Selective perturbation of the second electron transfer step in mutant bacterial reaction centers

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Abstract

In order to specifically perturb the primary electron acceptor B_A — a monomeric bacteriochlorophyll (BChl) a — involved in bacterial photosynthetic charge separation (CS), the protein environment of B_A in the reaction center (RC) of Rhodobacter sphaeroides was modified by site-directed mutagenesis. Isolated RCs were characterized by redox titrations, low temperature optical spectroscopy, ENDOR/TRIPLE resonance spectroscopy and femtosecond time-resolved spectroscopy. Two mutations were studied: In the GS(M203) mutant a serine is introduced near the ring E keto group of B_A, while in FY(L146) a phenylalanine near the ring A acetyl group of B_A is replaced by tyrosine. In all mutations the oxidation potential of the primary electron donor P as well as the electronic structure of both the P^+ radical cation and the radical anion of the secondary electron acceptor, H_A^-, are not significantly altered compared to the wild type (WT), while changes of the optical absorption spectra at 77 K in the BChl QX and QY regions are observed. The GS(M203) mutation only leads to a minor retardation of the CS reactions at room temperature, whereas for FY(L146) significant deviations from the native electron transfer (ET) rates could be detected: In addition to a faster first (2.9 ps) and a slower second (1 ps) ET step, a new 8-ps time constant was found in the FY(L146) mutant, which can be ascribed to a fraction of RCs with slowed down secondary ET. The results allow us to address the functional role of the acetyl group of B_A and question the role of the free energy changes as the main determining factor of ET rates in RCs. It is concluded that structural rearrangements alter the electronic coupling between the pigments and thereby influence the rate of fast CS. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

In photosynthetic purple bacteria, the primary reaction is a light-induced charge separation (CS) within a trans-membrane pigment–protein–complex called the reaction center (RC). In Rhodobacter sphaeroides, the complex contains three protein subunits (L, M and H) and the cofactors, consisting of four bacteriochlorophyll (BChl) a molecules, two bacteriopheophytins (BPh a), one carotenoid, one non-heme iron and two ubiquinones. Two BChl a molecules form an electronically coupled dimer (P) that serves as primary electron donor, while the other BChls (B_A and B_B), BPhs (H_A and H_B) and quinones (Q_A and Q_B) are arranged in two branches with an approximate C_2-symmetry called the A and B branches [1–3]. The two monomeric tetrapyroles B_B and H_B are apparently not utilized for electron transfer (ET).
The primary CS process begins with the photoexcitation of the BChl a dimer to its first excited singlet state, P*. The subsequent ET steps have been investigated by time-resolved spectroscopy over the last two decades [4–12] and for native reaction centers (WT RC), the following reaction sequence could be established:

\[
P^* \xrightarrow{3.5 \text{ps}} P^+ B_A^- \xrightarrow{0.9 \text{ps}} P^+ H_A^- \xrightarrow{220 \text{ps}} P^+ Q_A^- \]

Improved techniques yielded further details about the primary ET: Deviations from a monoexponential decay [11,13–15], oscillatory features [16,17] and unique temperature dependences of the individual ET steps [18–21] indicate that the standard non-adiabatic ET theory has to be expanded for a sufficient description of the observed phenomena [22].

The investigation of modified RCs — either site specific mutants or pigment exchanged RCs — has provided valuable information on the energetics and the mechanism of photosynthetic electron transport [15,23–29]. Different site-specific mutant RCs can vary in their rate of the primary CS by more than one order of magnitude [18]. Furthermore, particularly modified RCs helped to determine unambiguously the role of B_A as primary electron acceptor; others allowed to estimate the free energy difference between the various ET-intermediates [9,18,30–32].

Fig. 1. (A) Position of the exchanged residues G(M203) and F(L146) with respect to the primary donor P (BChl a dimer) and the primary acceptor (monomeric BChl a) B_A. Also shown is one of the axial ligands of P, H(M202), which is the residue next to G(M203), and a water molecule in the vicinity of the 131-keto group of B_A. (B) Position of F(L146) with respect to B_A and the secondary acceptor (monomeric BPh a) H_A. Data from Ref. [2].
The interplay between the photochemically active cofactors P, B_\alpha and H_\alpha is crucial for an effective CS. Several studies have shown that the individual intermediates are affected by an altered protein environment. For example, a change of the hydrogen bonding pattern between P and the protein drastically influences the oxidation potential of the primary donor \[33,34\]. The residue at position M210 has been shown to influence the free energy of the intermediate P^+\text{BA}_{C_0} [35], but also the oxidation potential of P [27,36] as well as the occurrence of conformational substates of the H_\alpha^- radical anion [37,38]. This raises the question of whether the intermediate P^+\text{BA}_{C_0} can be specifically perturbed in order to single out in detail the role of B_\alpha in the ET process.

The aim of the present paper is to address this question with experiments on \emph{R. sphaeroides} mutants that were designed to carefully modify the B_\alpha binding pocket. In GS(M203) a serine residue is introduced near the 131-keto carbonyl (ring E) of B_\alpha (Figs. 1A and 2), while in FY(L146) the native phenylalanine near the 3-acetyl carbonyl (ring A) of B_\alpha is replaced with tyrosine (Figs. 1B and 2). Both introduced residues, potential hydrogen bond donors, could change the polarity of the environment of the primary electron acceptor B_\alpha and may thus influence its spectral, energetic and dynamic properties. The experiments presented below indicate that the new mutations — in particular FY(L146) — have indeed a very specific effect on B_\alpha and on the primary reaction dynamics.

2. Materials and methods

2.1. Strain construction and protein isolation

The \emph{R. sphaeroides} strain \Delta LM1.1, which carries a deletion in the \textit{puf} operon and lacks the expression of the RCs, was complemented in trans by the \textit{puf} operon on the broad host range vector pRKSK, conveying the mutation [39]. Site-directed mutagenesis was performed according to the altered sites mutagenesis procedure (Promega, Heidelberg, Germany). Cells were grown semi-aerobically under non-photosynthetic conditions and RCs isolated by well-known procedures [40,41]. Finally, the detergent LDAO was removed by supplementing samples with Triton X-100 (Fluka, Biochemika) and dialysing extensively against 0.03% (w/v) Triton X-100 in 10 mM Tris/HCl (pH 8.0), if not indicated otherwise. The wild-type (WT) RCs were those from the deletion strain complemented with WT genes.

2.2. Redox titrations

To determine the oxidation midpoint potential of P, the flash-induced absorbance change at 876 nm was measured as a function of the ambient potential. Samples were excited at 800 nm. Purified RCs were diluted to OD_{800} = 0.1 in 10 mM Tris/HCl (pH 8.0), 100 mM KCl, 0.03% (w/v) Triton X-100. The redox titration was carried out by adding varying amounts of ferricyanide and ferrocyanide to a series of aliquots from the same RC stock solution. Immediately after each determination of the absorbance, the potential was measured using a combination Pt/Ag/AgCl electrode (Schott PT5900A), which was calibrated against the redox potential of a saturated solution of quinhydrone as a function of pH. A pH-meter (Knick PHM82) was used to read out the redox potential. All redox potentials are given relative to the normal hydrogen electrode (NHE).

2.3. ENDOR/TRIPLE resonance spectroscopy

EPR, ENDOR, and Special TRIPLE resonance spectra were recorded with a Bruker ESP 300E spectrometer as previously described [41–43] by using ENDOR accessories and an ENDOR TM_{110} resonator of local design. The P^{*+} state was generated by in situ-illumination of the samples with filtered light (600 nm < \lambda_{ex} < 1000 nm). In some experiments, the detergent LDAO was exchanged to 1 mM CHAPS by dialysis in order to avoid the formation of the second, detergent-induced conformation of the P^{*+} state [42]. ENDOR/TRIPLE resonance spectroscopy of H_\alpha^- was performed as described in Ref. [43].

2.4. Low temperature optical spectroscopy

Dark-adapted samples containing ~ 3 \mu M RC, 0.03% (w/v) Triton X-100 (Fluka, Biochemika), 10 mM Tris/HCl (pH 8.0) and 70% (v/v) glycerol in a plastic cuvette of

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![Fig. 2. Molecular structure of BChl a with IUPAC numbering scheme. In BPh a the central Mg ion is replaced by two protons.](image-url)
1-cm path length were cooled to ~ 77 K in a liquid nitrogen cryostat DN1704 (Oxford Instruments). The cryostat was mounted in the sample compartment of a Cary 0SE spectrophotometer (Varian). All spectra were recorded in the double-beam mode versus air in the reference compartment. The spectra were corrected by subtracting a spectrum of RC-free buffer recorded under identical conditions.

2.5. Time-resolved experiments

The femtosecond spectrometer used for the time-resolved measurements was based on a Ti:sapphire laser system and a regenerative chirped pulse amplifier operating at a repetition rate of 20 Hz. The output pulses of the amplifier, centered at 860 nm, had a duration of 120 fs and a typical pulse energy of 600 µJ. To exclude excitation induced nonlinear effects (i.e. two photon absorption), the energy of the pump pulse was adjusted to 0.2 µJ and the spot size to a diameter of 350 µm. These limits guarantee that less than 10% of the sample was excited with each laser shot.

The dye IR146 was used as saturable absorber to block the weak oscillator pulses. A white light continuum, generated in a 2-mm sapphire-plate, provided probe-pulses in the spectral range of 600–1300 nm. The different probe wavelengths were selected by a double-pass-spectrometer and additionally the spectrum was adjusted to a width of 10 nm (fwhm). The diameter of the probe beam was 210 µm. All experiments presented here were recorded with parallel polarisation between pump and probe pulses. Cross-correlation measurements were performed at the sample position; a typical value for the cross-correlation width was 300 fs.

The measurements were performed in a 1-mm quartz cuvette at room temperature. Sample concentrations were adjusted to have a transmission of 30% at the excitation wavelength. Continuous stirring of the RC solution avoided the accumulation of photoproducts. Before and after measurements at a particular probe-wavelength the spatial overlap of the pulses and the delay-time zero were checked with the dye KODAK 9860.

The transient absorption changes shown in Figs. 4–6 represent a small selection out of two independent sets of measurements. In the x-direction, the scaling of the plots is linear for the first picosecond before and after delay time zero, logarithmic for longer delay times. Each individual point in the curves is the average of about 1000 independent single measurements.

A sequential reaction model was applied for detailed data analysis. This model assumes that the recombination rates are negligible, which is reasonable in the case of fast forward ET. The fits were obtained by using a global fitting procedure with a set of time constants common to all data sets, whereas the cross-correlation time, the delay time zero and the amplitudes of the time constants could vary individually for each curve. The model function was a sum of exponential functions, convoluted with the instrumental response function. The global fit analysis yielded $\chi^2$ values below $3 \times 10^{-4}$ for all samples.

3. Results

3.1. Steady-state optical absorption spectra

At room temperature the spectra of the new mutants are quite similar to that of the WT. Only a shift of the $Q_Y$ band of the BChls from 804 to 809 nm is observed in FY(L146) (data not shown). At low temperature (77 K) changes in specific regions of the spectra can be resolved (Fig. 3A). In the $Q_X$ region two bands at 597 and 605 nm assigned to BChl $a$ are observed for WT RCs, which are not significantly shifted in GS(M203), although the 605-nm band is more intense in the mutant. For comparison, the mutant GD(M203) causes a somewhat larger splitting of the BChl $Q_X$ band into two bands, 15 nm apart [40]. In FY(L146) the two bands are located at 594 and 604 nm in the second derivative spectrum, with the latter being more intense than the former (Fig. 3B). The two bands at 533 and 546 nm that are assigned to H$_B$ and H$_A$, respectively, are not affected by the mutations. Note that broad bands of the main carotenoid spheroidenone are superimposed to the BPh absorptions.

In the $Q_Y$ region significant changes are observed only for bands assigned to the monomeric tetrapyrroles, whereas the low energy component of the absorbance of P essentially remains unaffected (Fig. 3A). In WT RCs two bands can be resolved at 804 and 815 nm (Fig. 3C). The former is shifted by 2 nm to the blue in GS(M203). A similar, but larger shift has been observed earlier in GD(M203) [40,44]. In contrast, only one band at 809 nm is found in FY(L146). The 760-nm band due to the $Q_Y$ absorptions of the two BPhs in the RC is not affected by the mutations. The shoulder at about 754 nm that is most prominent in the spectrum of FY(L146), but also visible in the other spectra, probably arises from unspecifically bound pigment, since it could be reduced significantly by additional purification of RCs using anion exchange chromatography.

In summary, the optical spectra indicate specific perturbation of monomeric BChl $a$ due to the mutations, while the pigment composition of the RCs and the overall excitonic interaction of the chromophores are not affected.

3.2. Oxidation midpoint potential of P/P$^{**}$

The oxidation midpoint potential of WT RCs was found to be approximately 490 mV (vs. NHE) in this study. Since the absolute value of the potential is somewhat dependent upon the used experimental method, our value is in reasonable agreement with literature data, ranging from 453 [33] to 506 mV [45]. For the two mutants, GS(M203) and FY(L146), we found values of 489 and 497 mV, respectively. In view of the estimated error of ±10 mV, the
deviations from the WT are not significant. In comparison, mutations at position M210 increase the oxidation potential by 30 to 50 mV [27,36], whereas in the mutant GD(M203) it is identical to that of the WT [40].

3.3. Special TRIPLE resonance spectroscopy of P**

Due to the electronic coupling of the two BChl moieties of P, the unpaired electron of the P** state is distributed over both dimer halves and interacts magnetically with nuclei of both cofactors. It is therefore possible to assess the probability of finding the electron on either dimer half, P_L or P_M, from a measurement of the hyperfine coupling (hfc) between the electron and certain protons of the two BChls by using ENDOR or Special TRIPLE resonance spectroscopy [46]. For WT RC the unpaired spin density is found to be 68% on P_L [47]. This value is not changed in the two mutants GS(M203) and FY(L146) (spectra not shown). In comparison, in mutations at position M210 that do not form or remove hydrogen bonds to either dimer half, the spin density on P_L varies between 67% and 71% [48]. Since the distribution of the electron is very sensitive to perturbations by either site-directed mutations [41,48] or detergents [42], these findings indicate that the primary donor is essentially unaffected by the mutants studied here.

Fig. 3. Optical absorption spectra of dark-adapted mutant and wild type RCs from *R. sphaeroides* (~ 3.5 µM) in 10 mM Tris/HCl (pH 8.0), 70% (v/v) glycerol and 0.03% (w/v) Triton X-100 at T = 77 K. Spectra are normalized to the long wavelength absorption of P around 890 nm. (A) Absorption spectra between 350 and 1000 nm. Note the changes in the BChl regions around 600 and 800 nm. (B) Second derivative of the Q_x region. The two bands at 533 and 546 nm are assigned to H_b and H_a, respectively, whereas the bands around 600 nm are due to the four BChls in the RC. (C) Second derivative of the Q_y region. The bands around 800–820 nm — that are affected by the mutations — mainly belong to the two monomeric BChls in the RC, while the BPhs absorb around 760 nm. The small shoulder at 754 nm is probably due to contamination of the samples with free pigment.
3.4. ENDOR spectroscopy of $H_A^\pm$

The unpaired electron trapped on the photoactive BPh in the $H_A^\pm$ state can also be investigated on the basis of proton hfc’s [46]. The distribution of the $\pi$-electron over the tetrapyrrole ring of $H_A$ is sensitive to pigment–protein interactions such as hydrogen bonding [43] and the orientation of the 3-acetyl group with respect to the macrocycle [37,38]. Conformational twists of the saturated rings B and D that might be visible in the ENDOR spectra usually have no large effect on the $\pi$-electron distribution. As will be discussed below, the electronic structure of the $B_A^\pm$ radical anion is assumed to depend in a similar manner on these factors, but cannot be trapped and investigated with ENDOR spectroscopy.

The ENDOR spectra of $H_A^\pm$ are similar for both the FY(L146) and the WT RC (spectra not shown), indicating that the mutation near the acetyl group of $B_A$ does not significantly perturb the unpaired $\pi$-electron distribution of $H_A^\pm$. This state has not been investigated in the GS(M203) RC. However, an earlier study of the mutant GD(M203) suggests that the $\pi$-electron distribution of $H_A^\pm$ is not changed by mutations at this position, whereas small conformational changes of the saturated rings of $H_A$ cannot be excluded [43].

3.5. Femtosecond time-resolved absorption spectroscopy

The experimental results presented in this section are summarized in Table 1. Selected transients at characteristic probing wavelengths from the fs-time resolved experiments are presented for the GS(M203) RC in Fig. 4. The points represent experimental data and the solid curves fits. These fits are compared with the best curve approximations for WT RC at the corresponding probing wavelengths (dotted curves). At $\lambda_{pr} = 780$ nm electrochromic shifts of monomeric BChls as a response to the formation of $P^+H_A/C_0$ are the dominating contributions to the signal, while at $\lambda_{pr} = 920$ nm the peak of the stimulated emission of $P^*$ is found, and hence the lifetime of the first excited state of the special pair can be monitored. The changes at $\lambda_{pr} = 1020$ nm reflect the population of the BChl $a$ anion $B_A^\pm$, and at $\lambda_{pr} = 1250$ nm mainly the formation of the cation $P^{++}$ is seen. In general, the dynamics of the mutated RCs at these special spectral positions of the probing light, indicative for different features of the ET reactions, are similar to those of the WT RC. There are only slight differences between GS(M203) and WT. From the results at $\lambda_{pr} = 920$ nm, we deduce the decay time of $P^*$ (monoexponential fit) to be in the order of $\tau_1 = 3.8$ ps, slightly above the value for WT RC ($\tau_1 = 3.5$ ps). The most pronounced differences are seen at 1020 nm (Fig. 4c), where the early part of the absorbance changes reflect the decay time $\tau_2$ of $P^B_A$. The modeling suggests a longer time constant of $\tau_2 = 1.3 \pm 0.2$ ps in the mutant than in WT RC ($\tau_2 = 0.9$ ps). The formation of $P^+$ (Fig. 4d) monitored at $\lambda_{pr} = 1250$ nm can be fitted well using the $\tau_1 = 3.8$ ps kinetic component. This transient also reveals a slower absorption change, which

Table 1
Summary of experimental results

<table>
<thead>
<tr>
<th>Strain</th>
<th>WT</th>
<th>GS(M203)</th>
<th>FY(L146)</th>
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<td>$\tau_1$/ps</td>
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<td>3.8</td>
<td>2.9</td>
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<td>$\tau_2$/ps</td>
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<td>1.3</td>
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<td>220</td>
<td>220</td>
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<tr>
<td>$\lambda_{max}$/nm</td>
<td>597, 605</td>
<td>597, 605</td>
<td>594, 604</td>
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<tr>
<td>$Q_X$</td>
<td>806, 815</td>
<td>804, 815</td>
<td>809</td>
</tr>
<tr>
<td>$E_{m}/mV$</td>
<td>490</td>
<td>489</td>
<td>497</td>
</tr>
<tr>
<td>$q_L$</td>
<td>0.68</td>
<td>0.68</td>
<td>0.68</td>
</tr>
</tbody>
</table>

a: Lifetime of $P^*$.  
b: Room temperature.  
c: Lifetime of $P^B_A$.  
d: Lifetime of $P^H_A$.  
e: Monomeric BChl absorption maxima at $T = 77$ K.  
f: Oxidation midpoint potential of $P$ at room temperature.  
g: Fraction of unpaired spin density on $P_L$ in the $P^{++}$ state at $T = 288$ K.

Fig. 4. Transient absorbance changes of the GS(M203) RC for selected spectral regions, where the electrochromic shift of the $Q_Y$ B-band (a), the $P^*$ decay (b), $B_A$ (c) and $P^+$ (d) formation can be observed. Solid points represent the experimental data; model functions of the data using the time constants given in the text are displayed as solid lines. For comparison, the WT RC curves at the corresponding probing wavelengths are plotted as dotted lines.
is well described by a weak component with $\tau_3 \approx 220$ ps. This latter time constant is assigned to the ET from HA to the quinone QA and is even more pronounced at $\lambda_{pr} = 780$ nm (Fig. 4a). The differences between WT and GS(M203) observed at 780 nm in the 10–200 ps time range are obviously related to $P^+HA$. The spectral change is most likely caused by a different electrochromic shift of the BA band, the main contribution at this wavelength.

The results on the GS(M203) RC can be summarized as follows: The primary reaction dynamics are nearly unaffected compared to WT RC. Both ET steps are slowed down, $\tau_3$ changes more than $\tau_1$. The electrochromic response of HA to the formation of $P^+HA$, appears to be slightly affected as judged from the observed changes at 780 nm in the 10–200 ps time range, indicating a modified environment near HA in the mutated RC.

In the case of FY(L146), larger differences of the fs-time resolved data with respect to the WT RC become evident. The decay of the excited electronic state $P^*$ (Fig. 5b) at $\lambda_{pr} = 920$ nm is slightly accelerated in FY(L146) to a time constant of $\tau_1 \approx 2.9$ ps. In the BChl-anion band at 1020 nm (Fig. 5c) the early and late parts of the signal trace are close to WT RC. However, there is a stronger induced absorption at delay times between 2 and 20 ps. The absorbance changes can be fit by a weak $\tau_2 = 1$ ps, a stronger $\tau_2a = 8$ ps kinetic component. The same kinetic differences between native and mutant RCs are observed at $\lambda_{pr} = 780$ nm: The strong deviation is again apparent in the 2–20 ps temporal range and the 8-ps kinetic component is necessary to correctly describe the data from FY(L146).

This 8-ps component also strongly contributes to the signal at $\lambda_{pr} = 800$ nm, i.e. in the monomeric BChl absorption band (Fig. 6, top). The spectral range of the BChl-anion band was investigated in greater detail (Fig. 6, bottom). Again, the absorption changes in FY(L146) are compared with WT RC (dotted lines). The 8-ps component is most clearly visible at 1040 nm, i.e. at a spectral position red shifted compared to the peak of the $B^A_{C0}$ band at 1020 nm in WT RC.

The amplitudes related to the two time constants $\tau_2a = 1$ ps and $\tau_2b = 8$ ps (deduced from the fits of the FY(L146) data) as a function of probing wavelength are plotted in Fig. 7 (top).
The most striking features are the same spectral characteristics but the opposite signs of the amplitudes. In the BChl absorption range at $\lambda_{pe} = 800$ nm the 1-and 8-ps kinetic components again have opposite signs. The close spectral correlation of the two kinetic components together with the finding that the 8-ps component was not observed outside the BChl and BChl$^\text{S}$/C0 bands leads to the assignment that $s_2b$ is related to the ET properties of BA.

It should be noted that the biphasic ET observed here is different from heterogeneities reported previously \[11,13–15\]. In many earlier studies, the changes representing the first ET step had to be modeled with (at least) two $\tau_1$ components, the amplitudes of which varied between preparations even for the WT RC. Such heterogeneities of the first ET step cannot be resolved in the samples used in the present study. Therefore, the two time constants assigned to $\tau_2$ reflect a new and special property of the FY(L146) RC.

In conclusion, the FY(L146) mutation has significant consequences for the primary CS: The first ET step is accelerated to $\tau_1 = 2.9$ ps, and the $\text{P}^\text{B}_\Lambda \rightarrow \text{P}^\text{H}_\Lambda$ step is biphasic with 1- and 8-ps kinetic components.

4. Discussion

4.1. GS(M203) RC

The effects of the GS(M203) mutation on the primary CS in the bacterial RC (Table 1) are much less pronounced, but point in the same direction, than the known results for the GD(M203) RC \[40\]. The introduction of an Asp residue results in a much slower P* decay ($\tau_1 = 9.4$ ps), but is also not correlated with a corresponding change in the P/P$^*$ oxidation potential. The extensive discussion of this mutation in the literature \[40,44,49\] focused on two questions,
namely (i) whether D(M203) is protonated or charged, and
(ii) whether there is a native hydrogen bond between a water
molecule and the 13\textsuperscript{1}-keto-group of B\textsubscript{A} [50].

In the case of GS(M203), it is unlikely that the introduced
serine is deprotonated, since this should result in a deceler-
ation of the first ET step at least as large as in GD(M203). If
S(M203) is protonated, then there are several possibilities:
The direct formation of a strong hydrogen bond from
S(M203) to the 13\textsuperscript{1}-keto group of B\textsubscript{A} also appears unlikely,
since this should again cause more drastic changes in the
time constants than observed. On the other hand, the side
chain of serine could disturb the water molecule near the 13\textsuperscript{1}-
keto group of B\textsubscript{A}, thereby either removing the putative
hydrogen bond or replacing it with a somewhat weaker
one from the hydroxyl oxygen of serine. In both cases, a
slight blue shift of the QY band of BA and a destabilization of
P+BA would occur. In addition, the polar character of the
side chain could lower \( G^0(P)^{\text{B}A} \), as demonstrated for a set
of different RC mutants (at position M210) in experiments
and electrostatic calculations [51].

Within standard ET theory [52], one also has to ask for
contributions from changes of the electronic coupling matrix
elements \( V \) and the reorganization energy \( \lambda \). Steric con-
straints produced by the replacement of glycine with the
more bulky serine as well as variations of the frontier
orbitals of B\textsubscript{A} by changed hydrogen bonding interactions
with the 13\textsuperscript{1}-keto group could affect both the electronic
coupling \( V_{\text{PB}} \) between P* and P\textsuperscript{+}B\textsubscript{A} and \( V_{\text{BH}} \) between
P\textsuperscript{+}B\textsubscript{A} and P\textsuperscript{+}H\textsubscript{A} [53–55]. In addition, the side chain
of S(M203) may be reoriented during ET, depending on
whether it is hydrogen bonded or not. Therefore, changes
of both \( V \) and \( \lambda \) could contribute to the observed rates. In
summary, it is not clear at present how the GS(M203)
mutant affects the primary CS, and different effects may
compensate for each other.

4.2. FY(L146) RC

The effects of replacing phenylalanine with tyrosine at
position L146 in the vicinity of the 3\textsuperscript{1}-acetyl group of B\textsubscript{A} on
the energetics of this cofactor can be assessed from a
comparison with a similar mutant near the acetyl group of
P\textsubscript{M}, namely FY(M197). This latter mutant has been inves-
tigated in detail [33,56–58]. X-ray crystallography in con-
junction with FTIR spectroscopy indicated the formation of
a hydrogen bond between Y(M197) and the 31-acetyl group
of BA, thereby either removing the putative hydrogen bond or replacing it with a somewhat weaker
one from the hydroxyl oxygen of serine. In both cases, a
slight blue shift of the QY band of BA and a destabilization of
P+BA would occur. In addition, the polar character of the
side chain could lower \( G^0(P)^{\text{B}A} \), as demonstrated for a set
of different RC mutants (at position M210) in experiments
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P\textsuperscript{+}B\textsubscript{A} and P\textsuperscript{+}H\textsubscript{A} [53–55]. In addition, the side chain
of S(M203) may be reoriented during ET, depending on
whether it is hydrogen bonded or not. Therefore, changes
of both \( V \) and \( \lambda \) could contribute to the observed rates. In
summary, it is not clear at present how the GS(M203)
mutant affects the primary CS, and different effects may
compensate for each other.

Using a recently refined model for the relationship between
the spin density distribution (over P\textsubscript{L} and P\textsubscript{M}) and the P/P\textsuperscript{+}
oxidation potential [59,60], we estimate that the HOMO of
P\textsubscript{M} is stabilized by 50–70 meV due to the FY(M197)
mutation.

Assuming the QY band of BChl \( a \) to be essentially due to a
HOMO→LUMO transition [61,62], we can estimate from the
observed 5-nm red-shift (Fig. 3C) that the HOMO/ LUMO difference is diminished by \( \sim 10 \) meV in FY(L146).
If the effect of replacing phenylalanine with tyrosine near
the acetyl group on the HOMO energy is essentially the
same for P\textsubscript{M} and B\textsubscript{A}, it would then follow that the LUMO
of B\textsubscript{A} and hence the state P\textsuperscript{+}B\textsubscript{A} is stabilized by at least
\( \sim 40 \) meV in FY(L146).

A kinetic analysis of the primary CS in a variety of
mutants [63] as well as in chemically modified RCs [32,33]
suggests, that for the first ET step \( \Delta G^0 = -60 \pm 23 \) meV and
\( \lambda = 100 \pm 30 \) meV in the WT RC. Using these values and
the assumption that the FY(L146) mutation only affects
\( G^0(P)^{\text{B}A} \), we find on the basis of the above estimates that the
experimental value of \( \tau_1 \) is in principal agreement with the
expected stabilization of the first intermediate state.
However, the effect of the FY(L146) mutation on \( \tau_2 \) cannot
be understood in this simple way.

The experimental results show a strong influence of the
FY(L146) mutation on the secondary ET: Besides a time
constant \( \tau_{2a} \approx 1 \) ps similar to the one in WT RC, we find an
additional 8-ps kinetic component, which is strongly con-
nected to the spectral range of the BChl and BChl\textsuperscript{−}
absorption bands. Therefore, we assign the 8-ps component
also to the second ET step. In this context, we discuss a
reaction model where we have a sample inhomogeneity
induced by the mutation: A fraction \( \eta \) of the reaction centers
has a secondary ET time \( \tau_{2a} \), while a fraction \( (1 - \eta) \) reacts
with \( \tau_{2b} \). Since no such inhomogeneity can be resolved for the
first and third ET step, we describe them for simplicity
by the same single lifetimes \( \tau_1 = 2.9 \) ps and \( \tau_2 = 220 \) ps,
respectively. For \( \tau_{2a} = 1 \) ps and \( \tau_{2b} = 8 \) ps, the population
densities of the different intermediates are obtained as given
in Fig. 7 (bottom). As mentioned in earlier publications, the
time constant \( \tau_2 \) is observed experimentally in the formation of
the intermediate P\textsuperscript{+}B\textsuperscript{−} for the case \( \tau_2 < \tau_1 \), while it is
found in the decay of P\textsuperscript{+}B\textsuperscript{−} for \( \tau_2 > \tau_1 \) [32,48]. As a
consequence, the amplitudes connected to \( \tau_{2a} \) and \( \tau_{2b} \) should
have opposite signs. The rate equation model allows to
estimate \( \eta \) (fraction of the relative concentration of the two
species) from the experimental data. For constant absorption
cross sections of the different RC, we can deduce \( \eta \) from the
fit parameters of Fig. 7 (top) to be \( \eta = 0.7 \), i.e. 70% of the
RC have a fast secondary ET (with \( \tau_{2a} = 1 \) ps) while 30%
react more slowly with \( \tau_{2b} = 8 \) ps.

To explain the occurrence of two distinct forms of the
FY(L146) RC within standard ET theory [52], we have to
ask for differences of the relevant parameters \( G^0(P)^{\text{B}A} \), \( \lambda \),
\( V_{PB} \) and \( V_{BH} \) between the two forms. Since the P/P\textsuperscript{+}
potential and the optical properties of P are not significantly
changed by the mutation, $G^0(P^*)$ is assumed to be unaffected. In addition, the constancy of $\tau_1$ indicates that any changes of $G^0(P^\Lambda H_{\Lambda})$ can also be neglected. If the major differences between the two forms would be a shift of $G^0(P^\Lambda B_{\Lambda})$, one would expect a strong change in both $\tau_1$ and $\tau_2$. Since $\tau_1$ is only weakly accelerated and since we do not observe an increased heterogeneity for the first ET step, the experimental observations argue against such a difference, i.e. the state $P^\Lambda B_{\Lambda}$ is stabilized in both forms of the FY(L146) RC by a similar amount with respect to the WT RC. Likewise, significant differences of $\lambda$ between the two forms should also result in a detectable inhomogeneity of the first step. Therefore, only a difference in $V_{BH}$, but not $V_{PB}$, between the two forms can explain the kinetics observed in the FY(L146) RC.

4.3. Role of electronic coupling and acetyl group orientation

Recent theoretical studies have shown that the electronic coupling matrix elements are highly sensitive to small changes in electron donor and acceptor position, and thus critically influence rate and unidirectionality of primary ET [54,55]. A translation or tilt of $B_{\Lambda}$ as a whole seems unlikely, since this would affect both $V_{PB}$ and $V_{BH}$. In order to explain the predominant effect on $V_{BH}$, one has to assume that the FY(L146) mutation somehow causes a distortion or bending of only one part of the $B_{\Lambda}$ macrocycle, such that the effective distance between $B_{\Lambda}$ and $H_{\Lambda}$ is decreased, whereas that between $P$ and $B_{\Lambda}$ is unaffected. Another interesting explanation — requiring less structural perturbations — is suggested by semi-empirical calculations of bacteriochlorin radical anions in vacuo. These calculations showed that the reorientation of the 31-acetyl group affects the distribution of the unpaired electron between rings $A$ and $C$ of the macrocycle and that the torsion potential of the acetyl group is in general a double-well potential [64]. Since these calculations did not take into account the protein environment, they can serve only as a qualitative guide for the following discussion. However, it can be concluded that a mixture of two distinct torsional isomers of the acetyl group might occur at elevated temperatures. How does this affect the electronic coupling matrix elements?

A possible relationship between the acetyl group orientation and the electronic structure of bacteriochlorinoids was revealed by recent ENDOR studies of RCs from $R. sphaeroides$, showing the existence of two distinct conformations of $H_{\Lambda}^\Lambda$ [43]. The occurrence of these conformations was found to be influenced by mutations near the 31-acetyl group of $H_{\Lambda}$, i.e. at position M210 [37]. It was concluded that the orientation of the acetyl group is the main difference between the two conformations. Of particular interest is the finding that the two forms of $H_{\Lambda}^\Lambda$ differ mainly in the electron density near ring $A$, whereas the wave function is perturbed to a lesser extent near ring $C$, indicating a predominantly local effect of acetyl reorientation on the LUMO of the bacteriochlorinoids. This feature was not so clearly born out by the earlier calculations [64]. However, similar calculations on the RC structure [53,54] showed that the electronic coupling between $B_{\Lambda}$ and $H_{\Lambda}$ is determined by the contact between rings $A$ of the two cofactors, probably involving hyperconjugation via the 21-methyl groups (Fig. 1B). Altogether, these studies point to the possibility that manipulations of the RCs leading to a reorientation of the acetyl group of either $B_{\Lambda}$, $H_{\Lambda}$ or both could change the LUMOs of the corresponding cofactors in a way that $V_{BH}$ is more strongly affected than other coupling matrix elements.

On the basis of the above considerations, we propose as a working model, that the two forms of the FY(L146) RC mainly differ in the orientation of the 31-acetyl group of $B_{\Lambda}$ (possibly accompanied by a distortion or bending of the macrocycle near ring $A$). This could cause a difference of $V_{BH}$ between the two conformations, while $V_{PP}$, $G^0(P^\Lambda B_{\Lambda})$ and $\lambda$ differ to a much lesser extent. This interpretation suggests that the mutation alters the torsion potential of the acetyl group from a single-well potential in the WT RC to a double-well potential in the FY(L146) RC. Since the acetyl group is a strong dipole, it is conceivable that the addition of the OH-dipole of tyrosine in its vicinity could affect the dynamics of the acetyl group in this way. Acetyl group reorientation also provides a simple explanation of the observed band shift in the $Q_Y$ region [61].

Our working model has several interesting implications: Although the observed change of $\tau_1$ in the FY(L146) RC is in principle agreement with the formation of a hydrogen bond to the 31-acetyl group of $B_{\Lambda}$ (see above), the occurrence of two time constants $\tau_{2a,b}$ for the second step argues against such an interpretation. On the basis of our working model, a strong hydrogen bond as observed for FY(M197) [58] would probably lock the acetyl group in one preferred orientation — similar to the acetyl group of $H_{\Lambda}$ in the mutant YH(M210) [37] — and only a single time constant $\tau_2$ would be observed. Note that hydrogen bonding is not necessary for a stabilization of the state $P^\Lambda B_{\Lambda}$ due to the introduced OH-dipole [51]. Therefore, our model does not exclude the possibility that a change of $G^0(P^\Lambda B_{\Lambda})$ contributes to the altered ET rates in the FY(L146) RC.

Because of the strong dipole of the CO fragment, the orientation of the acetyl group and hence the electronic coupling between pigments is sensitive to electric fields. This implies that applied electric fields could change ET rates not only via a shift of the free energies of radical pair states [65], but also via a modulation of electronic coupling matrix elements.

Deviations from monoexponential ET are usually explained by energetic heterogeneity of radical pair states [22]. Our data indicate that local structural perturbations of the chromophores can cause significant alterations of ET rates. Therefore, dispersive ET could well originate from a heterogeneity and uncertainty of electronic coupling matrix elements, which in turn to a large extent may be due to the torsional motion of the acetyl groups.
5. Conclusions

The primary photosynthetic CS has been investigated in RC mutants designed to specifically modify the environment of the first electron acceptor B_A without largely perturbing the functional properties of neighboring cofactors. The slight effect of the GS(M203) mutation on the CS cannot be explained unambiguously on the basis of the present data and more information such as a X-ray structure is required. In contrast, the effect of the FY(L146) mutation allows for some definite conclusions: Here, a biphasic secondary ET is observed with the two time constants differing by a factor of 8, whereas no such heterogeneity can be resolved for the first ET step. This results in a clearly increased population density of the first intermediate state P+BA (Fig. 7). Within the framework of standard non-adiabatic ET theory [52], this behavior can only be explained by significant differences in the electronic coupling matrix element V_{BH} between two forms of the FY(L146) RC.

The simplest explanation for a specific effect of a mutation on V_{BH} is suggested by ENDOR studies [37,38], emphasizing the possibility that a reorientation of the 3'-acetyl group of the bacteriochlorin macrocycle predominantly perturbs the LUMO near ring A (Fig. 2). Together with the results from semi-empirical calculations [53,54,64], these experimental findings indicate that the electronic coupling between B_A and H_A strongly depends on the orientation of the acetyl groups (Fig. 1B). The present data clearly support this view and show that both the energetics of the relevant intermediate states and their electronic coupling must be fine-tuned in order to optimize the photosynthetic CS. This fine-tuning not only requires a proper adjustment of inter-chromophore distances within the pigment–protein complex, but also an exact orientation of the acetyl groups. In view of the sensitivity of the torsional motion of the acetyl groups to environmental perturbations, it is then not surprising that dispersive and multiexponential ET is often observed.

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