Functional Consequences of the Organization of the Photosynthetic Apparatus in *Rhodobacter sphaeroides*

II. A STUDY OF PuF X - MEMBRANES*

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In the bacterium *R. sphaeroides*, the polypeptide PuFX is indispensable for photosynthetic growth. Its deletion is known to have important consequences on the organization of the photosynthetic apparatus. In the wild-type strain, complexes between the reaction center (RC) and the antenna (light-harvesting complex 1 (LH1)) are associated in dimers, and LH1 does not fully encircle the RC. In the absence of PuFX, the complexes become monomeric, and the LH1 ring closes around the RC. We analyzed the functional consequences of PuFX deletion. Some effects can be ascribed to the monomerization of the RC-LH1 complexes: the number of RCs that share a common antenna for excitation transfer or a common quinone pool become smaller. We examined the kinetic effects of the closed LH1 ring on quinone turnover: diffusion across LH1 entails a delay of ~1 ms, and the barrier appears to be located directly against the quinone-binding (secondary quinone acceptor (Qb)) pocket. The diffusion of ubiquinol from the RC to the cytochrome *bc* complex is ~2-fold slower in the mutant, suggesting an increased distance between the two complexes. The properties of the Qb pocket (binding of inhibitors, stabilization of Qb, and rate of Qb-H2 formation) appear to be modified in the mutant. Another specificity of PuFX is the accumulation of closed centers in the Qa resonance (where Qa is the primary quinone acceptor) state as the secondary acceptor pool becomes reduced, which is probably the origin of photosynthetic incompetence. We suggest that this is related to the Qb pocket alterations. The malfunction of the reaction center is probably due to a faulty association with LH1 that is prevented in the PuFX-containing structure.

In the preceding article (1), we examined some functional consequences of the supramolecular arrangement of the photosynthetic apparatus in the purple non-sulfur bacterium *Rhodobacter sphaeroides*. Two main issues were addressed concerning the excitation transfer between the RC1-LH1 complexes and the localization of the quinone pool. In both cases, the dimeric association of the RC-LH1 complexes was shown to play an important role. However, the dimeric arrangement of the RC-LH1 complexes is not a general feature in photosynthetic bacteria. For instance, monomeric RC-LH1 complexes from *Rhodospirillum rubrum* were resolved by electron microscopy from two-dimensional crystals (2, 3), whereas the same technique allowed the observation of dimers in native (4) or reconstituted (5) membranes of *R. sphaeroides*. The three-dimensional structure obtained from x-ray crystallography of complexes from *Rhodopsseudomonas palustris* is also monomeric (6). There are also atomic force microscopy data that confirm the dimeric arrangement in *R. sphaeroides* (7), whereas monomers were observed by this technique in *Blastochloris viridis* (8) and *Rhodospirillum photometricum* (9).

Another important structural feature of the RC-LH1 complexes that differs in the various cases described so far is the open/closed character of the LH1 ring surrounding the RC. LH1 is an oligomeric ring-shaped structure whose building block is a heterodimer of two subunits named α and β (each a transmembrane helix) and binds two bacteriochlorophylls and one carotenoid. In *R. sphaeroides*, LH1 does not form a closed ring, but an open C-shaped structure. Several αβ-heterodimers with the associated bacteriochlorophylls are missing with respect to a complete closed ring. From biochemical evidence, the number of αβ-heterodimers/RC has been estimated in the range of 10–13 (4, 10–13), whereas the "complete" ring is expected to contain 16 heterodimers, as in *R. rubrum*. From electron microscopy images, a figure of 12 was estimated (4, 5). An open ring has also been obtained in the crystallographic structure of *R. palustris* (6), which includes 15 αβ-heterodimers. The other known structures display closed LH1 rings. In such a case, it is not obvious how the quinone acceptors can efficiently shuttle between their reducing (Qb) site on the RC and their oxidizing site on the *bc* complex.

To understand the structural causes and functional consequences of the particular supramolecular arrangement found in *R. sphaeroides*, it is very useful to examine the effects of the deletion of PuFX. This small polypeptide, coded in the *puf* operon (which also contains the genes for the RC and LH1 subunits), appears to play an important role in controlling the supramolecular structure. It is found at a 1:1 ratio with the RC in purified RC-LH1 complexes (14). The sequence is indicative of a single transmembrane α-helix with hydrophilic C- and N-terminal loops; a study based on gradual clipping investigated the functional roles of each loop (15). The available evidence indicates that the deletion of PuFX suppresses the formation of RC-LH1 dimers and that about four additional αβ-units are incorporated to form a closed ring (5, 13, 14, 16). Such mutants have lost the
ability to grow photosynthetically under anaerobic reducing conditions. Photosynthetic growth is recovered, however, when oxidants such as trimethylamine N-oxide and Me$_3$SO are added to the medium (17, 18). When examined on a single turnover basis, the electron transfer kinetics appear normal. However, a dramatic slowing down of the cyclic flow is observed under steady-state illumination under anaerobic conditions (17, 18). It was suggested that the closed LH1 ring around the RC obstructs the shuttling of quinol/quinone between the RC and bc$_1$ complexes.

In this work, we compare the functional behavior of membranes from a PufX$^-$ mutant and from the WT. The consequences of the monomeric structure appear to be a greater confinement of the quinone pool and more restricted excitation transfer. Special attention is given to the question of quinone quenches of the monomeric structure appear to be a greater confinement of the quinone pool and more restricted excitation transfer. We propose that the main role of PufX is to prevent an incorrect interaction of the RC with LH1, which is responsible for this malfunction.

**EXPERIMENTAL PROCEDURES**

We used the same materials and procedures as described in the preceding article (1), except those specified below. The strain denoted as WT was **R. sphaeroides** Ga. The PufΔX$^-$ strain, with PufX deleted (denoted here as PufX$^-$), was a kind gift of Prof. D. Oesterhelt. Its construction has been described (17). It was grown under semi-aerobic conditions in Hutner's medium containing kanamycin (25 µg/ml). As reported previously, this strain was unable to grow under photosynthetic conditions (anaerobiosis + light). This strain contains both the LH1 and LH2 antenna complexes.

RC-LH1 complexes were isolated as described in the preceding article (1). In agreement with previous work (14), the dimer band found in the sucrose gradient with the WT was totally absent in preparations from the PufX$^-$ strain. A single RC-LH1 band was observed, and these complexes displayed the characteristic monomer behavior as described in the previous article with respect to excitonic connectivity or sharing of the quinone pool. A difference with respect to monomers prepared from WT membranes was the amount of endogenous quinone retained in the complexes, which was larger (typically 14 Q molecules/RC) than in WT complexes (typically 7 Q molecules/RC), in line with the larger quinone pool found in membranes from this mutant compared with those from the WT (grown under the same conditions).

For monitoring absorption changes at subzero temperatures (see Fig. 6), we used our Joliot-type spectrophotometer equipped with the low temperature attachment as described (19). The cuvette accommodates a thin sample layer (~0.25 mm) between a glass slide and a reflecting metal sheet on a Peltier element. The transmission is measured from the reflected light. The membrane samples used were 10-fold more concentrated than those used in the setup for room temperature experiments, where the optical path was 15 mm. Due to the small optical path, absorption changes can be detected in frozen aqueous samples without addition of glycerol or antifreeze. The temperature is measured with a thermocouple in close thermal contact with the sample.

**RESULTS**

Excitation Transfer—Fig. 1 shows a plot of the normalized variable bacteriochlorophyll fluorescence yield (Φ) as a function of the amount of closed centers (c). The curvature of such a plot is indicative of the degree of “connectivity” of the RC-LH1 complexes, i.e. the probability that the excitation visiting a complex with a closed RC can be made available for other RCs (20, 21). The data were obtained during the P$_0^+$ Q$_B^-$ recombination following a saturating flash to stigmatellin-inhibited membranes. The relative fluorescence yield and the amount of P$_0^+$ (603 nm absorption change) were recorded at discrete times, and each data point is a couple of these values. The Φ(c) relationship obtained with PufX$^+$ membranes (Fig. 1, closed circles) displays a smaller curvature than that obtained with WT membranes (open circles and plus signs for membranes from photosynthetic and semi-aerobic cultures, respectively). These curves can be characterized (22) by a parameter (J) obtained by a fit with the following function (Equation 1).

$$
Φ = \frac{c}{1 + J - Jc}
$$

(Eq. 1)

This relation is appropriate for a large and reasonably homogeneous array of RC-antenna complexes. As previously shown (see Ref. 1 and the associated Supplemental “Experimental Procedures”), it may also be used in a broader context as a phenomenological fitting function. The parameter J expresses the degree of connectivity of RC-LH1 units: the trapping section of an open RC is enhanced by a factor of J + 1 when the neighboring centers become closed. The allowed interval for J ranges from 0 for isolated units to $J_{lake} = F_m/F_0 - 1$ (where $F_m$ and $F_0$ are the fluorescence yields for closed and open RCs, respectively) for unrestricted excitonic diffusion (“lake model”). J depends both on the efficiency of antenna connectivity and on the trapping properties of the RC, notably the quenching efficiency of the closed RC. The fit of the PufX$^+$ data (Fig. 1, solid line) corresponds to J = 0.32, which may be compared with a value of 0.81 for the WT. This indicates that the RC-LH1 monomers in the PufX$^+$ membrane are midway between the situation of completely isolated units and the connectivity observed in the WT, which was determined as essentially due to efficient transfers between the two partners within an RC-LH1 dimer. A likely interpretation is that there is a distribution of the distances between monomers, so some of them behave as isolated units, whereas others are close enough to allow excitation transfer.

Photosreduction of the Quinone Pool—Fig. 2 shows the photoreduction kinetics of TMPD (603 nm absorption change) during continuous illumination of PufX$^+$ (closed circles) and WT (open circles) membranes. The reaction medium included the set of inhibitors and uncouplers indicated in the preceding article (1). In particular, the bc$_1$ inhibitors myxothiazol and myxothiazol and myxothiazol and

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2 In a more general way, the enhancement factor is the derivative $\frac{d\Phi}{dc}$ for $c = 1$ (the slope of Φ(c) at the top right corner of the plot), which can be estimated experimentally, irrespective of any particular model.
antimycin A were used for blocking quinol oxidation. As explained previously (1), the photo-oxidation of TMPD under such conditions is a mirror image of the photoreduction of the acceptor pool, and we will use these terms as equivalents. The TMPD concentration was 10 mM, allowing reduction of $P^+$ in the 100-μs range; as documented below, this reaction is not rate-limiting in these experiments. The vertical scale indicates the number of electrons transferred per RC, using for calibration the absorption change induced by a single turnover flash on a dark-adapted sample.

The major difference in Fig. 2 between the PuFX− and WT kinetics is the overall size of the acceptor pool. It is $\sim 92$ electrons/RC (46 quinones) in the mutant, which is $\sim 2$-fold larger than in the WT. These figures were found to be somewhat variable depending on preparations, but the enlargement of the pool in the mutant was always obvious. Typical figures are 40–60 quinones/RC in PuFX− membranes and 25–30 and 15–25 quinones/RC in WT membranes from semi-aerobic and photosynthetic cultures, respectively. The experiments in Fig. 2, which were run at moderate illumination intensity, reveal no obvious slowing of the kinetics in the mutant (in the region where both kinetics can be compared) that could be ascribed to the closed LH1 ring. The experiments described below investigate this issue in a more quantitative manner.

Fig. 3 shows a magnification of the initial part of the photoreduction kinetics under high illumination intensity. The kinetics in the PuFX− membrane are clearly slower than those in the WT membrane, especially beyond the first 2 electrons (dashed lines). The initial slope (e.g., for the first electron) corresponds (WT curve) to $\sim 1$ electron/700 μs. This is $\sim 4$-fold slower than $P^+$ reduction by TMPD. This initial rate is thus still essentially limited by the photochemical reaction, depending on the light intensity and its collection and trapping efficiencies. The initial rate for the PuFX− curve is slower, probably because of the lower LH2 content. Beyond this initial part of the kinetics, a roughly linear section can be identified, as shown by the dashed lines. The slope of this line can be taken as the quasi steady-state rate of quinone reduction in the presence of the oxidized pool. It is representative of the full turnover of the quinone cycle on the $Q_B$ pocket, including the transfer of 2 electrons and 2 protons, the release of the reduced quinol, and the binding of a fresh oxidized quinone.

Fig. 4A shows plots of this rate as a function of the illumination intensity for WT and mutant membranes. Clearly, the rate-limiting step for quinone turnover is 2–3-fold slower in the mutant. To analyze these data, we made the assumption that the turnover of the centers involves sequentially a photochemical reaction, with rate constant $k_\text{r}$ (where $I$ is the light intensity and $a$ is an efficiency constant), and a dark recovery reaction, with rate constant $k_\text{d}$. The intensity dependence of the turnover rate should then be as in Equation 2.

$$v = \frac{k_\text{r}}{1 + k_\text{d}/aI}$$  \hspace{1cm} (Eq. 2)

The lines in Fig. 4 are best fits of the data using Equation 2. The accessible intensity range was not sufficient to reach the saturated rate $k_\text{r}$, in the WT membranes, but this may be deduced from the fits, which give $k_\text{r} \sim 0.57$ (PuFX− mutant) and 1.29 (WT) electrons per ms and per RC. Therefore, the full turnover of one quinone (2 electrons) on the RC corresponds to time constants $(2/k_\text{r})$ of 3.5 and 1.6 ms for the mutant and WT membranes, respectively. This is slower than the electron transfer reactions on the donor side (135 μs) and acceptor side ($<100$ μs) (23–26) and must predominantly reflect the time required for releasing $Q_B$ and binding an oxidized ubiquinone to the $Q_B$ pocket.

Fig. 4B shows the results of similar experiments run with isolated RC-LH1 complexes. When applied to WT membranes, the isolation procedure described in the preceding article (1) yielded a major fraction of dimeric complexes and a small amount of monomeric complexes. With PuFX− membranes, the RC-LH1 complexes were strictly monomeric, in agreement with Ref. 14. Both types of complexes retained a 25–30% fraction of the native Q pool. As may be seen, the saturation curves obtained for the dimeric complexes from the WT and for the monomeric complexes from PuFX− are similar to those of the corresponding membranes. With RC-LH1 monomers from the WT, we obtained different results depending on the preparation procedure. Fresh WT monomers collected directly from the sucrose gradient had the same maximum rate as the dimers. Further purification on a chromatography column or aging resulted in complexes with a slower turnover, similar to the PuFX− complexes. (For the latter, there was no difference between “fresh” and “purified” preparations.) In a preliminary report (27), we declared that PuFX− and WT monomers behave similarly, but we have now realized that this does not apply to fresh preparations.

When first tackling these experiments, we considered it likely that the closed LH1 ring in the PuFX− mutant would delimit two quinone pool domains. An internal pool within the ring could react directly with the RC at a fast rate, whereas the...
The results of Figs. 3 and 4 do not support the first proposition since a slower rate-limiting step was observed in the PufX− mutant from the very first quinone turnovers (excluding the initial reduction of the QB bound in the dark). On the other hand, the photoreduction kinetics in the PufX− membranes (see Fig. 2) slow down markedly in the final part, which might be a sign of a kinetic barrier due to the closed ring. The experiment in Fig. 5 was devised to test this possibility. The idea is to compare the kinetics during a continuous illumination pulse (curve 1) or after a dark interruption of several hundred milliseconds (400 ms for curve 2 and 700 ms for curve 3) inserted after reducing a large fraction (about two-thirds) of the total pool. If the slower rate observed during this later part of the photoreduction kinetics were limited by oxidized quinone crossing the LH1 barrier, whereas the "internal pool" would be reduced, then the dark period would allow this diffusional process to continue, reconstituting an oxidized proximal pool. This would predict significantly faster kinetics when resuming the illumination compared with the uninterrupted control. The data of Fig. 5 do not support this prediction. The traces observed when resuming the illumination can be superimposed (open symbols) on curve 1 after a suitable horizontal translation. Fig. 5B shows a zoom of curve 1 and the translated curves 2 and 3. A small fast phase is actually observed (arrow) with an amplitude of ~2 electrons. It corresponds to the binding of an oxidized Qb in the RC pocket during the dark period.

The preceding results show that the only kinetic barrier resulting from the PufX− deletion appears to be located directly at the opening of the Qb pocket and that there is no proximal pool, besides Qb itself, with privileged access to the RC. This suggests that the LH1 ring is in close contact with this region of the RC. Moreover, this barrier exerts a significant but moderate effect, slowing the turnover rate by slightly >2-fold. This is far from the dramatic blocking suggested by previous reports (17, 18), but it makes the photosynthetic competence of other bacteria with a native closed LH1 ring easier to understand.

We wondered whether lowering the temperature would lock the LH1 subunits more tightly and inhibit the quinone turnover in the PufX− mutant. This was not really the case, as illustrated in Fig. 6, which shows photoreduction kinetics run at −22 °C. As described elsewhere (28), in this experiment, the bulk medium was frozen, but it appears that a liquid water phase was still in contact with the donor side of the RC, allowing submillisecond reduction of P+ by TMPD over many turnovers. Briefly stated, this view is substantiated by the following observations. Fig. 6 (inset) shows the kinetics of P+ reduction by TMPD following a single turnover flash on both sides (−5 and −10 °C) of the freezing transition of bulk medium. The freezing temperature was around −7 °C when cooling because of a supercooled state of the medium. (Thawing occurred around 0 °C.) The bulk freezing was accompanied by a 5-fold acceleration of the reaction, which presumably reflects an increase in the TMPD concentration in the liquid phase. The reaction rate was still proportional to the concentration of
the initial negative change is due to P$_b$561 (29). (Oxidized/reduced spectra of this heme can be difference (561–569 nm), reflecting the reduction of changes triggered by the second flash of a series for the wave-
inhibit the reoxidation of the b hemes on the quinone-reducing assay medium was as an electron donor. In both types of membranes, the extent of the change was indicative of the reduction of ~0.25 hemes/RC. The inset shows the absence of a significant change on the first flash, ensuring that the process is trig-
ergated by the release of the quinol formed on the RC on the second flash (arrows).

The quinone turnover is also preserved at ~22 °C, although very much slowed down with respect to room temperature. As pointed out in the preceding article (1), the demonstration of quinone turnover at low temperature supports our proposal that quinones are not diluted among the lipids, but form a particular phase where the Q molecules are still mobile at subzero temperatures and remain accessible to the RC. The slopes of Fig. 6 correspond to turnover rates of ~100 ms$^{-1}$ and (180 ms$^{-1}$) electron/RC for the WT and PufX$^-$ mutant, respectively, hence a slowing by ~100 with respect to room temperature. As seen from the break occurring after the first electron, the Q$_A$Q$_B$Q$_n$ electron transfer has become partly respon-
sible for the slower quinone turnover, as shown elsewhere (28). Irrespective of this complication, the results of Fig. 6 show that the diffusion of quinone across the LH1 ring of the PufX$^-$ mutant still occurs at an appreciable rate at ~22 °C.

**Diffusion of Quinol to the Cytochrome bc$_1$ Complex**—When the quinone pool is oxidized in the dark, the quinol oxidation reaction on the oxidizing site of the cytochrome bc$_1$ complex becomes rate-limited by the arrival of a quinol reduced by the RC (29). To monitor this process, we used ferrocene (E$_{m}^{0}$ = 420 mV) as an electron donor for the RC. The redox potential of the assay medium was ~380 mV, so the high potential chain of the bc$_1$ complex (Fe-S center and cytochrome c$_1$ with E$_{m}^{0}$ = 270–300 mV) was oxidized in the dark. Antimycin A was added to inhibit the reoxidation of the b hemes on the quinone-reducing site of the complex. Fig. 7 shows the kinetics of the absorption changes triggered by the second flash of a series for the wave-
length difference (561–569 nm), reflecting the reduction of heme b$_{561}$ (29). (Oxidized/reduced spectra of this heme can be found in Refs. 30 and 31.) As shown in the inset, no heme b reduction was observed on the first flash: this is a check that no quinol was present in the dark, so the kinetics observed on the second flash correspond to the release of the quinol formed on the Q$_B$ pocket of the RC and its diffusion to the bc$_1$ complex. The half-time thus obtained for the WT was ~7 ms, in agreement with literature data (29). For the PufX$^-$ membranes, the process was slowed by 2-fold ($t_{1/2}$ ~14 ms). We thus again observed a “significant but moderate” effect of the PufX deletion. Similar experiments were reported by Barz et al. (18) and Francia et al. (15), who found a larger slowing (>10-fold) and a pronounced lag of ~9 ms before the onset of the reduction kinetics. In our experiments, the time course of heme b reduc-
tion is sigmoidal, with a lag of only ~2 ms. We do not know the origin of this discrepancy. The major differences in the exper-
imental procedures are the use of a mixture of redox mediators (1–10 μM), but no addition of an exogenous donor to P$_b$ in the experiments of Barz et al. and Francia et al., whereas we added ferrocene and no other mediator. Tentatively, we suggest that there may be a problem with the use of endogenous cytochrome c$_2$ as a donor. These authors noted that the PufX$^-$ membranes were not sealed vesicles, which should entail considerable dilution of the soluble cytochrome.

**Quinone Domains**—As described in the preceding article (1), one can estimate a “size” of the domains where the quinone acceptors can diffuse in the 100-ms time range by measuring the decrease in the extent of the photo reducible pool (denoted as $P$) when inhibiting a variable fraction of the RCs. This decrease is due to quinones that cannot access an active RC anymore. If the diffusion domain includes n centers, this means that all n centers are inhibited: the probability for this is $f^n$, where f is the probability of having one center blocked by the inhibitor. Fig. 8 (closed circles) shows the results of such an experiment with PufX$^-$ membranes. The WT data already shown in the preceding article are plotted for comparison (plus signs). The solid line is a best fit with the function 1 $– f^n$, yielding n = 1.7, which suggests that most quinone domains in the PufX$^-$ membranes include only one or two RCs. The average domain size is clearly smaller than in the WT membranes. As previously noticed, the WT data do not match the theoretical function for a single n, suggesting a more complex distribution of domain sizes than in the mutant.

**Q$_A$ Accumulation during the Photoreduction Process**—In the preceding article (1), we studied the accumulation of Q$_A$ during pool photoreduction in WT membranes and isolated RC-LH1 complexes. The relevant quantity here is the amount of Q$_A^-$ present after the relaxation of the electron transfer equilibrium. Because of the large equilibrium constant implied by the $\Delta E_m^{0}$ ~130 mV between Q$_A^-$ and the pool, one expects that the accumulated Q$_A^-$ reflects the fraction of RCs whose available quinone pool is entirely reduced. In this manner, the
distribution of the quinone/RC stoichiometry can be estimated. The results obtained with the WT were indicative of a relatively broad distribution of this ratio, which appeared to be roughly consistent with the statistical fluctuations corresponding to the quinone domain size. Fig. 9 (closed circles) shows the results obtained with the mutant. As described previously for the WT, we used variable periods of strong illumination to reduce variable fractions of the pool. The electron transfer relaxation of QA after putting the illumination off was monitored to determine the extent of the slow phase reflecting the equilibrated fraction of QA. This determination is unambiguous because the relaxation was completed in <100 ms, whereas the slow phase extended over several seconds. The extent of the slow phase is plotted as a function of the reduced pool (closed circles). Comparison with the WT results (open circles) reveals a dramatic difference between the two strains, with a much larger accumulation of QA in the mutant.

Fig. 9 indicates a surprisingly small (i.e. close to 1) “apparent equilibrium constant” between QA and the Q pool in the PufX mutant. In the interpretation framework that we have developed thus far, this would imply a very broad distribution function for the Q/RC ratio. This distribution would have to be much broader than that of the WT. Fig. 8 shows that, in terms of the number of RCs sharing a common pool, the quinone domains were significantly smaller in the PufX mutant. In itself, this would tend to broaden the distribution, but the effect should be offset by the increased number of quinones/RC present in the PufX membranes. Even if the quinone domains included a single RC (although Fig. 8 indicates the average size is close to two), the statistical fluctuations about a mean of 45 quinones would still fail by far to account for the data. The analysis of the pool distribution in isolated complexes (see preceding article (1)) led us to suggest a cooperative association, implying a self-affinity of the isoprenoid quinones. The consequence of such cooperativity is to broaden the distribution. It seems rather unlikely, however, that the deletion of PufX would result in a major enhancement of this cooperative trend. It thus appears difficult to account for the results of Fig. 9 in terms of a drastic modification of the pool distribution in the mutant, and an alternative explanation is called for. As described below, we found out that the deletion of PufX caused important modifications of the equilibria on the acceptor side of the RC. We believe that this is probably the explanation for the results of Fig. 9 and, more importantly, for the inability of the PufX strain to grow under anaerobic conditions.

The QA Pocket Is Modified in the PufX Mutant—When performing the experiments of Fig. 8, we noticed that the affinity for stigmatellin was significantly lower in the PufX membranes than in the WT. This effect is documented in Fig. 10A, which shows plots of the fraction of inhibited centers as a function of the concentration of added stigmatellin. These membranes contained little cytochrome c, so, in the absence of stigmatellin, the reduction of P− following a flash took place mostly in the seconds range. The fraction of centers with stigmatellin bound to the QA pocket in the dark was estimated from the extent of the 50-ms decay phase due to the P−QA recombination. The stigmatellin concentration for half-inhibition is ~6-fold higher in the mutant (0.89 μM) than in the WT (0.16 μM). Marked differences in the same direction were also observed with other inhibitors of the QA pocket (terbutryn, atrazine, and α-phenanthroline) (data not shown).

Another difference between the WT and PufX mutant concerns the rate of P−QA recombination, which is ~4-fold slower in the mutant. This is illustrated in Fig. 10B, which shows the kinetics obtained in RC-LH1 complexes. (The effect was also observed in membranes, but the unambiguous determination of the P−QA− kinetics was complicated, on this slow time scale, by the possible interference of cytochrome c2.) No difference was found between monomeric (open circles) and dimeric (open inverted triangles) WT complexes. On the other hand, the rate of P−QA recombination in the presence of a saturating concentration of stigmatellin was the same in complexes from the WT and PufX (inset). This indicates that the reason for the slower P−QA− recombination is a larger equilibrium constant ($K_a = (Q_A Q_B)/(Q_A Q_B + 2H^+)$) in the PufX strain.

An increased stabilization of QA− is expected, all things being equal, to decrease the equilibrium constant for the “second electron transfer,” $Q_A \rightarrow Q_B + 2H^+ \rightarrow Q_A Q_B H_2$. This prompted us to search for possible kinetic modifications concerning this step. Fig. 10C shows the decay kinetics of QA−Q_B following the second flash, monitored from the absorption change in the semiquinones at 450 nm. The kinetics of the WT (open circles) have a global half-time of ~60 μs. They are satisfactorily fitted as a sum

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**Fig. 8. Size of quinone domains in PufX membranes.** The extent of the photoreducible pool (P) is plotted as a function of the fraction (f) of centers inhibited by stigmatellin added at variable concentrations. The PufX data (●) are compared with the WT data (○). The solid line is a fit with the function $1 - f^n$, yielding $n = 1.7$. The dashed line is for $n = 2$, and the dotted line is for $n = 1$.

**Fig. 9. Accumulation of QA during pool photo reduction.** The fraction of RCs in the closed state (QA−) was determined as explained below after various durations of strong illumination. The relaxation of QA was probed by measuring the P− absorption change induced by a saturating flash triggered at various times after putting off the continuous illumination. This relaxation phase was completed in <100 ms and was followed by a much slower phase. The data points indicate the extent of this slow phase, reflecting the “equilibrium” of QA− with its acceptor pool. The extent of pool photo reduction (horizontal scale) was determined from the TMPD absorption changes. ● data for PufX membranes; ○ data for WT membranes.
of two exponentials with a half-time of 40 μs (80% of the amplitude) and a slow phase with a half-time of 250 μs. In the PuF− membranes (closed circles), the kinetics are markedly slower, with \( t_{1/2} \approx 330 \mu s \) (single exponential).
either the binding or release step (or both). In the 
PufX
tens of microseconds, so the rate limitation must originate from
transfer reactions are known to occur with half-times of some
Thus, under saturating illumination, the average time for a full
rate as a function of light intensity, we could estimate the rate
investigation involving a modified equilibrium constant in the RC.
seems very unlikely. We propose below an alternative expla-
nation involving a modified equilibrium constant in the RC.
**Quinone Diffusion Rate**—From a study of the pool reduction
rate as a function of light intensity, we could estimate the rate
of the process limiting quinone turnover on the 

much lower apparent equilibrium constant in the PufX−
membranes. This difference is not due to a kinetic effect: it was
observed under quasi-equilibrium conditions in the 100-ms
range after putting the illumination off. In the interpretation
framework developed in the preceding article (1), this would
indicate a much broader distribution of the Q/RC stoichiometry
in the mutant compared with the WT, which, as argued above,
seems very unlikely. We propose below an alternative expla-
nation involving a modified equilibrium constant in the RC.

**Alterations of the RC**—The accepted view concerning the
PufX− mutant was that the RC was unaffected by the deletion
of PufX and that its handicap was the permeation of quinones
through LH1. It was thus a surprise to realize that a number of
features concerning the QA pocket were in fact modified in
the mutant. We found an increased stabilization of the QA−
state, a decreased affinity for the QB pocket inhibitors, and a
6-fold slower rate for the second electron transfer (decay of the
QA− QB− state). In contrast, the P⇌QA− recombination rate and
the E′′ of QA were the same as in the WT.

Our results on the rate of P⇌QB− recombination do not
fully agree with those recently reported by Francia et al. (34).
These authors observed a relatively slow recombination (t1/2 ~
2.3 s) in RC-LH1 complexes for WT dimers as well as for
PufX− monomers, in comparison with the faster rate (t1/2 ~
0.69 s) observed in isolated RCs without LH1 (experiments
performed at pH 7.8). Their conclusion is that the interaction
of the RC with LH1, irrespective of the presence of PufX,
causes a stabilization of the semiquinone QB−. In our experi-
ments, the P⇌QB− recombination rate in fresh WT RC-LH1
complexes (monomeric or dimeric), with t1/2 ~ 1.2 s (at pH 8),
is close to that observed in chromatophores of PufX-containing
strains (determined using strain CYC17 (35) devoid of
cytochrome c5 and of its isofrom). On the other hand, we
found a 4-fold slower rate (t1/2 ~ 4.9 s) in the RC-LH1 com-
plexes from the PufX− mutant. Thus, we believe that the LH1
interaction with the RC is dependent on the presence of PufX.
We have no discrepancy, however, regarding the fact that the
P⇌QB− recombination rate is faster in isolated RCs. (This

![Fig. 11. Two models for quinone access to the RC in the PufX−
RC-LH1 complex. A, a fraction of the quinone pool is present within
the internal space between the LH1 ring and the RC, and these quino-
nes have direct access to the QB pocket. B, LH1 is applied against the
mouth of the QB pocket, and there is no internal pool directly accessible
to QB. This does not exclude the possible presence of quinones in the
internal space, as pictured in B.](http://www.jbc.org/fig11.png)
was also observed for *R. capsulatus* (26). There are a number of functional differences between isolated RCs and membranes (21, 23), and further investigation is required to determine which features are specifically controlled by the interaction with LH1 or by the lipid/detergent environment.

Can the increased stabilization of $Q_B$ in the *PufX* strain have significant functional consequences? Titrations of the redox couples involved in the $Q_AQ_B$ system were reported by Rutherford and Evans (36). When lowering the ambient redox potential, one first observes the appearance of a semiquinone wave, ascribed to the $Q_B/QB$ couple, with $E_m \approx 40$ mV (at pH 8). A semiquinone decrease then takes place around $-40$ mV, ascribed to the second reduction step ($Q_B^*/QB-H_2$). A final reduction wave occurs around $-80$ mV, reflecting $Q_A$ reduction. These $E_m$ values predict an equilibrium constant for the second electron transfer ($K_{sec} = [Q_AQ_B-H_2]/[Q_A^*QB]$) of $\sim 5$. If such is the case, the decay kinetics of $Q_A^*QB$ should be biphasic, with a fast phase of relative amplitude 5/6 reflecting the relaxation of the above equilibrium and a slower 1/6 phase caused by the dissociation of $Q_AH_2$. Our results with WT membranes (Fig. 10C, open circles) are consistent with this prediction (40- and 250-$\mu$s phases with 80:20 weights). In the *PufX* membranes, the higher potential of the $Q_A/QB$ couple* indicated by the slower $P^*QB$ recombination is expected (all things being equal, e.g. the binding energy for the quinol form) to result in a lower potential for the $Q_A/QB-H_2$ couple, thus decreasing $K_{sec}$ possibly below 1. The slowing of the second electron transfer observed in the mutant (Fig. 10C, closed circles) may tentatively be explained in this manner. Rather than a slowing of the electron transfer rate proper, this would be due to an increase in the slow phase reflecting $Q_BH_2$ release. We could not resolve a fast phase, however, which, in this framework, implies that $K_{sec}$ has indeed decreased much below 1, so the reoxidation of $Q_A$ is entirely limited by the release of the quinol.

If the above hypothesis is correct, it may explain the "anomalous" accumulation of $Q_A$ during the photoreduction of the pool in the *PufX* mutant. In domains where a large fraction of the pool is reduced, there would be a significant fraction of $Q_A$ due to the diminished $K_{sec}$. This does not contradict the fact that the overall equilibrium constant between $Q_A$ and the pool is large. As pointed out previously (37), the $Q_AQ_B$ system does not establish spontaneously a global electron transfer equilibrium with the quinone pool. Partial equilibria occur within two families of states, which do not equilibrate with each other. The Even family includes states $Q_AQ_B$, $Q_AQ_B-H_2$, $Q_A^*Q_B$, and $Q_A^*Q_B^*$ (where * stands for the empty $Q_B$ pocket); the Odd family includes $Q_AQ_B^*$, $Q_A^*Q_B$, and $Q_A^*Q_B^*$. Both families will equilibrate only in the presence of a redox mediator able to handle single electron transfer ("global equilibrium"). Then, the equilibrium fractions of $Q_A^*$ and reduced pool, as well as the distribution of the RCs between the two families, will be set according to the large equilibrium constant implied by $\Delta F_m \sim 130$ mV (see preceding article (1)). In the absence of such a mediator, the distribution among the two families is not thermodynamically defined, but depends on the history of the system (e.g. illumination). An excess (with respect to global equilibrium) of RCs in the Even family will result, if $K_{sec}$ is small, in an excess of $Q_A^*$. During an illumination, as a significant fraction of the pool becomes reduced, there will be precisely an accumulation of the RCs in the Even family because of the larger fraction of closed centers in this family. We suggest that this may be the origin of the over-accumulation of $Q_A^*$ in the experiment in Fig. 9.

The *PufX* Phenotype—Deletion of the *PufX* polypeptide leads to the inability of the bacterium to grow photosynthetically under reducing conditions, i.e. in anaerobic Hutner's or Sistrom's medium. Photosynthetic growth remains possible, however, in media that are likely to maintain a relatively oxidizing poise, such as trimethylamine N-oxide and Me$_3$SO (18). In fact, in such media, the growth was found to be quite similar to WT growth. On the other hand, a recovery of photosynthetic growth under reducing conditions has been observed in *PufX* strains devoid of LH1 (38) or with impaired LH1 (10). This was taken as evidence that the *PufX* handicap is the barrier to quinone diffusion put up by the closed LH1 ring. This does not explain, however, the full recovery of photosynthesis under oxidizing conditions, which should not affect the rate limitation exerted by the closed ring on the steady-state cyclic electron flow.

As argued above, it turns out that the slowing of quinone diffusion due to the closed LH1 ring is quite modest. It entails a penalty of $\sim -1$ ms when going into or out of the RC-LH1 complex, which cannot account for the loss of photosynthetic competence. A larger effect is observed for the diffusion time between the RC and bc$_1$ complex. Here, the time penalty due to *PufX* deletion is $-2 \times 7 = 14$ ms on a round trip (meaning a doubling with respect to WT). It is still unlikely that this 2-fold slowing of the diffusion time for quinone shuttling is mainly responsible for the phenotype.

Whereas the quinone turnover rate on the RC is little affected by the *PufX* deletion as long as the quinone pool is mostly oxidized, things are different as the pool becomes substantially reduced. Slow phases are observed in the later part of the photoreduction kinetics, which have no counterpart in the WT. This is not mainly due to a kinetic limitation since this slowing is still observed upon light limitation; the light interruption/resumption experiment shown in Fig. 5 confirms this point. The measurements in Fig. 9 showing the accumulation of $Q_A^*$ in relation to the pool reduction state provide additional information. In these experiments, the amount of $Q_A^*$ was determined after relaxation was achieved, again ruling out a possible distortion due to kinetic limitation. The accumulation of $Q_A^*$ accompanying the reduction of the pool is much larger in the *PufX* mutant than in the WT. For instance, when the pool is 80% reduced, there is $\sim -70\%$ $Q_A^*$ in the mutant compared with 20% in the WT. We propose that, as explained above, this $Q_A^*$ congestion is due to the lowered equilibrium constant $K_{sec}$ of the second electron transfer. This phenomenon would be mainly responsible for the inability of the *PufX* strain to sustain photosynthetic growth in a reducing context. In this respect, it is likely that, under such conditions, some threshold of photosynthetic efficiency is required for growth. Reducing conditions tend to inhibit photosynthesis by accumulating $Q_A^*$. On the other hand, once started, the photosynthetic activity will maintain a partially oxidized acceptor pool by the $\Delta \mu$-driven "reverse" functioning of the NADH dehydrogenase. But this positive feedback cannot be initiated if the RCs are abnormally sensitive to the presence of a reduced pool.

The finding that the *PufX* deletion causes quite significant alterations of the $Q_A$ pocket was unexpected because it is clear that the RC may function perfectly well in the absence of this polypeptide. Such is the case, for instance, in the isolated RC solubilized with detergents. It is also the case in LH1-lacking strains, where the deletion of *PufX* has no consequence. This leads to the idea that *PufX* acts as an *antidote* to some *harmful effect* of LH1. We suggest that the closed LH1 ring is a problem not because it blocks quinone diffusion, but because its association with the RC induces a conformational change that results in a malfunction of the $Q_B$ pocket. The role of *PufX* is to prevent

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*Here, the oxidized partner of the couple, denoted "$Q_{in}$" covers in fact the equilibrated bound and dissociated states of the secondary quinone. The same applies to "$Q_{in}H_2" when denoting the $Q_{in}/Q_{in}H_2$ couple.*
this faulty interaction by nucleating an appropriate supramolecular arrangement. This may also be the role of polypeptide W in *R. palustris*, resulting in a different structure of the RC-LH1 complex. If this view is correct, this problem must have been solved in another way by bacterial strains with a closed LH1 ring and no equivalent of PuF X or polypeptide W, such as *B. viridis*, *R. rubrum*, *R. photometricum*, etc. This could be achieved by modifying the RC-LH1 interface or by reinforcing the Qb pocket.

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Functional Study of PuF X—Membranes

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