We report the observation of two conformational states of closed RCs from *Rhodobacter sphaeroides* characterized by different P$^+\text{H}_\Lambda^-$ → PH$_\Lambda$ charge recombination lifetimes, one of which is of subnanosecond value ($700 \pm 200$ ps). These states are also characterized by different primary charge separation lifetimes. It is proposed that the distinct conformations are related to two protonation states either of reduced secondary electron acceptor, Q$_\Lambda^-$, or of a titratable amino acid residue localized near Q$_\Lambda$. The reaction centers in the protonated state are characterized by faster charge separation and slower charge recombination when compared to those in the unprotonated state. Both effects are explained in terms of the model assuming modulation of the free energy level of the state P$^+\text{H}_\Lambda^-$ by the charges on or near Q$_\Lambda$ and decay of the P$^+\text{H}_\Lambda^-$ state via the thermally activated P$^+\text{B}_\Lambda^-$ state.

**Materials and Methods**

RCs isolated from a His-tagged version of *Rh. sphaeroides* strain 2:4:1 were prepared according to the procedure described in ref 12. To remove the quinone from the Q$_\Lambda$ site, we applied the procedure described in refs 13 and 14. Reduction of Q$_\Lambda$ was realized by permanent continuous illumination of the sample during the transient absorption experiment, with a halogen lamp, after addition of 10 mM sodium ascorbate. Sodium ascorbate rereduced P$^+$ to P after formation of P$^+\text{Q}_\Lambda^-$ as a result of continuous illumination. The intensity of this illumination was set on a level which ensured elimination of the features characteristic of open RCs from the transient spectra. The effect of *o*-phenanthroline was studied after addition of 100 μL of...
stock solution of this herbicide yielding its final concentration of 10 mM. The experiments on open RCs, then on the QA-reduced RCs, first without and then in the presence of o-phenanthroline, were performed each time sequentially on the same sample and without any changes in the alignment of the setup.

For the transient absorption measurements, the stock solution containing RCs of OD$_{800\text{ nm}}$ $\approx$ 15 cm$^{-1}$ was 5-fold diluted in a 15 mM Tris-HCl buffer (pH = 8.2 and pH = 6) containing 0.025% N,N-dimethyldodecylamine-N-oxide (LDAO) and 1 mM EDTA. During the transient absorption experiment, the sample ($\sim$2.5 mL) was placed in a spinning quartz wheel of 10 cm in diameter, rotating at about 10 Hz in order to ensure full relaxation of the sample between laser flashes. The optical path of the laser beam in the sample was about 1.5 mm.

The transient absorption spectrometer, containing a 1 kHz femtosecond laser system (Ti:sapphire, Spectra Physics) and a grating polychromator (Spectra Pro 150, Acton Research Corp.) with a thermoelectrically cooled CCD camera (Back Illum., Princeton Instruments) was described in detail in ref 15. Laser pulses of $\sim$2 µJ energy, $\sim$100 fs duration (full width at half-maximum, fwhm), and 15 nm spectral bandwidth centered at 800 nm were used to excite the sample with 1 kHz repetition rate. The pulses of white light generated in a calcium fluoride plate were used to probe absorption changes induced by the excitation pulses, in the spectral window from 330 to 700 nm. The transient absorption spectra were collected for pump–probe delay times up to 1 ns. The temporal step between 57 consecutive transient spectra was gradually increased from 100 fs for the shortest delays to 100 ps for the longest delays. The data were subjected to global analysis$^{16,17}$ by using the ASUFIT program (available at www.public.asu.edu/~laserweb/asufit/asufit.html).

Results

Figure 1 shows the decay associated spectra (DAS) of *Rb. sphaeroides* RCs with both free electron transfer (open RCs; panel A) and with blocked electron transfer from H$_{A^-}$ to Q$_A$ (closed RCs) either by removing Q$_A$ (Figure 1B) or by reducing it (Figure 1C,D). In all four cases, we limited the number of exponential components to three plus a constant (nondecaying component, ND). The interpretation of the two fastest components was the same for all four sets of data: the subpicosecond component was assigned to the excitation energy transfer from B$^*$ to P$_r$,

\[ B^* \rightarrow P_r \]

and the 5.2–6.8 ps component was assigned to the primary

\[ P_r \rightarrow P_i \]
charge separation forming \( \text{P}^+\text{H}_\text{A}^- \).

The 230 ps component associated with electron transfer from \( \text{H}_\text{A}^- \) to \( \text{Q}_\Lambda \) in open RCs was replaced in RCs with reduced quinone \( \text{Q}_\Lambda \) by the \( \sim 700 \) ps component assigned to the fast phase of the \( \text{P}^+\text{H}_\text{A}^- \) charge recombination. In RCs without \( \text{Q}_\Lambda \), the respective component had very low amplitude. The nondecaying component was assigned to the \( \Delta \text{OD}(\text{P}^+\text{Q}_\Lambda^-\text{PQ}_\Lambda) \) signal in open RCs (Figure 1A),

and to the slow phase of the \( \text{P}^+\text{H}_\text{A}^- \) charge recombination in closed RCs (Figure 1B–D). The results presented in Figure 1 were obtained for RCs dissolved in the buffer of pH = 8.2, but very similar spectra and lifetimes associated with these spectra were obtained at pH = 6 and can be found in the Supporting Information.

**Comparison of the Primary Charge Separation in Open and Closed RCs.** Signal to noise ratio of our experiment was insufficient to resolve more than one lifetime associated with the primary charge separation in whole investigated spectral range. Thus, the lifetimes of the few picosecond components presented in this paper should be regarded as average values from the literature.

The spectrum of charge separation is of similar shape for open and closed RCs (Figure 1A–D). The lifetime associated with this spectrum increases by \( \sim 30\% \) from 5.2 ps in open RCs to 6.8 ps in closed RCs (Figure 1A,C). The same \( \sim 30\% \) increase in the primary charge separation time after chemical reduction of \( \text{Q}_\Lambda \) by sodium dithionite was reported before.

Also, similar 6.1 ps lifetime of charge separation in \( \text{Q}_\Lambda \)-reduced RCs from *Rb. sphaeroides* was measured from decay of stimulated emission of \( \text{P}^+ \) between 870 and 1000 nm. Addition of 10 mM \( \alpha \)-phenanthroline to \( \text{Q}_\Lambda \)-reduced RCs slightly accelerates the primary charge separation to \( \sim 6.4 \) ps. The effect of \( \alpha \)-phenanthroline is not very big but reproducible as checked in a few independent experiments (also at pH = 6, see Supporting Information). We propose that the deceleration of the primary charge separation in \( \text{Q}_\Lambda \)-reduced RCs is caused by the negative charge on \( \text{Q}_\Lambda \) which is expected to influence the energetics of this reaction. This explanation is in line with the effect of \( \alpha \)-phenanthroline which is known to mediate the protonation of titratable groups near \( \text{Q}_\Lambda \),

which partly screening the negative charges.

In RCs without \( \text{Q}_\Lambda \), the charge separation time is 6.2 ps and is insignificantly changed after addition of \( \alpha \)-phenanthroline. The value of 6.2 ps is greater than 5.2 ps characteristic of open RCs but this difference may be related to small structural changes caused by the removal of quinone \( \text{Q}_\Lambda \).

**230 ps DAS and Nondecaying Spectrum in Open Reaction Centers.** Within 230 ps electron is transferred from \( \text{H}_\Lambda^- \) to \( \text{Q}_\Lambda \). Therefore, absorption changes visible in the 230 ps DAS (Figure 1A) are a sum of the absorption changes caused by reoxidation of \( \text{H}_\Lambda^- \) and by reduction of \( \text{Q}_\Lambda \). Similarly, the nondecaying spectrum is a sum of absorption changes caused by oxidation of \( \text{P} \), \( \Delta \text{OD}(\text{P}^+\text{P}) \), and reduction of \( \text{Q}_\Lambda \), \( \Delta \text{OD}(\text{Q}_\Lambda^-\text{Q}_\Lambda) \). It should be noted that the contribution of absorption changes originating from reduction of \( \text{Q}_\Lambda \) in both spectra is much smaller than those related to oxidation of \( \text{P} \) and \( \text{H}_\Lambda^- \) and limited to the blue spectral range.

Since the contributions of \( \Delta \text{OD}(\text{Q}_\Lambda^-\text{Q}_\Lambda) \) to the 230 ps DAS and to nondecaying spectra are identical but of opposite signs (the 230 ps DAS and ND component depict the kinetics of appearance and infinitesimally slow decay of \( \text{Q}_\Lambda^- \), respectively), the sum of the two latter spectra represents absorption difference spectrum of the states \( \text{P}^+\text{H}_\Lambda^- \) and \( \text{PH}_\Lambda^- \), \( \Delta \text{OD}(\text{P}^+\text{H}_\Lambda^-\text{P}) \), well-known from the literature.

**Subnanosecond and Nondecaying Charge Recombination Components in Closed Reaction Centers.** The shape of the nondecaying spectrum of RCs with \( \text{Q}_\Lambda^- \) (Figure 1C) is identical to that of the sum of the 230 ps DAS and the nondecaying spectrum of open RCs (Figures 1A and 2A). Since the latter sum has been assigned to the difference of absorption spectra of the states \( \text{P}^+\text{H}_\Lambda^- \) and \( \text{PH}_\Lambda^- \), the identity of the shapes mentioned above allows the assignment of the nondecaying component in \( \text{Q}_\Lambda \)-reduced RCs to \( \Delta \text{OD}(\text{P}^+\text{H}_\Lambda^-\text{PH}_\Lambda) \). Since \( \text{P}^+\text{H}_\Lambda^- \rightarrow \text{PH}_\Lambda^- \) charge recombination in closed RCs occurs in the nanosecond time range, we assign the component nondecaying on 1 ns time scale to charge recombination. On the other hand, the shape of the 700 ps spectrum is similar to that of the nondecaying spectrum in \( \text{Q}_\Lambda 

\)reduced RCs (Figure 2A), indicating that the subnanosecond component is due to the fast phase of the \( \text{P}^+\text{H}_\Lambda^- \rightarrow \text{PH}_\Lambda^- \) charge recombination. This phase is visualized in Figure 3, in which the kinetic traces at 369, 668, and 420 nm are presented. The traces at the two former wavelengths represent relatively pure decay of the \( \Delta \text{OD}(\text{H}_\Lambda^-\text{H}_\Lambda) \) signal whereas the trace at 420 contains additionally contribution from the decay of the \( \Delta \text{OD}(\text{P}^+\text{P}) \) signal. The relationships between these two contributions at particular wavelengths are assessed from the amplitudes of the 230 ps DAS (representing mostly decay of the \( \Delta \text{OD}(\text{H}_\Lambda^-\text{H}_\Lambda) \) signal) and nondecaying component (representing mostly decay of the \( \Delta \text{OD}(\text{P}^+\text{P}) \) signal) in open RCs (Figure 1A).

The shapes of the subnanosecond and slow (ND) charge recombination spectra deviate somewhat from each other (Figure...
The subnanosecond spectrum of the \( Q_A \)-removed RCs is characterized by significantly smaller amplitude, shorter lifetime and the shape somewhat different than those of \( Q_A \)-reduced RCs (Figure 1B). Careful comparison of the shapes of the few hundred picosecond spectra in panels A, B, and C leads to the conclusion that the 430 ps DAS (Figure 1B) has the shape intermediate between that of the 230 ps DAS (Figure 1A) and that of the 700 ps DAS (Figure 1C). Also the value of 430 ps is between 230 and 700 ps. Both these observations indicate that the small 430 ns component is due to a mixture of two processes. One of them is electron transfer from \( H_A^- \) to \( Q_A \) in a very minor fraction of the "\( Q_A \)-removed RCs" still containing \( Q_A \). This fraction is estimated to be below 10\%. Another process possibly contributing to this DAS is the fast, subnanosecond, charge recombination occurring in a very minor fraction of RCs, estimated to be also below 10\%.

The shape of the nondecaying component for the \( Q_A \)-removed RCs is very similar to the respective component of the \( Q_A \)-reduced RCs (Figure 1B,C). This similarity demonstrates the same process of slow charge recombination, occurring on the time scale longer than that of our experiment. Minor differences between these two spectra are due to a small admixture of the (\( P^+Q_A^-\) $\rightarrow$ \( P^+Q_A^- \)) signal in "\( Q_A \)-removed" RCs resulting from a minor fraction of RCs still containing \( Q_A \). Addition of 10 mM \( o \)-phenanthroline to the sample with \( Q_A \)-removed RCs has no significant effect on the lifetimes, shapes, and relative amplitudes of the DAS and of the nondecaying components resulting from the global analysis (data not shown).

In general, for all closed RCs under study, the spectral shapes of both phases of the charge recombination resemble the mirror reflections of the charge separation spectra (Figure 1B–D). This symmetry is expected since the charge separation and charge recombination are two opposite processes involving, respectively, the formation and the decay of the same species, \( P^+ \) and \( H_A^- \) (eq 1). The symmetry is somewhat broken mostly by the contribution of absorption changes from \( P^+ \) (in the Soret and \( Q \), regions) in the charge separation DAS.

**Discussion**

The primary charge recombination in purple bacterial RCs was, until recently, regarded as an essentially monophasic process occurring from the relaxed form of the primary radical pair \( P^+H_A^- \) with 10–30 ns lifetime.\(^{5–7} \) The relaxation of the \( P^+H_A^- \) state was inferred from the multiphasic fluorescence decay occurring on the subnanosecond to nanosecond time scales.\(^{14,33,34} \) Recently, we have reported results of the transient absorption measurements performed on the nano- to microsecond time scales with the 1 ns temporal resolution, demonstrating the multiexponential decay of the \( \Delta OD(P^+\rightarrow P) \) signal at 970 nm and 1300 nm modeled with the \( <1, 3–4, \) and \( 9–12 \) ns time constants.\(^{5} \) These data were interpreted in terms of the coexistence of three conformational states of RCs characterized by different charge recombination dynamics (an alternative interpretation of this multiphasic kinetics related to relaxation of the free energy level of the state \( P^+H_A^- \), also was discussed in ref 9). The equilibrium between the three conformational states was proposed to be controlled by the electrical charges in the site \( Q_A \); both the negative charge on \( Q_A^- \) and the charges of the ionizable amino acid side chains in the \( Q_A \) pocket. In the following we extend this model to explain the results of ultrafast transient absorption measurements performed in the 1 ns temporal window.

**Charge Recombination.** The subnanosecond charge recombination was clearly resolved in these studies for \( Q_A \)-reduced

---

**Figure 3.** Kinetic traces of absorbance changes recorded at three different probe wavelengths at 800 nm excitation for three samples with closed RCs. The fits were extracted from the global analysis presented in Figure 1.

2A). This suggests that the two phases of the \( P^+H_A^- \rightarrow PH_A \) charge recombination are related to RCs differing in their conformational states. Moreover, it is the fast phase that deviates from the shape of the "230 ps + ND" open RCs spectrum (Figure 2A), suggesting that the conformational state related to this phase and not to the slow one is different from the state characteristic of open RCs. The most pronounced spectral differences between the two charge recombination phases are different relative amplitudes of the \( \sim 544 \) and \( \sim 600 \) nm bands (the amplitude ratio of the 600/544 nm bands is about twice greater in the 700 ps spectrum) and a larger amplitude of the strong anionic band around 670 nm in the 700 ps spectrum (Figure 2A).

The integrated area of the 700 ps spectrum is somewhat smaller than that of the nondecaying component in \( Q_A \)-reduced RCs (47% vs 53%; Figure 1C and Table 2). Addition of 10 mM \( o \)-phenanthroline causes a significant decrease in the contribution of the fast charge recombination phase (to \( \sim 27\% \); compare panels C and D in Figure 1). On the other hand, the addition of this herbicide does not affect the shapes of the fast and slow charge recombination spectra (Figure 2).
 TABLE 1: Results of Multiexponential Fitting of the Combined Absorption Changes Measured at 369, 420, 454, and 668 nm in 1 ns Temporal Window and at 970 nm in 80 ns Temporal Window (Data Taken from Ref 9)*

<table>
<thead>
<tr>
<th>sample</th>
<th>lifetime (ns)</th>
<th>rel amplitude (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q$_A$</td>
<td>0.5–0.9</td>
<td>23–29</td>
</tr>
<tr>
<td></td>
<td>3.7–4.3</td>
<td>56–64</td>
</tr>
<tr>
<td></td>
<td>12–16</td>
<td>9–13</td>
</tr>
<tr>
<td></td>
<td>const</td>
<td>3–4</td>
</tr>
<tr>
<td>Q$_A$  + o-phenanthroline</td>
<td>0.4–0.7</td>
<td>10–16</td>
</tr>
<tr>
<td></td>
<td>7.6–8.2</td>
<td>68–81</td>
</tr>
<tr>
<td></td>
<td>19–30</td>
<td>6–11</td>
</tr>
<tr>
<td></td>
<td>const</td>
<td>∼5</td>
</tr>
<tr>
<td>without Q$_A$</td>
<td>0.2–0.3</td>
<td>0–4</td>
</tr>
<tr>
<td></td>
<td>9–12</td>
<td>65–90</td>
</tr>
<tr>
<td></td>
<td>17–24</td>
<td>0–26</td>
</tr>
<tr>
<td></td>
<td>const</td>
<td>5–9</td>
</tr>
</tbody>
</table>

*The ranges of the values reflect somewhat different fit parameters resulting from combining the data at 970 nm with those obtained at four different wavelengths in the visible.

 TABLE 2: Contributions of the Subnanosecond P$^+$H$_A$ $\rightarrow$ PH$_A$ Charge Recombination in Closed RCs with and without Addition of 10 mM o-Phenanthroline*

<table>
<thead>
<tr>
<th>sample</th>
<th>Q$_A$</th>
<th>Q$_A$  + o-phenanthroline</th>
<th>without Q$_A$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gibasiewicz and Pajderska, 2008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>contribution of subnanosecond charge recombination (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 ns range</td>
<td>47</td>
<td>27</td>
<td>&lt;10</td>
</tr>
<tr>
<td>1 ns + 80 ns range</td>
<td>∼26</td>
<td>∼13</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

*The contributions in 1 ns range were calculated from the formula $A_{90}(A_{700} + A_{80})$, where $A_{90}$ is the integrated area of the ∼700 ps charge recombination spectrum, and $A_{80}$ is the integrated area of the nondecaying charge recombination spectrum (Figure 1B–D). The contributions in the combined “1 ns + 80 ns range” are taken from Table 1.

The relative contributions of the subnanosecond charge recombination found from the fits performed in 1 ns range and combined 1 ns plus 80 ns range should also be compared to those published previously (Table 2). In these previous studies, the subnanosecond component was found from the deconvolution procedure applied in the analysis of the data collected with the 1 ns temporal resolution. Apparently, contributions of this component were overestimated in ref 9 (Table 2) but it is not much surprising since the temporal resolution in the previous study was insufficient to determine accurately the amplitude of such a fast phase. A common important feature of all the data sets in Table 2 is that the contribution of the subnanosecond charge recombination is modulated by the electrical charges at the site Q$_A$ (Figure 1B–D): it is the highest for RCs with Q$_A$ (Figure 1C), intermediate for RCs with Q$_A$ after addition of o-phenanthroline (Figure 1D), and the lowest for RCs without Q$_A$ (Figure 1B).

The remaining two, 3–4 and 12 ns, phases of the charge recombination, reported in ref 9 for both Q$_A$-reduced and Q$_A$-removed RCs, naturally could not be resolved in the present studies performed in 1 ns temporal window. Instead, they are lumped together and seen as one nondecaying component (Figure 1B–D). In the combined fits (Figure 4, Table 1), the lifetimes originating from the multieponential analysis and associated with slow charge recombination are somewhat different from those published previously due to constraints superimposed on the fits by the experimental results measured within first nanosecond. RCs with Q$_A$, Q$_A$ plus o-phenanthroline, and without Q$_A$ are dominated by ∼4, ∼8, and ∼11 ns.
The two charge recombination phases resolved in 1 ns temporal window (Figure 1C,D), in line with the previous model,9 are assigned to separate subpopulations of RCs characterized by conformational states differing from each other also spectrally (Figure 2). The size of the “fast charge recombination” subpopulation is correlated with the load of the negative charge at the site QA. Both of them are the highest when QA is protonated, lowered after addition of o-phenanthroline, and very low when there is no QA. However, even for the QA-reduced RCs, a large fraction of RCs show slow charge recombination kinetics similar to that one observed in the QA-removed RCs (Figure 1C,D). All these observations can be consistently explained by the simple model relating the two conformational states of RCs with a protonation state of a single titratable group in the QA site. If this group is protonated, and thus the negative charge on QA is neutralized, the slow charge recombination occurs. Otherwise, the fast (subnanosecond) charge recombination takes place. Thus, the lack of the negative charge on QA in the QA-removed RCs explains the domination of the slow charge recombination in this case. Also, the lack of the negative charge on QA in open RCs explains the identical shapes of the sum of $\Delta OD(H_\alpha^+ - H_\alpha)$ and $\Delta OD(P^+ - P)$ spectra and the slow charge recombination spectrum (Figure 2A). The slightly different shape of the fast charge recombination spectrum (Figure 2A,B), in addition to the short lifetime, reveals the effect of the noncompensated negative charge. The coexistence of both phases in the QA-reduced RCs indicates that $pK$ value of the hypothetical titratable group is close to the local pH at the QA site and, thus, similar fractions of RCs remain in the protonated and unprotonated states. In line with this reasoning, relative contributions of the fast and slow charge recombination (Table 2, 1 ns range) can be used to estimate the $pK$ value of the titratable group from the Henderson–Hasselbach equation

$$pK = pH - \log(A/B)$$

where $A$ and $B$ are the concentrations of the unprotonated and protonated forms of the titratable group. By substituting $A$ and $B$ with the relative amplitudes of the fast and slow phase, respectively, and assuming $pH$ at the site QA the same as that of the buffer used (8.2), one can obtain $pK \approx -8.3$ for the QA-reduced RCs and $\sim 8.7$ after addition of o-phenanthroline. Using contributions of fast and slow recombination from an alternative combined fit (Table 1, 1 ns + 80 ns range) makes only a small difference in the respective $pK$ shift from 8.5 to 8.9. A positive shift of $pK$ of similar value ($\sim 0.4 pK$ unit), from $\sim 9.7$ to $\sim 10.1$, after addition of o-phenanthroline was previously reported for QA in Rhodobacter sphaeroides.10 On the other hand, a positive shift of $pK$, from 7.5 to 8.7 and from 8.5 to 9.0, of the hypothetical titratable amino acid residue situated close to QA in, respectively, two conformational states of RCs from Rhodo pseudomonas viridis was reported after addition of o-phenanthroline.11,13 These reports suggest that the titratable group responsible for two conformational states of RCs reported by us may be either QA itself or a side chain of a nearby amino acid residue.

In order to verify the hypothesis that the protonation state of the titratable site near QA modulates the relative contributions of the fast and slow phases of charge recombination we performed a series of experiments at pH 6 (see Supporting Information). At low pH we expected full protonation of the titratable group discussed above. Unexpectedly, the contributions of both phases of charge recombination was almost the same as at pH = 8.2. A possible explanation of this observation is that pH outside RC has no significant effect on the local pH near QA.

On the basis of our data we cannot completely rule out the possibility that the biphasic charge recombination observed in 1 ns temporal window (and three-phasic charge recombination observed on longer time scale) originates from the pH-independent static heterogeneity of RC conformation or dynamic relaxation of the state $P^+H_\alpha^+$, $P^+H_\beta^+$, and $PQ^+H_\alpha^+$ charge recombination.14,33,34 However, the observed $pK$ value of the QA site, $pK = 8.7-8.9$, suggests static heterogeneity of RCs related to different protonation states of the titratable residues.11,30 More detailed discussion on dynamic relaxation vs static heterogeneity model was presented in ref 9.

**Primary Charge Separation.** The coexistence of two conformational states of RCs in preparations with QA reduced, clearly detected on the charge recombination time scale (Figure 1C,D), is more difficult to observe experimentally on the time scale of the primary charge separation. A possible reason for this difficulty may be that the temporal separation of the charge separation lifetimes related to the two conformational forms of RCs is much smaller than that of the subnanosecond and nanosecond charge recombination phases. Moreover, the charge separation is in fact multieponential even for homogeneous conformational state of RCs,26,27,29,30 that makes the trials to resolve components belonging to different conformational states practically hopeless. Nevertheless, the increase in the average lifetime from 5.2 to 6.8 after reducing the RCs may be interpreted as the effect of an appearance of two conformational states, both with similar slow charge separation or with two different charge separation lifetimes. We favor the latter possibility for the following reason. As we noticed above, the “slow charge recombination” conformation assigned to the state with neutralized charge on QA has got identical long-time spectral properties as the conformation characteristic of the open RCs (Figure 2A). Thus, it is reasonable to assume that the charge separation in this conformation, even after prereduction of QA, remains unchanged and is still close to 5.2 ps. Then, the value of 6.8 ps observed in QA-reduced RCs would be an average of the $\sim 5.2$ ps and a few picoseconds longer another charge separation lifetime characteristic of the “fast charge recombination” conformation, weighted with relative contributions of the two subpopulations of RCs. Consistent with this reasoning, addition of o-phenanthroline, which decreases the contribution of the “fast charge recombination” subpopulation, should result in the decrease of the experimentally measured averaged primary charge separation lifetime, and this is indeed observed (Figure 1, C and D).

$P^+B_\alpha^- \leftrightarrow P^+H_\alpha^-$ **Equilibrium Model.** The three-exponential decay of $P^+H_\alpha^-$ measured on the nanosecond time scale has been recently proposed to occur via the thermally accessible $P^+B_\alpha^-$ state.3 Earlier, a similar thermally activated mechanism was proposed both for $P^+H_\alpha^+$2 and for $P^+Q\alpha^-$ and $PQ^+H_\beta^+$ charge recombination.14,33,34 Hereby, we adopt the idea of $P^+B_\alpha^- \leftrightarrow P^+H_\alpha^-$ thermal equilibrium to model the charge recombination reaction studied in the 1 ns temporal window. This model may be applied independently of the interpretation of the different phases of charge recombination (static distribution of conformational states or dynamic relaxation of the state $P^+H_\alpha^-$) as it was discussed in ref 9.

In order to roughly estimate an equilibrium concentration of the states $P^+H_\alpha^-$ and $P^+B_\alpha^-$ on the 700 ps charge recombination time scale we have made a few following assumptions. First, we assumed that the steady-state absorption band at 544 nm is
Figure 5. Comparison of the relative amplitudes and shapes of the 544 and 600 nm bands of the two charge recombination spectra in QA-reduced RCs: subnanosecond (700 ps) and nondecaying (ND; see Figure 1C,D) may serve to estimate the equilibrium populations of the states P^+H^_A^- and P^+B^_A^- in the respective time scales. The decay of H^_A^- (due to charge recombination) should be manifested by the decay of photobleaching of the 544 nm band, whereas the decay of P^+ and B^_A^- should result in decay of photobleaching of the 600 nm band. In consequence, the decay of P^+B^_A^- should show a single negative band at ~600 nm, whereas the decay of P^+H^_A^- should show two negative bands: at 544 and at ~600 nm. We propose that the different relative amplitudes of these two negative bands for the ~700 ps and nondecaying spectra (Figures 1C,D and 2A,B) are a result of different contributions of the state P^+B^_A^- in these two spectra. Our second assumption, necessary to perform simple calculations, is that the amount of the RCs in the state P^+B^_A^- , being in equilibrium with the state P^+H^_A^-, may be neglected on the long time scale corresponding to the nondecaying component but is significant on 700 ps time scale. As shown below this assumption is justified: ~30% of QA-reduced RCs is in the state P^+B^_A^- on 700 ps time scale, and only ~6% on longer time scale. This implicates that the negative band at ~600 nm in the nondecaying spectra is contributed exclusively by P^+ from P^+H^_A^- state (Figure 1C,D). On the other hand, the band at ~600 nm in the ~700 ps DAS (greater than that in the nondecaying spectrum relative to the 544 nm band) contains an additional contribution from B^_A^- and P^+ originating from the state P^+B^_A^-.

Table 3: Contributions of the States P^+H^_A^- and P^+B^_A^- to the 600 nm Band of the 700 ps Charge Recombination Spectra and Free Energy Difference between These States (See Figure 5 and Text for Details)

<table>
<thead>
<tr>
<th>State</th>
<th>Q^-</th>
<th>Q^+ + o-phenanthroline</th>
</tr>
</thead>
<tbody>
<tr>
<td>A_iod(P^+H^_A^-)/A_iod(P^+B^_A^-) (%)</td>
<td>70/30</td>
<td>74/26</td>
</tr>
<tr>
<td>∆G (meV)</td>
<td>21</td>
<td>27</td>
</tr>
</tbody>
</table>

B^_A^- and P^+ originating from the other subpopulation of RCs being in the P^+B^_A^- state. Half of this remaining part was attributed to A_iod(P^+H^_A^-)/A_iod(P^+B^_A^-) and half to ∆G(B^_A^- - B^-) (assuming the same differential extinction coefficients for P^+H^- and for B^-). From the integrated areas related to P^+ from P^+H^_A^- and from P^+B^_A^- within the 600 nm band we calculated relative populations of the RCs with P^+H^_A^- and P^+B^_A^- . Next, using the Boltzmann distribution, we estimated free energy difference between these states in the fraction of RCs undergoing the ~700 ps charge recombination (Table 3). The same procedure was applied for QA-reduced RCs with addition of o-phenanthroline (Table 3). It turned out that despite significant drop in the population of RCs with P^+H^_A^- recombining in 700 ps in response to the addition of o-phenanthroline (Figure 1C,D and Table 2) the free energy gap between P^+H^_A^- and P^+B^_A^- was not significantly altered by this herbicide. Interestingly, the values of this gap, 21–27 meV, are small, comparable with those of the initial gap between these states of 0–75 meV42–48 (on the charge separation time scale) suggesting that both P^+H^_A^- and P^+B^_A^- states undergo similar energetic relaxation within first nanosecond after excitation.

Assumptions underlying our calculations described above should be confronted with earlier experimental observations. It has been shown that direct excitation of the B band at 800 nm leads, in a small fraction of RCs, to formation of the primary radical pairs, possibly including B^_A^-H^_A^-, without intermediate formation of P^+.49,50 However, even if such a radical pair is formed it is very unlikely that it persists on a time scale of charge recombination and influences our calculations. A possible small bleaching at 600 nm caused by reduction of H^_A^- has been neglected resulting in a possible underestimation of the concentration of the P^+B^_A^- state being in equilibrium with the P^+H^_A^- state.

The deep internal electrostatic modulation of the P^+H^_A^- → PHA charge recombination kinetics together with the modulation of the P^+B^_A^- → P^+B^_A^- equilibrium shown above indicate that the charge recombination occurs predominantly via the thermally activated state P^+B^_A^-, at least at room temperature. Having the 21–27 meV value of ∆G(P^+B^_A^- → P^+H^_A^-) for the RCs showing the ~700 ps charge recombination, and knowing the lifetimes of the two longer charge recombination phases from the combined fits (Figure 4), we may estimate the parameters of the model assuming the coexistence of RCs in three conformational states characterized by different free energy gaps between P^+H^_A^- and P^+B^_A^- . Figure 6 summarizes our calculations. The intrinsic charge recombination lifetime from the state P^+B^_A^- to the ground state, τ_i ≈ 200 ps, was estimated from the formula

$$\tau_i = \tau[1 + \exp(\Delta G/k_B T)]^{-1}$$

where ∆G = 21–27 meV, τ = 600–800 ps, k_B is the Boltzmann constant, and T is absolute temperature. The value of τ_i ≈ 200 ps is more precisely determined than previously (15–400 ps).9
between P to PHA charge recombination were taken from Figure 4.

This ~200 ps value is similar to that of the P → B charge recombination reported for the BCHl (Bb → BPh) mutant and smaller than the 675 ps charge recombination of P in the Phe to Asp(L121) mutant of Rb. spheeroides. The same eq 3 was then used to calculate the free energy gaps, ∆G, between P→ and lower lying P states. Equilibrium concentrations of the RCs in the state P→ estimated from these gaps for the fastest, intermediate, and slowest charge recombination conformations are respectively 30%, 6%, and 1% for Q→-reduced RCs, and 26%, 2%, and 0.7% for Q→-reduced RCs with of -phenanthroline. Assuming that in open RCs charge recombination occurs within ~10 ns, similarly as in RCs with removed Q→ whereas electron from H to Q is transferred within ~200 ps, it is easy to calculate that the efficiency of the forward reaction is ~98%.

The two or three subpopulations or conformational states of RCs (resolved in 1 ns and 1 ns + 80 ns temporal windows, respectively) differing in the rate of charge recombination and free energy levels of P→ were impossible to resolve kinetically and spectrally (as discussed above), and also energetically on the charge separation time scale. The overall effect of the negative charge on Q→ on the charge recombination time scale is an upshift of the average free energy of the state P→ (weighted with the populations of particular P→ states) relative to that of the P→ state. This upshift was observed before and may be easily concluded from the comparison of the data for Q→-removed and Q→-reduced RCs (Table 1). On the other hand, the screening effect of -phenanthroline decreases this upshift both by decreasing the population of quickly recombining conformation (in ~700 ps) and by slowing down recombination lifetime associated with the intermediate conformation from ~4 to ~8 ns (Table 1, Figure 6). Similar upshift of the averaged P→ level in Q→-reduced RCs is expected on the charge separation time scale and is consistent with the observed increase of the charge separation lifetime in Q→-reduced RCs.

The free energy upshift of the averaged state P→ on the time scales of both charge separation and charge recombination gives opposite effects on the rates of these two reactions: deceleration of the primary charge separation and acceleration of the charge recombination. These opposite effects are expected since the upshift decreases the driving force, ∆G(P→−P→), of the former reaction and at the same time shifts the free energy level of the P state closer toward the quickly recombining P→ state.

Figure 6. Free energy gaps between P→ and P→ calculated on the charge recombination time scales for Q→-reduced RCs (without and with 10 mM -phenanthroline). The lifetimes of the P→ PHA charge recombination were taken from Figure 4.

Abbreviations: B, B accessory bacteriochlorophyll; BCHl, bacteriochlorophyll; BPh, bacteriopheophytin; DAS, decay-associated spectrum; H, HA, primary electron acceptor; ND, nondecaying; P, primary electron donor; RC, reaction center.

Acknowledgment. The authors are very thankful to Neal Woodbury and Haiyu Wang for providing the samples, reading the manuscript, and valuable comments. K.G. acknowledges financial support from the Polish government (project entitled “Electrostatic control of electron transfer in purple bacteria reaction center”) and the technical assistance of Margareta Gibasiewicz.

Supporting Information Available: Figure showing decay-associated spectra of RCs at pH = 6. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes
