevant, because the saturation rate could be enhanced by change of the ionic strength of the solution. The highest rate was established at 30–40 mM ionic strength, where the cytochrome exchange rate (binding of reduced form + release of the oxidized form) had maximum (Gerencsér et al. 1999). The observation of significant ionic strength dependence of the saturation rate excluded the possibility of rate limitation of any of the acceptor side reactions. It can be concluded, that the release of UQ_0H_2 is similarly fast as the other quinols (UQ_6H_2, $UQ_{10}H_2$).

As the saturation rate is clearly not correlated exclusively with acceptor side processes, the description of measured quinone concentration dependence of cytochrome turnover by Michaelis–Menten model (as proposed earlier (Paddock et al. 1988; Gerencsér and Maróti 2004)) is insufficient and should be extended. From theoretical point of view, the Michaelis–Menten mechanism describes the reaction of UQ_0 (substrate) and RC (enzyme), but the assay of cytochrome turnover is suitable to monitor this reaction only if the enzymatic reaction is the rate-limiting step of the photocycle. Therefore, we extended the Michaelis–Menten mechanism (including binding and unbinding of quinone (k_{Qon}) and (k_{Qoff}), respectively and unbinding of quinol (k_{QH2})) to a working model by introduction of donor side reactions [primary photochemistry (k_p) and donation of cytochrome (k_C)], and other acceptor side reactions (first ($k_{AB}^{(1)}$) and second electron transfer ($k_{AB}^{(2)}$)). The model can be simplified (Fig. 8) by introduction of new rate constants defined by rate constants taken either from literature ($k_{AB}^{(1)}, k_{AB}^{(2)}$, Kleinfeld et al. 1984) or measured directly (k_p) or obtained from fitting (k_C, Gerencsér et al. 1999):

$$k_1 = \frac{k_C k_p}{k_C + k_p}$$

$$k_2 = \frac{k_{AB}^{(1)} k_p k_C}{k_p k_C + k_{AB}^{(1)} k_C + k_{AB}^{(1)} k_p}$$

$$k_3 = \frac{k_p k_C k_{AB}^{(1)}}{2k_C k_{AB}^{(1)} + 2k_p k_{AB}^{(1)} + k_p k_C}.$$

The dependence of the rate of cytochrome donation on the concentration of reduced cytochrome and RC was determined previously (Gerencsér et al. 1999) and was adapted to our case according to this relationship [$k_C = 2{,}500\ s^{-1}$ (1 μM RC and 80 μM cyt c^{2+} (Fig. 2)] or $k_C = 820\ s^{-1}$ [35 nM RC and 100 μM cyt c^{2+} (30 mM NaCl) and $k_C = 240\ s^{-1}$ (35 nM RC and 15 μM cyt c^{2+}) (Fig. 3)]. The quinone concentration dependences of the rate of cytochrome turnover were fitted according to an expression (not shown) derived by solution of system of kinetic equations from our model with fitting parameters of $k_{Qon} = 2.1 \times 10^5\ M^{-1}\ s^{-1}$, $k_{Qoff} = 100\ s^{-1}$ and $k_{QH2} \sim 8 \times 10^3\ s^{-1}$ $\pm 4 \times 10^3$. The fitting parameters can be uniquely determined from the global fit of quinone dependences measured under different conditions (notice the saturation levels and the difference of half rise quinone concentration values of cytochrome turnover on Fig. 3). Unlike the quinone binding parameters, the rate of quinol release was rather insensitive fitting parameter. The dissociation constant of UQ_0 can be easily calculated using the fitting values ($K_D = k_{Qoff}/k_{Qon} = 480$ μM). Although the fitting parameters should be independent of concentration of RC,

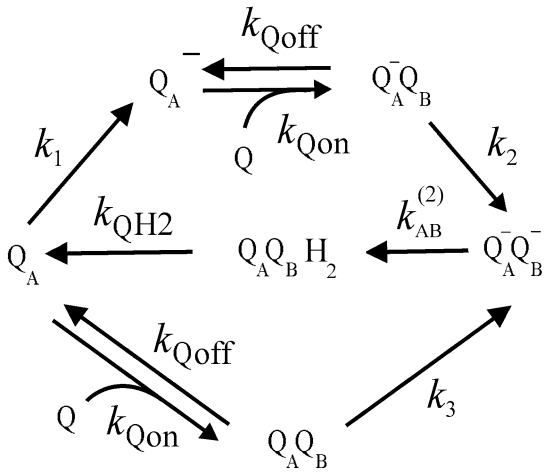

Fig. 8 Kinetic scheme to model the quinone concentration dependence of the observed rate of cytochrome photo-oxidation. To minimize the model, several reactions were unified to a single reaction with rate constants k_1, k_2 and k_3 (see the definitions in the text) and the intermediate states were omitted. The values of k_1, k_2 and k_3 can be calculated from k_p (measured directly), from $k_{AB}^{(1)}$ and $k_{AB}^{(2)}$ (Kleinfeld et al. 1984) and from k_C (obtained from the fit of light-intensity dependence of cytochrome turnover rate), and k_{Qon}, k_{Qoff} and k_{QH2} can be obtained from the global fit of quinone concentration dependences (Figs. 2b, 3)

the fit fails on Fig. 2 if the values obtained from Fig. 3 are kept constant. However, the quality of the fit can be remarkably improved by increase of k_{Qon} and k_{Qoff} but keeping K_D constant. This modification can be interpreted by the altered detergent concentration if larger amount of RC is added to the solution.

In earlier studies, the binding affinity of UQ_0 was determined by measuring the rate of formation of absorption transient at 402 nm after single flash excitation (Wraight and Stein 1983). The absorption change can be attributed to the transition of $Q_A^-Q_B \rightarrow Q_AQ_B^-$ [the contribution of charge separation ($PQ_A \rightarrow P^+Q_A^-$) can be neglected at 402 nm or it can be kinetically separated]. The electron transfer can occur only after the binding of quinone to the Q_B site, thus, this assay effectively monitors the rate of quinone binding. The measured rate of electron transfer showed saturation by increase of the quinone concentration and it was slightly smaller (1,400 s^{-1}) than the corresponding saturation value of the cytochrome turnover (see Fig. 2). The quinone concentration at half value of the saturation rate was 420 μM in 0.06% LDAO. It corresponds to 175 μM after correction to the detergent concentration (0.0025% LDAO). However this value is twice larger, than the reported value of 90 μM (in 0.0025% LDAO) determined from cytochrome turnover study (Paddock et al. 1988). Similar value of the dissociation constant of UQ_0 after correction to the LDAO concentration ($K_D = 600$ μM in 0.1% LDAO) was obtained on the basis of amplitude analysis of charge recombination kinetics (McComb et al. 1990). As the UQ_0 is quite hydrophilic, the correction of K_D values to the detergent concentration gives lower estimate of the true value. The K_D value derived from our measurements is 2–3 times larger than the corresponding value determined from flash experiment. This difference indicates that the light-intensity dependent acceptor side effect on the binding affinity of quinone at the Q_B site can be operative even in RC, where the quinone turnover is the rate-limiting step. However, the extent of the effect is far not so strong than observed on RC, where the proton binding was rate limiting and the binding affinity of quinone equivalents at the Q_B site dropped 1–5 orders of magnitudes.

What does the anomalous light-intensity dependence of the rate of cytochrome turnover at saturating quinone concentration regime reflect (Fig. 4)? The light intensity dependence clearly deviates from the linearity, which needs different interpretation than suggested in our previous paper (Gerencsér and Maróti 2004). Indeed, the observed light-intensity dependence can be adequately described by light-intensity dependence of the classical RC photocycle. Actually even the simplified version of the photocycle is sufficient, where the primary photochemistry (k_p) and the rate of cytochrome donation (k_C) are involved only and all other reactions were supposed to be fast and omitted (Fig. 9). The measured data can be fitted well by a simple equation derived from the model: $k_{cyt} = k_C k_p/(k_C + k_p)$. The k_C was chosen as fitting parameter and the obtained value ($k_C = 2{,}500$ s^{-1}) is in fine accordance with our previous result (Fig. 4; Gerencsér et al. 1999). Why could not be seen the additional and linear light-intensity dependent acceleration, although the light-intensity dependent change of the binding affinity of quinone was simultaneously observed? The answer is straightforward: in case of photocycle where the quinone turnover is rate limiting, the RC is not trapped in long-living biradical

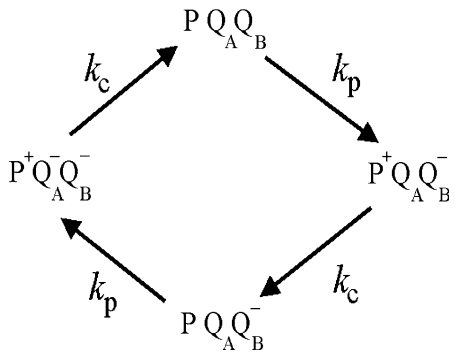

Fig. 9 The minimal model of the photocycle to describe the light intensity dependence of the rate of cytochrome photo-oxidation (in Fig. 4). All other reactions in the photocycle were neglected, as they were faster compared to the primary photochemistry (k_p) and cytochrome donation (k_C)

semiquinone state, therefore no semiquinone release occurs and there is no electron shunt in the photocycle.

Light-intensity dependent "acceleration" of cytochrome turnover by use of different types of quinones

It has long been believed that the binding of oxidized and unbinding of reduced forms of quinone at the acceptor side are not connected to the light excitation of the RC. Very recently it was shown that the binding affinity of quinone type molecules (inhibitors, different redox forms of quinones) to the Q_B site was decreased by increasing light-intensity of the continuous excitation (Gerencsér and Maróti 2006). The mechanism of the effect is far not clearly understood, but the high-frequency ($\sim$5 kHz) production of short-living ($\sim$10 ns) charge pairs of P^+Pheo^- may offer additional interaction (activation) energy to the acceptor site to shift the binding equilibrium of the quinone to the direction of unbinding. In untreated RC, the manifestation of the effect is highly time-limited but in RC of inhibited proton uptake by metal ions, significant change of binding parameters of UQ_6 was observed (Gerencsér and Maróti 2006).

The fast turnover of RC was severely hindered by inhibition of proton binding in presence of transition metal ions and/or by substitution of the longtail native quinone pool by the tailless UQ_0 pool. Since the binding rate constants of the quinones were different, it can be expected that the rate of the semiquinone release are also different, whose modification is the primary factor responsible for the acceleration. The slope of light-intensity dependence of rate of cytochrome turnover was 3.2 times smaller in UQ_0 than in UQ_6 (Fig. 6b). As it depends not only on the rate of semiquinone release, but also on the size ("pressure") of the quinone pool that can be quantified by the binding rate of the quinone ($k_{Qon}[Q]$, Gerencsér and Maróti 2006). The applied concentration of UQ_0 (3 mM) was not sufficiently larger than that of the UQ_6 (40 μM) to compensate the large difference in the bimolecular binding rate constants of different quinones ($k_{Qon}(UQ_0) = 2.1 \times 10^5\ M^{-1}\ s^{-1}$ and $k_{Qon}(UQ_6) = 1 \times 10^8\ M^{-1}\ s^{-1}$), therefore the "pressure" of the quinone pool was four times smaller in UQ_0 ($k_{Qon}(UQ_0) \times [UQ_0] = 630\ s^{-1}$) than in UQ_6 ($k_{Qon}(UQ_6) \times [UQ_6] = 4 \times 10^3\ s^{-1}$). As a consequence of high light-intensity excitation, similarly large increase of the dissociation constant (and the rate of release) of UQ_0^- occurs as for UQ_6^- at the Q_B site of the RC.

The kinetics of cytochrome turnover deviates from the straight line by using large UQ_0 pool. The problem becomes especially significant at high light-intensity, where the dark reaction is rather efficient (Fig. 7a). This reaction can be attributed to the re-reduction of photo-oxidized cytochrome by unbound semiquinone itself or by other free radicals that produced by the semiquinone in solution. Under semiaerobic conditions, the semiquinone can react with unsaturated O_2 in solution and generates super oxid radical (O_2^-). The presence of super oxid radical could be detected by super oxid assays (Gerencsér and Maróti 2006). The question arises, why is the dark reaction so efficient in presence of UQ_0? First, it is well known that UQ_0 is more hydrophilic than the other ubiquinones, therefore the reaction with water-soluble oxidized cytochrome is more favored. Secondly, it was used in much higher concentration, therefore the semiquinone can collide by higher frequency with an oxidized quinone, consequently, and the electrons diffuse much faster in solution to the cytochromes. Third, the difference in the chemical structure can be relevant: terpenylnaphtoquinones can accelerate the O_2 consumption (super oxid production) by different extent: the 6-(4-methyl-pentyl)[1,4]naphtoquinone (MPNQ) was more effective than the 2-hexylamino-6-(4-methyl-pentyl)-[1,4]naphtoquinone (HAPNQ) (Alegria et al. 2002). To exclude the possibility that the dark reaction was initiated by redox agent available in solution in the dark, the effect of E_h on the kinetics of cytochrome turnover was investigated. The decrease of E_h enhanced the rate of dark reaction, but in different extent: in the control experiment, where no semiquinone release was expected (Fig. 7a), the observed dark reaction was not so efficient. We cannot exclude the possibility completely in recent state of our study that the E_h was not relevant but the amount of ascorbate used to set the E_h. Ascorbate induced semiquinone production was reported for 1,4-benzoquinones (Roginsky et al. 1998). However this is unlikely in the case of UQ_0 as the E_m value of UQ_0/UQ_0^- pair (−404 mV) measured in dimethylformamide was significantly lower than that of BQ(1,4-benzoquinone)/BQ^- (−160 mV) (Prince et al. 1983). It is more likely that ascorbate reduces quinone to quinol on the basis of available data: UQ_{10} was dissolved in ethanol-water mixture of 4:1 and at pH 8.4 following data were measured E_m(UQ/UQH_2) $\sim$0 mV (Dryhurst 1982), and 30 mV (Morrison et al. 1982).

In order to understand qualitatively the nature and mechanism of cytochrome related redox reactions in the light and in the dark, a redox agent should be found that is produced in the light phase and can act both as reductant and oxidant (Fig. 7). The generation of superoxide was identified with SOD assay in our experiments. The superoxide has high reactivity acting as a reductant and an oxidant depending on the conditions. However, the superoxide is not particularly reactive in biological systems and does not by itself cause much oxidative damage. It is a precursor to other oxidizing agents, including singlet

oxygen and other highly reactive molecules. The conditions of singlet oxygen formation from superoxide are revealed (Koppenol 1976) and should be checked whether are appropriate to our system.

Conclusions

Increase of dissociation constant of ubiquinone-0 at the Q_B site was observed under high light-intensity of excitation. Similar effect was described earlier with ubiquinone-6 in RC of inhibited proton transfer. Here, a different system was presented where the light-intensity dependent acceptor side effect operated and ultimately excluded the possibility of the increase of the rate of inhibited proton binding as major source of the acceleration effect. We obtained, however, smaller (2–3 times) change of K_D due to the shorter time scale that confirms the time-dependent character of light-intensity dependent acceptor side effect. Furthermore, we can conclude that the biradical semiquinone state is not indispensable precursor of the effect, as in our condition the single semiquinone radical state (PQ_A^-) was present dominantly. These observations support our hypothesis that the high-frequency generation of short-living P^+Pheo^- charge separated state evokes the modification of the binding affinity of quinone equivalents on the Q_B site, because the production of the charge-pair needs only fast electron donor to P^+ (cyt c^{2+}) and slow acceptor side process (inhibited proton transfer or slow quinone turnover at the Q_B site) to trap the RC in PQ_A^- state. As the light-intensity dependent acceleration did not depend on the type of quinones, the release rate and the dissociation constant of semiquinone is not influenced by the length of the isoprenoid chain of the quinone, but likely exclusively by the reduced keto group of the hydrophilic headgroup.

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