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ARTICLE

Kinetic limitations in turnover of photosynthetic bacterial reaction center protein

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ABSTRACT The membrane-bound reaction center from purple nonsulfur photosynthetic bacterium *Rhodobacter sphaeroides* performs light-induced charge separation and exports two molecules of oxidized cytochrome and one molecule of fully reduced quinone (quinol) from two opposite (periplasmic and cytoplasmic) sites of the protein during a single turnover. The rate of the turnover was measured based on cytochrome photooxidation under intense and continuous illumination of a laser diode that mimicks the open field conditions for photosynthetic organisms. At the highest intensity of illumination, the primary photochemistry was driven by a rate constant of 4,800 s^{-1}. Factors limiting the steady-state turnover rate of the reaction center were studied under wide variety of conditions of light intensity, ionic strength, heavy metal ion binding and pH. The reaction center was solubilized in detergent, and reduced mammalian cytochrome c and oxidized ubiquinone were the exogenous electron donor and acceptor to the reaction center, respectively. Depending on the conditions, the kinetic limitation was attributed to light intensity, or to donor and acceptor side reactions. At low light intensities (<1,000 s^{-1}, in terms of initial rate of photochemistry), the turnover rate was limited only by the light intensity. At higher intensities, however, the unbinding of the oxidized cytochrome at the donor side (acidic and neutral pH range) or the proton coupled interquinone electron transfer (alkaline pH range) were the bottlenecks of the turnover of the reaction center. The possible entries and pathways of protons to the secondary quinone are discussed based on multiple turnover of the reaction center. **Acta Biol Szeged 44(1-4):45-52 (2000)**

KEY WORDS

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light-induced charge separation
electron transfer
proton uptake

During photosynthesis, conversion of light energy into electrochemical energy occurs in membrane proteins of plants and bacteria. In bacterial photosynthesis, the energy transduction is mediated by a pigment-protein complex called reaction center (RC) that spans the bacterial cell membrane. This protein performs the primary photochemistry and couples electron and proton transfer across the bacterial membrane (for review see: Shinkarev and Wraight 1993; Maróti 1993; Okamura and Feher 1995; Sebban et al. 1995). It is the best characterized photosynthetic system, which serves as an obvious and effective model for the photosystems of higher plants. The bacterial RC has also been established as the premier system for studying biological electron transfer, and mechanisms of dynamic charge compensation in proteins. Considering that one-third of all known proteins are redox active and almost all known enzymatic mechanisms involve proton transfers, these are issues of major significance for understanding protein function in general.

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Structure of the RC

The RC of photosynthetic purple bacterium *Rhodobacter (Rb.) sphaeroides* consists of three polypeptide subunits (L, M and H), and nine prosthetic groups – 4 bacteriochlorophylls, 2 bacteriopheophytins, one non-heme iron, and 2 ubiquinones (Q_A and Q_B). Its three dimensional atomic structure has been determined by several groups using X-ray diffraction (Allen et al. 1986; Chang et al. 1986), and the details of the protein-chromophore interaction have been determined to 2.65 Å resolution (Ermler et al. 1994) that was further improved to 2.2 Å resolution recently (Stowell et al. 1997). The L and M subunits each have 5 transmembrane helices. The H subunit caps the RC on the cytoplasmic side of the membrane and is more hydrophilic than L and M. It contains mixed structural motifs, including a single transmembrane helix. The cofactors are all non-covalently bound to the L and M subunits. The primary electron donor (P) is a dimer of bacteriochlorophyll and is located towards the periplasmic side of the membrane. The two quinones of the acceptor complex on the cytoplasmic side of the membrane are chemically identical but functionally very different due to their highly different protein environment (Stein et al. 1984; Shinkarev and Wraight 1993; Wraight 1998). The

primary quinone (Q_A) functions only as a one-electron carrier under physiological conditions. It is tightly bound into a relatively hydrophobic part of the M subunit. In contrast, the secondary quinone (Q_B) is reversibly bound in a more polar region of the L subunit and functions as a two-electron acceptor. The Fe^{2+} atom, hexacoordinated by five amino acids, is symmetrically positioned between the quinones.

Basic function of the RC

The energy of photons absorbed in the antenna is transferred with high quantum yield to the RC, where it initiates a series of electron transfer (ET) reactions coupled to proton translocation across the photosynthetic membrane. The first steps of establishment of the transmembrane proton gradient are taking place in the RC and are schematically presented in Figure 1. ET in the RC and uptake of two protons from the cytoplasm leads to the formation of loosely bound quinol, Q_BH_2, which is released from the RC into the bacterial membrane. The quinol is subsequently oxidized by the cytochrome bc_1 complex, releasing the two protons into the periplasm. The gradient of protons formed in this way across the bacterial membrane supplies energy for various cellular functions, including solute transport, flagellar rotation and synthesis of ATP from ADP.

Reduction cycle: turnover of the RC

In the RC, light-induced ET proceeds from P to Q_B through a series of electron donor and acceptor molecules. The charge separation is initiated by excitation of P ($P \rightarrow P^*$) followed by three sequential ET reactions to bacteriopheophytin ($P^* \rightarrow P^+I^-$), primary quinone ($P^+I^- \rightarrow P^+Q_A^-$) and secondary quinone ($P^+Q_A^- \rightarrow P^+Q_B^-$) that take place on the few picosecond, hundred picosecond and hundred microsecond time scales, respectively. If external electron donor (reduced cytochrome c_2, cyt c^{2+}) is available on the donor side, then it can re-reduce the oxidized dimer (Overfield and Wraight 1986; Moser and Dutton 1988; Tiede et al. 1993)

$$P^+ \text{cyt } c^{2+} \rightarrow P \text{ cyt } c^{3+} \qquad \text{(D1)}$$

The RC can then be excited again and similar ET from P to Q_B^- is initiated. Considering the acceptor side redox reactions, the first interquinone ET to Q_B

$$Q_A^- Q_B \xrightarrow{\text{1st electron}} Q_A Q_B^- \qquad \text{(A1)}$$

does not involve direct protonation of the quinone (Kleinfeld et al. 1984; Maróti and Wraight 1988, 1997). However, the second ET is coupled to direct protonation of Q_B (Maróti and Wraight 1988; Graige et al. 1996; Abresch et al. 1998; Gopta et al. 1999; Miksovska et al. 1999):

$$Q_A^- Q_B^- + H^+ \xrightarrow{\text{1st proton}} Q_A^- (Q_BH) \xrightarrow{\text{2nd electron}} Q_A (Q_BH)^- \qquad \text{(A2)}$$

The uptake of the 2nd proton leads to the formation of quinol:

$$Q_A (Q_BH)^- + H^+ \xrightarrow{\text{2nd proton}} Q_A (Q_BH_2) \qquad \text{(A3)}$$

The quinol dissociates from the RC and is replaced by an oxidized quinone from the quinone pool:

$$Q_A (Q_BH_2) + Q_{pool} \xrightarrow{\text{quinol release+ quinone binding}} Q_A Q_B + QH_2 \qquad \text{(A4)}$$

Thus, the quinone reduction cycle is completed: two electrons are transferred and two protons are bound to Q_B as a result of two separate photochemical reactions yielding the oxidation of two cytochrome molecules. The RC separates reducing (QH_2) and oxidizing (cyt c^{3+}) equivalents that are exported on the acceptor and donor sides, respectively.

The turnover can be driven by a series of saturating light flashes or by onset of continuous illumination (Osváth and Maróti 1997; Larson et al. 1998; Gerencsér et al. 1999). Whereas the former method is suited for experiments in laboratories, the latter technique is similar to open field conditions where the plants are exposed to continuous excitation of sunshine. The two methods differ in the rates of the observed charge separation. Using saturating single flash excitation, the charge separation occurs immediately (within the duration of the flash) but upon onset of continuous excitation, the rate constant of primary photochemistry (k_I) depends on the intensity of the illumination:

$$PQ_A \xrightarrow{k_I} P^+ Q_A^- \qquad \text{(L1)}$$

Here, the rate constant (k_r) of the reverse ($P^+Q^- \rightarrow PQ$) reaction (charge recombination) is neglected relative to that of the photochemistry ($k_r << k_I$). In this case, 37% of the RCs are able to absorb photon from the excitation (*i.e.* get into charge-separated state) after onset of illumination within the time interval of reciprocal of the photochemical rate constant (k_I^{-1}).

As the turnover of the RC should involve all reactions of the bioenergetic cycle, the slowest component will determine the overall turnover rate. From the point of view of research in molecular biophysics and plant physiology, it will be interesting to explore the conditions determining the bottleneck of the turnover. In the majority of practical cases, the rate limiting step is the light intensity (L1), however, depending on the conditions, the limitation may also occur on the donor side (D1) or on the acceptor side ((A1)-(A4)).

The present study will utilize the unique advantages of the bacterial system: 1) the knowledge of the RC's three dimensional structure, 2) the existence of well established methods for modifying the protein and the distinct reaction

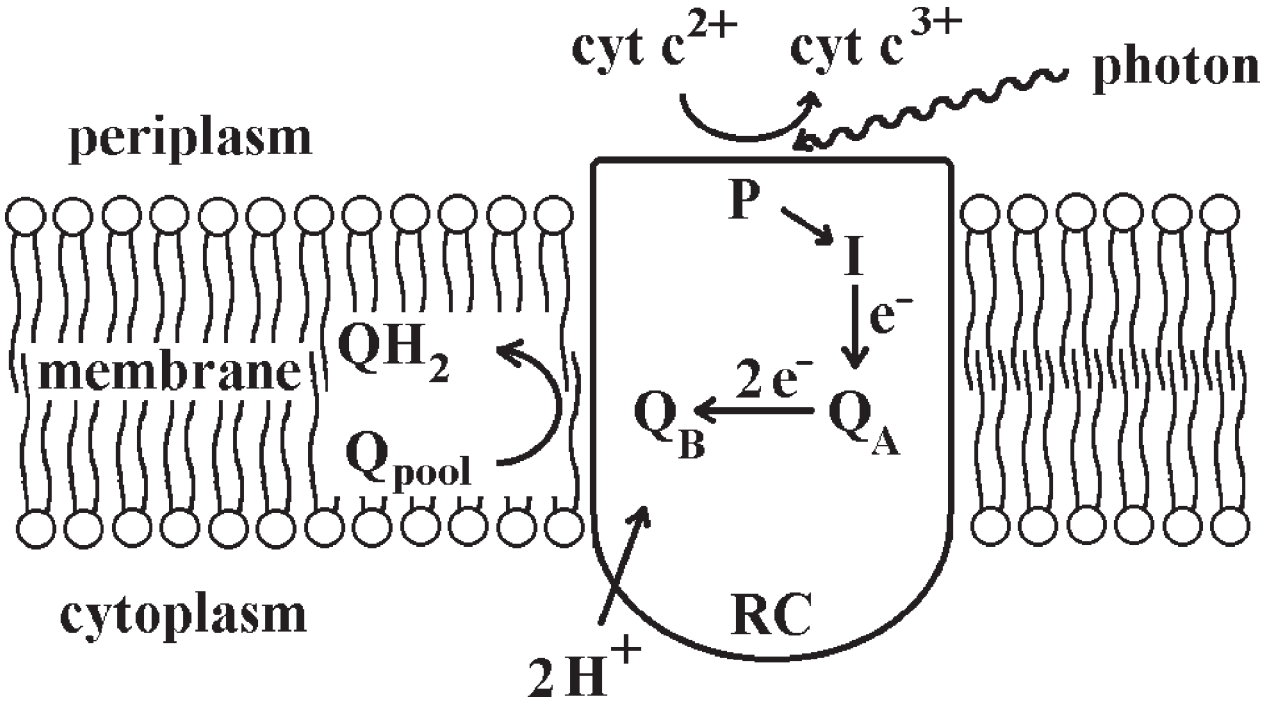

Figure 1. Schematic arrangement of RC protein and redox cofactors including bacteriochlorophyll dimer (P), bacteriopheophytin (I) and primary (Q_A) and secondary (Q_B) quinones in the photosynthetic membrane of *Rb. sphaeroides*. Photon absorption by P leads to a series of electron transfer reactions and uptake of H^+ ion coupled to interquinone electron transfer. The turnover of the RC consists of subsequent oxidation and release of two molecules of cytochrome c at the periplasmic side and full (two-electron and two-proton) reduction and release of one quinone molecule at the cytoplasmic side of the membrane.

steps listed above and 3) the diversity of methods of absorption spectroscopy for investigating the ET events.

Materials and methods

Reagents, cytochromes and quinones

Ubiquinone UQ_{10} (coenzyme Q_{10}; 2,3-dimethoxy-5-methyl-6-decaisoprenyl-1,4-benzoquinone, Sigma) was sonicated in 30% non-ionic detergent Triton X-100 (octyl-phenol-polyethylenglycol-ether, Sigma) and ubiquinone UQ_6 (coenzyme Q_6, 2,3-dimethoxy-5-methyl-6-hexaisoprenyl-1,4-benzoquinone, Sigma) was solubilized in ethanol. Terbutryne (Chem Service, Inc., West Chester, USA), a Q_B site electron transfer inhibitor, was prepared in ethanol before use. In most of our measurements, mammalian (horse heart) cytochrome c (Sigma) was used as an external donor to the oxidized dimer (P^+) of the RC because the mitochondrial cytochrome c behaves similarly to cytochrome c_2, the native electron donor to P^+ in *Rb. sphaeroides* (Overfield and Wraight 1986; Moser and Dutton 1988; Tiede et al. 1993). The cytochrome c was reduced (>95%) by hydrogen gas on palladium or by sodium ascorbate. The degree of reduction of the cytochrome c was monitored by the increase of the optical density at 550 nm in a spectrophotometer (Unicam UV4). The amount of cytochrome c oxidized by the RC was determined using the extinction coefficient difference of:
$\xi_{red} - \xi_{ox} = 21.1 \pm 0.4\ mM^{-1}xcm^{-1}$ (Van Gelder and Slater 1962).

Isolation and preparation of RCs

RC protein was solubilized by ionic detergent LDAO (N,N'-dimethyl-dodecylamine N-oxide) from blue-green (carotenoidless) mutant (strain R-26) of photosynthetic purple bacterium *Rb. sphaeroides* as described earlier (Maróti and Wraight 1988). For alternate non-ionic detergent (Triton X-100) preparation and to remove excess salt that influences the kinetics of cytochrome photooxidation, the RC preparation was dialyzed against 1 mM Tris buffer (pH 8.0) and 0.03% Triton X-100 overnight at 4 °C before use. The RC isolated in this way was depleted of secondary quinone as checked by biexponential analysis of the kinetics of charge recombination evoked by single saturating flash, where the slow component indicated the presence of functional Q_B (Kleinfeld et al. 1984; Stein et al. 1984). The secondary quinone activity was reconstituted by ubiquinones added to the sample in excess from 10 mM stocks. The degree of reconstitution was above 90%.

Transient absorption spectroscopy

Charge recombination kinetics were measured by monitoring the recovery of the oxidized dimer (P^+) at 430 nm or 865 nm after bleaching with a saturating Xe flash of light by using a single-beam spectrophotometer of local design (Maróti and Wraight 1988). The bandwidth of the measuring beam was 2 nm in the visible spectral range and 4 nm at 865 nm. The concentration of the oxidized dimer was determined from the magnitude of the flash-induced absorption

change at 430 nm or 865 nm using extinction coefficients of 26 $mM^{-1}xcm^{-1}$ and 288 $mM^{-1}xcm^{-1}$, respectively (Kleinfeld et al. 1984; Maróti and Wraight 1988).

The kinetics of the transfer of the first electron from Q_A to Q_B was determined by monitoring the shift of the bacteriopheophytin band at 397 nm. The monitoring bacteriopheophytin is situated at dielectrically different distances from the two quinones, therefore its absorption is sensitive to which of the quinones (Q_A or Q_B) is reduced. To improve the signal-to-noise ratios, 64 traces were averaged.

The kinetics of the proton-coupled second electron transfer from Q_A to Q_B was measured by monitoring the decay of the semiquinone absorption at 450 nm after the second saturating flash in the presence of external donor (reduced cytochrome c).

The kinetics of photo-oxidation of reduced cytochrome c under rectangular shape of laser excitation was measured with a single beam kinetic spectrophotometer of local design (Osváth and Maróti 1997; Gerencsér et al. 1999). The probe light crossed the 3x3 mm rectangular quartz cuvette horizontally and was measured with a photomultiplier (EMI 9558) through a Corning 4-96 filter in the visible spectrum and with a photodiode (UDT Sensors, Inc. PIN 10DI) through an 850 nm high-pass filter in the near infrared spectral range. The actinic illumination was provided by a fiber coupled laser diode (Opto Power; type OPC-A002-796-FC/150, emission wavelength 796 nm, emission bandwidth (FWHM) < 3 nm, maximum power 1.2 W) controlled by a home-made driver (operating current 1.2 A). The duration of the laser emission (typically 5 ms) was controlled by a digital pulse generator (Híradástechnika, Type TR 0360), the rectangularity of its shape was checked by a photodiode connected to an oscilloscope through an amplifier and the light intensity was measured by a pyroelectric joulemeter (Molectron, Model J25). The measuring beam was focussed on a small area (3 mm x 3 mm) of the sample. The cuvette was held in a massive brass block channeled for liquid circulation to control the temperature. The temperature in the cuvette was measured with a NiCr–Ni thermocouple (TH 3010) coupled to a digital thermometer (Vermer VE 305K) and, unless stated otherwise, was 23 °C. The sample was kept under nitrogen atmosphere.

Experimental data were recorded by a digital oscilloscope (Hitachi VC 6025) interfaced with an IBM PC where data analysis were carried out. The kinetic analysis of the absorption transients was performed using the Levenberg-Marquardt nonlinear least-squares fitting algorithm.

Results

Comprehensive kinetic study of the RC turnover needs experimental determination of the rates of intraprotein ET steps and the quinone reduction cycle. The short description of the spectroscopic assays used here and the major results for isolated RC of purple nonsulfur photosynthetic bacterium *Rb. sphaeroides* solubilized in detergent are summarized below.

Primary photochemistry

A convenient kinetic spectrophotometric assay was used to follow the primary photochemistry (see L1). The near infrared absorption band at 865 nm is sensitive to the redox state of the dimer: it is bleached if P gets oxidized.

After excitation of the dimer, the $P^* \rightarrow P^+Q_A^-$ charge separation is an extremely fast process (<200 ps) that is well above our time resolution. Under conventional (not saturating picosecond laser) illumination, the observed rate of charge separation depends on the intensity of light excitation (and not on the primary $P^* \rightarrow P^+Q_A^-$ step), *i.e.* how fast the dimers in the population of the RCs get excited. If rectangular shape of excitation is applied, the accumulation of the $P^+Q_A^-$ charge separated state of the RC will follow an exponential function: the rate of initial rise is k_I and 1/e of the saturation level is obtained within k_I^{-1} after onset of the excitation (see L1). The photochemical rate constant is proportional to the intensity of the illumination and to the overlap of the absorption spectrum of the sample and the spectrum of the exciting light. We used monochromatic (796 nm) emission of a laser diode that was very close to the main absorption peak (802 nm) of the monomeric bacteriochlorophylls of the RC. The efficient absorption and the high intensity of the exciting laser light (about 1 W light power) assured a very high rate of primary photochemistry. We could adjust k_I as high as 4,800 s^{-1} which means that under this condition, half of the RCs became photochemically closed within about 200 ms after onset of laser diode excitation.

Charge recombination

The electron on the semiquinones (Q_A^- and Q_B^-) can recombine with the positive charge on the dimer (P^+). The reverse ETs can decrease the effectiveness of the photo-chemistry if their rates are comparable to those of the forward ET rates. The charge recombination rates for the reactions $P^+Q_A^- \rightarrow PQ_A$ (k_{PA}) and $P^+Q_B^- \rightarrow PQ_B$ (k_{PB}) were measured at 865 nm

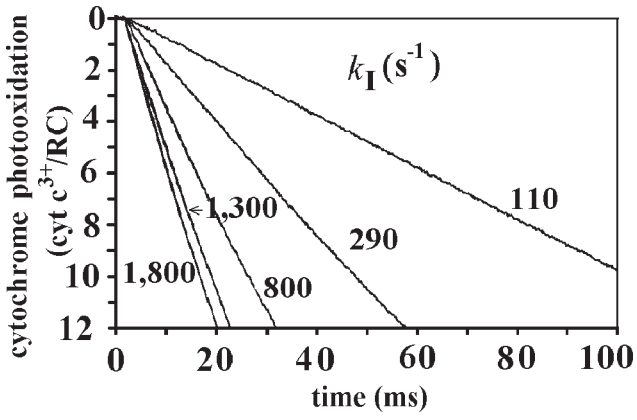

Figure 2. Kinetics of cytochrome photooxidation upon onset of steady-state illumination of different intensities expressed in terms of photochemical rate constant k_I (see L1). The different light intensities of the laser diode (emission wavelength 796 nm) were adjusted by calibrated neutral density filters. The concentration of the photooxidized cytochrome was related to that of RC. Conditions: 1.5 mM RC, 0.05% Triton X-100, 30 mM UQ_6, 57 mM cyt c^{2+} and 5 mM Tris buffer at pH 7.3.

(or 430 nm) after single flash excitation. The observed values of $k_{PA} = 9\ s^{-1}$ and $k_{PB} = 0.8\ s^{-1}$ at pH 8 were much smaller than the rate of primary photochemistry (see above). Therefore, only the forward ET step is considered in reaction scheme (L1) under our experimental condition of high light excitation.

Cytochrome photooxidation

The rate of cyt c^{2+} photooxidation under continuous illumination was measured in the presence of high concentration (>50 mM) of exogenous UQ. The amount of photooxidized cytochrome relative to that of the RC was monitored optically at 550 nm as function of time (Fig. 2). In the presence of a cyt c^{2+} pool, the oxidized dimer is immediately reduced again allowing more electrons to flow from P to Q_B. For each electron cycled through the RC, one cyt c^{2+} is oxidized to cyt c^{3+} (Fig. 1). Thus, the oxidation of cyt c^{2+} provides a measure of the turnover rate (in cyt $c^{3+}.RC^{-1}.s^{-1}$) determined as the slope of the kinetics.

In Figure 2, the kinetics of cytochrome photooxidation are given when the RCs are exposed to rectangular shape of light excitation of different intensities. The light intensity of the laser diode was adjusted by neutral density filters and was expressed in terms of the corresponding rate constant (k_I). The light intensities ranged from 110 s^{-1} to 1,800 s^{-1} in this experiment. The pools of (reduced) cytochrome and (oxidized) quinone were large enough to allow several turnovers of the system. Slight deviation from linearity (indicating exhaustion of any of the pools) can be observed only after more than 10 turnovers. The slopes of the curves (*i.e.* the rate constants of cytochrome photooxidation) depend clearly on light intensity. It can be shown that the rate of cytochrome turnover is linearly proportional to the light intensity (Osváth and Maróti 1997). This result implies that, at relatively low light intensities, the turnover rate of the RC is limited by the light intensity of the excitation.

The linear proportionality between the light intensity and the rate of the turnover will be lost if much higher light intensity is applied. Using photochemical rate constant (light intensity) as high as $k_I = 4{,}800\ s^{-1}$, the rate of cytochrome photooxidation will not follow this value but significantly lower rates are observed (Fig. 3). Clearly, the light intensity will not be the rate limiting factor any more. To find the bottleneck of the observed turnover, the steady-state cytochrome photooxidation was measured as a function of ionic strength of the solution. Contrary to the expectations, the rate was retarded at low salt concentration and accelerated as the salt concentration increased. The highest turnover rate was measured at 30 mM ionic strength. At even higher salt concentrations, the rate declined sharply. These observations support the donor side limitation of the turnover rate. 1) Although the excitation light intensity was kept constant, the cytochrome turnover rates were smaller than that of the photochemistry and showed complex dependence on the ionic strength of the solution. 2) The set of observed kinetics is in good agreement with the electrostatic nature of association and dissociation of RC and cytochrome (Gerencsér et al. 1999). 3) The use of different types of quinones (UQ_6 and UQ_{10}) in high excess did not influence the measured turnover rate.

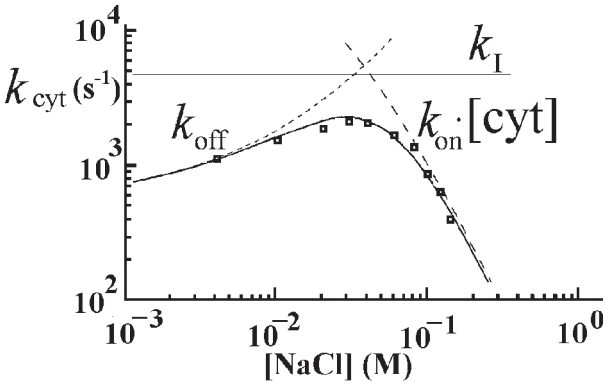

Figure 3. Steady-state rate constant of cytochrome photooxidation as a function of NaCl concentration of the solution. The photochemical rate constant (k_I, thin line) and the ionic strength-dependence of the binding (k_{on}◊[cyt]) and unbinding (k_{off}) rates (dotted lines) are also presented (see for more details of the simulation in Gerencsér et al. 1999). Conditions were the same as in Figure 2.

Conditions for acceptor side limitation of the turnover can easily be adjusted and demonstrated by decreasing the rates of quinone-related electron and proton transfers. These can be achieved by mutating one or more key amino acids in the vicinity of the quinones (Maróti et al. 1995; Okamura and Feher 1995; Sebban et al. 1995; Tandori et al. 1999). Here we present a complementary approach to site-directed mutagenesis as similar decrease in turnover rate can be established in wild type RC by increase of pH to the alkaline range and by inhibition of proton transfer by binding of heavy metal ions (Fig. 4). Both the increase of pH and the presence of Cd^{2+} ions decrease the turnover rate dramatically. The very recent interquinone ET measurements using flash excitation yielded similar results for Zn^{2+} ions (Utschig et al. 1998) as well as for Cd^{2+} ions (Paddock et al. 1999).

First ET rate $k_{AB}^{(1)}$

The rate of transfer for the first electron to Q_B was measured at 397 nm after single flash excitation (see (A1)). The observed kinetics was complex *i.e.* it could not be described with a single exponential phase. The rate of the dominating fast component was plotted in function of the pH (Fig. 4). It did not show much pH dependence below pH 8, but decreased with increasing pH above pH 8 with a slope proportional to H^+ concentration. In contrary to recent publications

(Utschig et al. 1998; Paddock et al. 1999), we did not observe significant effect of heavy metal ion (Cd^{2+}) on the kinetics of the first electron transfer even if we used Cd^{2+} in great excess over RC ([Cd^{2+}]/[RC] ª 100). No deceleration of the first interquinone transfer was noticed. The disagreement is not clear presently but cannot be attributed to different monitoring wavelengths used in these studies (397 nm (here) and 750 nm (in referred works)).

Second ET rate $k_{AB}^{(2)}$

The rate of transfer for the second electron to Q_B was determined by monitoring the decay of the semiquinone absorption at 450 nm after a second saturating flash in the presence of cytochrome. As the disappearance of semiquinones includes reactions (A2) and (A3), the observed rate will reflect the slowest step in proton coupled electron transfer. In wild type RC, the uptake of the first proton is very fast, thus the observed rate will reflect that of the subsequent ET (Graige et al. 1996). In the presence of Cd^{2+}, however, the rate of the proton transfer becomes so low that the proton transfer will be the rate limiting process in the interquinone second ET (Paddock et al. 1999). The pH profile of $k_{AB}^{(2)}$ differs from that of $k_{AB}^{(1)}$ (Fig. 4). It decreases monotonously in the whole pH range and can be approximated with exponential functions with fractional exponents. The drop occurs in two steps: $k_{AB}^{(2)}$ is proportional to $[H^+]^{0.3}$ in the acidic and neutral pH range and to $[H^+]^{0.7}$ in the alkaline pH region.

Discussion

Experimental evidences were given in the previous section providing that the rate of turnover of the RC was determined by several factors including light intensity of excitation (see reaction (L1)), and rates of electron transfer reactions at the donor (see (D1)) and acceptor (see (A1)-(A4)) sides of the protein. We were able to find conditions where their overlapping effects could be separated and investigated in more detail. The discussion will focus on major limitations in kinetics of turnover as light intensity, cytochrome exchange and proton-assisted second electron transfer with special emphasis on inhibitory effects of heavy metal cations.

Light limitation

The arrangement of the cofactors in the protein matrix and the individual electron and proton transfer steps are well suited for fast turnover of the RC: the machinery works fine even at a steady-state turnover rate as high as 1,000 s^{-1} under physiological conditions. Consequently, if the light intensity is not high enough to drive the primary photochemistry with higher rate, then the turnover will be limited by the light intensity itself. We found that this threshold intensity was about 1 $Wxcm^{-2}$ using a laser diode emitting at 796 nm. This value can be expressed in more convenient units used mainly in plant physiology: $6.7x10^4$ $mExs^{-1}xm^{-2}$, which is very high as compared to the normal physiological conditions. However, we must not forget about the light harvesting mechanism of intact photosynthetic organisms. Practically all the light energy captured by the light harvesting pigments is funneled to the RC. Due to the antenna system, the absorption cross section area and, consequently, the rate of excitation are at least two orders of magnitudes higher than in RC without antenna. As our experiments were carried out in isolated RCs without additional harvesting pigments, a limiting value of about 700 $mExs^{-1}xm^{-2}$ may be deduced for native organisms. This value may already have practical significance as it falls in the range of light intensities where photoinhibition occurs in plants. The light limitation of the turnover serves as natural protection against harmful effects due to extremely intense excitation.

Donor side limitation

The ionic strength dependence of the turnover rate demonstrated the limitation due to donor side reactions (Fig. 3). The observed steady state turnover rate of the cytochrome was clearly not light intensity limited as its values (<2,300 s^{-1}) were everywhere below the line representing the photochemical rate constant of 4,800 s^{-1}. In good accordance with recent findings (Larson et al. 1998; Gerencsér et al. 1999), the turnover rate increased with increasing bulk ionic strength in the range of 0-30 mM NaCl and then decreased at high ionic strength. These observations can be explained by the electrostatic interaction between cytochrome c and RC including pH-dependent attraction/repulsion and ionic strength dependent screening of the interaction. At neutral and acidic pH and at low or moderate ionic strength, the turnover rate of the RC was limited primarily by the low release rate of the photooxidized cytochrome c (product inhibition). At high ionic strength, however, the binding rate of the reduced cytochrome c decreased dramatically and became the bottleneck.

Using high light intensity for excitation at neutral pH, the rate of continuous turnover of the RC is not proportional any more to the light intensity but exhibits saturation due to slow dissociation of cyt c^{3+} from the RC. It is important to stress, however, that this upper limiting rate of turnover (P^+ reduction, ~2,000 s^{-1}) does not limit the photosynthetic growth of the bacterial cells. This conclusion may be supported by light intensity independent doubling times at illuminations comparable to that used in our experiments with isolated RCs. This means that not the rate of P^+ reduction but the rate of generation of transmembrane proton electrochemical potential (~200 s^{-1}) is likely to be the growth-limiting process in native bacterial cultures.

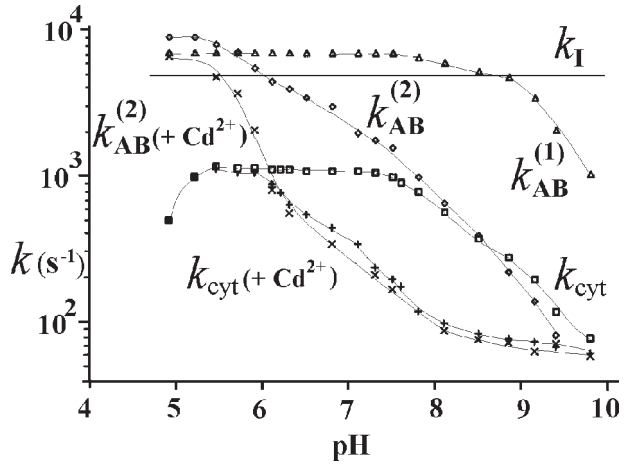

Figure 4. PH-dependence of rates of the first electron transfer ($k_{AB}^{(1)}$, Δ), second electron transfer ($k_{AB}^{(2)}$, with (◊) and without ( ) 100 mM Cd^{2+} ion) and cytochrome photooxidation (k_{cyt}, with (+) and without (~) 100 mM Cd^{2+} ion) together with pH-independent photochemical rate constant (k_I, thin horizontal line). Conditions were the same as in Figure 2.

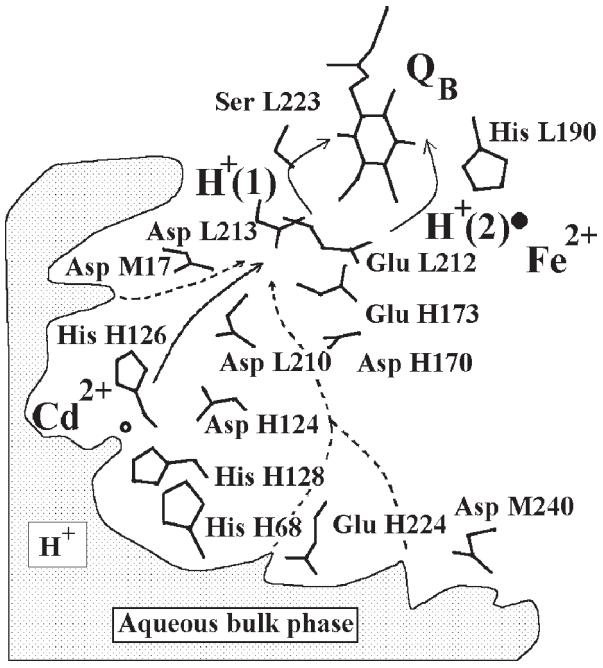

Figure 5. Proton delivery pathways from aqueous bulk phase to Q_B and nearby key acidic residues derived from crystallographic structure of the RC in light-adapted state (Stowell et al. 1997). The first proton (H^+(1), see also (A2)) is directed to the distal (relative to the non-heme iron) carbonyl oxygen of Q_B located near Ser L223 and the second proton (H^+(2), see also (A3)) binds to the proximal carbonyl oxygen atom of Q_B located near His L190. The Cd^{2+} ion replaces a water molecule (W72) in the gate of the dominant pathway (solid line) and blocks the entrance of protons into the RC. Alternative proton channels (dashed lines, see Abresch et al. 1998) can deliver protons to Q_B at significantly lower rates.

Acceptor side limitation

This kind of limitation can be observed if the rates of quinone-related reactions become comparable to those of primary photochemistry (light intensity) and cytochrome exchange. Conditions like alkaline pH and stoichiometric binding of heavy metal ions to the RC set the stage for dominance of the quinone side in limiting the turnover rate of the RC. Although realization of these conditions does not have any practical importance for cultivation of bacteria, it can provide a significant insight to the structure/function relationship of a light energy converting protein on molecular level (Fig. 5).

The protein matrix of the RC maintains negative potential in the vicinity of the secondary quinone in order to pull protons from the aqueous bulk phase to Q_B. The negative electrostatic profile is produced primarily by a large cluster of carboxylic acidic residues located 10 Å from Q_B (Stowell et al. 1997; Abresch et al. 1998). This cluster is supposed to act as a local proton reservoir. This theory of the partial proton sponge may have widespread physiological importance if the local proton accumulation is shared by other similar energy converting complexes in the membrane. The connectivity would demonstrate a high adaptability of these membrane proteins, probably reflecting a consequence of evolution. However, the negative electrostatic potential is not sufficient to ensure fast proton delivery inside the protein. A good connectivity of an extended H-bond network composed by water molecules in the cytoplasmic side of the RC, and the side chains of polar and charged residues are also necessary for fast uptake and transport of protons.

Studies on revertant protonation mutants showed that the proton pathways to Q_B were much less specific than expected (Maróti et al. 1995; Okamura and Feher 1995; Miksovska et al. 1999). Protons can penetrate into the protein by different entries and are delivered by alternative routes which can easily be bypassed (Fig. 5). In contrary to these results, the electron and proton transfer kinetics of RC modified by stoichiometric binding of bivalent heavy metal cations (e.g. Cd^{2+}) adjacent to one of the proton delivery pathways has led to the conclusion that there is one dominant site of proton entry into the RC from which proton transfer to Q_B occurs (Paddock et al. 1999). Our studies showing dramatic decrease of turnover rate upon metal ion binding confirm this hypothesis. The Cd^{2+} ion is ligated to His H126, His H128 and Asp H124 and replaces a crucial water molecule (W72) that functions as a gate between the aqueous water phase and the protein interior. The replacement causes dramatic changes in the electrostatics of the entry (becomes more positive) and in the polarizability of internal water molecules and polar residues along the delivery path of the protein. The effect of cation binding on the turnover rate of the RC reveals the central role of the organization of the H-bond network in proton delivery. Water molecules are essential part of the H-

bond network structure as they are more mobile than side chains, and therefore may ensure good connectivity and high cooperativity for efficient proton conduction. The motions and rearrangements of water molecules may be functionally coupled to the electron transfer reaction within the protein (Alexov and Gunner 1999). This might be of general interest in proteins involved in redox reactions.

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References

Abresch EC, Paddock ML, Stowell MHB, McPhillips TM, Axelrod HL, Soltis SM, Rees DC, Okamura MY, Feher G (1998) Identification of proton transfer pathways in the X-ray crystal structure of the bacterial reaction center from *Rhodobacter sphaeroides*. Photosynth Res 55:119-125.

Alexov EG, Gunner MR (1999) Calculated Protein and Proton Motions Coupled to Electron Transfer: Electron Transfer from Q_A^- to Q_B in Bacterial Photosynthetic Reaction Centers. Biochemistry (in press).

Allen JP, Feher G, Yeates TO, Rees DC, Deisenhofer J, Michel H, Huber R (1986) Structural homology of reaction centers of *Rb. sphaeroides* and *R. viridis* as determined by X-ray diffraction. Proc Natl Acad Sci USA 83:8589-8593.

Chang C-H, Tiede D, Tang J, Smith U, Norris J, Schiffer M (1986) Structure of *Rhodopseudomonas sphaeroides* R-26 reaction center. FEBS Letters 205:82-86.

Ermler U, Fritzsch G, Buchanan SK, Michel H (1994) Structure of the photosynthetic reaction center from *Rhodobacter sphaeroides* at 2.65 Å resolution: Cofactors and protein-cofactor interactions. Structure 2:925-936.

Gerencsér L, Laczkó G, Maróti P (1999) Unbinding of Oxidized Cytochrome c from Photosynthetic Reaction Center of *Rhodobacter sphaeroides* Is the Bottleneck of Fast Turnover. Biochem 38:16866-16875.

Gopta OA, Cherepanov DA, Junge W, Mulkidjanian AY (1999) Proton transfer from the bulk to the bound ubiquinone Q_B of the reaction center in chromatophores of *Rhodobacter sphaeroides*. Retarded conveyance by neutral water. Proc Natl Acad Sci USA 96:13159-13164.

Graige MS, Paddock ML, Bruce JM, Feher G, Okamura MY (1996) Mechanism of Proton-Coupled Electron Transfer for Quinone (Q_B) Reduction in Reaction Centers of *Rb. sphaeroides*. J Am Chem Soc 118:9005-9016.

Kleinfeld D, Okamura MY, Feher G (1984) Electron transfer in reaction centers of *Rhodopseudomonas sphaeroides*. Biochim Biophys Acta 785:126-140.

Larson JW, Wells TA, Wraight CA (1998) Product inhibition of the bacterial photosynthetic reaction center from *Rhodobacter sphaeroides* by steady-state continuous cytochrome oxidation. Biophys J 74(2):A76.

Maróti P, Wraight CA (1988) Flash-induced H^+ binding by bacterial photosynthetic reaction centers: Comparison of spectrophotometric and conductimetric methods. Biochim Biophys Acta 934:314-328.

Maróti P (1993) Flash-induced proton transfer in photosynthetic bacteria (minireview). Photosynth Res 37:1-17.

Maróti P, Hanson DK, Schiffer M, Sebban P (1995) Long-range electrostatic interaction in the bacterial photosynthetic reaction centre. Nature Struc Biol 2:1057-1059.

Maróti P, Wraight CA (1997) Kinetics of H^+-ion binding by the $P^+Q_A^-$ state of the bacterial photosynthetic reaction centers: Rate limitation within the protein. Biophys J 73:367-381.

Miksovska J, Schiffer M, Hanson DK, Sebban P (1999) Proton uptake by bacterial reaction centers: The protein complex responds in a similar manner to the reduction of either quinone acceptor. Proc Natl Acad Sci USA 96:14348-14353.

Moser CC, Dutton PL (1988) Cytochrome c and c_2 Binding Dynamics and Electron Transfer with Photosynthetic Reaction Center Protein and Other Integral membrane Redox Proteins. Biochemistry 27:2450-2461.

Okamura MY, Feher G (1995) Proton-Coupled Electron Transfer Reactions of Q_B in Reaction Centers from Photosynthetic Bacteria. In Blankenship RE, Madigan MT, Bauer CE, eds., Anoxygenic Photosynthetic Bacteria. Dordrecht, Kluwer Academic Publishers, 577-594.

Osváth Sz, Maróti P (1997) Coupling of Cytochrome and Quinone Turnovers in the Photocycle of Reaction Centers from the Photosynthetic Bacterium *Rhodobacter sphaeroides*. Biophys J 73:972-982.

Overfield RE, Wraight CA (1986) Photooxidation of mitochondrial cytochrome c by isolated bacterial reaction centers: Evidence for tight-binding and diffusional pathways. Photosynth Res 9:167-179.

Paddock ML, Graige MS, Feher G, Okamura MY (1999) Identification of the proton pathway in bacterial reaction centers: Inhibition of proton transfer by binding of Zn^{2+} or Cd^{2+}. Proc Natl Acad Sci USA 96:6183-6188.

Sebban P, Maróti P, Hanson DK (1995) Electron and proton transfer to the quinones in bacterial photosynthetic reaction centers: Insight from combined approaches of molecular genetics and biophysics. Biochimie 77:677-694.

Shinkarev VP, Wraight CA (1993) Electron and Proton Transfer in the Acceptor Quinone Complex of Reaction Centers of Photosynthetic Bacteria. In Deisenhofer J, Norris J, eds., The Photosynthetic Reaction Center. Vol I. Academic Press, New York, 193-255.

Stein RR, Castellvi AL, Bogacz JP, Wraight CA (1984) Herbicide-quinone competition in the acceptor complex of photosynthetic reaction centers from *Rhodopseudomonas sphaeroides*. A bacterial model for PSII-herbicide activity in plants. J Cell Biochem 24:243-259.

Stowell MHB, McPhillips TM, Rees DC, Soltis SM, Abresch E, Feher G (1997) Light-Induced Structural Changes in Photosynthetic Reaction Center: Implications for Mechanism of Electron-Proton transfer. Science 276:812-816.

Tandori J, Sebban P, Michel H, Baciou L (1999) In *Rhodobacter sphaeroides* Reaction centers, Mutation of Prolin L209 to Aromatic Residues in the Vicinity of a Water Channel Alters the Dynamic Coupling between Electron and Proton Transfer Processes. Biochemistry 38:13179-13187.

Tiede DM, Vashishta AC, Gunner MR (1993) Electron-Transfer Kinetics and Electrostatic Properties of the *Rhodobacter sphaeroides* Reaction Center and Soluble c-Cytochromes. Biochemistry 32:4515-4531.

Utschig LM, Ohigashi Y, Thurnauer MC, Tiede DM (1998) A New Metal-Binding Site in Photosynthetic Bacterial Reaction Centers That Modulates Q_A to Q_B Electron Transfer. Biochemistry 37:8278-8281.

Van Gelder BF, Slater EC (1962) The extinction coefficient of cytochrome c. Biochim Biophys Acta 58:593-595.

Wraight CA. (1998) Functional linkage between the Q_A and Q_B sites of photosynthetic reaction centers. In Garab Gy, ed., Photosynthesis: Mechanisms and Effects. Vol. II. Kluwer Academic Publishers, Dordrecht, 693-698.