Role of the PufX Protein in Photosynthetic Growth of *Rhodobacter sphaeroides*. 2. PufX Is Required for Efficient Ubiquinone/Ubiquinol Exchange between the Reaction Center Q_B Site and the Cytochrome bc_1 Complex†

Wolfgang P. Barz,‡,§ André Verméglio,|| Francesco Francia,⊥ Giovanni Venturoli,⊥ B. Andrea Melandri,⊥ and Dieter Oesterhelt*,‡

Max-Planck-Institute for Biochemistry, Department of Membrane Biochemistry, 82152 Martinsried, Germany, CEA Centre d'Etudes de Cadarache, Département de Physiologie Végétale et Ecosystemes, Section de BioEnergétique Cellulaire, 13108 Saint Paul lez Durance, France, and University of Bologna, Department of Biology, Laboratory of Biochemistry and Biophysics, Via Irnerio 42, 40126 Bologna, Italy

Received April 6, 1995; Revised Manuscript Received September 19, 1995⊗

ABSTRACT: The PufX membrane protein is essential for photosynthetic growth of *Rhodobacter sphaeroides* because it is required for multiple-turnover electron transfer under anaerobic conditions [see accompanying article; Barz, W. P., Francia, F., Venturoli, G., Melandri, B. A., Verméglio, A., & Oesterhelt, D. (1995) *Biochemistry 34*, 15235−15247]. In order to understand the molecular role of PufX, light-induced absorption spectroscopy was performed using a *pufX*$^-$ mutant, a *pufX*$^+$ strain, and two suppressor mutants. We show that the reaction center (RC) requires PufX for its functionality under different redox conditions than the cytochrome bc_1 complex: When the kinetics of flash-induced reduction of cytochrome b_{561} were monitored in chromatophores, we observed a requirement of PufX for turnover of the cytochrome bc_1 complex only at high redox potential (E_h > 140 mV), suggesting a function of PufX in lateral ubiquinol transfer from the RC. In contrast, PufX is required for multiple turnover of the RC only under reducing conditions: When the Q pool was partially oxidized *in vivo* using oxygen or electron acceptors like dimethyl sulfoxide or trimethylamine *N*-oxide, the deletion of PufX had no effect on light-driven electron flow through the RC. Flash train experiments under anaerobic *in vivo* conditions revealed that RC photochemistry does not depend on PufX for the first two flash excitations. Following the third and subsequent flashes, however, efficient charge separation requires PufX, indicating an important role of PufX for fast Q/QH_2 exchange at the Q_B site of the RC. We show that the Q/QH_2 exchange rate is reduced approximately 500-fold by the deletion of PufX when the Q pool is nearly completely reduced, demonstrating an essential role of PufX for the access of ubiquinone to the Q_B site. The fast ubiquinone/ubiquinol exchange is partially restored by suppressor mutations altering the macromolecular antenna structure. These results suggest an indirect role of PufX in structurally organizing a functional photosynthetic apparatus.

The purple non-sulfur bacterium *Rhodobacter sphaeroides* is able to grow photosynthetially by using a light-driven, cyclic electron transport pathway. Two light-harvesting complexes, LH1 and LH2 [reviewed in Zuber & Brunisholz (1991)] capture and transfer energy to the reaction center (RC)[1] where photochemistry takes place. The RC is surrounded by a ring of LH1 complexes (Monger & Parson, 1977), while the LH2 antennae build a more peripheral light-harvesting system. Light-induced charge separation across the membrane involves the transfer of an electron from a bacteriochlorophyll dimer (the primary donor) to the initial ubiquinone acceptor Q_A and finally to the secondary ubiquinone acceptor Q_B. After rereduction of the primary donor by cytochrome (cyt) c_2, a second turnover of the RC leads to reduction of the semiquinone molecule at the Q_B site. Following binding of two protons, the resulting ubiquinol (QH_2) rapidly exchanges with a pool of ubiquinone (Q) molecules in the hydrophobic part of the membrane (McPherson et al., 1990; Paddock et al., 1990). This so-called Q pool consists of about 25−30 molecules per RC (Takamiya & Dutton, 1979). Another intrinsic membrane complex, the cyt bc_1 complex, oxidizes QH_2 molecules and transfers electrons to cyt c_2 to complete the light-driven electron cycle. Redox reactions are coupled with vectorial translocation of protons across the membrane, forming a proton electrochemical gradient that is used for ATP synthesis (Crofts & Wraight, 1983; Robertson et al., 1990).

The *pufX* gene encodes a 9 kDa membrane protein which is located in the intracytoplasmic membrane and is associated with the RC−LH1 core complex (Farchaus et al., 1992). Although previous studies have established the essentiality of PufX for anaerobic, photosynthetic growth in both *R.*

† This work was supported by the Deutsche Forschungsgemeinschaft Sonderforschungsbereich 143. W.P.B. had a doctoral fellowship by the Boehringer Ingelheim Fonds. F.F., G.V., and B.A.M. acknowledge the support of Consiglio Nazionale delle Ricerche and Ministero dell'Universita e della Ricerca Scientifica e Tecnologica.

* Author to whom correspondence should be addressed.
‡ Max-Planck-Institute for Biochemistry.
§ Present address: Department of Biochemistry and Biophysics, University of California Medical School, San Francisco, CA 94143-0448.
|| CEA Centre d'Etudes de Cadarache.
⊥ University of Bologna.

⊗ Abstract published in *Advance ACS Abstracts,* November 1, 1995.

[1] Abbreviations: BQ, benzoquinone; cyt, cytochrome; DMSO, dimethyl sulfoxide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; P, primary electron donor; PIPES, 1,4-piperazinediethanesulfonic acid; Q, ubiquinone; QH_2, ubiquinol; RC, reaction center; TMAO, trimethylamine *N*-oxide; WT, wild type.

0006-2960/95/0434-15248$09.00/0

sphaeroides (Farchaus et al., 1992) and *R. capsulatus* (Lilburn et al., 1992), the precise role of PufX is not known. Spectroscopic studies with trains of flashes demonstrated an impairment of cyclic electron flow in the absence of PufX, despite normal primary charge separation in the RC (Farchaus et al., 1992; Lilburn et al., 1992).

A recent study demonstrated that spontaneous point mutations in *pufB* or *pufA* suppress the requirement of PufX in *R. sphaeroides* by rearranging the macromolecular antenna structure around the RC (Barz & Oesterhelt, 1994). Another work has shown that PufX is not required for photosynthetic growth in a mutant strain lacking all antenna complexes (McGlynn et al., 1994). These observations have led to proposals that PufX facilitates, either directly or indirectly, the flow of QH_2 between the Q_B site of the RC and the Q_o site of the cyt bc_1 complex.

In the accompanying article, we provide strong evidence that PufX plays a key role in the multiple-turnover cyclic electron flow, although it has no apparent role following single-flash excitation. Although these *in vivo* data clearly show the essentiality of PufX for an effective photosynthetic electron flow, these results did not allow us to precisely localize the role of PufX. In order to understand the molecular function of PufX, the redox dependence of the PufX function was analyzed in the work reported here. The green strains PUFC/g, PUFΔX/g, Sup101/g, and Sup102/g (Barz et al., 1995) were used to reveal which step of energy transduction is impaired in the absence of PufX and can be restored by suitable point mutations in the LH1 genes. We show that, under anaerobic conditions, PufX is involved in the efficient interaction between the RC Q_B site and the Q pool.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. The *R. sphaeroides* strains used in this study and their growth conditions are described in the preceding article (Barz et al., 1995).

Membrane Isolation and Analysis. Chromatophores were isolated from semiaerobically grown cells that were harvested at a defined cell density of 100 ± 5 Klett units. A French press cell was used to prepare chromatophores as described previously (Bowyer et al., 1979). Concentrations of bacteriochlorophyll a were determined as described by Clayton (Clayton, 1973).

Determination of Reaction Center Turnover Rates in Vitro. RC−LH1 membrane complexes were isolated from semiaerobically grown cells with *n*-octyl-β-glucopyranoside as described previously (Gray et al., 1990). Cyt *c* turnover assays were performed as described before (Okamura et al., 1982; Paddock et al., 1988). RC−LH1 complexes were used in a final RC concentration of 30 nM (determined spectroscopically using $\epsilon_{802} = 288\ mM^{-1}\ cm^{-1}$; Straley et al., 1973). Horse heart cyt *c* (Type VI, Sigma Chemical Co., Munich, FRG) was prereduced by incubation with an excess of sodium ascorbate until no additional increase at 550 nm was observed. The ascorbate was removed by gel filtration chromatography over Sephadex G-10 (Pharmacia, Piscataway, NJ). The intensity of the actinic light (defined by a Schott OG590 filter) was 500 W/m^2. This intensity was not saturating as determined by using neutral density filters.

Measurement of Light-Induced Absorption Changes in Vitro. Flash-induced absorption changes in chromatophores were determined as described in detail in the preceding article (Barz et al., 1995).

Flash-induced formation of semiquinone (Q_B^-) at the Q_B site of the RC (Verméglio, 1977; Wraight, 1977) was measured at 446 nm, close to the peak in the Q_B^-/Q_B difference spectrum (Bowyer et al., 1979). The concentration of chromatophores was adjusted so that a single flash induced turnover in more than 90% of the RC (as determined with a series of 10 flashes). In addition to the use of redox mediators described in the preceding article, the sample was supplemented with 10 μM antimycin A and 60 μM *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. At a redox poise of 280 mV, this mediator has been proven to act as an efficient reductant of the flash-oxidized primary donor (P^+) and as a slow oxidant of the reduced secondary acceptor (Q_B^-), allowing a complete dark adaptation of the acceptor quinone species over the time scale of minutes (Mulkidyanian et al., 1986). Before flash excitation, the samples were dark adapted for 20 min to allow complete equilibration of the RC with the redox mediators.

The kinetics of flash-induced cyt b_{561} reduction through the Q_o site of the cyt bc_1 complex were measured in a nitrogen atmosphere under controlled redox conditions as described previously (Venturoli et al., 1986). Chromatophores were suspended in 100 mM KCl, 50 mM MOPS (pH 7.0), and supplemented with redox mediators as described in the preceding article. In addition, 10 μM valinomycin, 7 μM nigericin, and 10 μM antimycin A were added in this assay.

Measurements of cyt b_{561} reduction through the Q_i site of the cyt bc_1 complex were performed in 100 mM KCl, 50 mM CHES (pH 9.3) or in 100 mM KCl, 50 mM CAPS (pH 10.4). The same redox mediators (see preceding article) were added except that the final concentrations of phenazine methosulfate, phenazine ethosulfate, pyocyanin, and 2,3,5,6-tetramethyl-*p*-phenylenediamine were decreased to 0.2 μM. In addition, 10 μM valinomycin, 7 μM nigericin, and 3.5 μM myxothiazol were added in this assay. The redox potential was poised at values (E_h = 160−220 mV) which maximize the extent of cyt b_{561} reduction through the Q_i site (Glaser et al., 1984).

Numerical Analysis of Cytochrome b_{561} Reduction Kinetics in Vitro. Kinetic traces of cyt b_{561} reduction were analyzed in terms of pseudo-first-order kinetics following an initial lag period. The signals were fitted to an exponential function using a nonlinear least-squares minimization routine based on a modified Marquardt algorithm (Bevington, 1969). In order to determine the best fitting kinetic parameters, the lag time before the onset of first-order kinetics was varied stepwise: for each lag period, the parameters of the exponential function were optimized as described (Bevington, 1969) and a plot of χ^2 vs the lag period was constructed for each curve. For all traces, this procedure yielded a minimum reduced χ^2 between 0.8 and 1.2. The uncertainty in the lag period, estimated from the χ^2 vs lag curve, is presented in Figure 3B as the time interval correspondent to 1% increase in χ^2 around the best fitting value.

Light-Induced Difference Spectroscopy in Vivo. For *in vivo* spectroscopy, we used cell cultures that were grown semiaerobically for 20 h. Cells were harvested by centrifugation (6 000*g*, 5 min), resuspended in fresh medium, and transferred to the thermostated (30 °C) measuring cuvette. Prior to the anaerobic measurements, cell suspensions were

incubated anaerobically in the cuvette for at least 30 min. In order to study oxygen-saturated cells, air was pumped through the dark-incubated cell suspension immediately before transferring the cells into the measuring cuvette. For treatment with *p*-benzoquinone (BQ), cells were incubated for 10 min at room temperature with 200 μM BQ. The treated cells were centrifuged (6 000*g*, 5 min) to remove excess BQ and resuspended in fresh medium containing 1 mM KCN and 20 μM myxothiazol.

The flash-detection measurements were carried out using a spectrophotometer similar to that described previously (Joliot et al., 1980; Joliot & Joliot, 1984). Continuous light excitation was performed using a 24 V quartz lamp. For flash excitation, a saturating xenon actinic flash (2 μs duration at half-maximal intensity) was provided by a FX-199 flash tube (EG & G, Electro Optics, Salem, MA). The actinic light was defined by a Wratten 89B filter (Kodak) and the photodetectors were protected from the actinic light by a Corning CS 4-97 filter. The light intensity was determined to be saturating by using neutral density filters. Light-induced absorption changes were detected over a wide time range (20 μs to 60 s) using a train of nonactinic detection flashes. The dark adaptation time between two measurements was at least 60 s.

RESULTS

In the accompanying article, we demonstrate that PufX is essential for multiple-turnover cyclic electron transfer under anaerobic conditions. Although, after single-flash excitation, PufX is not required for electron flow through the RC or cyt bc_1 complex, PufX is essential for the generation of a photosynthetic membrane potential during prolonged illumination (Barz et al., 1995). In order to understand the molecular role of PufX in photosynthetic growth, we studied the functionality of the RC and the cyt bc_1 complex under different redox conditions.

Binary Oscillation of the Reaction Center Semiquinone in Vitro. The two-electron gate at the secondary ubiquinone acceptor, Q_B, was characterized in PUFC/g (containing *pufX*) and PUFΔX/g (lacking *pufX*). In chromatophores supplemented with electron donors capable of rapidly reducing the photooxidized primary donor, stable semiquinone anions Q_B^- are formed on odd-numbered flashes and disappear on even-numbered flashes. The binary oscillations of Q_B^- can be observed as flash-induced absorption changes at 446 nm (Verméglio, 1977; Wraight, 1977; Bowyer et al., 1979). The disappearance of Q_B^- is attributed to the formation of ubiquinol, Q_BH_2, which exchanges with a ubiquinone molecule in the membrane Q pool.

Figure 1 shows the absorption changes at 446 nm in chromatophores of PUFC/g and PUFΔX/g induced by a series of four saturating flashes. In addition to stable semiquinone signals, transient absorption changes in the millisecond time range were observed that were caused by the photooxidation and rapid rereduction of P870. As shown in Figure 1, binary oscillation of the formation and disappearance of the Q_B^- anion could be observed for both strains. Using an extinction coefficient of $\epsilon_{450} = 8.5$ mM^{-1} cm^{-1} (Wraight et al., 1975), it was calculated that approximately one Q_B^-/RC was formed upon the first flash. These results demonstrate that under the conditions of this experiment the semiquinone molecules formed upon the first and third flash

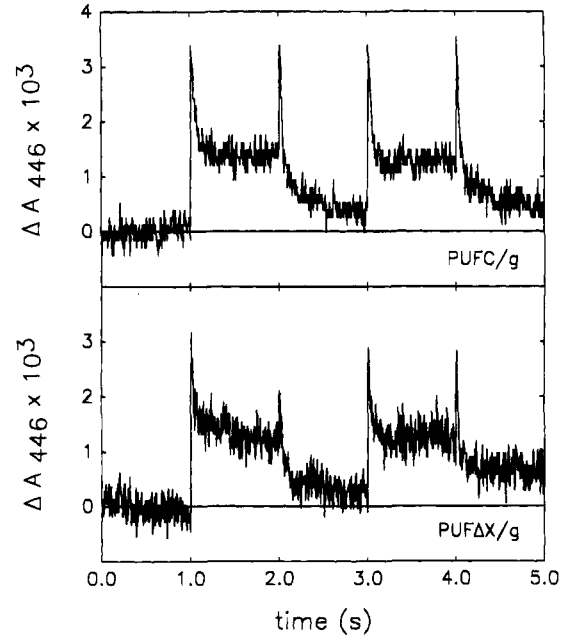

FIGURE 1: Flash-induced binary oscillations in semiquinone formation, measured at 446 nm with chromatophores isolated from PUFC/g (A) and PUFΔX/g (B). Chromatophores were suspended in an anaerobic redox cuvette to a final RC concentration of 0.17 μM. The samples were supplemented with redox mediators (see Materials and Methods), redox poised to E_h = 280 mV and dark adapted for 20 min before flash excitation. Actinic illumination was provided by four flashes spaced 1 s apart, as indicated by the arrows. The instrument response time was 5 ms.

were stable in both strains on a second time scale and that the two electron gate operates normally even in the absence of PufX.

Reaction Center Photochemistry in Vitro during Continuous Illumination. The efficiency of RC turnover was studied under continuous illumination. To this aim, the light-induced oxidation of mammalian cyt *c* by solubilized RC−LH1 complexes was measured in the presence of Q_0 (ubiquinone lacking the isoprenoid chain). Using such an assay, the rate-limiting step of RC turnover can be studied *in vitro* (Paddock et al., 1988). Instead of isolated RCs, solubilized RC−LH1 complexes were used since PufX is known to be associated with RC−LH1 complexes, but not with isolated RCs (Farchaus et al., 1992). Using β-octyl glucoside, RC−LH1 complexes were solubilized from PUFC/g, PUFΔX/g, and Sup101/g membranes. The resulting protein complexes gave very similar absorption spectra and protein gels (data not shown) showing that PufX has no important structural role in binding the LH1 antennae to the RC.

The turnover rates of the solubilized RC−LH1 complexes were constant during a 1 s period of illumination and were similar in the presence or absence of PufX. The traces (not shown) revealed the following turnover numbers: 76 cyt c RC^{-1} s^{-1} for PUFC/g, 85 cyt *c* RC^{-1} s^{-1} for PUFΔX/g, and 95 cyt *c* RC^{-1} s^{-1} for Sup101/g. These values differed from the turnover number reported earlier (270 cyt *c* RC^{-1} s^{-1}; Paddock et al., 1988) because light excitation was not saturating in this experiment. The similarity of the cyt *c* oxidation rates demonstrates that the rate-limiting step in the turnover of detergent-solubilized RC−LHI complexes does not require PufX when Q_0 is used as the electron acceptor.

Kinetics of cyt b_{561} Reduction Induced by a Single-Turnover Flash in Vitro. In order to study flash-induced

redox interactions between the RC and the cyt bc_1 complex, the kinetics of cyt b_{561} reduction were monitored in chromatophores following single-flash illumination. In chromatophores inhibited with antimycin A, electron transfer through the quinol-oxidizing site (Q_o site) of the cyt bc_1 complex can be determined by measuring the kinetics of flash-induced cyt b_{561} reduction (Crofts et al., 1983). In addition, the redox potential can be easily controlled in chromatophores for studying the dependence of this reaction on the redox poise of the Q pool.

In WT, the reduction of cyt b_{561} presents a lag period prior to the onset of the reaction at maximal rate (Crofts et al., 1983). The initial rate of reduction after the lag has been shown to increase progressively up to 10-fold upon decreasing the redox potential from 250 to 100 mV (Crofts et al., 1983). When the redox poise was lowered over this range, the duration of the lag period (approximately 1 ms at 200−250 mV) was shortened to 200 μs. This kinetic behavior has been shown to reflect the prereduction of the Q pool and consequently the increased availability of QH_2 as substrate to the Q_o site of the cyt bc_1 complex (Crofts et al., 1983; Venturoli et al., 1986). At redox potentials positive enough to keep the Q pool completely oxidized, the only reductant of cyt b_{561} is the QH_2 produced by photoexcitation of the RC. Under this condition the maximal duration of the lag (about 1 ms) was observed (Crofts et al., 1983).

The flash-induced reduction of cyt b_{561} was determined *in vitro* by measuring absorbance changes $\Delta A_{561\text{-}569}$ in chromatophores isolated from PUFC/g, PUFΔX/g, Sup101/g, and Sup102/g. Figure 2 (panels A and B) shows the kinetic traces obtained for PUFΔX/g chromatophores at two different redox potentials. Figure 3 demonstrates the redox dependence of the initial rate of cyt b_{561} reduction (Figure 3A) and of the lag duration (Figure 3B) for all four strains investigated. The data obtained for PUFC/g chromatophores (◆) exhibited the typical features observed previously for *R. sphaeroides* WT (Crofts et al., 1983; Snozzi & Crofts, 1984; Venturoli et al., 1986). However, the absence of PufX had a dramatic effect on the kinetics of cyt b_{561} reduction. In PUFΔX/g chromatophores (●) the initial rate of cyt b_{561} reduction was decreased by approximately one order of magnitude when compared to PUFC/g chromatophores (Figure 3A). At low redox poise (E_h < 180 mV), the inhibitory effect of the mutation was less evident, the ratio of the rates being between 3 and 5.

At high redox potential, the dramatic decrease in the rate of cyt b_{561} reduction observed in PUFΔX/g chromatophores was paralleled by a similarly large increase in the duration of the lag, which was close to 10 ms at E_h > 200 mV (as compared to about 1 ms in PUFC/g) (Figure 3B). At lower E_h values the lag was progressively shortened and below 130 mV reached a value close to that observed in PUFC/g chromatophores (about 300 μs). These data show that, when the only QH_2 reacting at the Q_o site was that released by the RC, the reduction of cyt b_{561} was severely impaired by the absence of PufX. This impairment appeared to be due to a reduced availability of QH_2 coming from the RC and not to a decreased activity of the Q_o site (see Discussion).

A possible explanation for the extremely slow reduction kinetics of cyt b_{561} observed in PUFΔX chromatophores could be that the absence of PufX affected the stability of semiquinone at the Q_B site of the RC. Under the conditions used to study the kinetics of cyt b_{561} reduction (E_h < 250 mV), the secondary quinone Q_B is in the semiquinone form (Q_B^-) in approximately half of the RCs before the flash (Crofts et al., 1983). The deletion of PufX might in principle affect the redox state of Q_B, leading to flash-induced release of less than 0.5 QH_2 molecules per RC and therefore drastically decreasing the initial rate of cyt b_{561} reduction at E_h > 140 mV. Although this explanation seemed unlikely in the light of the observation that, in the absence of PufX, Q_B^- was stably formed upon odd-numbered flashes (see Figure 1), the requirement of PufX for semiquinone stability was further investigated. To this aim, we analyzed the kinetics of cyt b_{561} reduction induced by two closely spaced flashes (500 μs apart) in chromatophores of PUFC/g or PUFΔX/g. The open symbols in Figure 3A show that, following double-flash excitation, the initial rate of cyt b_{561}

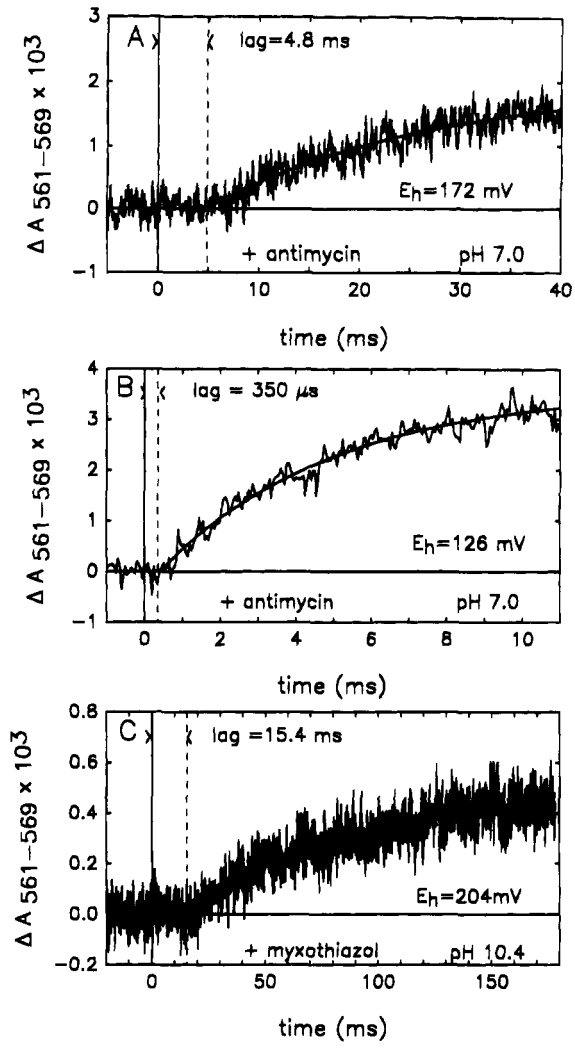

FIGURE 2: Kinetic traces of cyt b_{561} reduction induced by a single-turnover flash in PUFΔX/g chromatophores. Chromatophores were suspended to a final bacteriochlorophyll concentration of 30 μM and poised to the indicated redox potentials (see Materials and Methods). Flash-induced cyt b_{561} reduction through the Q_o site of the cyt bc_1 complex was measured at pH 7.0 in the presence of 10 μM antimycin (panels A and B); traces are the average of 25 measurements. Reduction of cyt b_{561} through the Q_i site was measured at pH 10.4 in the presence of 3.5 μM myxothiazol (panel C); the signal is the average of 32 measurements. The instrument response time was 100 μs. The continuous curves are best fits to an exponential function (see Materials and Methods). The distance between the vertical lines indicates the lag between the flash and the onset of cyt b_{561} reduction. The estimated lag periods are given in the panels.

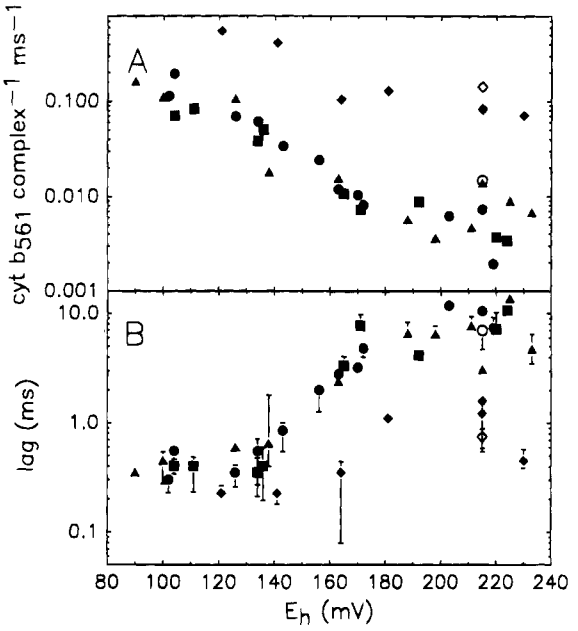

FIGURE 3: Redox titration of the kinetics of flash-induced cyt b_{561} reduction in chromatophores isolated from PUFC/g (◆), PUFΔX/g (●), Sup101/g (■), and Sup102/g (▲). The initial rate of the reaction (panel A) and the lag period (panel B) were obtained in the presence of 10 μM antimycin from traces similar to those in Figure 2. The assay conditions and the best fitting procedure are described under Materials and Methods. The initial rate of cyt b_{561} reduction was normalized to the concentration of total photoreducible cyt b_{561}. Error bars in panel B represent the time period corresponding to a 1% increase of χ^2 around the best-fitting value. For each strain, the figure includes data from two different chromatophore preparations. Open symbols represent the kinetic data obtained with two closely spaced flashes (500 μs apart) in chromatophores of PUFC/g (◇) or PUFΔX/g (○).

reduction was approximately doubled when compared to the rates observed after a single saturating flash (PUFC/g, 0.142 versus 0.083 ms^{-1}; PUFΔX/g, 0.015 versus 0.0073 ms^{-1}). The same result was obtained when the dark time between the two flashes was increased up to 2 ms (data not shown). These results show that the stability of Q_B^- was not affected by the deletion of *pufX* since two closely spaced flashes should otherwise stimulate cyt b_{561} reduction to a much larger extent (see Discussion).

Interestingly, for the suppressor mutants Sup101/g and Sup102/g the same kinetic parameters as in PUFΔX/g were obtained over the whole E_h range investigated (Figure 3). Therefore, in this single-turnover experiment, the impaired redox coupling between the RC and the cyt bc_1 complex observed in the absence of PufX was not restored by the suppressor mutations (see Discussion).

Flash-Induced cyt b_{561} Reduction through the Q_i Site of the cyt bc_1 Complex. Due to the location of the Q_o site at the periplasmic side of the membrane (Degli Esposti et al., 1993), the polar head groups of the QH_2 molecules formed at the RC Q_B site have to cross the hydrophobic membrane core ("flip-flop"). The observed requirement of PufX for the flash-induced reduction of cyt b_{561} under oxidizing conditions could be explained by involvement of PufX in facilitating the transmembrane translocation of the quinol head group. To examine this possibility, we studied electron flow between the Q_B and Q_i catalytic sites, both facing the cytoplasmic side of the membrane. In the presence of myxothiazol (an inhibitor of the Q_o site), flash-induced

Table 1: Kinetic Parameters of Flash-Induced cyt b_{561} Reduction through the Antimycin-Sensitive Site (Q_i) of the cyt bc_1 Complex in PUFC/g, PUFΔX/g, and Sup102/g Chromatophores[a]

mutant strain	pH	initial reduction rate (cyt b_{561} complex^{-1} s^{-1})	lag (ms)
PUFC/g	9.4	9.81	4.5 (3.8−5.4)
PUFΔX/g		1.34	12.1 (10.7−15.3)
Sup102/g		4.97	8.7 (7.8−9.4)
PUFC/g	10.4	6.18	5.4 (3.5−6.9)
PUFΔX/g		0.75	15.4 (12.0−17.6)
Sup102/g		1.06	12.2 (9.6−14.2)

[a] Data were obtained from traces similar to those presented in Figure 2C. The assay conditions and best fitting procedure are described under Materials and Methods. Values in parentheses represent the uncertainty in determination of the lag (see legend of Figure 3).

reduction of cyt b_{561} has been demonstrated to occur via an antimycin-sensitive route involving the Q_i site of the cyt bc_1 complex (Glaser et al., 1984; Robertson et al., 1984). Cyt b_{561} reduction via Q_i is thermodynamically unfavored at physiological pH values and could only be observed upon decreasing the size of the quinone pool or, in native chromatophores, at alkaline pH values in the range 8.5−10.5 (Glaser et al., 1984; Robertson et al., 1984).

Flash-induced cyt b_{561} reduction through the Q_i site was studied at pH 9.4 and 10.4 in chromatophores that were inhibited with myxothiazol (see Figure 2C for a typical trace). As shown in Table 1, the absence of PufX caused an almost 10-fold decrease in the initial reaction rate of cyt b_{561} at both pH's used. In parallel an almost 3-fold increase of the duration of the lag was observed in PUFΔX/g chromatophores as compared to PUFC/g membranes (Table 1). Both effects were partially reverted in Sup102/g chromatophores, the recovery being more prominent at pH 9.4 (see Table 1). Thus, the redox interaction between the Q_B site and the Q_i site was markedly impaired in the absence of PufX, excluding an rate-limiting role of PufX in translocating the quinol head group across the hydrophobic membrane core. In addition, these experiments revealed a significant reversion of these effects in Sup102/g, in contrast to the observations for the Q_o site.

Light-Induced Cyclic Electron Transfer in Vivo under Oxidizing Conditions. In intact cells PufX was found to be required for multiple-turnover cyclic electron transfer under anaerobic conditions where the Q pool should be reduced (Barz et al., 1995). To reveal the redox dependence of the PufX function, oxygen-saturated cells of all four strains were used to study cyclic electron flow in continuous light. Under aerobic conditions, electrons are delivered to oxygen through the electron transport chain, thus raising the redox poise of the Q pool (Jones et al., 1990). As shown in Figure 4 (panels A and B), under aerobic conditions both the light-driven generation of the membrane potential and the steady-state photooxidation of cyt c were similar in continuously illuminated PUFC/g and PUFΔX/g cells. In contrast to anaerobic conditions, the absence of PufX thus has no consequences on the light-driven cyclic electron transport under aerobic conditions. Similar traces were obtained for aerobically incubated Sup101/g and Sup102/g cells (data not shown).

The observation that PufX affects light-driven electron flow *in vivo* only under anaerobic conditions suggests that the requirement of PufX can be observed only under certain

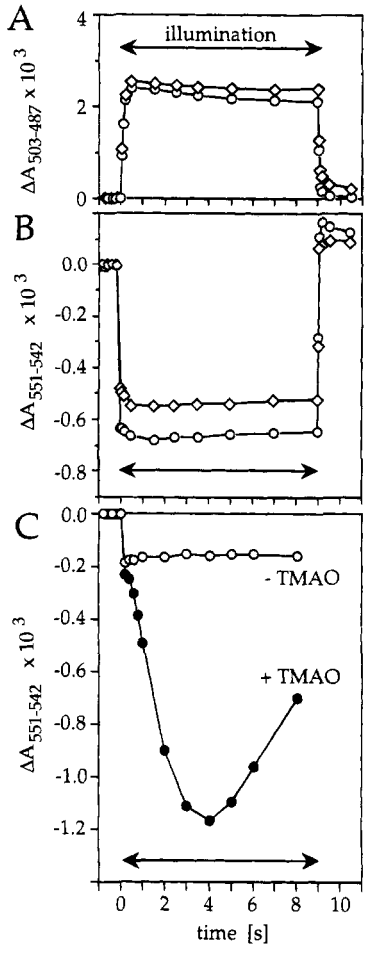

FIGURE 4: Electrochromic carotenoid band shift (A) and cyt *c* oxidation (B and C) induced by continuous illumination of intact cells of PUFC/g (◇) and PUFΔX/g (○ or ●). Light-induced absorbance changes were measured using cells saturated with oxygen (open symbols) or anaerobic cells supplemented with 20 mM TMAO (●). The arrow indicates the period of illumination (50 W/m^2).

redox states of the Q pool. To confirm this interpretation, cyclic electron transfer was studied in anaerobically incubated PUFΔX/g cells supplemented with dimethylsulfoxide (DMSO) or trimethylamine *N*-oxide (TMAO). These oxidants can act as terminal electron acceptors in dark anaerobic growth (Yen & Marrs, 1977; Ferguson et al., 1987). DMSO and TMAO are also known to raise the redox state of the Q pool (Richardson et al., 1988; Jones et al., 1990). After the addition of TMAO, both the light-driven generation of membrane potential (data not shown) and the steady-state photooxidation of cyt *c* (Figure 4C) were restored to values similar to those observed for unsupplemented PUFC/g cells (see Figures 8 and 9 in the accompanying article). This result suggests that (at least partial) oxidation of the Q pool effectively restores cyclic electron flow in the absence of PufX. To directly prove this interpretation, photosynthetic growth of PUFΔX was studied in the presence of DMSO or TMAO. After the addition of 20 mM DMSO, the photosynthetic growth rates were independent of PufX (0.32 ± 0.02 h^{-1} at 100 W/m^2). In the presence of 20 mM TMAO, PUFΔX/g had a growth rate (0.32 h^{-1} at 100 W/m^2), which was slightly smaller than that of PUFC/g (0.39 h^{-1}). Dark controls (in the presence of 20 mM DMSO or TMAO) showed only very limited growth (<0.05 h^{-1}), excluding that any reaction of the dark anaerobic respiration pathway was

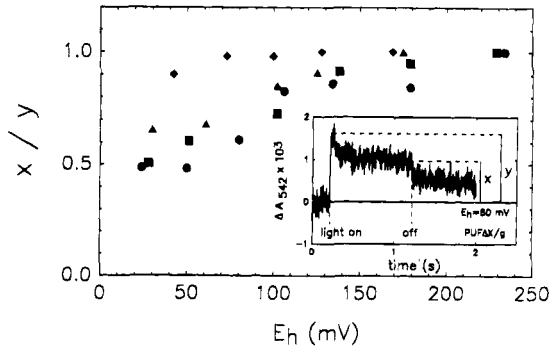

FIGURE 5: Redox titration of the absorbance changes of the primary RC electron donor induced by a 1 s period of illumination. Chromatophores of PUFC/g (◆), PUFΔX/g (●), Sup101/g (■), and Sup102/g (▲) were supplemented with uncouplers and redox mediators as indicated under Materials and Methods. The concentration of photooxidizable RC was 0.42 μM. The *X*/*Y* values represent the ratio of the steady-state level of photooxidation (*X*) and the transient maximum photooxidation (*Y*) (see inset). Data were obtained from averaged traces (four to eight measurements), the instrument time constant was 0.5 ms.

causing the observed effect of DMSO/TMAO. Therefore, the strong stimulation of photosynthetic growth of PUFΔX/g was indeed due to oxidation of the Q pool by these oxidants.

In aerobically incubated cells without electron flow inhibitors, the photooxidation of cyt *c* cannot be analyzed quantitatively due to cyt *c* oxidation by oxygen and due to cyt *c* reduction by the cyt bc_1 complex. In order to study the efficiency of cyt *c*−RC interaction under oxidizing conditions, PUFC/g and PUFΔX/g cells were treated with benzoquinone (BQ), a treatment known to oxidize the Q pool (Joliot et al., 1989), and supplemented with inhibitors of the cyt bc_1 complex (myxothiazol) and of the cyt *c* oxidase (KCN). Under these conditions, cyt *c* oxidation induced by continuous illumination was identical in the presence or absence of PufX (data not shown) clearly demonstrating that PufX was not required for an effective cyt *c*−RC interaction when the Q pool was largely oxidized. Under reducing conditions, however, the absence of PufX caused an impairment of cyt *c* photooxidation (Barz et al., 1995) indicating a role of PufX for RC function under these conditions.

Redox Dependence of Multiple Reaction Center Turnover in Vitro. In order to reveal the precise redox dependence of the PufX function, multiple-turnover RC photochemistry was studied by illuminating chromatophores for a 1 s period (Figure 5). Over the whole range of redox poises tested (20−240 mV), the primary donor P of PUFC/g reached a maximum level of photooxidation within a few tens of milliseconds. This level of P photooxidation was steadily maintained for all the photoexcitation period. Different kinetics were observed in PUFΔX/g chromatophores, where, below approximately 100 mV, a transient maximum of P oxidation is attained on the millisecond time scale after the onset of illumination, followed by relaxation to a lower steady-state level (see inset of Figure 5). The ratio between this steady-state value and the initial maximum of photooxidation (*X*/*Y* in Figure 5) decreased upon decreasing the redox potential, suggesting a progressive impairment in RC photoactivity when the fraction of oxidized Q molecules in the Q pool is decreasing. When chromatophores isolated from Sup101/g or Sup102/g were investigated, a partial reversion of this effect was observed (Figure 5).

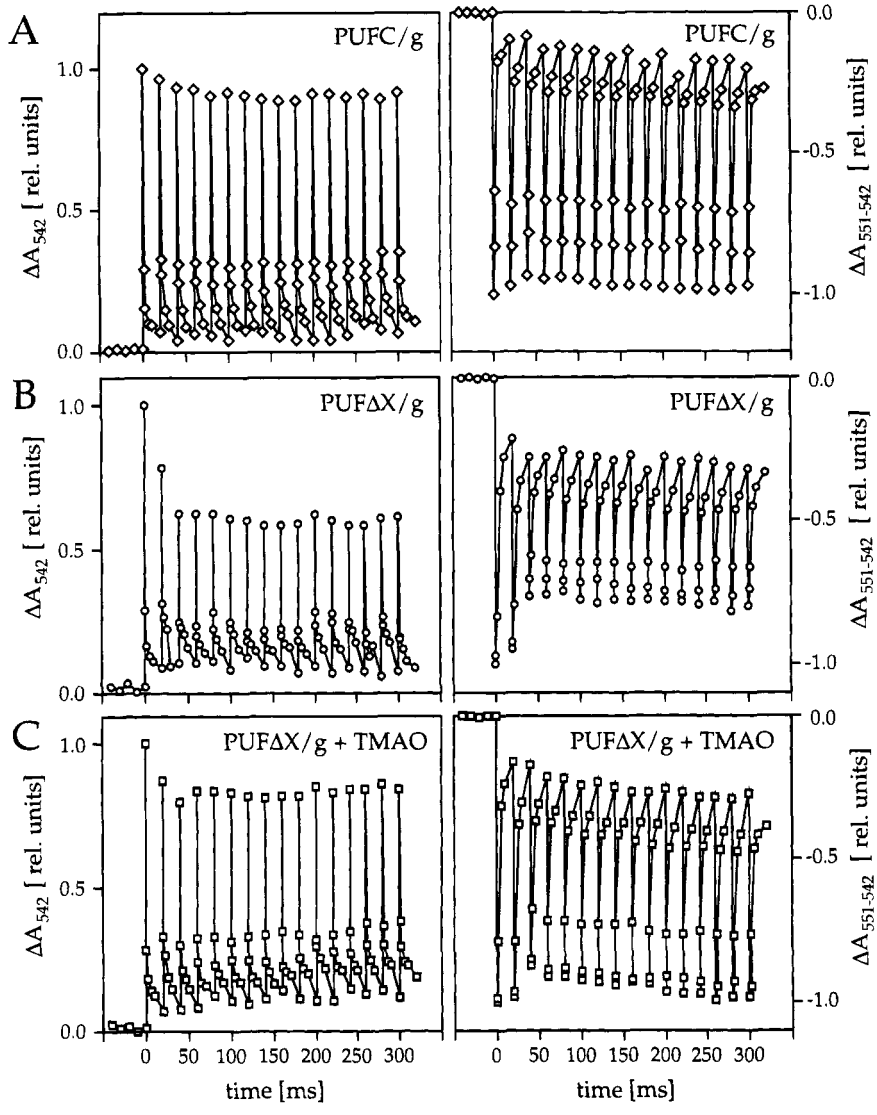

FIGURE 6: Flash-induced oxidation of primary electron donor P (left panel) and of cyt *c* (right panel) in intact cells of PUFC/g (A), PUFΔX/g (B), as well as in PUFΔX/g cells supplemented with 20 mM TMAO (C). Dark adapted, anaerobically incubated cells were excited using a train of 16 saturating flashes fired 20 ms apart.

Flash-Induced Multiple-Turnover Electron Transfer in Vivo. Under anaerobic conditions, PufX was shown to be required for multiple-turnover but not for single-turnover electron transfer (Barz et al., 1995). However, the continuous illumination used for the multiple-turnover studies was not suitable to determine the molecular role of PufX because of the unknown excitation frequency of the RC. In order to further understand the PufX function, a train of 16 saturating actinic flashes (spaced 20 ms apart) was used to excite PUFC/g and PUFΔX/g cells. Figure 6 shows the absorption changes due to RC (left panels) and cyt *c* (right panels) photooxidation in anaerobic cells. For PUFC/g, the extent of RC photooxidation was similar for each of the 16 excitations (Figure 6A), showing that the 20 ms between two flashes were sufficient to allow complete turnover of the cyclic electron transfer chain. The same conclusion could be drawn when the oxidation of cyt *c* was measured although a limited accumulation of oxidized cyt *c* was apparent (Figure 6A). In PUFΔX/g cells, however, the photooxidation of both RC and cyt *c* were significantly impaired following the third and subsequent flashes (Figure 6B). In PUFΔX/g cells supplemented with 20 mM TMAO, the photooxidation pattern observed in the presence of PufX was partially restored (Figure 6C).

This behavior indicates that, in the absence of PufX, primary photochemistry is drastically inhibited following more than two flashes when the Q pool is largely reduced. When the redox components are oxidized, however, PufX is apparently not required for efficient cyclic electron flow. These results suggest that, under reducing conditions, PufX might be involved in the replacement of QH_2 by Q at the Q_B site of the RC. An impairment of the fast QH_2/Q exchange would prevent a stable charge separation in the RC following the third and subsequent flashes because of fast charge recombination.

Quinol/Quinone Exchange Rate at the Reaction Center in Vivo. The results described above suggest an important role of PufX for an effective QH_2/Q exchange at the RC Q_B site under anaerobic conditions (indicative of a reduced Q pool). In order to confirm this interpretation directly, we varied the frequency of excitation flashes and measured the amplitude of the fast phase of the carotenoid band shift *in vivo*. Figure 7 shows the extent of charge separation in the RC following the third flash (relative to the extent induced

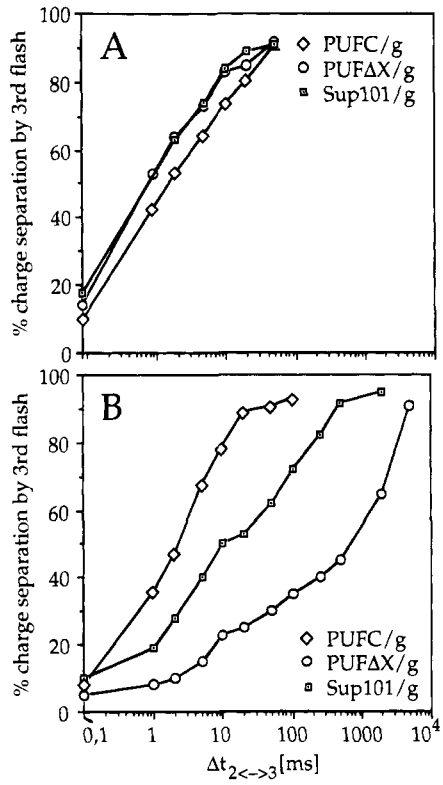

FIGURE 7: Flash-induced RC photochemistry as a function of the dark time $\Delta t_{2\leftrightarrow3}$ between the second and third flashes. Aerobically (A) and anaerobically (B) incubated cells were excited using three saturating single-turnover flashes. The dark time between the first and second flash was 10 ms. Absorption changes at 487 and 503 nm were detected 50 μs before and after each flash. The flash-induced absorption changes $\Delta A_{503-487}$ were plotted as the ratio of the signals induced by the third and the first flashes. The traces are the average of five measurements.

by the first flash) as a function of the dark time between the flashes. In oxygen-saturated cells, the QH_2/Q exchange rate at the Q_B site was similar for PUFC/g, PUFΔX/g, and Sup101/g ($t_{1/2} \approx 2$ ms; Figure 7A). In anaerobically incubated cells, however, RC photochemistry following the third flash drastically depended on PufX (Figure 7B). While the QH_2/Q exchange rate was unchanged in the presence of PufX, it was reduced by more than two orders of magnitude in PUFΔX/g cells ($t_{1/2} \approx 500$ ms). Interestingly, the fast QH_2/Q exchange was partially restored in the suppressor mutant Sup101/g ($t_{1/2} \approx 10$ ms) demonstrating that the requirement of PufX for fast RC turnover under reducing conditions could be suppressed by suitable point mutations in the genes coding for LH1 antennae.

DISCUSSION

In order to determine the role of PufX for photosynthetic growth, light-driven electron flow was studied in detail in this and the preceding article. In contrast to the accompanying article in which anaerobic cells were studied (Barz et al., 1995), the redox potential was varied in this work to determine the role of PufX more precisely.

To reveal the importance of PufX for the redox interaction between the RC and the cyt bc_1 complex, we studied the kinetics of cyt b_{561} reduction in redox-poised chromatophores in the presence of antimycin (Figure 3). The absence of PufX drastically impairs the kinetics of cyt b_{561} reduction at $E_h > 150$ mV, where all the QH_2 molecules reacting at the Q_o site are those released by the RC (Crofts et al., 1983). Thus, the availability of QH_2 molecules coming from the RC seems to be drastically reduced in the absence of PufX. The lag period (observed before the onset of cyt b_{561} reduction) has been proposed to represent the time needed for the dissociation of QH_2 from the Q_B site and/or for its diffusion to the Q_o site of the cyt bc_1 complex (including the time for the translocation of the polar QH_2 head group across the membrane) (Crofts et al., 1983; Snozzi & Crofts, 1984; Venturoli et al., 1986). Since the total size of the Q pool is essentially unaffected by the deletion of *pufX* (Barz et al., 1995), the increased duration of the lag period at $E_h > 140$ mV indicates that the release and/or diffusion of the QH_2 (formed at the RC) to the Q_o site of the cyt bc_1 complex was drastically slowed down in the absence of PufX. This result is consistent with *in vitro* data from *R. capsulatus* showing a requirement of PufX for the slow phase of the carotenoid band shift under oxidizing but not under reducing conditions (Lilburn et al., 1992). Although the precise redox dependence of cyt bc_1 turnover in *R. capsulatus* was not reported, these results are indicative of a similar function for the PufX proteins of these two species.

At low redox poise ($E_h < 140$ mV), the importance of PufX is different for the cyt b_{561} reduction rate and the lag (Figure 3). Although the deletion of PufX does not affect the lag period under these redox conditions (where the Q pool is partially reduced), the rate of cyt b_{561} reduction is three to five times lower than in WT. *In vivo*, however, single turnover of the cyt bc_1 complex is independent of PufX under anaerobic conditions indicative of a reduced Q pool (Barz et al., 1995). This result, together with the observed redox dependence of the lag period, demonstrates that the activity of the Q_o site is not affected by the deletion of *pufX*. Therefore, at $E_h < 140$ mV, the lowered rate of cyt b_{561} reduction in the absence of PufX is likely to reflect a slower oxidation of the high potential chain of the cyt bc_1 complex. If *pufX*$^-$ chromatophores are unsealed vesicles (Barz et al., 1995), the effective concentration of cyt c_2 in these *in vitro* experiments is very low (1 to 2 μM), in spite of the high cyt c_2/RC ratio. This concentration is not saturating for the binding to the RC, and therefore cyt c_2 is oxidized in a slow bimolecular reaction (Venturoli et al., 1990, 1993). Indeed, cyt c_2 is only slowly photooxidized in chromatophores lacking PufX (see Figure 6 of the accompanying article). Therefore, the flash-induced oxidation rate of cyt c_1, the Rieske iron sulfur cluster, and the Q_o must also be reduced in the absence of PufX. Due to the concerted character of QH_2 oxidation at the Q_o site (Crofts et al., 1983), the photooxidation of cyt c_2, if slow, can limit the reduction rate of cyt b_{561}. This does not occur under oxidizing conditions ($E_h = 150-250$ mV), when the frequency of QH_2 collisions at Q_o is low, but may well be the case below 140 mV. This interpretation is also consistent with the observation that the minimum duration of the lag, attained at redox poises of 120−90 mV, seems to be approximately 150 μs longer in the absence of PufX than in WT.

The $Q_B \rightarrow Q_o$ transfer of QH_2 can be regarded as a combination of a lateral diffusion step and a "flip-flop" translocation across the membrane. Therefore, the requirement of PufX for cyt b_{561} reduction at high redox potential could be caused by a role of PufX in facilitating one (or both) of these steps. To distinguish these possibilities, we studied the flash-induced reduction of cyt b_{561} via the Q_i site

of the cyt bc_1 complex, a nonphysiological reaction occurring only in the presence of myxothiazol at high pH values (Glaser et al., 1984; Robertson et al., 1984). The data of Table 1 clearly show that, in the absence of PufX and at high redox poise, the redox interaction between the Q_B site and the cyt bc_1 complex is dramatically impaired, independent of the catalytic site (Q_i or Q_o) involved. Since the deletion of PufX decreases the rate of both reactions to a similar extent (approximately 10-fold), a significant (i.e., rate-limiting) function of PufX in flip-flop translocation of QH_2 is very unlikely. Instead, PufX seems to be clearly required for lateral diffusion of QH_2.

Most interestingly, in both suppressor strains investigated, the kinetics of cyt b_{561} reduction through the Q_o site were not restored to WT levels, but showed the same drastic impairment as observed in the *pufX*$^-$ mutant (Figure 3). This fact excludes any functional correlation between the impaired release and/or diffusion of QH_2 and the photosynthetic growth incompetence (see below). However, cyt b_{561} reduction via Q_i was partially recovered in Sup102/g chromatophores (Table 1). It is tempting to speculate that this partial recovery is related to the relative topology of the Q_B and Q_i catalytic sites, both facing the same side of the membrane.

Two control experiments demonstrated that the requirement of PufX for the reduction of cyt b_{561} at high redox potential is not caused by a reduced stability of the semiquinone Q_B^- in the absence of PufX. Following excitation with two closely spaced flashes, cyt b_{561} was reduced with a rate approximately double of that observed after single-flash excitation (open symbols in Figure 3A). This is the expected behavior when 0.5 Q_B^- molecules per RC are present in dark-adapted RCs (Crofts et al., 1983). If the stability of Q_B^- were significantly lower in the absence of PufX, two closely spaced flashes would have caused a much higher stimulation in the reduction of cyt b_{561}, leading to a value close to that observed in WT after single-flash excitation. The stability of Q_B^- is thus not affected by the deletion of PufX. The same conclusion was drawn when the flash-induced formation and disappearance of Q_B^- was detected spectroscopically at high redox poise (Figure 1). This experiment rules out an important role of PufX for the functionality of the RC two electron gate under oxidizing conditions. This conclusion was confirmed when the rates of light-induced cyt *c* reduction were measured with solubilized RC−LH1 complexes in the presence of excess Q_0 (corresponding to a completely oxidized Q pool). Since release of QH_2 is the rate limiting step of electron flow from cyt c_2 to the Q pool, the activity of the two electron gate can be studied well with such an assay (Paddock et al., 1988).

Taken together, these *in vitro* results clearly show a requirement of PufX for the $Q_B \rightarrow Q_o$ transfer of QH_2 at high redox potential, although the activities of both the RC and cyt bc_1 complex are not affected by the absence of PufX under these conditions. However, these experiments allow no conclusion on the RC−cyt bc_1 interaction at low redox poise, where the Q pool is partially reduced. This topic was therefore addressed in another set of experiments.

In the accompanying article, we present evidence that PufX is essential *in vivo* for multiple turnover (but not for single turnover) of the light-driven electron cycle under anaerobic conditions. Under such conditions, the Q molecules of the membrane Q pool are known to be in their reduced redox state (Jones et al., 1990). In this study, however, multiple-turnover electron flow was found not to require PufX under high redox conditions *in vivo*. When cells were saturated with oxygen or supplemented with TMAO, the light-induced generation of a photosynthetic membrane potential was independent of PufX (Figure 4). In oxygen-saturated cells, the Q pool is partially oxidized (Jones et al., 1990) because cytochrome oxidase activity drains electrons from the electron transfer chain. TMAO also oxidizes the Q pool of anaerobic cells only partially since TMAO did not affect the half-time of the slow electrochromic phase (data not shown). Thus, PufX is not required for cyclic electron flow when the Q pool is partially oxidized. When at least some Q molecules per RC are present in the membrane, multiple turnover of the RC is independent of PufX. This conclusion was directly confirmed by the observation that *pufX*$^-$ cells supplemented with DMSO or TMAO can grow photosynthetically. Since these electron acceptors did not stimulate cell growth in the dark (as shown by dark controls), the light-induced growth in the absence of PufX could be clearly correlated to the partial oxidation of the Q pool by these oxidants. Interestingly, the growth rate of a semiaerobically growing *pufX*$^-$ culture was also increased significantly by strong illumination (although not to WT levels), showing that PufX is essential for photosynthetic growth only in the complete absence of oxygen (data not shown).

The precise redox dependence of multiple turnover electron flow became apparent, when the steady-state level of RC photobleaching was studied in redox-poised chromatophores that were illuminated for 1 s. Below 100 mV, maximal RC photooxidation was only transiently attained in membranes lacking PufX and was followed by relaxation to a lower steady-state level (Figure 5). In addition to precisely defining the redox dependence of RC photochemistry, this *in vitro* result strongly supports the conclusion that PufX is not required for the first few turnovers of the RC, but for a high efficiency of multiple turnover. This conclusion was directly confirmed by multiple-flash experiments *in vivo*. Under anaerobic conditions indicative of a reduced Q pool (but not in oxygen-saturated cells), primary photochemistry in the absence of PufX is drastically inhibited following the third and subsequent flashes (Figure 6). These results suggest that PufX is required for the fast QH_2/Q exchange at the Q_B site of the RC when the Q pool is completely reduced. Under these conditions, Q_BH_2 (formed following the first two flashes) is apparently not efficiently replaced by Q. Therefore, RC photochemistry is impaired following the third and subsequent flashes because of fast charge recombination in the ns time range (Parson et al., 1987).

To directly correlate this impairment to the low redox state of the Q pool, redox-poised chromatophores were used to determine the precise redox dependence of RC photochemistry following multiple flashes. When flash-induced absorption changes of the RC primary donor (at 542 nm) were studied in the absence of PufX, the extent of photooxidation following the third flash was markedly decreased below 80 mV (data not shown). This clearly confirms that PufX is functionally required for RC photoactivity only at a redox poise indicative of a very low concentration of oxidized Q. However, these *in vitro* data could not be analyzed quantitatively because the RC was rereduced with different rates in the presence or absence of PufX. Since *pufX*$^-$ chromatophore are unsealed vesicles (Barz et al., 1995), the concen-

tration of cyt c_2 is kinetically limiting for RC rereduction in the absence of PufX, thus causing slower kinetics of cyt c photooxidation and RC rereduction (data not shown). Due to this problem, intact cells allow a more reliable analysis of primary photochemistry inhibition. By varying the frequency of flash excitation, the role of PufX for fast QH_2/Q exchange could be measured quantitatively (Figure 7). As expected, PufX was not required for efficient RC photochemistry in the presence of oxygen. Under reducing conditions, however, the efficiency of QH_2/Q exchange is drastically lowered by the absence of PufX (more than 200-fold).

Several complementary lines of evidence now indicate an important role of PufX for efficient interaction between the Q_B site and a largely reduced Q pool. This conclusion is consistent with the *in vitro* results showing that PufX does not affect RC turnover under oxidizing conditions (Figure 1). Apparently, only when the concentration of Q is very low, PufX is required for the replacement of Q_BH_2 by Q. Therefore, PufX could be involved in release of Q_BH_2 from the RC or in rebinding Q to the RC (or in both reactions). The Q/QH_2 exchange rate at the RC is influenced by the concentrations of both quinone species (defined by the redox potential), the affinity of Q and QH_2 to the Q_B site and by the diffusion constants for Q and QH_2. Both the impaired Q transfer to the Q_B site (at very low redox potential) as well as the slow diffusion of QH_2 to the Q_o site (at very high redox poise) could be caused by a role of PufX in lowering the affinity of the Q_B site for QH_2. This would allow faster binding of the few available Q molecules under reducing conditions and would also increase the rate of QH_2 release under oxidizing conditions.

A *direct* role of PufX in facilitating Q_BH_2/Q exchange can be considered (e.g., as a carrier protein that binds Q and/or QH_2); the rate of such exchange would be affected by a low Q concentration. The requirement of PufX for Q_BH_2/Q exchange under reducing conditions might also be caused by a direct role of PufX in facilitating flip-flop translocation of the oxidized quinone species (generated at the Q_o site) to the cytoplasmic side of the membrane.

Alternatively, PufX could have an *indirect* role in allowing efficient access of the Q molecules to the Q_B site. Under very reducing conditions, the only oxidized Q molecules are those generated at the Q_o site (following photoexcitation). PufX might be required to allow efficient diffusion of these Q molecules to the Q_B site. We suggest that PufX structurally arranges the RC surrounding thus enabling efficient access of the few available Q molecules to the RC. It is feasible that PufX interacts with the RC in a way that prevents LH1 molecules from physically blocking the Q_B site (see below). This suggestion is consistent with the facts that PufX was detected in solubilized RC−LH1 complexes (Farchaus et al., 1992) and that many different LH1 mutations allow photosynthetic growth in the absence of PufX (Barz & Oesterhelt, 1994).

Under oxidizing (but not under reducing) conditions, we observed a strict requirement of PufX for lateral QH_2 diffusion to the cyt bc_1 complex (see above). On the other hand, efficient turnover of the RC requires PufX only under reducing conditions. Although the different redox dependence observed for RC and cyt bc_1 activity might seem contradictory at first sight, these results do not contradict each other. Apparently, PufX is important for *both* the transfer of QH_2 (observed under oxidizing conditions when the concentration of QH_2 is limiting) and for the access of Q to the RC (revealed under reducing conditions). This fact strongly suggests an indirect function of PufX in lateral, intramembrane transfer of Q and QH_2 (e.g., by structurally organizing the photosynthetic membrane). This function only becomes apparent at very low or very high redox potential, when either Q or QH_2 is limiting for efficient cyclic electron flow. Thus, PufX is required for anaerobic, photosynthetic growth because it has an essential role for the access of Q to the RC when the Q pool is almost completely reduced. At higher redox poise, PufX is no longer required for Q transfer because the Q pool is partially oxidized. On the other hand, the importance of PufX for QH_2 transfer can only be observed at $E_h > 140$ mV, a redox potential that is nonphysiological for anaerobic, photosynthetic growth. This interpretation is consistent with the observation that, under oxidizing conditions, the transfer of QH_2 to the cyt bc_1 complex is not restored by suppressor mutations in the antenna structure (Figure 3). Indeed, anaerobic conditions were used to isolate these suppressor mutants (Barz & Oesterhelt, 1994), and such reducing redox conditions select only for efficient Q access to the RC.

The suppressor strains Sup101/g and Sup102/g also point to a central role of PufX in structurally organizing the photosynthetic apparatus. The requirement of PufX for access of Q to the RC (under reducing conditions) is partially restored in these strains which contain a mixture of WT and mutant *pufBA* genes (Barz & Oesterhelt, 1994) that drastically reduces the amounts of antenna complexes (Table 1). Apparently, the suggested role of PufX in keeping the Q_B site physically accessible can be suppressed by specific LH1 mutations that cause severe changes in the macromolecular antenna structure. We suggest that the coexpression of WT and point-mutated *pufBA* genes in Sup101/g and Sup102/g leads to rearrangements in the antenna structure around the RC thus partially restoring a fast Q/QH_2 exchange and, consequently, photosynthetic growth under reducing conditions. This interpretation is clearly supported by a recent study showing that PufX has no significant effect on the photosynthetic growth rates of *R. sphaeroides* strains lacking both types of antenna complexes (McGlynn et al., 1994). The fact that photosynthetic growth of such strains has no absolute requirement of PufX strongly supports our conclusion that PufX has an indirect effect on Q/QH_2 exchange between the RC and the cyt bc_1 complex.

The recently determined crystal structure of LH2 of *Rhodopseudomonas acidophila* shows a perfectly symmetrical, closed ring of nine identical heterodimeric protein units and their associated pigments (McDermott et al., 1995). Using electron diffraction data obtained from two-dimensional LH1 crystals of *Rhodospirillum rubrum*, another recent publication (Karrasch et al., 1995) suggested a similar ring-like structure for LH1 (built of 16 identical, heterodimeric subunits) surrounding the RC. In light of this suggested continuous ring of LH1 molecules, it is hard to imagine a role of PufX in catalyzing a fast Q/QH_2 exchange. However, the LH1 structure was determined using purified LH1 possibly devoid of PufX. In addition, the 16-fold symmetry of LH1 was obtained by filter imaging of the diffraction data, and therefore some asymmetry due to the insertion of PufX into the ring could have been lost. In our opinion, there is some possibility that PufX is an integral part of the LH1

ring, possibly in close proximity of the RC Q_B site, thereby catalyzing proper shuttling of Q and QH_2 through the antenna ring. Deletion of PufX might therefore impair the fast Q/QH_2 exchange. This would explain why drastically reduced antenna levels (Barz & Oesterhelt, 1994) or the complete absence of antennae (McGlynn et al., 1994) allow photosynthetic growth without PufX.

Spectroscopic studies have shown previously that the RC and cyt bc_1 complex are not randomly arranged in the membrane but form macromolecular "supercomplexes" (Joliot et al., 1989). These studies demonstrated that the diffusion of cyt c_2 is restricted to a small number of cyt bc_1 complexes. Due to the observed requirement of PufX for an efficient RC−cyt bc_1 interaction, PufX might in principle be involved in structurally forming such supercomplexes. Preliminary measurements showed that the diffusion of cyt c_2 is equally restricted in the presence or absence of PufX (A. Verméglio, unpublished results) thus making such a role of PufX very unlikely. However, this conclusion is limited by the fact that a spatial restriction of electron transfer has only been shown for the donor side of the photosynthetic chain, and it has not yet established for the acceptor side. Further work will be necessary to study a possible role of PufX in restricting Q/QH_2 diffusion between the RC and cyt bc_1 complex.

In conclusion, the results presented in this and the preceding article point to a central role of PufX in organizing the macromolecular antenna structure around the RC. We suggest that PufX builds a functional arrangement of the membrane complexes, thus allowing efficient electron flow even under redox conditions where either Q or QH_2 is limiting. Although the molecular mechanism of the PufX function is not yet known, the present data clearly demonstrate that PufX enables photosynthetic growth of *R. sphaeroides* by allowing efficient access of Q to the RC under anaerobic conditions.

ACKNOWLEDGMENT

The authors thank Uta Schimanko for synthesis of the oligonucleotides, Dr. Romana Fato for assistance in HPLC determinations of the ubiquinone content, and Dr. Josef Wachtveitl for help with the spectroscopic cytochrome-turnover assay.

REFERENCES

Barz, W. P., & Oesterhelt, D. (1994) *Biochemistry 33*, 9741−9752.
Barz, W. P., Francia, F., Venturoli, G., Melandri, B. A., Verméglio, A., & Oesterhelt, D. (1995) *Biochemistry 34*, 15235−15247.
Bevington, P. R. (1969) in *Data Reduction and Error Analysis for the Physical Sciences*, pp 204−246, McGraw-Hill, New York.
Bowyer, J. R., Tierney, G. V., & Crofts, A. R. (1979) *FEBS Lett. 101*, 201−206.
Bowyer, J. R., Meinhardt, S. W., Tierney, G. V., & Crofts, A. R. (1981) *Biochim. Biophys. Acta 635*, 167−186.
Clayton, R. K. (1973) *Biochim. Biophys. Acta 75*, 312−323.
Crofts, A. R., & Wraight, C. A. (1983) *Biochim. Biophys. Acta 726*, 149−185.
Crofts, A. R., Meinhardt, S. W., Jones K. R., & Snozzi, M. (1983) *Biochim. Biophys. Acta 723*, 202−218.
Degli Esposti, M., De Vries, S., Crimi, M., Ghelli, A., Patarnello, T., & Meyer, A. (1993) *Biochim. Biophys. Acta 1143*, 243−271.
Farchaus, J. W., Barz, W. P., Grünberg, H., & Oesterhelt, D. (1992) *EMBO J. 11*, 2779−2788.
Ferguson, S. J., McEwan, A. G., & Jackson, J. B. (1987) *FEMS Microbiol. Rev. 46*, 117−143.
Glaser, E. G., Meinhardt, S. W., & Crofts, A. R. (1984) *FEBS Lett. 178*, 336−342.
Gray, K. A., Farchaus, J. W., Wachtveitl, J., Breton, J., & Oesterhelt, D. (1990) *EMBO J. 9*, 2061−2070.
Joliot, P., & Joliot, A. (1984) *Biochim. Biophys. Acta 765*, 210−218.
Joliot, P., Béal, D., & Frilley, B. (1980) *J. Chim. Phys. 77*, 209−216.
Joliot, P., Verméglio, A., & Joliot, A. (1989) *Biochim. Biophys. Acta 975*, 336−345.
Jones, M. R., Richardson, D. J., McEvan, A. G., Ferguson, S. J., & Jackson, J. B. (1990) *Biochim. Biophys. Acta 1017*, 209−216.
Karrasch, S., Bullough, P. A., & Ghosh, R. (1995) *EMBO J. 14*, 631−638.
Lilburn, T. G., Haith, C. E., Prince, R. C., & Beatty, J. T. (1992) *Biochim. Biophys. Acta 1100*, 160−170.
McDermott, G., Prince, S. M., Freer, A. A., Hawthornthwaite-Lawless, A. M., Papiz, M. Z., Cogdell, R. J., & Isaacs, N. W. (1995) *Nature 374*, 517−521.
McGlynn, P., Hunter, C. N., & Jones, M. R. (1994) *FEBS Lett. 349*, 349−353.
McPherson, P. H., Okamura, M. Y., & Feher, G. (1990) *Biochim. Biophys. Acta 1016*, 289−292.
Monger, T. G., & Parson, W. W. (1977) *Biochim. Biophys. Acta 460*, 393−407.
Mulkidjanyan, A. Ya., Shinkarev, V. P., Verkhovsky, M. I., & Kaurov, B. S. (1986) *Biochim. Biophys. Acta 849*, 150−161.
Okamura, M. Y., Debus, R. J., Kleinfeld, D., & Feher, G. (1982) in *Function of Quinones in Energy Conserving Systems* (Trumpower, B. L., Ed.) pp 299−317, Academic Press, New York.
Paddock, M. L., Rongey, S. H., Abresch, E. C., Feher, G., & Okamura, M. Y. (1988) *Photosynth. Res. 17*, 75−96.
Parson, W. W. (1987) in *Photosynthesis* (Amesz, J., Ed.) pp 43−61, Elsevier Science Publishers B.V., Amsterdam.
Richardson, D. J., King, G. F., Kelly, D. J., McEwan, A. G., Ferguson, S. J., & Jackson, J. B. (1988) *Arch. Microbiol. 150*, 131−137.
Robertson, D. E., Giangiacomo, K. M., de Vries, S., Moser, C. C., & Dutton, P. L. (1984) *FEBS Lett. 178*, 343−350.
Robertson, D. E., Daldal, F., & Dutton, P. L. (1990) *Biochemistry 29*, 11249−11260.
Snozzi, M., & Crofts, A. R. (1984) *Biochim. Biophys. Acta 766*, 451−463.
Straley, S. C., Parson, W. W., Mauzerall, D. C., & Clayton, R. K. (1973) *Biochim. Biophys. Acta 305*, 597−609.
Takamiya, K., & Dutton, P. L. (1979) *Biochim. Biophys. Acta 546*, 1−16.
Venturoli, G., Fernández-Velasco, J. G., Crofts, A. R., & Melandri, B. A. (1986) *Biochim. Biophys. Acta 851*, 340−352.
Venturoli, G., Melandri, B. A., Gabellini, N., & Oesterhelt, D. (1990) *Eur. J. Biochem. 189*, 105−112.
Venturoli, G., Mallardi, A., & Mathis, P. (1993) *Biochemistry 32*, 13245−13253.
Verméglio, A. (1977) *Biochim. Biophys. Acta 459*, 516−524.
Wraight, C. A. (1977) *Biochim. Biophys. Acta 459*, 525−531.
Wraight, C. A., Cogdell, R. J., & Clayton, R. K. (1975) *Biochim. Biophys. Acta 396*, 242−249.
Yen, H.-C., & Marrs, B. (1977) *Arch. Biochem. Biophys. 181*, 411−418.
Zuber, H., & Brunisholz, R. A. (1991) in *The Chlorophylls* (Scheer, H., Ed.) pp 627−703, CRC Press, Boca Raton, FL.

BI950777I