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$P^+Q_A^-$ and $P^+Q_B^-$ Charge Recombinations in *Rhodopseudomonas viridis* Chromatophores and in Reaction Centers Reconstituted in Phosphatidylcholine Liposomes. Existence of Two Conformational States of the Reaction Centers and Effects of pH and *o*-Phenanthroline

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ABSTRACT: The $P^+Q_A^-$ and $P^+Q_B^-$ charge recombination decay kinetics were studied in reaction centers from *Rhodopseudomonas viridis* reconstituted in phosphatidylcholine bilayer vesicles (proteoliposomes) and in chromatophores. P represents the primary electron donor, a dimer of bacteriochlorophyll; Q_A and Q_B are the primary and secondary stable quinone electron acceptors, respectively. In agreement with recent findings for reaction centers isolated in detergent [Sebban, P., & Wraight, C. A. (1989) *Biochim. Biophys. Acta 974*, 54-65] the $P^+Q_A^-$ decay kinetics were biphasic (k_{fast} and k_{slow}). Arrhenius plots of the kinetics were linear, in agreement with the hypothesis of a thermally activated process (probably via P^+I^-; I is the first electron acceptor, a bacteriopheophytin) for the $P^+Q_A^-$ charge recombination. Similar activation free energies (ΔG) for this process were found in chromatophores and in proteoliposomes. Significant pH dependences of k_{fast} and k_{slow} were observed in chromtophores and in proteoliposomes. In the pH range 5.5-11, the pH titration curves of k_{fast} and k_{slow} were interpreted in terms of the existence of three protonable groups, situated between I^- and Q_A^-, which modulate the free energy difference between P^+I^- and $P^+Q_A^-$. In proteoliposomes, a marked effect of *o*-phenanthroline was observed on two of the three p*K*s, shifting one of them by more than 2 pH units. On the basis of recent structural data, we suggest a possible interpretation for this effect, which is much smaller in *Rhodobacter sphaeroides*. The decay kinetics of $P^+Q_B^-$ were also biphasic. Marked pH dependences of the rate constants and of the relative proportions of both phases were also detected for these decays. The major conclusion of this work comes from the biphasicity of the $P^+Q_B^-$ decay kinetics. We had suggested previously that biphasicity of the $P^+Q_A^-$ charge recombination in *Rps. viridis* comes from nonequilibrium between protonation states of the reaction centers due to comparable rates of the protonation events and charge recombination. This hypothesis does not hold since the $P^+Q_B^-$ decays occur on a time scale ($\tau \approx 300$ ms at pH 8) much longer than protonation events. This leads to the conclusion that k_{fast} and k_{slow} (for both $P^+Q_A^-$ and $P^+Q_B^-$) are related to conformational states of the reaction centers, existing before the flash. In addition, the fast and slow decays of $P^+Q_B^-$ are related to those measured for $P^+Q_A^-$, via the calculations of the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ apparent equilibrium constants, K_2. Finally, these two "conformations", which could arise from different interactions between Fe^{2+} (situated between Q_A and Q_B) and the quinone acceptors or between I and Q_A, behave as two independent components. They have their own ΔG, pH dependence, pH dependence of K_2, and slightly different absorption change spectra, which can be separated near the isosbestic point at 833 nm. Ionic conditions, pH, and *o*-phenanthroline have notable effects on the relative proportions of the two phases. However, the meaning of these two populations is still unclear, as is their possible importance in the reaction center's function.

The electromagnetic energy absorbed by the antenna of photosynthetic organisms is transferred to the photochemical reaction centers. This energy is then stabilized as a transmembrane charge separation. A main step for the understanding of this process has been accomplished with the crystallization and X-ray structural analysis of the reaction center proteins from the purple bacteria *Rhodopseudomonas viridis* (Deisenhofer et al., 1985; Michel & Deisenhofer, 1988; Michel et al., 1986) and *Rhodobacter sphaeroides* (Allen et al., 1988; Chang et al., 1986; Ducruix & Reiss-Husson, 1987; Komiya et al., 1988; Yeates et al., 1988). The reaction center consists of three polypeptides, L, M, and H, with molecular weights between 30 000 and 35 000. In *Rps. viridis*, a tightly bound cytochrome (40 kDa) containing four *c*-type hemes

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(Weyer et al., 1987) is found. A high sequence homology is found between the L and M polypeptides and the equivalent polypeptides (D1 and D2) of the reaction centers from the PSII of plants (Komiya et al., 1988; Michel & Deisenhofer, 1988).

The L and M polypeptides contain all the pigments involved in the primary electron transport: four bacteriochlorophyll molecules, two bacteriopheophytins, two quinone molecules, one non-heme iron, and one carotenoid molecule. The first charge separation is accomplished between the primary electron donor, a dimer of bacteriochlorophylls (P), and the primary electron acceptor, a bacteriopheophytin molecule (I) (Kirmaier & Holten, 1987). The electron is then transferred to a primary quinone molecule (Q_A, located in M) and then to a secondary quinone molecule (Q_B, located in L). In *Rb. sphaeroides*, Q_A and Q_B are ubiquinones, whereas in *Rps. viridis* Q_A is a menaquinone and Q_B a ubiquinone. In the absence of electron donors to P^+ or when they are oxidized, charge recombination between P^+ and Q_B^- is observed. In *Rps. viridis*, at pH 7, this proceeds in about 100 ms in reaction centers purified in detergent (Shopes & Wraight, 1987) and in about 400 ms in chromatophores (D. E. Fleischman, personal communication). When Q_B is absent or extracted, or in the presence of an inhibitor of the Q_A to Q_B electron transfer, charge recombination between P^+ and Q_A^- is induced. This occurs in about 1.5 ms at pH 8 in *Rps. viridis* reaction centers (Sebban & Wraight, 1989; Shopes & Wraight, 1987). Because of the lower energy barrier between the $P^+Q_A^-$ and P^+I^- states in *Rps. viridis*, compared to *Rb. sphaeroides*, this recombination takes place in *Rps. viridis* via a thermally activated state, probably a relaxed form of the P^+I^- state (Kleinfeld et al., 1985; Gopher et al., 1985; Gunner et al., 1986; Sebban, 1988a; Sebban & Wraight, 1989; Shopes & Wraight, 1987; Woodbury et al., 1986). It has been shown recently that the $P^+Q_A^-$ recombination kinetics are not monophasic (Sebban & Wraight, 1989), at variance with what is observed in the reaction centers from *Rb. sphaeroides*. The relative amplitudes of the two observed components (k_{slow} and k_{fast}) were found to be very pH sensitive and were dependent on the presence of *o*-phenanthroline. Since the $P^+Q_A^-$ kinetics were nearly monoexponential at low pH, but were becoming more and more heterogeneous as the pH was raised, these authors interpreted their results in terms of nonequilibrium of different protonation states of the reaction center, the protonation events being must faster than the recombination at low pH, but not when pH was >8.

In this work, we have studied these processes in chromatophores and in reaction centers incorporated in phosphatidylcholine bilayers. In addition to $P^+Q_A^-$, we do find the same type of heterogeneity in $P^+Q_B^-$. In contrast to the above hypothesis, our data suggest that the two states exist before the flash and are not induced during the charge separation.

Materials and Methods

Wild-type *Rps. viridis* cells were grown anaerobically (N_2 and CO_2) in light in Hunter medium. Reaction centers were prepared as previously described (Prince & Dutton, 1976). Liposomes were prepared by using egg yolk phosphatidylcholine from Lipid Products. Proteoliposomes were prepared following the same method as for reaction centers from *Rb. sphaeroides* (Sadler et al., 1984). Dried lipids were mixed with 10 mM Tris, 100 mM NaCl, and 0.1 mM EDTA,[1] pH 8 (buffer), at a concentration of 50 mg/mL, shaken in a vortex, and then ultrasonicated. The suspension was then centrifuged at 100000*g* during 1 h to eliminate the multilayer vesicles. The reconstitution of the reaction center proteins into the liposomes was achieved as follows. The LDAO reaction center solution (≈20 μM) was added to the liposomes suspended in the buffer solution to produce a lipid to protein ratio of 4. The success of the reconstitution was first checked by an increase of the sample turbidity which was commonly observed wihin 5–10 min. It must be noted that the completion of the reconstitution was very dependent on the degree of purity of the reaction center preparation. Typically, the A_{280nm}/A_{830nm} ratio had to be in the range 2.1–2.3. Moreover, the presence of NaCl was apparently important to get a good reconstitution. Attempts to reconstitute reaction centers into liposomes without NaCl led to substantial precipitation. Another parameter to monitor was the final LDAO concentration. It has to be maintained above 0.12% before the preparations are loaded on the Sepharose CL-4B column (see below).

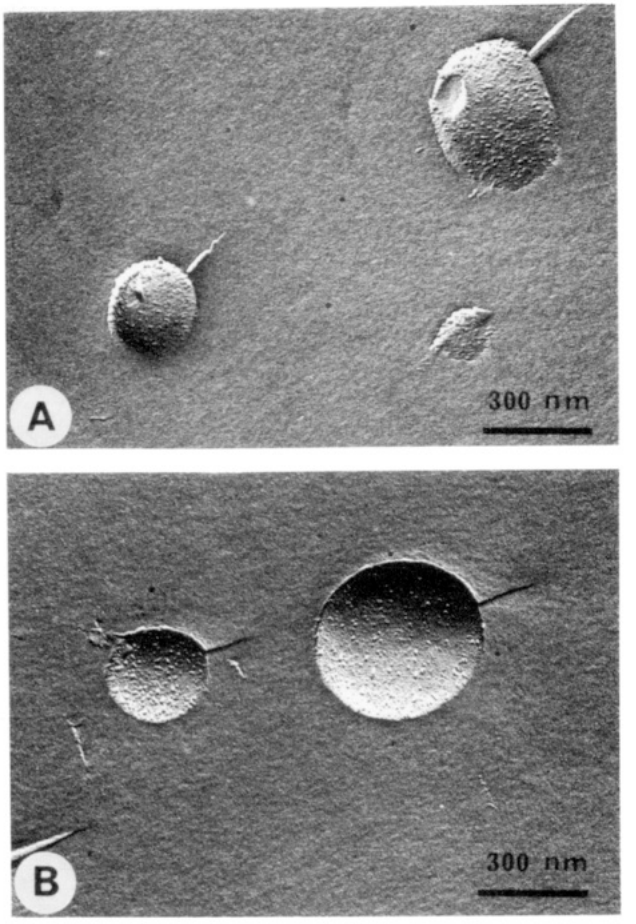

FIGURE 1: Freeze-fracture electron micrographs of the proteoliposomes. The protein to lipid ratio was 0.25 (w/w). Convex (A) and concave (B) fracture faces. Magnification 49100×.

The detergent–lipid mixed micelles were separated from the reconstituted liposomes by gel chromatography. The proteoliposome solution was loaded on a Sepharose CL-4B column previously equilibrated with the buffer and then saturated with the liposome solution, and equilibrated again with the same buffer. The reconstituted proteoliposomes were eluted in the void volume. A freeze-fracture electron micrograph of these vesicles is represented in Figure 1.

Phosphorus content was determined by the method of Barlett (1959) and phosphatidylcholine content calculated with a multiplying factor of 25. Protein concentration was measured by the method of Peterson (1977) with bovine serum

[1] Abbreviations: Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)-methylamino]propane; LDAO, lauryl dimethylamine *N*-oxide; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

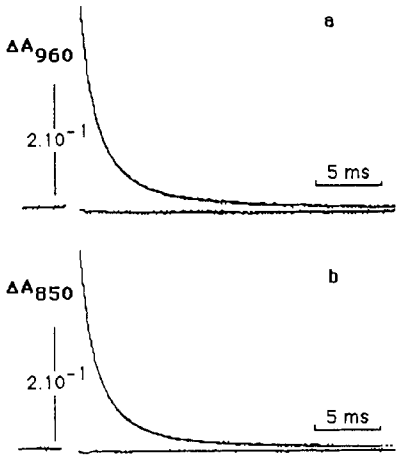

FIGURE 2: $P^+Q_A^-$ charge recombination decay kinetics from *Rps. viridis* reaction centers reconstituted in phosphatidylcholine vesicles (a) and in chromatophores (b), at 960 and 850 nm, respectively. The data are fitted by the sum of two exponentials with lifetimes of 0.68 ± 0.05 (50%) and 2.25 ± 0.2 ms (50%) in proteoliposomes and 0.54 ± 0.05 (40%) and 2.2 ± 0.2 ms (60%) in chromatophores. The differences between the experimental and the fitting curves are shown below. Conditions: 20 mM Bis-Tris propane (pH 9)/100 mM NaCl/4 mM *o*-phenanthroline. 50 μM ferricyanide was added to keep the $E_h \approx 440$ mV, to maintain the bound cytochromes oxidized.

albumin as a standard. Under these conditions, the lipid/protein ratio was estimated to be 245 mol of phospholipid/mol of reaction center.

The light–dark difference absorption spectrum of the proteoliposomes was superimposable to that of the reaction centers isolated in detergent. To determine the amount of cytochromes exposed to the outside of the vesicles membrane, the oxidized (1 mM potassium ferricyanide plus 1 mM potassium ferrocyanide)–reduced ($Na_2S_2O_4$ plus 1 mM ferrocyanide) absorbance difference spectra were measured near 550 nm. From the comparison with the amount of photochemically oxidized dimer, it was deduced that 1 ± 0.15 cytochrome was oxidized per oxidized dimer. As charged molecules such as ferricyanide are hardly likely to penetrate the bilayer membrane, it seems probable that the cytochrome units are mostly oriented to the outside of the membrane.

The flash absorption spectroscopy apparatus was the same as previously described (Sebban, 1988a,b).

pH buffers used were 2-(*N*-morpholino)ethanesulfonic acid (MES) (Sigma) between pH 5.5 and 6.5, 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris propane) (Sigma) between pH 6.3 and 9.5, and 3-(cyclohexylamino)-propanesulfonic acid (CAPS) (Calbiochem) above pH 9.5.

For the $P^+Q_B^-$ decay measurements, ubiquinone 10 (Sigma) was added with an excess of about 20.

The temperature was monitored by using a NiCr–Ni thermometer with a precision of ±0.3 °C. For the activation energy measurements, pH was measured in line and readjusted at different temperatures.

Results

$P^+Q_A^-$ Charge Recombination Kinetics in Proteoliposomes and Chromatophores. (a) Biphasicity of the Recombination Kinetics. The $P^+Q_A^-$ charge recombination kinetics, observed in proteoliposomes at 960 nm and in chromatophores at 850 nm, at pH 9, in the presence of *o*-phenanthroline, are shown in parts a and b of Figure 2, respectively. In chromatophores the decay is fit by the sum of two exponentials ($\tau_{fast} = 1/k_{fast}$ and $\tau_{slow} = 1/k_{slow}$) with lifetimes of 0.54 ± 0.05 (40%) and 2.2 ± 0.15 ms (60%). For proteoliposomes, these lifetimes are 0.68 ± 0.05 (50%) and 2.2 ± 0.15 ms (50%). The decay rate

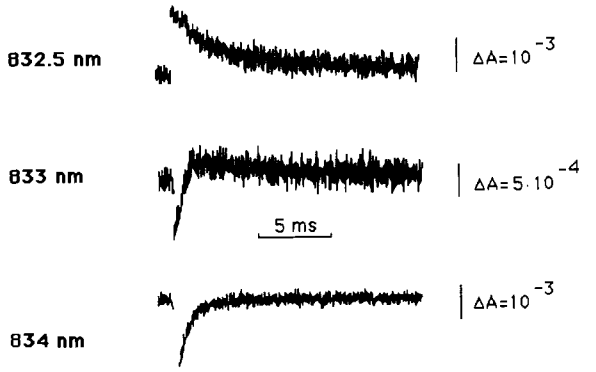

FIGURE 3: Room temperature decay kinetics of the $P^+Q_A^-$ charge recombination measured near the isosbestic point at 833 nm in chromatophores, at pH 9. At 832.5 nm, the decay is nearly exponential with lifetime of the slow component (see the text). At 833 nm, both components are present, with opposite contributions to the absorption changes. At 834 nm, the decay is again close to exponential, but with lifetime of the fast component (see text). Conditions as in Figure 2.

constants are slightly accelerated compared to those of reaction centers isolated in LDAO (0.83 and 2.8 ms) (Sebban & Wraight, 1989). The calculated exponential fitting of Figure 2 was the same at 800, 850, and 960 nm. However, as was already shown by these authors, the ratio of the two components changes dramatically near the isosbestic point at 833 nm. We have presented in Figure 3 the decay kinetics observed in chromatophores just above this point, at this point, and just below (these data were obtained in collaboration with P. Parot and A. Verméglio). At the isosbestic point, the two decays are observed with opposite signs, suggesting a slightly different spectrum for the fast and the slow components. At 832.5 nm the slow component could be isolated almost pure. Similarly, at 834 nm, the observed decay was close to exponential with the lifetime of the fast component [similar decays were obtained in proteoliposomes (data not shown)]. The exponential fitting analysis was performed with a program using the Marquardt algorithm. However, we have also fitted the decay kinetics with a nonminimization program based on the Laplace transform method (Yeramian & Claverie, 1986) which does not need any hypothesis on the number of exponentials. Even in that case, the program focused on a two exponentials fit with nearly the same values as mentioned above. This supports the existence of two well-defined kinetic states for the $P^+Q_A^-$ charge recombination in *Rps. viridis*.

(b) Arrhenius Plots of k_{fast} and k_{slow}. The Arrhenius plots of k_{total} (the overall rate constant), k_{slow}, and k_{fast} for chromatophores and proteoliposomes are shown in Figure 4a,b. Low-temperature (down to 80 K) behavior of the $P^+Q_A^-$ charge recombination decays in reaction centers from *Rps. viridis* has previously been reported (Sebban & Wraight, 1989; Shopes & Wraight, 1987). These authors observed a thermally activated process down to about 240 K and a plateau from 240 to 80 K. The former phenomenon, which dominates at room temperature, was suggested to arise from reexcitation of the P^+I^- state from $P^+Q_A^-$. The latter, with no activation energy, was attributed by Shopes and Wraight to a direct recombination to the ground state, via an electron-tunneling process. For these reasons, and because P^+I^- and $P^+Q_A^-$ are in rapid equilibrium (Holten et al., 1978), it was proposed that

$$k_{total} = k_d \exp(-\Delta G_{total}/k_B T) + k_T$$
$$k_{slow} = k_d \exp(-\Delta G_{slow}/k_B T) + k_{Tslow} \quad (1)$$
$$k_{fast} = k_d \exp(-\Delta G_{fast}/k_B T) + k_{Tfast}$$

where ΔG_{total}, ΔG_{slow}, and ΔG_{fast} are the free energy differences

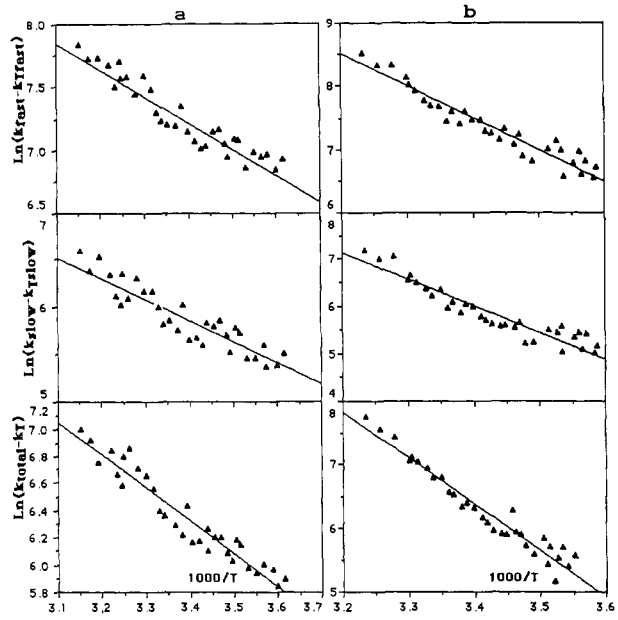

FIGURE 4: Arrhenius plots of the $P^+Q_A^-$ charge recombination kinetics in *Rps. viridis* reaction centers reconstituted in liposomes (a) and in chromatophores (b). According to eq 1, the limiting rates reached a low temperature are subtracted from the observed rate constants. For both samples, we used the values obtained for chromatophores at 80 K, without glycerol (Sebban and Mathis, unpublished data): $k_T = 170\ s^{-1}$, $k_{Tslow} = 150\ s^{-1}$, and $k_{Tfast} = 600\ s^{-1}$. Conditions as in Figure 2.

between $P^+Q_A^-$ and P^+I^- and k_B is Boltzmann's constant. k_d is the kinetic rate constant of charge recombination from the P^+I^- state, after back electron tranfer from $P^+Q_A^-$. It must be pointed out that the Arrhenius plots of the total decay are only indicative and do not have a true physical meaning since the decay is not exponential and cannot be considered as a true first-order kinetic rate constant. We consider it here as an average value that helps in comparing our data with previous data which did not take into account the biphasicity of the charge recombination decays.

For the same reasons as previously discussed (Sebban & Wraight, 1989; Shopes & Wraight, 1987), we used $k_d = 2 \times 10^7\ s^{-1}$. However, taking $k_d = 7 \times 10^7\ s^{-1}$, the direct measurements of the P^+I^- decay rate constant (Holten et al., 1978), would only increase the ΔG values by about 30 meV. The thermodynamic parameters derived from the data of Figure 4 are represented in Table I. We used for k_T, k_{Tslow}, and k_{Tfast} the values obtained from the low-temperature (80 K) $P^+Q_A^-$ charge recombination kinetics from chromatophores in the presence of *o*-phenanthroline and without glycerol (Sebban and Mathis, unpublished results): $k_T = 170\ s^{-1}$, $k_{Tslow} = 150\ s^{-1}$, and $k_{Tfast} = 600\ s^{-1}$. Since the $P^+Q_A^-$ decay lifetimes measured at room temperature in chromatophores are close to that in reaction centers incorporated in vesicles, we took the above k_T, k_{Tfast}, and k_{Tslow} values for both samples. As shown in Table I, we find close ΔG values in chromatophores and in proteoliposomes: $\Delta G_{total} = 0.253 \pm 0.01$ and 0.259 ± 0.01 eV, $\Delta G_{slow} = 0.263 \pm 0.02$ and 0.271 ± 0.02 eV, $\Delta G_{fast} = 0.227 \pm 0.03$ and 0.237 ± 0.03 eV, respectively. These figures are slightly smaller, by about 30 meV, than the ΔG values measured for the reaction centers isolated in LDAO. These small differences are inside the error bars. However, it is possible that the electrostatic effect of the LDAO molecules surrounding the reaction center, and interacting with the $P^+Q_A^-$ state, accounts for these small differences. At variance with the free energy values, the enthalpic and entropic contributions are extremely different in chromatophores and

Table I: Activation Parameters, at pH 9, for the $P^+Q_A^-$ Charge Recombination in Reaction Centers from *Rps. viridis* Reconstituted in Phosphatidylcholine Vesicles and in Chromatophores[a]

component	ΔH (eV)	$-T\Delta S$ (eV)	ΔG (eV)
	Proteoliposomes		
total	0.204 (±0.012)	0.055 (±0.012)	0.259 (±0.010)
fast	0.176 (±0.030)	0.062 (±0.020)	0.237 (±0.030)
slow	0.186 (±0.025)	0.085 (±0.025)	0.271 (±0.020)
	Chromatophores		
total	0.556 (±0.012)	−0.303 (±0.012)	0.253 (±0.010)
fast	0.427 (±0.030)	−0.200 (±0.020)	0.227 (±0.030)
slow	0.470 (±0.025)	−0.206 (±0.025)	0.263 (±0.020)

[a] $T = 295$ K. For both samples, k_T, k_{Tfast}, and k_{Tslow}, the low-temperature limiting values for the rate constants, were taken at 170, 600, and 150 s^{-1} as determined by Sebban and Mathis (unpublished data) in the chromatophores at low temperature (80 K) in the absence of glycerol.

vesicles. The difference between both samples in $T\Delta S$ terms is about 0.26–0.3 eV. This may suggest a strong influence of the surrounding lipids on the degree of motion of the electron carriers. The chromatophore membrane is mainly composed by phospholipids, only about 25% of total lipids being phosphatidylcholine (Niedermann & Gibson; 1978; Rivas et al., 1987). It is possible that the difference of the head-groups charge and area between the phospholipids of the in vivo membrane and those of the vesicles cause different flexibility of the hydrocarbon chains inside the bilayers. This could affect the motion of the polypeptides of the reaction centers. The above effect could also be induced by the differences between the length of the aliphatic chains of the egg yolk phosphatidylcholine (used here to prepare the proteoliposomes) and the length of the natural phospholipids of the chromatophore membrane. Alternatively, the above difference between proteoliposomes and chromatophores may arise from interactions, in the chromatophore membrane, between the reaction centers and the surrounding antenna proteins. It has been suggested that, in *Rps. viridis*, close spatial relationships or direct interaction may exist between the reaction center and the B1020 antenna (Drews, 1985; Jay et al., 1984; Peters et al., 1984; Takemoto et al., 1982). Thus, it is possible that the reaction center surrounding antenna play a role in the charge stabilization process.

The ΔG values of both phases are consistent with the independent measurements of the rate constants at pH 9. According to eq 1, one would expect the ratio $(k_{fast} - k_{Tfast})/(k_{slow} - k_{Tslow})$ to match the term $\exp(\delta\Delta G/k_BT)$, where $\delta\Delta G = \Delta G_{slow} - \Delta G_{fast}$. Taking $k_{Tslow} = 150\ s^{-1}$ and $k_{Tfast} = 600\ s^{-1}$, as justified above, leads to an equivalence of both terms within 5% in chromatophores and 15% in proteoliposomes. This consistency between the kinetic and the energetic parameters for k_{fast} and k_{slow} suggests again that they are related to two well-defined $P^+Q_A^-$ states which are not in equilibrium, at least during the time scale of the lifetime of the $P^+Q_A^-$ state.

(c) pH Dependence of the $P^+Q_A^-$ Charge Recombination Kinetics in Proteoliposomes. The pH dependences of the $P^+Q_A^-$ charge recombination kinetics of the reaction centers from *Rps. viridis*, reconstituted in phosphatidylcholine vesicles, in the presence and absence of *o*-phenanthroline, are shown in parts a and b of Figure 5, respectively. These curves were obtained by a two-component decomposition of the decays measured at 960 nm. These curves display a shape similar to that for solution of reaction centers in detergent (Sebban & Wraight, 1989; Shopes & Wraight, 1987).

In the presence of *o*-phenanthroline, at pH above about 9.5, k_{total} accelerates. In fact, the pH values at which k_{fast} and k_{slow} accelerate are not the same. k_{fast} is roughly constant from pH

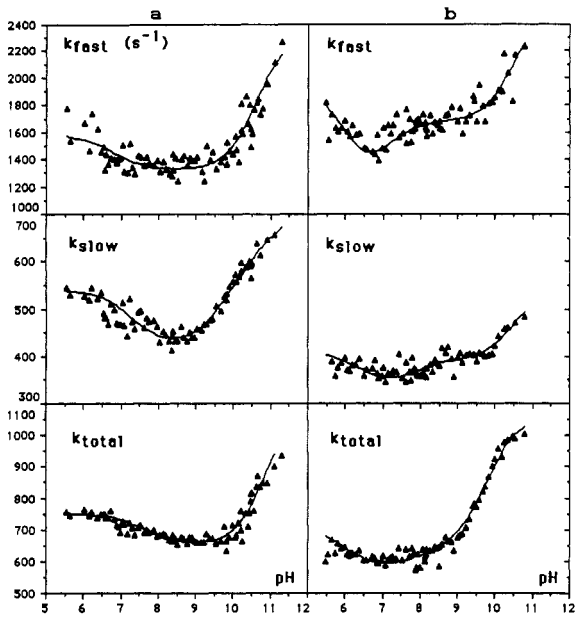

FIGURE 5: pH dependence of the $P^+Q_A^-$ charge recombination kinetics in proteoliposomes, in the presence (a) and absence (b) of *o*-phenanthroline. The lines are drawn according to eq 2 (see Discussion). Conditions: 100 mM NaCl, 21 °C, buffers (see Materials and Methods).

7.5 to about pH 9.5, whereas k_{slow} never reaches a plateau and increases above pH 9. Both rate constants display a nearly 2-fold increase from pH 8.5 to pH >11. At low pH, k_{fast} and k_{slow} both slow down from pH 5.5 to pH 8. As shown in Figure 7a, the relative amplitudes of k_{fast} and k_{slow} (A_{fast} and A_{slow}) are nearly pH independent, except maybe some slight relative increase of A_{fast} at high pH (>10.5).

In the absence of *o*-phenanthroline, the pH at which k_{total}, k_{fast}, and k_{slow} start to increase is substantially lower. This occurs at pH values of about 7.5, 7, and 7.3, respectively. Similarly, the observed decrease of the rate constant at low pH is observed at lower pH values than in the presence of *o*-phenanthroline. At variance with what occurs when *o*-phenanthroline is present, there is a marked effect of pH on A_{fast} and A_{slow} (Figure 7b). Whereas A_{slow} dominates below pH 8, both components become roughly equal from pH 8 to pH 9, and A_{fast} increases up to about 65% at pH 10.5. This behavior for the amplitudes of the two phases is similar to what we observed previously in the reaction centers from *Rps. viridis* in LDAO, in the presence or absence of *o*-phenanthroline (Sebban & Wraight, 1989).

(d) pH Dependence of the $P^+Q_A^-$ Charge Recombination Kinetics in Chromatophores. The pH dependence of k_{total}, k_{fast}, and k_{slow} in chromatophores, in the presence of *o*-phenanthroline, is shown in Figure 6. The different titration waves observed in these curves occur at slightly lower pH values than in proteoliposomes, in the presence of *o*-phenanthroline. This is especially verified for k_{slow}. The A_{fast} and A_{slow} titration curves are shown in Figure 7c. They display the same behavior as for reaction centers in detergent. k_{slow} is by far the main component (75%) below pH 8. As pH increases, the amplitudes start inverting. Above pH 10, A_{fast} dominates. A plateau seems to be reached at pH 10.8, where A_{fast} and A_{slow} are equal to about 60% and 40%, respectively.

$P^+Q_B^-$ Charge Recombination in Reaction Centers, in Proteoliposomes, and in Chromatophores. (a) Biphasicity of the Recombination Kinetics. The $P^+Q_B^-$ charge recombination kinetics also were found to be biphasic in reaction centers, proteoliposomes, and chromatophores. The $P^+Q_B^-$ decay kinetics from proteoliposomes and chromatophores, at

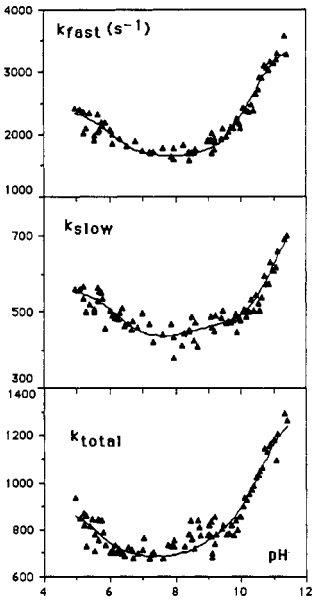

FIGURE 6: pH dependence of the $P^+Q_A^-$ charge recombination kinetics in chromatophores, in the presence of 4 mM *o*-phenanthroline. The lines are drawn according to eq 2. Conditions as in Figure 5.

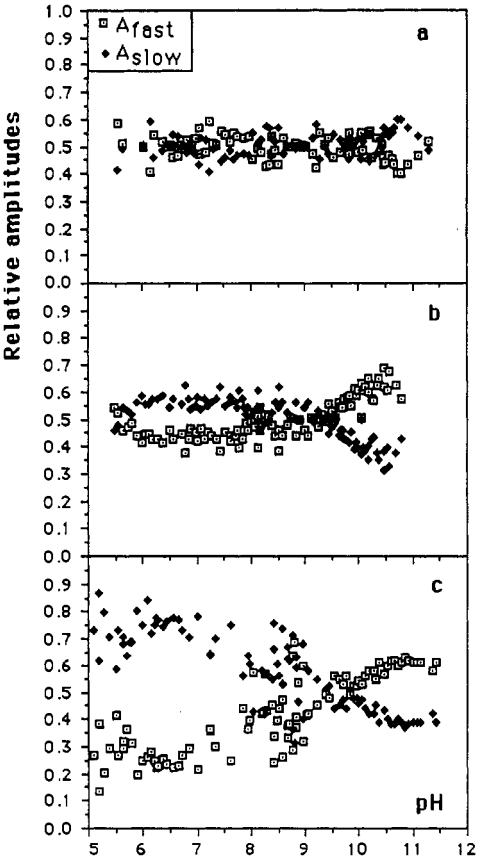

FIGURE 7: pH dependence of the relative amplitudes of the slow and the fast kinetic phases of the $P^+Q_A^-$ charge recombination in *Rps. viridis* reaction centers reconstituted in phosphatidylcholine vesicles: (a) +4 mM *o*-phenanthroline; (b) no *o*-phenanthroline; (c) in chromatophores in the presence of 4 mM *o*-phenanthroline. Conditions as in Figure 5.

pH 9, are shown in parts a and b of Figure 8, respectively. Except for the presence of a long component ($\tau > 3$ s) with an initial amplitude of <5%, the decays are fit by a two-exponential analysis. For proteoliposomes this decomposition gives $1/k_{fast} = 120 \pm 10$ ms (40%) and $1/k_{slow} = 456 \pm 50$ ms (60%). In chromatophores the curves are decomposed into $1/k_{fast} = 115 \pm 10$ ms (30%) and $1/k_{slow} = 510 \pm 50$ ms

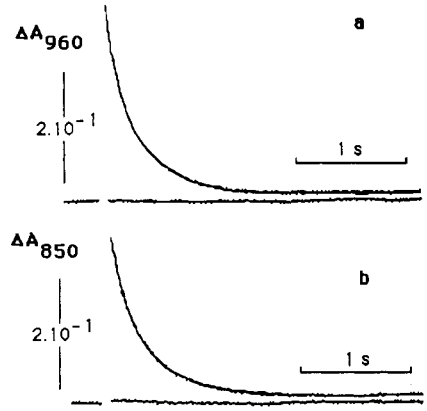

FIGURE 8: $P^+Q_B^-$ charge recombination decay kinetics in proteoliposomes (a) and chromatophores (b), at pH 9 and 21 °C. Except for the presence of a very long component ($\tau > 3$ s) with an initial amplitude of ≈5%, the decays are fitted by the sum of two exponentials. The best fit is obtained with $\tau_{fast} = 120 \pm 10$ ms (40%) and $\tau_{slow} = 456 \pm 50$ ms (60%) in proteoliposomes and with $\tau_{fast} = 115 \pm 10$ ms (30%) and $\tau_{slow} = 510 \pm 50$ ms (70%) in chromatophores. The differences between the experimental and the fitting curves are shown below. Conditions as in Figure 2.

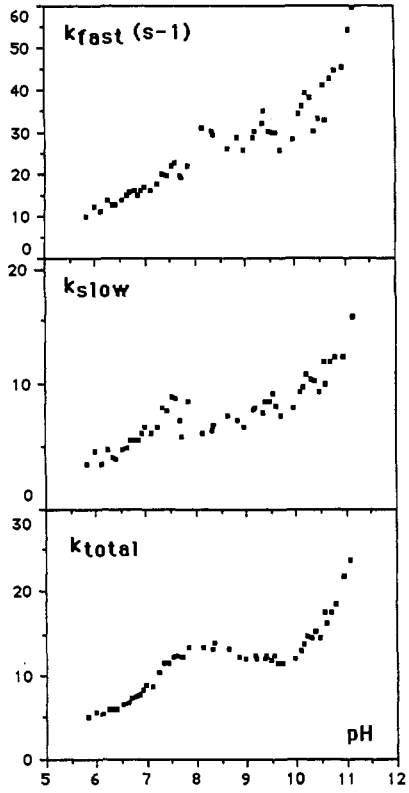

FIGURE 9: pH dependence of the $P^+Q_B^-$ charge recombination kinetics in the reaction centers from *Rps. viridis* isolated in LDAO, 21 °C. Conditions: 100 mM NaCl/0.1% LDAO/100 μM EDTA. For buffers, see Materials and Methods.

(70%). At the same pH, the observed rates are substantially faster in the reaction centers in LDAO. In that case, the analysis gives $1/k_{fast} = 41 \pm 5$ ms (55%) and $1/k_{slow} = 142 \pm 15$ ms (45%). This difference in the $P^+Q_B^-$ charge recombination kinetics between chromatophores and isolated reaction centers was already mentioned by Shopes and Wraight (1985). Since similar recombination rates are observed here in proteoliposomes and chromatophores, it seems that the above difference probably arises from interaction of the detergent with Q_B^- (or with its environment) rather than from a change of the Q_B site during the purification of the reaction centers.

(b) pH Dependence of the $P^+Q_B^-$ Charge Recombination Kinetics. The pH dependence of the $P^+Q_B^-$ charge recombination kinetics in reaction centers in detergent, in proteoliposomes, and in chromatophores is shown in Figures 9, 10, and 11, respectively. Although small differences may be detected, the overall aspect of the curves is roughly the same for the three samples. A substantial increase of the $P^+Q_B^-$ re-

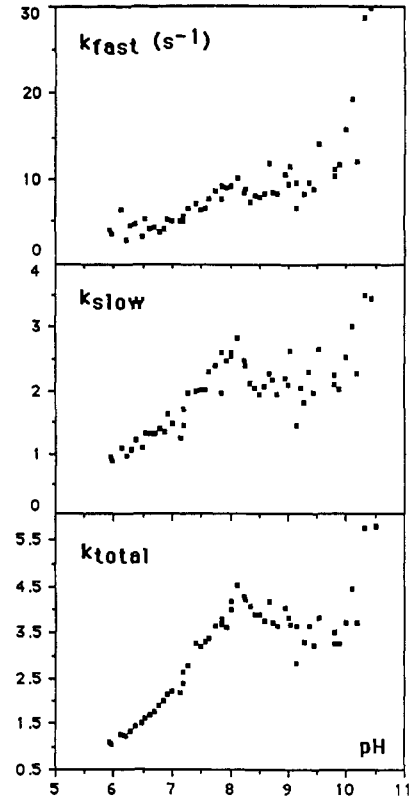

FIGURE 10: pH dependence of the $P^+Q_B^-$ charge recombination kinetics in reaction centers from *Rps. viridis*, reconstituted in phosphatidylcholine vesicles. Conditions as in Figure 5.

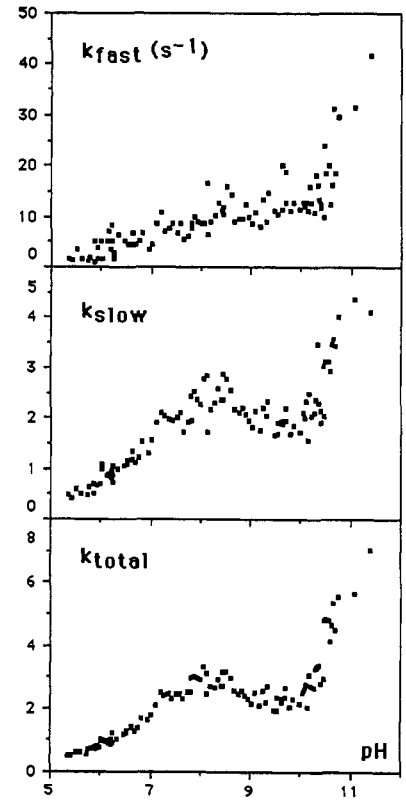

FIGURE 11: pH dependence of the $P^+Q_B^-$ charge recombination kinetics in chromatophores from *Rps. viridis*. Conditions as in Figure 5.

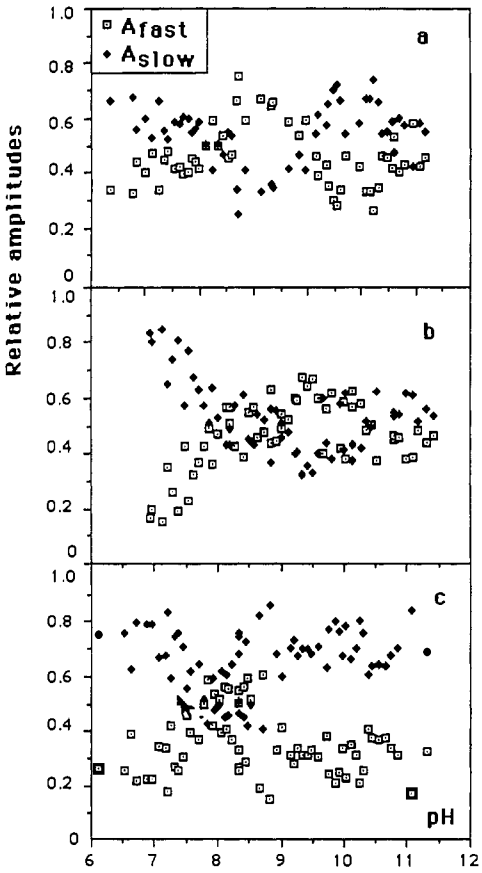

FIGURE 12: pH dependence of the relative amplitudes of the fast and slow decay kinetics from the $P^+Q_B^-$ charge recombination, in *Rps. viridis* reaction centers isolated in LDAO (a), in proteoliposomes (b), and in chromatophores (c). Conditions as in Figures 9–11, respectively.

combination rates occurs between pH 5.5 and pH 7.5–8. Then a plateau or a decrease of the rates is observed from pH 8 to pH 10. Then the rates increase again above pH 10. The relative amplitudes of the two phases for the three samples are displayed in Figure 12. A common behavior is observed. The slow phase dominates at low pH (<6.5 in reaction centers and proteoliposomes; <7.5 in chromatophores) and at high pH (>9.5 in reaction centers and proteoliposomes; >9 in chromatophores). Between these pH values, some changes occur in the reaction centers that lead to an inversion of the two populations or at least to a relative decrease of A_{slow}.

It was already suggested that in *Rb. sphaeroides* P^+ and Q_B^- recombine mainly via the $P^+Q_A^-$ state (Kleinfeld et al., 1984)9. This could also be the case in *Rps. viridis*. Thus, the substantial acceleration of the $P^+Q_B^-$ charge recombination observed above pH 10 probably reflects a diminution of the free energy difference between $P^+Q_A^-$ and $P^+Q_B^-$ caused by the deprotonation of some side chains of amino acids in the vicinity of Q_B. This is in agreement with the data available on *Rb. sphaeroides*, for which a pK of 11.3 was suggested for Q_B (Kleinfeld et al., 1984). In the low pH range, a 2–3-fold increase of the rates is also observed with pH for the reaction centers in detergent. This effect is more pronounced in proteoliposomes and in chromatophores, for which a 4–5-fold acceleration of the $P^+Q_B^-$ recombination is observed in the same pH range (5.5–8). These changes may arise from deprotonation of the side chains (with pK values in the range 6–7) of some amino acids that lie closer to Q_B^- than to Q_A^-.

(c) $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ Apparent Equilibrium Constant of the Two Phases, pH Dependence. As mentioned above, the $P^+Q_B^-$ charge recombination kinetics are substantially slower in chromatophores and in vesicles than in reaction centers in

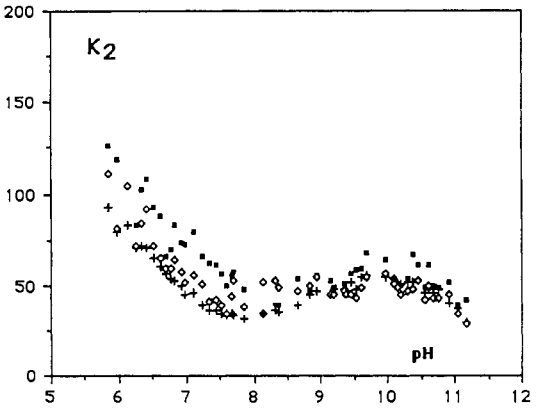

FIGURE 13: pH dependence of the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ apparent equilibrium constant (K_2) in reaction centers in LDAO. K_2 was obtained for each component by using the formula $1 + K_{2i} = k_{fP^+Q_A^-}/k_{fP^+Q_B^-}$ (Wraight, 1981), where $k_{fP^+Q_A^-}$ and $k_{fP^+Q_B^-}$ denote the rate constants of charge recombination from $P^+Q_A^-$ and $P^+Q_B^-$, for the total, fast, or slow phase (i). (+) K_{2total}; (■) K_{2fast}; (◇) K_{2slow}.

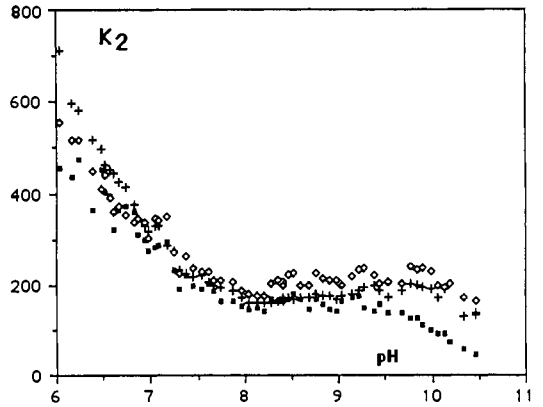

FIGURE 14: pH dependence of the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ apparent equilibrium constant (K_2) in reaction centers reconstituted in liposomes. The calculation of these curves was achieved as in Figure 13. (+) K_{2total}; (■) K_{2fast}; (◇) K_{2slow}.

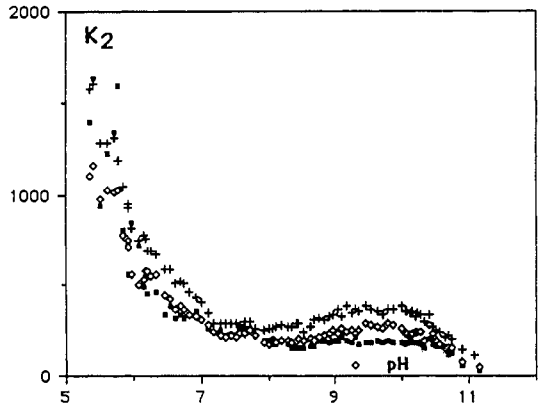

FIGURE 15: pH dependence of the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ apparent equilibrium constant (K_2) in the chromatophores from *Rps. viridis*. The calculation of these curves was achieved as in Figure 13. (+) K_{2total}; (■) K_{2fast}; (◇) K_{2slow}.

LDAO. Interestingly, the $P^+Q_A^-$ kinetics were also slowed down in detergent compared to chromatophores or proteoliposomes.

It is of interest to plot the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ apparent equilibrium constant (K_2) as a function of pH. These curves are displayed in Figures 13, 14, and 15 for reaction centers in detergent, for proteoliposomes, and for chromatophores, respectively. Considering the two phases as independent components, in each case, K_2 was determined by using the formula suggested by Wraight (1981): $1 + K_{2i} = k_{fP^+Q_A^-}/$

Table II: Protonation Parameters Associated with the $P^+Q_A^-$ State in *Rps. viridis*[a]

	proteoliposomes + *o*-Phe	proteoliposomes − *o*-Phe	chromatophores + *o*-Phe	reaction centers + *o*-Phe[b]	reaction centers − *o*-Phe[b]
k_{total}	$pK_3 = 7.65$, $\delta\Delta G = +3$	$pK_3 = 6$, $\delta\Delta G = +6$	$pK_3 = 5.6$, $\delta\Delta G = 9$	$pK_3 = 6.4$, $\delta\Delta G = +9$	$pK_3 = 6.1$, $\delta\Delta G = +23$
	$pK_2 = 9.6$, $\delta\Delta G = -2$	$pK_2 = 7.9$, $\delta\Delta G = -4$	$pK_2 = 9$, $\delta\Delta G = -4.5$	$pK_2 = 8.45$, $\delta\Delta G = -18$	$pK_2 = 8.8$, $\delta\Delta G = -39$
	$pK_1 = 10.95$, $\delta\Delta G = -15$	$pK_1 = 9.8$, $\delta\Delta G = -15$	$pK_1 = 10.6$, $\delta\Delta G = -15$	$pK_1 = 10.7$, $\delta\Delta G = -8$	$pK_1 = 10.9$, $\delta\Delta G = -14$
k_{fast}	$pK_3 = 6.8$, $\delta\Delta G = +7.5$	$pK_3 = 6.1$, $\delta\Delta G = 13$	$pK_3 = 6$, $\delta\Delta G = 14$	$pK_3 = 7$, $\delta\Delta G = +16$	$pK_3 = 7.2$, $\delta\Delta G = +30$
	$pK_2 = 9.8$, $\delta\Delta G = -9$	$pK_2 = 6.9$, $\delta\Delta G = -7$	$pK_2 = 9.6$, $\delta\Delta G = -14.5$	$pK_2 = 8.7$, $\delta\Delta G = -21.5$	$pK_2 = 7.5$, $\delta\Delta G = -26$
	$pK_1 = 10.6$, $\delta\Delta G = -13$	$pK_1 = 10.6$, $\delta\Delta G = -16$	$pK_1 = 10.5$, $\delta\Delta G = -11$	$pK_1 = 10.3$, $\delta\Delta G = -10$	$pK_1 = 9$, $\delta\Delta G = -10$
k_{slow}	$pK_3 = 7.3$, $\delta\Delta G = +9$	$pK_3 = 6.4$, $\delta\Delta G = 6.5$	$pK_3 = 6.2$, $\delta\Delta G = 10$	$pK_3 = 6.8$, $\delta\Delta G = +9$	$pK_3 = 6.1$, $\delta\Delta G = +38$
	$pK_2 = 9.55$, $\delta\Delta G = -12$	$pK_2 = 7.85$, $\delta\Delta G = -5$	$pK_2 = 9$, $\delta\Delta G = -3$	$pK_2 = 9$, $\delta\Delta G = -12$	$pK_2 = 8.5$, $\delta\Delta G = -54$
	$pK_1 = 10.6$, $\delta\Delta G = -5$	$pK_1 = 10.6$, $\delta\Delta G = -13$	$pK_1 = 11.0$, $\delta\Delta G = -17$	$pK_1 = 10.25$, $\delta\Delta G = -5$	$pK_1 = 10.3$, $\delta\Delta G = -12$

[a] pKs and associated ΔG changes ($\delta\Delta G$) (meV) related to the three protonation waves derived from eq 2 applied to the curves in Figures 5 and 6. [b] Fitting of the data from Sebban and Wraight (1989).

$k_{i P^+Q_B^-}$, where i denotes the total, fast, or slow component.

The K_{2i} values measured for reaction centers in detergent are 3 times smaller than those in proteoliposomes or chromatophores, reflecting a much higher stabilization of Q_B^- (compared to Q_A^-) in the lipidic membranes compared to the detergent. A common behavior of the three samples is the 3-fold decrease of K_2 from pH 6 to pH 8. It is likely that, in this pH range, amino acid side chains closer to Q_B^- than to Q_A^- deprotonate. Less clear is the behavior from pH 8 to pH 10, where K_2 stays constant or increases. However, above pH 10, all K_2 curves decrease, suggesting again the existence of, at least, one protonation site with a pK above 11. The true value of this pK is difficult to obtain because of the loss of the signal above about 11.3.

The similarities of the K_{2fast} and K_{2slow} pH dependence curves obtained independently for the three samples support again the coexistence of two states of the reaction center protein. In addition, they suggest a correspondence between the two reaction center populations associated with the biphasic decay of $P^+Q_A^-$ and of $P^+Q_B^-$.

Discussion

Our data show that the charge recombination kinetics either from $P^+Q_A^-$ or from $P^+Q_B^-$ are heterogeneous (probably biphasic) and that the relative amplitudes of the two components (for $P^+Q_A^-$ and $P^+Q_B^-$) are sensitive to pH. These effects are observed in reaction centers either in detergent or reconstituted in phosphatidylcholine liposomes or in chromatophores. As the $P^+Q_B^-$ decay time is much longer than any protonation event, we suggest that these conformational states exist in the dark and are not created during the flash. According to that hypothesis, both phases must be treated as independent components, with their own thermodynamic, kinetic, and structural properties. Thus, to assign pK values to the pH titration curves of the $P^+Q_A^-$ rate constants (Figures 5 and 6), we have considered k_{fast} and k_{slow} as pure components. We have assumed the simplest hypothesis, with regard to our data, that three protonation sites could account for these curves. Under these conditions, we may apply to the curves of Figures 5 and 6 the equation derived by Sebban and Wraight (1989)

$$k_{obs} = \frac{k_A[A] + k_B[B] + k_C[C] + k_D[D]}{[A] + [B] + [C] + [D]}$$

$$k_{obs} = [\{(k_A \times 10^{(pH-pK_1)} + k_B) \times 10^{(pH-pK_2)} + k_C\} \times 10^{(pH-pK_3)} + k_D] / \prod_{i=1}^{3}(1 + 10^{(pH-pK_i)}) \quad (2)$$

where pK_3, pK_2, and pK_1 represent the pK values of some lateral chains of amino acid residues between I^- and Q_A^-, respectively, from lower to higher pH. D and C, C and B, and B and A are the protonated and unprotonated states associated with pK_3, pK_2, and pK_1, respectively. Any protonation site closer to I^- than to Q_A^- will reduce the ΔG when protonated and increase it when deprotonated. According to eq 1, this leads to a decrease of the $P^+Q_A^-$ charge recombination rate constant on increasing pH. Any protonation site closer to Q_A^- than to I^- will have the opposite effect on the kinetics. The results of the fitting of the curves of Figures 5 and 6 are presented in Table II. We have also included the parameters obtained for the fitting of the total component, although it may be not meaningful because of the heterogeneity of the kinetics. However, it helps in comparing our results with previous data obtained either by redox titration (Prince & Dutton, 1978) or by proton uptake measurements (Marôti & Wraight, 1988a,b; McPherson et al., 1988). In Table II, we added the fitting of the data obtained on reaction centers in LDAO (Sebban & Wraight, 1989). Clearly, the data fit reasonably well with the presence of three protonation sites, one of which (D to C, pK_3) is closer to I^- than to Q_A^- and the two others (C to B, pK_2; B to A, pK_1) are closer to Q_A^- than to I^-. From the comparison of the data obtained in the presence (or in the absence) of *o*-phenanthroline, it arises that the corresponding pK values for the reaction centers in detergent, in phospholipid vesicles, or in chromatophores are not the same. This suggests indirect electrostatic influence of the detergent or the type of lipid constituting the membrane on the side chains of some amino acids. It must be noted that the ΔG changes associated with each deprotonation event are generally larger in isolated reaction centers than in vesicles. This may come from a different accessibility of the protonable sites to the protons, in detergent or in lipids, or, alternatively, to a screening effect of ionizable groups, more pronounced in the membrane than in detergent. The observed pK values are notably different for k_{fast} and k_{slow} (Table II), suggesting structural differences in the surrounding of I and Q_A, between the two populations of reaction centers.

The main trend suggested by the data is the marked effect of *o*-phenanthroline on pK (Table II). It must be noted that because of the noise in the data and also because the end of each titration is not well-defined, the calculated pK values are obtained with a poor precision, ±0.3 pH unit. The pK_1 values are less sensitive (except for k_{fast} in reaction centers) to the inhibitor than pK_2 and pK_3. pK_1 is also less sensitive to the environment. This suggests that the group involved in that protonation lies in a region whose structure is not much changed either by the replacement of lipids by detergent or by the presence of *o*-phenanthroline.

The amplitudes of the pK shifts induced by the presence of *o*-phenanthroline are greater in proteoliposomes than in detergent. For reaction centers in detergent, for the fast component, pK_2 and pK_1 are shifted from 8.7 to 7.5 and from 10.3 to 9, respectively. pK_3 does not seem to be affected. The situation is different for k_{slow} for which pK_1 is not affected, whereas pK_3 and pK_2 are shifted from 6.8 to 6.1 and from 9

to 8.5, respectively. In vesicles, pK_3 and pK_2 are shifted from 6.8 to 6 and from 9.8 to 6.9, respectively, for k_{fast}. For k_{slow}, pK_3 and pK_2 are shifted from 7.3 to 6.4 and from 9.55 to 7.85, respectively.

Recent proton uptake measurements on *Rb. sphaeroides* reactions centers (Marõti & Wraight, 1988a,b; McPherson et al., 1988) have led to the conclusion that four protonable groups may be involved in the protonation of the $P^+Q_A^-$ state, in the pH range 4.5–11. If we except the lowest pH group (pH ≈4.5–5) observed by these authors, our data obtained by a different technique and on a different species lead to the same conclusions, in the pH range 5.5–11. Equilibrium redox titration measurements on *Rb. sphaeroides* and *Rps. viridis* chromatophores (Dutton et al., 1973; Prince & Dutton, 1976, 1978) have shown that the $E_m(Q_A/Q_A^-)$ vs pH plots exhibits a −60 mV/pH slope until the pK, reached around 10. This figure differs from that observed for reaction centers in detergent, where $E_m(Q_A/Q_A^-)$ is pH independent from 6 to 9 (Dutton et al., 1973). The pH dependence of the $E_m(Q_A/Q_A^-)$ observed in the reaction centers from *Rb. sphaeroides* reconstituted in phospholipid vesicles (Wraight, 1981) was similar to that in chromatophores. These data would not suggest the presence of the protonable groups mentioned above since a −60 mV/pH slope is significant of the protonation of a single group. However, if we focus on the total component (that must be taken into account to compare our data with the above), Table II shows that in chromatophores or in proteoliposomes the $\delta\Delta G$ associated with the highest protonation wave (pK_1) is systematically higher than for pK_2 or pK_3. This is not the case for reaction centers in detergent solution, for which the three $\delta\Delta G$ values are higher than in lipids. So, it seems that in *Rps. viridis*, the protonable groups that one can detect on reaction centers are also observed in the membrane of either chromatophores or proteoliposomes, but with an associated $\delta\Delta G$ (or redox potential change) greater on the highest pK group than on groups with pK_2 or pK_3. This could partly explain the above discrepancies between equilibrium redox potential measurements in reaction centers in detergent and in lipid membrane.

Similarly to what was measured by Prince and Dutton (1978) in chromatophores from *Rps. viridis*, we observe here a notable effect of *o*-phenanthroline on the protonation pK values of Q_A^-, in reaction centers reconstituted in phospholipid vesicles (Table I). For reaction centers in detergent, this effect is much weaker. pK values observed on chromatophores by the above authors and those measured here (pK_1 and pK_2) by a different technique and on proteoliposomes agree for a pK shift of 1–2 pH/units, or more, when *o*-phenanthroline is present, compared to when it is absent. According to Prince and Dutton (1978), this effect was also found to be important in *Chromatium vinosum* (>2 pH unit shift), but weaker in *Rhodospirillum rubrum* (1 pH unit shift) or almost absent in *B. sphaeroides*. However, in *Rps. viridis* and in *Chr. vinosum*, Q_A is a menaquinone, whereas in *R. rubrum* and in *Rb. sphaeroides* it is a ubiquinone. In *Rps. viridis* (Michel et al., 1986) and in *Rb. sphaeroides* (Allen et al., 1988), the two nitrogen atoms of *o*-phenanthroline were suggested to form a shared hydrogen bond with the imidazole nitrogen of histidine L190, which is a ligand for the iron atom. In *Rps. viridis*, a carbonyl oxygen of Q_A was suggested (Michel et al., 1986) to form a hydrogen bond with the imidazole of histidine M217, whereas in *Rb. sphaeroides* the distance between the oxygen and the imidazole of histidine M219 (4.5 Å) seems too large to allow such a bond. These structural differences could explain the different influences of *o*-phenanthroline on the two strains. One may think that if the two hydrogen bonds between *o*-phenanthroline and His L190 lead to changes in the interaction between His L190 and the Fe, this change will be more easily transmitted to the Q_A pocket in *Rps. viridis* than in *Rb. sphaeroides*. The large pK shift observed in *Chr. vinosum* would then suggest that in the strains where a menaquinone acts as Q_A the interaction *o*-phenanthroline–Fe–Q_A is stronger than when ubiquinone acts as Q_A.

Conclusion

In this work, we have reconstituted the reaction centers from *Rps. viridis* in phosphatidylcholine vesicles and compared some electron-transfer kinetics measured in such systems to what they are in the chromatophore membrane and in reaction centers isolated in detergent. From most results, proteoliposomes appeared to have properties closer to chromatophores than to reaction centers in detergent. That was the case for the pH titration curves of the $P^+Q_A^-$ charge recombination rate constants, for the ΔG measurements, or for the $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ apparent equilibrium constant, which was found to be in proteoliposomes and in chromatophores at least 4 times as high as in isolated reaction centers. In addition, the big effect of *o*-phenanthroline on the protonation pK of some amino acid groups in the vicinity of Q_A^- was comparable in proteoliposomes and in chromatophores (Prince & Dutton, 1978) and much more pronounced than in isolated reaction centers. Thus, it results that for the measurements of kinetic or energetic parameters of electron-transfer processes in relation to the quinones system (such as the Q_A^- to Q_B electron-transfer rate), in *Rps. viridis*, it is rather preferable to work on chromatophores or reaction centers incorporated in phospholipid bilayers than isolated in detergent.

In a previous study, we have demonstrated the heterogeneity of the $P^+Q_A^-$ charge recombination kinetics in *Rps. viridis* reaction centers (Sebban & Wraight, 1989). The monophasicity of these kinetics at low pH, and the inversion of the amplitudes of the two phases at high pH, had led us to conclude that the observed biphasicity was arising from nonequilibrium of different protonation states of the reaction centers during the time of the charge recombination. These protonation states were supposed to be induced by the flash.

We confirm here the above observations in chromatophores and in reaction centers reconstituted in phospholipid vesicles. However, the $P^+Q_B^-$ decay kinetics are also shown to be biphasic, with marked pH dependences of the relative amplitudes of both phases. However, the lifetime of these decays is about 500 ms at pH 7, a time scale which should allow any protonation event to complete and the induced protonation states to equilibrate. In other words, if our previous hypothesis was right, one would not expect to see any heterogeneity in the $P^+Q_B^-$ charge recombination decays. So, we come to the conclusion that rather than flash-induced protonation states, we are in the presence of conformational states existing before the flash. Assuming the above hypothesis, the observation that the low-temperature (80 K) kinetics of $P^+Q_A^-$ recombination (Sebban & Wraight, 1989) were closer to exponential than at room temperature is more easily understood. It is possible that one of the two conformations is more favorable at low temperature. In isolated reaction centers and chromatophores from *Rb. sphaeroides* and *R. rubrum*, Parot et al. (1987) have detected biphasicity of the $P^+Q_A^-$ charge recombination kinetics at low temperature. The relationship between the phenomenon they observed at low temperature (and not at room temperature) on wild type *Rb. sphaeroides* and what we observe at room temperature on *Rps. viridis* and on AQ's reconstituted reaction centers from *Rb. sphaeroides* (Sebban,

1988b) is unclear, except, as we previously mentioned, for the splitting of the spectra of the two components near the isosbestic point. Working at low temperature on chromatophores from the G9 mutant strain from *R. rubrum*, in the presence of *o*-phenanthroline, Parot and Verméglio (unpublished results) observed the $P^+Q_A^-$ charge recombination kinetic after a second flash delayed from the first one by a time period intermediate between the lifetimes of the two components. Under these conditions, the decay kinetics was enriched in the fast component. Thus, it appears that the two states corresponding to the slow and the fast decays as well as their relative amplitudes are determined before the flash and are not induced by the flash light. This suggests some similitude between the two components detected at room temperature on *Rps. viridis* or *Rb. sphaeroides* reaction centers with anthraquinone acting as Q_A (Sebban, 1988b) and those detected at low temperature in wild-type *Rb. sphaeroides* and *R. rubrum* (Parot et al., 1987).

We have shown here that the two states observed in *Rps. viridis* can be distinguished by their kinetic, spectroscopic, or energetic properties.

At the isosbestic point, near 833 nm, a slight difference in their light-induced absorption spectra allows a separation of both phases since they contribute with opposite signs to the absorption change. The measure of $\delta\Delta G$ between both components is in agreement with the independent measure of the ratio of their kinetic rate constants, suggesting that each state possesses its own ΔG. Biphasicities of the charge recombination kinetics from $P^+Q_A^-$ and from $P^+Q_B^-$ seem to be related, since both phases get close $Q_A^-Q_B \rightleftarrows Q_AQ_B^-$ apparent equilibrium constants and a somewhat similar pH dependence of this parameter. Finally, the environment (detergent or different lipid bilayer) of the reaction center protein as well as *o*-phenanthroline, pH, and ionic conditions (Sebban and Wraight, unpublished data) influences the relative proportion of the two conformations.

In PSII preparations and in *R. rubrum*, it has been observed that the $Q_A^-Fe^{2+}$ and $Q_B^-Fe^{2+}$ EPR signals split into two components attributed to two reaction center populations with different semiquinone–iron interactions (Beijer & Rutherford, 1987; Zimmermann & Rutherford, 1986). The distribution of the two states is influenced by pH and the binding of herbicides. It was suggested that different interactions between Q_A^-, Q_B^-, and Fe^{2+} could be at the origin of the such heterogeneity. Some relationship with the phenomena observed in our work is possible.

Low-temperature experiments that freeze the two conformations and measurements of the above parameters in different environments of the protein would help in understanding these phenomena that could be of importance for the reaction center function.

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Membrane Contact, Fusion, and Hexagonal (H_{II}) Transitions in Phosphatidylethanolamine Liposomes†

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ABSTRACT: The behavior of phosphatidylethanolamine (PE) liposomes has been studied as a function of temperature, pH, ionic strength, lipid concentration, liposome size, and divalent cation concentration by differential scanning calorimetry (DSC), by light scattering, by assays measuring liposomal lipid mixing, contents mixing, and contents leakage, and by a new fluorometric assay for hexagonal (H_{II}) transitions. Liposomes were either small or large unilamellar, or multilamellar. Stable (impermeable, nonaggregating) liposomes of egg PE (EPE) could be formed in isotonic saline (NaCl) only at high pH (>8) or at lower pH in the presence of low ionic strength saline (less than 50 mOsm). Bilayer to hexagonal (H_{II}) phase transitions and gel to liquid-crystalline transitions of centrifuged multilamellar liposomes were both detectable by DSC only at pH 7.4 and below. The H_{II} transition temperature increased, and the transition enthalpy decreased, as the pH was raised above 7.4, and it disappeared above pH 8.3 where PE is sufficiently negatively charged. H_{II} transitions could be detected at high pH following the addition of Ca^{2+} or Mg^{2+}. No changes in light scattering and no lipid mixing, mixing of contents, or leakage of contents were noted for EPE liposomes under nonaggregating conditions (pH 9.2 and 100 mM Na^+ or pH 7.4 and 5 mM Na^+) as the temperature was raised through the H_{II} transition region. However, when aggregation of the liposomes was induced by addition of Ca^{2+} or Mg^{2+}, or by increasing [Na^+], it produced sharp increases in light scattering and in leakage of contents and also changes in fluorescent probe behavior in the region of the H_{II} transition temperature (T_H). Lipid mixing and contents mixing were also observed below T_H under conditions where liposomes were induced to aggregate, but without any appreciable leakage of contents. We conclude that H_{II} transitions do not occur in liposomes under conditions where intermembrane contacts do not take place. Moreover, fusion of PE liposomes at a temperature below T_H can be triggered by H^+, Na^+, Ca^{2+}, or Mg^{2+} or by centrifugation under conditions that induce membrane contact. There was no evidence for the participation of H_{II} transitions in these fusion events.

The ability of phosphatidylethanolamine and other phospholipids to assume nonbilayer configurations has been well documented. Evidence for the occurrence of hexagonal (H_{II}) phases in phospholipids comes from X-ray diffraction (Luzzati et al., 1968; Rand et al., 1971; Harlos & Eibl, 1981; Gruner et al., 1988), ^{31}P NMR studies (Cullis & de Kruijff, 1978, 1979; Hui et al., 1981), freeze-fracture electron microscopy (Deamer et al., 1970; de Kruijff et al., 1979; van Venetie & Verkleij, 1981), infrared spectroscopy (Mantsch et al., 1981), electron spin resonance spectroscopy (Hardman, 1982), and differential scanning calorimetry (Harlos & Eibl, 1981; Seddon et al., 1983). The evidence for hexagonal (H_{II}) phases for phosphatidylethanolamine comes primarily from studies in which multibilayers of phospholipid have been studied at very high phospholipid concentrations. These conditions allow for the presence of large areas of bilayer in close proximity, and may favor formation of nonbilayer lipid under the appropriate thermodynamic conditions given the extensive three-dimensional character of the H_{II} phase.

Nonbilayer lipid has been postulated to play an important biological role in cell membranes particularly in fusion and transport functions (Hope & Cullis, 1981; Cullis et al., 1980; Venetie & Verkleij, 1982; Ellens et al., 1989). However, suspensions of phospholipid vesicles, or liposomes, are a closer approximation to biological membranes than are closely packed multilayers. Therefore, a study of unilamellar liposomes under conditions leading to nonbilayer structures or H_{II} transitions should provide us with relevant information about the occurrence and role of nonbilayer lipid in biological membranes.

The difficulty in forming stable liposomes of phosphatidylethanolamine (PE)[1] at physiological pH in isotonic buffers has

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[1] Abbreviations: PE, phosphatidylethanolamine; EPE, egg yolk PE; TPE, PE prepared from egg phosphatidylcholine by transesterification; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rh-PE, *N*-(lissamine rhodamine B sulfonyl)phosphatidylethanolamine; EDTA, ethylenediaminetetraacetic acid; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; DPX, *p*-xylylenebis(pyridinium bromide); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; DSC, differential scanning calorimetry; SUV, small unilamellar vesicle(s); LUV, large unilamellar vesicle(s); MLV, multilamellar vesicle(s); T_c, gel to liquid-crystalline phase transition temperature; T_H, liquid-crystalline to hexagonal (H_{II}) phase transition temperature.

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