Modulation of Primary Radical Pair Kinetics and Energetics in Photosystem II by the Redox State of the Quinone Electron Acceptor QA

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ABSTRACT Time-resolved photovoltage measurements on destacked photosystem II membranes from spinach with the primary quinone electron acceptor QA either singly or doubly reduced have been performed to monitor the time evolution of the primary radical pair P680⁺Pheo⁻. The maximum transient concentration of the primary radical pair is about five times larger and its decay is about seven times slower with doubly reduced compared with singly reduced QA. The possible biological significance of these differences is discussed. On the basis of a simple reversible reaction scheme, the measured apparent rate constants and relative amplitudes allow determination of sets of molecular rate constants and energetic parameters for primary reactions in the reaction centers with doubly reduced QA as well as with oxidized or singly reduced QA. The standard free energy difference ΔG° between the charge-separated state P680⁺Pheo⁻ and the equilibrated excited state (Chl₆P680⁺) was found to be similar when QA was oxidized or doubly reduced before the flash (−50 meV). In contrast, single reduction of QA led to a large change in ΔG° (−40 meV), demonstrating the importance of electrostatic interaction between the charge on QA and the primary radical pair, and providing direct evidence that the doubly reduced QA is an electrically neutral species, i.e., is doubly protonated. A comparison of the molecular rate constants shows that the rate of charge recombination is much more sensitive to the change in ΔG° than the rate of primary charge separation.

INTRODUCTION

Photosystem II (PS II) is a membrane-bound protein complex that catalyzes the conversion of light energy into a more stable form of electrochemical energy during the primary processes of oxygenic photosynthesis (for a review see Diner and Babcock, 1996). With respect to the structure and function, the core subunits of PS II are thought to be similar to those of the better characterized purple bacterial reaction centers (RCs), both belonging to the family of quinone-type RCs (Rutherford and Nitschke, 1996). Light absorption by chlorophylls in antenna proteins (light-harvesting complexes) leads to the creation of excited singlet states, called excitons. The excitons are rapidly equilibrated within the antenna as well as between the antenna and RC (McCaulay et al., 1989; Holzwarth, 1991). The excited states can be depopulated either by trapping in the RC (photochemical quenching), by internal conversion, or by emission of fluorescence (Geacintov and Breton, 1987). The trapping is defined as the conversion of an excited state into a charge-separated state in the RC. This primary charge separation occurs by electron transfer from an excited primary donor, a chlorophyll species (P680), to a primary electron acceptor, a pheophytin (Pheo), creating the primary radical pair P680⁺Pheo⁻. The primary radical pair (RP) decays by forward electron transfer to the first quinone electron acceptor QA (τ ≈ 500 ps) when the latter is oxidized (open RC), or by charge recombination in the nanosecond range when QA is already reduced (closed RC). This charge recombination may occur by several competing pathways such as reformation of the excited singlet state P680⁺, direct recombination to the ground state, or via singlet-triplet mixing to populate the triplet state of the primary donor.

PS II is generally considered as a shallow trap, with the free energy of the charge-separated state being close to the one of the excited state (van Gorkom, 1985). There are two main reasons why PS II is different from the other photosystems in this respect. First, the energy transfer between the antenna chlorophylls and the primary donor is largely reversible due to similar energy levels of their excited states Chl* and P680* In a first approximation, full equilibration of the excited states leads to a decrease of the free energy of the state (Chl₆P680⁺) compared with that of Chl₆P680⁺, due to an entropy term $S = k_B T \ln N_{\text{eff}}$, where $N_{\text{eff}}$ is the effective number of pigments over which the excitation is equilibrated (see below) (Trissl, 1993; van Miegem et al., 1995). At physiological temperatures and due to the relatively large antenna size, this entropy contribution amounts to $\sim 120$ meV. Second, the extremely positive redox midpoint potential of the primary donor $E_m(P680⁺/P680)$, of the order of +1.1 eV, necessary to drive the oxidation of water, causes its excited singlet state redox potential $E_m(P680⁺/P680⁺⁺)$ to be less negative than in all other systems. Both effects bring the free energy of the equilibrated excited state (Chl₆P680⁺) close to the level of the radical pair state P680⁺Pheo⁻. As a consequence, trapping has to be considered as a reversible reaction and is usually described by an exciton/radical pair equilibrium model (Schatz et al., 1988; Leibl et al., 1989).
The formation of the primary radical pair is a crucial step of photosynthetic energy conversion as it has to be kinetically competitive with wasteful loss processes depopulating the excited states. The small driving force makes the yield of primary charge separation in PS II particularly sensitive to small changes in the energetics. A well-known example is the large increase in fluorescence yield upon reduction of QA. It is interpreted as a shift of the equilibrium between the charge-separated state and the equilibrated excited state toward the excited state (Schatz et al., 1988). From the kinetic point of view, equilibration of the excited state is much faster than its lifetime. Therefore, the trapping is considered as limited by the primary charge separation in the RC, i.e., trap-limited (e.g., Schatz et al., 1988; Leibl et al., 1989). The shallow-trap properties of PS II make this system interesting to study the influence of small energy changes induced by electrostatic interactions within the proteins on the kinetics of intra-protein electron transfer.

The efficiency of charge stabilization on QA depends to a large extent on the ratio of the rate constants for electron transfer from Pheo to QA and backward to P680. However, the latter rate constant is not easily accessible. One approach to assess the intrinsic rate of back-reaction is to block the forward electron transfer to QA. In principle this can be realized by pre-reducing QA to QA, either by light or chemically. However, according to various authors, the single reduction of QA makes the primary charge separation slower and less efficient (Schatz et al., 1988; Roelofs et al., 1992) and/or makes the charge recombination faster compared with RCPs with oxidized QA (Leibl et al., 1989; Roelofs et al., 1992). As mechanisms responsible for these effects, electrostatic interaction between the negatively charged QA and Pheo (van Mieghem et al., 1995) or changes of local protein conformation (van Mieghem et al., 1992) have been proposed. On the other hand, several observations indicate that strongly reducing conditions can cause double reduction of QA and that this state is probably stabilized by double protonation leading to the electrically neutral state QAH2 (van Mieghem et al., 1992, 1994, 1995; Vass et al., 1992; Liu et al., 1993). Such a state with blocked forward electron transfer but electrostatic conditions similar to open RCs should be well suited for determination of the intrinsic rate constant of charge recombination of the primary pair.

The efficiency of creation and stability of the RP in various experiments with blocked electron transfer from Pheo to QA has been usually assessed by two quantities discussed in the literature: RP yield and lifetime. As the RP is a transient state, its yield is not easily defined. The definition adopted in the following is the maximum transient concentration of the radical pair (RPmax) relative to the number of photons absorbed at low excitation energy. The RP lifetime τ is the apparent time constant in the exponential function exp(−t/τ), which fits the RP decay deduced from the analysis of the experimental kinetics. These quantities were examined by several groups mainly for samples with singly reduced QA (QAH or state) (Nuijs et al., 1986; Schatz et al., 1987, 1988; Schlodder and Brettel, 1988; Hansson et al., 1988; Leibl et al., 1989; Liu et al., 1993; van Mieghem et al., 1995) but also for samples with doubly reduced QA (referred to in this paper as QAH state) (Liu et al., 1993; van Mieghem et al., 1995). The results of these studies are ambiguous. Reported RP yields at room temperature for the QAH state vary from 10% (Leibl et al., 1989) to 60% (Schlodder and Brettel, 1988) depending on the antenna size, species, and probably the procedure of preparation and the experimental method. At low temperature (20 K), a RP yield of 100% has been determined (van Mieghem et al., 1995). The published values of the RP lifetime in the QAH state at room temperature vary from ~1 ns (Leibl et al., 1989) to 11 ns (Schlodder and Brettel, 1988) or even longer (Takahashi et al., 1987; Hansson et al., 1988; Liu et al., 1993). In samples with QA doubly reduced (in core complexes from Synecococcus containing ~40 chlorophyll molecules per RC), van Mieghem et al. (1995) reported at room temperature a RP lifetime of 13 ns and a yield of 100%. Liu et al. (1993) measured the RP decay of PS II core complexes from spinach in this state to be biphasic with lifetimes of 4 ns (40%) and 30 ns (60%). It is worth noting that RP lifetimes measured in isolated RCs are generally longer than in more intact preparations and extend to several tens of nanoseconds (Takahashi et al., 1987; Hansson et al., 1988; Booth et al., 1991).

In most kinetic studies of the primary RP in PS II, absorption change measurements or fluorescence decay measurements have been applied. Both techniques have some drawbacks. In the former a limited time resolution and/or contribution from excited states often do not allow correct measurements of the fastest decay components. With pump-probe techniques, lifetimes longer than a few nanoseconds are difficult to detect due to the limitations in the delay time. Fluorescence measurements might suffer from contributions from unconnected antenna chlorophylls or they might, in the case of a distribution or relaxation of radical pair states, over-represent the states lying energetically closer to the excited state. In addition, fluorescence measurements do not give any direct information on the RP yield. The latter can in principle be determined from molecular rate constants; however, even with global target analysis it is difficult to obtain unambiguous results (Roelofs et al., 1992).

Direct detection of RP formation and decay by time-resolved photovoltage measurements is an attractive alternative that can give complementary information. In the present work we applied a fast photovoltage technique (Wulf and Trissl, 1995; Trissl and Wulf, 1995) that allows selective measurements of yield and dynamics of the electronic states P680-Pheo and P680QA. The main aim was to investigate the effect of electrostatic interactions...
within the RC protein on primary charge separation and recombination. From the photovoltage response after inhibition of charge stabilization, it is possible to determine both the relative yield of RP formation (from the amplitude) and the lifetime of the RP (from the decay time of the signal). These quantities are compared for the states \( Q_A \) and \( Q_A H_2 \).

On the basis of a first-order reversible reaction scheme (Scheme 1, below), molecular rate constants and free energy differences between the state P680\(^{+}\)Pheo\(^{-}\) and the equilibrated excited state (Chl\(_N\)P680\(^{+}\))* for the three redox states of \( Q_A \) are calculated and discussed.

**MATERIALS AND METHODS**

**Sample preparation**

PS II membrane fragments were prepared from spinach according to the procedure described by Berthold et al. (1981) with slight modifications. To obtain single, destacked membrane fragments suitable for electrical orientation, a mild trypsin treatment was performed on the sample (Leibl et al., 1989; Pokorny, 1994). Concentrated PS II membranes (4 mg of chlorophyll/ml) were diluted in a buffer containing 10 mM 2-[N-morpholino]ethanesulfonic acid (MES) (pH 6.0), 10 mM NaCl, and 0.3 M sucrose to a final chlorophyll concentration of 100 \( \mu \)g/ml. Then trypsin (Sigma Chemical Co., St. Louis, MO) was added from a stock solution (5 mg/ml in 20 mM CaCl\(_2\)) to yield a concentration of 2 \( \mu \)g/ml. After 5 min of incubation in the dark at room temperature, the proteolysis was stopped by addition of a fivefold excess of trypsin inhibitor (Sigma) from a stock solution (10 \( \mu \)g/ml in 20 mM CaCl\(_2\)). The solution was then centrifuged (20,000 \( \times \)g for 10 min). A, B, and C denote the respective states. (Chl\(_N\)P680\(^{+}\))* designates the excited state equilibrated between \( N \) antenna chlorophylls and P680. Molecular rate constants are as follows: \( k_1 \), rate for primary charge separation; \( k_1 \), rate for RP recombination with repopulation of the excited state; \( k_3 \) in \( a \), overall rate for charge stabilization and for recombination to the ground or triplet P680 state; \( k_3 \) in \( b \), rate for primary RP recombination reactions not repopulating the excited state; \( k_3 \), rate for decay of excited states (fluorescence emission and radiationless deactivation).

**Fluorescence measurements**

Fluorescence kinetics were measured on samples (chlorophyll concentration \(-2 \) mg/ml) placed in a flat glass cuvette with an optical path length of 1 mm. Excitation was by flashes from a frequency-doubled picosecond Nd:YAG laser (532 nm, 20 ps; Continuum, Santa Clara, CA). To minimize nonlinear effects (such as singlet-singlet annihilation) the energy density was reduced by neutral density filters to 10–100 \( \mu \)J/cm\(^2\). Fluorescence kinetics was detected with a microchannel photomultiplier (FWHM 150 ps; Hamamatsu, Hamamatsu City, Japan) and a 7-GHz digitizing oscilloscope (INT7000, Intertechnique, Les Ulis, France). The detection wavelength was selected by an interference filter centered at 680 nm. The apparatus response was determined by measuring the response to 20-ps flashes of green scattered light. The fluorescence traces were fitted by a sum of two or three exponential components \( FL = \sum \frac{a_i}{\tau_i} \exp(-t/\tau_i) \), \( n = 2 \) or 3, convoluted with the apparatus response. The values of the parameters \( a_i \),
and \( \tau_i \) were determined by a fit procedure minimizing the sum of the unweighted squared residuals.

**Time-resolved photovoltage measurements**

A detailed description of the photovoltage technique has been given elsewhere (Wulf and Trissl, 1995; Trissl and Wulf, 1995). Briefly, the transmembrane electron transfer during primary charge separation builds up a membrane potential, which can be detected as a photovoltage signal if the sample in the capacitative measuring cell is oriented. The sample (chlorophyll concentration \( \sim 4 \, \text{mg/ml} \)) was placed in a small coaxial cell between two platinum electrodes (Trissl and Wulf, 1995). The destacked PS II membranes were oriented in multilayers on the lower electrode by applying a short electric pulse (\( \sim 200 \, \text{ms}, 800 \, \text{V/cm} \)). From the amplitude of the photovoltage upon a saturating flash (\( \sim 300 \, \text{mV} \)) it can be estimated that this procedure leads to effective orientation of \( \sim 10 \) layers of membranes stacked on top of each other. In the case of experiments with samples in the states QA and QAH\(_2\), chemical reduction was achieved after electrical orientation by adding a reducing buffer under an argon atmosphere. Excitation was as in the fluorescence experiments. For detection of the photovoltage kinetics, a 6-GHz preamplifier (Nucletude, Les Ulis, France) and the same digitizing oscilloscope as for fluorescence measurements were used. The apparatus response was determined experimentally by measuring the ultrafast charge separation in oriented purple membranes from *Halobacterium halobium* (Trissl and Wulf, 1995; see inset in Fig. 2 A). The primary and secondary radical pair kinetics was modeled by a reversible reaction scheme (Scheme 1) that, in the low-energy limit, results in a function \( PV(t) \) containing two exponential components with two apparent time constants and a relative amplitude \( A_{mp} \) (Eqs. A1–A3 in the Appendix). The relations between these apparent parameters and the five molecular parameters (four molecular rate constants and the relative electrogenicity \( e_j/e_i \); see below) are given by Eqs. A4–A6. The kinetic traces were analyzed by a convolution of the apparatus response and \( PV(t) \) (see Trissl and Wulf, 1995). The apparent parameters were determined by an iterative fit procedure as described before for analysis of the fluorescence kinetics.

**RESULTS**

Fluorescence and photovoltage kinetics were measured on destacked PS II membrane fragments pretreated to prepare them in three different initial redox states of QA: oxidized, singly reduced, and doubly reduced. Fluorescence decay kinetics in these redox states have been reported by several groups on comparable samples using a single photon-counting technique (van Mieghem et al., 1992; Vass et al., 1993). It has been demonstrated that the fluorescence decay kinetics are characteristic of the redox state of QA. On the basis of these published data, the fluorescence measurements in this work were primarily performed to serve as a tool and control of establishment of the desired redox states of the sample.

**Fluorescence kinetics**

Fig. 1 shows typical fluorescence kinetics detected for the samples with QA oxidized, QA singly reduced, and QA doubly reduced (see Materials and Methods). For comparison, the experimental traces are normalized to equal initial amplitudes. The parameters resulting from a fit by a sum of two or three exponential functions are given in Table 1. Taking into account the limited time resolution of the fluorescence measurements in this work, our results agree fairly well with the more precise data from single photon-counting experiments (Table 1) (van Mieghem et al., 1992; Vass et al., 1993). In agreement with the observations reported in the literature, kinetics in the states QA and QAH\(_2\) are dominated by fast phases of \( \sim 100–200 \, \text{ps} \), whereas in the state QA\(_2\), a slower phase of \( \sim 1.5 \, \text{ns} \) is dominant. In the state QAH\(_2\), there is a very slow phase of 6 ns, which is not present in the two other redox states of QA. For preparation of the doubly reduced state, we optimized the conditions (time and intensity of illumination, dark re-adaptation time, and concentration of sodium dithionite) so as to obtain fluorescence kinetics with a maximum contribution of the 6-ns component (see Discussion). The double reduction of QA was almost completely reversible after addition of 10 mM ferricyanide (data not shown).

**Photovoltage kinetics**

The same preparation and the same protocol to establish the initial redox states of QA as the one described above were used for photovoltage experiments. Fig. 2 A presents the photovoltage kinetics obtained upon excitation with a picosecond flash of the same oriented PS II membranes prepared successively with QA oxidized (QA) and either QA singly reduced (QA\(_r\)) or QA doubly reduced (QAH\(_2\)). Clear differences between the three traces are seen both in amplitudes and in kinetics. For better comparison of the kinetics in the
states $Q_A$ and $Q_AH_2$, these traces were normalized in Fig. 2 B. Normalization was done by multiplying the photovoltage signal in the state $Q_A$ (Fig. 2 A) by a constant so as to obtain the same maximal amplitudes for both traces.

Owing to the limited sensitivity of the photovoltage setup and the necessity of correlation of the results of both techniques, relatively high excitation energies ranging from 10 to 70 $\mu J/cm^2$ were applied for both fluorescence and photovoltage. In some experiments an even higher excitation energy, up to 100 $\mu J/cm^2$, was applied to record photovoltage signals in the state $Q_A$ because of the low signal-to-noise ratio in this state. No significant differences were observed for excitation within this energy range. As shown in Fig. 3, the photovoltage generated by samples in the state $Q_AH_2$ for these energies is still in the linear range of the saturation curve. This justifies the application of the simplified model, which neglects nonlinear effects (Scheme 1) for a quantitative analysis of photovoltage results.

In the case of initially oxidized $Q_A$, not only the transient primary radical pair ($P680^+\text{Pheo}^-$) is monitored, but also subsequent formation of the secondary radical pair ($P680^+\text{Pheo}Q_A$) leads to a further increase of the membrane potential and appears as a second positive electrogenic step in the photovoltage. According to a two-step sequential forward reaction the photovoltage in samples with oxidized $Q_A$ increases biphasically, reaches its maximum value at $\sim$2–3 ns after the flash, and is stable on the time scale of the measurement (5–10 ns). Both radical pair states involved are weighted with electrogenicity factors, which reflect the (dielectrically weighted) transmembrane distances between the separated charges (Eqs. A3 and A4). As it has been shown previously, the electrogenicity factor of the secondary radical pair is about twice as large as the one of the primary radical pair, indicating a transmembrane position of Pheo about midway between P680 and $Q_A$ (Leibl et al., 1989; Pokorny, 1994).

Single reduction of $Q_A$ before the flash by addition of dithionite (Fig. 2 A, trace $Q_A$) causes a drastic decrease in the photovoltage amplitude and a completely different shape of the kinetics. As expected, the second rising phase, due to $Q_A$ reduction, is lost and replaced by a back-reaction. In addition, the amplitude of the photovoltage signal is diminished compared with that for the samples with oxidized $Q_A$ by much more than the expected factor of two, indicating a significant reduction of the yield of RP formation.

Double reduction of $Q_A$ by illumination and subsequent dark incubation of dithionite-treated samples (Fig. 2 A, trace $Q_AH_2$) leads to a strong increase of the amplitude of the photovoltage signal relative to the trace $Q_A$. The increase in the amplitude is accompanied by a slower decay of the photovoltage. It is necessary to stress that this evolution does not result from reoxidation of $Q_A$ in part of the centers, which would lead to a mixture of $Q_A$ and $Q_AH_2$ states and consequently could give rise to a similar effect of apparent slower decay and increased amplitude. To exclude this possibility two tests were performed that, if some reoxidation of $Q_A$ had occurred, would regenerate the state $Q_A$. The tests consisted in either application of continuous background light or in re-addition of freshly prepared dithionite to the sample. Neither of these control experiments showed a significant change (acceleration of the decay or decrease of the amplitude) of the photovoltage response, thus demonstrating that reoxidation of $Q_A$ was not the origin of the observed changes. It can therefore be concluded that the described photovoltage response is characteristic of RCs with doubly reduced $Q_A$. Qualitatively, it indicates that in samples with doubly reduced $Q_A$ the yield of RP formation and the lifetime of the RP are increased relative to those for the samples with singly reduced $Q_A$.

Kinetic analysis of the photovoltage data revealed that the kinetics in all three redox states of $Q_A$ could be well fitted with two exponential components (Eq. A3). The results of this analysis are collected in Table 2. In the state $Q_A$ the relative amplitude $A_2^{app}$ equals 3.4 (corresponding to an electrogenicity of the state $p^+Q_A^-$ of $\sim$1.85 relative to the electrogenicity of the state $p^+\text{Pheo}^-$ (see below); Eq. A4),
and the time constants of the two phases were $\tau_1 = 220$ ps and $\tau_2 = 620$ ps. These time constants are in good agreement with values of 170 ps and 520 ps measured for PS II membranes from peas (Leibl et al., 1989). The kinetics in the state QAH$_2$ is characterized by a similar time constant for formation of the primary RP ($\tau_1 = 180$ ps) and a time constant of $\tau_2 = 5.5$ ns for its decay. The data are well fitted by a value of $A_{2\text{app}} = 3.4$ (corresponding to $e_2/e_1 = 1.85$ (Eq. A4)), which is expected for a monophasic radical pair recombination process. The peak amplitude of the photovoltage signal observed in different experiments ranged from 30% to 46% of the one observed on the same preparation with oxidized QA. These values may be underestimated due to a slight loss of amplitude, which could be caused by the procedure of dithionite addition disturbing somewhat the orientation of the sample. However, it is clear that the amplitude in the state QAH$_2$ was significantly (about five

![FIGURE 2](image_url)  
**FIGURE 2** (A) Photovoltage kinetics of PS II membrane fragments with QA in three different initial redox states as indicated. An exponential decay due to the apparatus response (inset) has been deconvoluted for clearer presentation. Best fits are superimposed on the experimental traces and residual plots are shown below with upper and lower straight lines indicating ±2% deviation for the states QA and QAH$_2$ and ±5% deviation for the state Q$_A$. (Inset) Photovoltage kinetics of purple membranes used to determine the exponential decay due to the apparatus response used for deconvolution. (B) Photovoltage traces for the states QA and QAH$_2$ with best fits superimposed and normalized to the same maximal amplitude. Additionally, for the state Q$_A$H$_2$ two model curves (1 and 2) are presented obtained by fixing $k_1 = 2$ ns$^{-1}$ (trace 1) or $k_1 = 10$ ns$^{-1}$ (trace 2) and optimizing $k_2$. As can be seen, values of $k_2$ different from those in Table 3 do not allow a fit to the experimental traces. In the states Q$_A$H$_2$ and QA, similar deviations can be observed (not shown) when fixing the values of other molecular rate constants outside the ranges given in Table 3. In the state Q$_A$, fixing one molecular rate constant outside the range of values in Table 3 may be compensated by the other rate constants so that the experimental trace can be well fit. However, in these cases other constraints (e.g., relative peak amplitude of the photovoltage signal; see Appendix 2) would be violated.

![FIGURE 3](image_url)  
**FIGURE 3** Double logarithmic plot of the dependence of the peak amplitude of the photovoltage response in the state Q$_A$H$_2$ on the excitation energy. The measurements used for kinetic analysis were performed at an excitation energy of less than 100 $\mu$J/cm$^2$.

<table>
<thead>
<tr>
<th>Initial redox state</th>
<th>Kinetics components</th>
<th>Relative peak amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\tau_1$ (ns)</td>
<td>$\tau_2$ (ns)</td>
</tr>
<tr>
<td>QA</td>
<td>0.22 ± 0.03</td>
<td>0.62 ± 0.03</td>
</tr>
<tr>
<td>Q$_A$H$_2$</td>
<td>0.05 ± 0.02</td>
<td>0.80 ± 0.30</td>
</tr>
<tr>
<td>Q$_A$</td>
<td>0.18 ± 0.02</td>
<td>5.5 ± 0.5</td>
</tr>
</tbody>
</table>

$A_{2\text{app}} = 3.4$ corresponds to a relative electrogenicity $e_2/e_1 = 1.85$ (Eq. A4). Values were obtained by a best fit according to Eq. A3. The relative peak amplitudes of the photovoltage responses (after deconvolution of the apparatus response function) are given in the right column.
times) higher when compared with that obtained for the state \( Q_A \). It is worth noting that the transition from the single to the doubly reduced state did not require a change of the redox buffer solution covering the stacked membrane layer in the measuring cell (such a buffer change is necessary for the transition from the oxidized to the single reduced state; see Materials and Methods) but was realized only by the application of continuous light. Therefore, the observed increase of the amplitude by a factor of five when going from the state \( Q_A \) to \( Q_{A\text{H}_2} \) is clearly due to an increased yield of RP formation. The kinetics in the state \( Q_A \) is characterized by a rising phase of \( \sim 50 \) ps and a decay phase of \( 800 \) ps, both time constants being significantly faster than in the case of doubly reduced \( Q_A \). As in the case of \( Q_{A\text{H}_2} \), a relative amplitude of \( A_2^{\text{pp}} = -1 \) fit well in the analysis. It is interesting to note that on the basis of a simple phenomenological description by two consecutive reactions the decrease in the maximum RP concentration in the state \( Q_A \) could be easily explained by assigning the 800-ps component to the formation of the RP and the 50-ps component to its decay. A more detailed analysis in terms of molecular rate constants will be given below.

**DISCUSSION**

**Preparation of the redox states of \( Q_A \)**

In general, the methods of preparing samples with \( Q_A \) oxidized and singly reduced seem well established. The oxidized state is the one that is easiest to obtain in pure form. To assure that all RCs were in the state \( Q_A \), a small amount (up to 100 \( \mu \)M) of potassium ferricyanide was added to the sample in addition to dark adaptation. This artificial electron acceptor reoxidizes residual \( Q_A \) between flashes during data acquisition.

Preparation of samples with all \( Q_A \) singly reduced is more difficult. It was suggested, for example, that addition of sodium dithionite could cause some double reduction of \( Q_A \) already in the dark, although more purified preparations such as core complexes seem to be more susceptible to this than the intact membrane preparations used in this work (van Mieghem, 1994). Single reduction of \( Q_A \) could in principle be performed also by a preillumination of the sample in the presence of DCMU, an inhibitor of reoxidation of \( Q_A \) by electron transfer to \( Q_B \). However, it was found that chemical reduction gives the most reliable results. The other methods (DCMU plus saturating preflash or continuous background light) might leave \( Q_A \) in the oxidized state in a small fraction of RCs. On the other hand, due to the instability of sodium dithionite there remains also some uncertainty about the establishment of a stable \( Q_A \) state by chemical reduction. Fortunately, the very properties of the photovoltage response offer sufficient arguments against a significant contribution from the states \( Q_A \) and \( Q_{A\text{H}_2} \). The characteristic kinetics and especially the very small amplitude are inconsistent with a significant contribution of the other states, which are characterized by quite different kinetics and much larger amplitudes. On the other hand, because the signal from the state \( Q_A \) is small, even minor contamination by the larger signal from the other redox states would distort it significantly. However, simulations show that such a contamination would clearly manifest itself by the presence of a slow component in the photovoltage decay (5.5 ns or a constant). This is not observed. Furthermore, the fluorescence kinetics for the states \( Q_A \) and \( Q_A \) (Fig. 1; Table 1) agreeing well with those in the literature (Hodges and Moya, 1986; Schatz et al., 1988; Leibl et al., 1989; van Mieghem et al., 1992; Vass et al., 1993) also confirm the validity of the methods of preparation of the different redox states.

To verify the preparation of the state \( Q_{A\text{H}_2} \), we compared our fluorescence kinetics to published data (Table 1) for the same kind of preparation, i.e., PS II membrane fragments from spinach (van Mieghem et al., 1992; Vass et al., 1993). These data had been obtained by the technique of single photon counting and analyzed with four exponential functions. The published characteristics of the fluorescence decay kinetics in the state \( Q_{A\text{H}_2} \) are very similar to those presented in this paper with a dominant fast phase (150–220 ps), a middle component of smaller contribution (600 ps), and a very slow phase (7–10 ns). Irrespective of the number of kinetic phases and their exact lifetimes, it appears clearly that the relative fluorescence yields, \( \Phi \), for the three redox states of \( Q_A \) (Table 1) are in good agreement with literature data. Compared with the oxidized state, the values of \( \Phi \) increase by about a factor of 6 and 3–4 for the singly and doubly reduced state, respectively. In the work of van Mieghem et al. (1992), fluorescence experiments were performed on samples for which the redox state of \( Q_A \) was monitored by electron paramagnetic resonance (EPR). PS II membranes with \( Q_A \) singly reduced showed a large \( Q_B \) EPR signal, which was absent both in samples with \( Q_A \) oxidized and in samples doubly reduced. Based on the correlation of the \( Q_A \) redox state monitored by EPR and the fluorescence kinetics characterized by van Mieghem et al. (1992) we used in our work the presence of a long (6–10 ns) fluorescence component with significant amplitude, together with the large amplitude of the fast phase (60–70%) and the value of \( \Phi(Q_{A\text{H}_2})/\Phi(Q_A) = 3–4 \) as indicators for the state \( Q_{A\text{H}_2} \).

**Molecular rate constants**

In principle, fluorescence and photovoltage data give complementary kinetic information, and the experimental parameters (lifetimes and amplitudes) from both techniques could be used to determine molecular rate constants. In the framework of Scheme 1 the kinetics in all redox states of \( Q_A \) should be characterized by two exponential phases, and the values for time constants for photovoltage and fluores-
cence kinetics should be identical (see Appendix 1). However, some time constants measured by fluorescence, both in this work and in work by other authors, are significantly different from time constants measured by photovoltage (compare Tables 1 and 2). This is the most evident in the state QAH2, where more than two phases are necessary to describe the fluorescence decay. But also in the other states some differences, especially concerning the time constant of the fast phases, are observed.

The reason for these discrepancies is probably that Scheme 1 is too simple to give a correct description of the fluorescence kinetics because it does not take into account additional reaction steps that are expected to give additional kinetic phases of fluorescence decay. Several studies of PS II preparations with high temporal and amplitude resolution reported fluorescence decay kinetics with more than two phases (Roelofs and Holzwarth, 1990; van Mieghem et al., 1992; Roelofs et al., 1992; Vass et al., 1993). These results were interpreted either in terms of heterogeneity of photosystems (contribution of PS I and/or different behavior of PS IIα and PS IIβ; Roelofs et al., 1992) or in terms of a relaxation of the primary radical pair (Vass et al., 1993, Yruela et al., 1996). The samples used in this work contain no detectable amounts of PS I and, judged from the values for molecular rate constants determined from the photovoltage kinetics, resemble PS IIβ (see below). In other words, the photovoltage response probably originates from a homogeneous sample. It is not completely sure that this applies also to the fluorescence response, which could possibly contain a contribution from still stacked PS II membranes behaving like PS IIα.

Concerning a possible relaxation of the primary pair, the doubly reduced state should be the one in which it is the most easily detectable due to the long lifetime of the RP in this state. In fact, fluorescence transients in this state showed a third phase of ~600 ps, which could correspond to an energetic relaxation of the primary RP. The photovoltage kinetics, however, which monitors directly the time dependence of the radical pair concentration, was well described by a single decay time. This could mean that within the precision of the photovoltage measurements, a possible radical pair relaxation is not connected with sufficient change in electrogenicity (due to charge movement perpendicular to the membrane plane) to be observed.

Another additional reaction step that is neglected in Scheme 1 is exciton equilibration within the PS II antenna complexes. Such processes had been detected as fast fluorescence phases (15–20 ps) of significant relative amplitude (0.35), both for open and closed RCs (McCaulley et al., 1989; Roelofs et al., 1992). The fluorescence data in the present work were not of sufficient precision to resolve such phases. As a consequence the lifetimes and amplitudes of the faster fluorescence phases observed in this work may be distorted.

The additional reaction steps discussed above, like exciton equilibration and primary radical pair relaxation, are expected to affect fluorescence much more than photovoltage kinetics, for which Scheme 1 seems sufficient. Certainly a global analysis of kinetic data from both techniques would be the best approach. However, the use of a more complicated reaction scheme makes it impossible to determine all involved molecular rate constants. For these reasons the analysis presented in the following will be mainly based on the photovoltage data and the simple scheme (Scheme 1).

One advantage of the photovoltage is that the amplitudes give information about relative concentrations of the RP, which can be compared for different redox states. This allows us to derive an important conclusion about the rate constant of primary charge separation independently of the other molecular rate constants. As can be seen from Eq. A7 the maximum concentration of the RP depends only on $k_1$ and the directly measured quantities $\tau_1$ and $\tau_2$. Taking into account the relative amplitudes of the photovoltage traces in the states QAH2 and QA as well as the apparent rates (Table 2), the ratio $k_1(QAH2)/k_1(QA) \approx 1.25$ can be deduced. This is an important result, which does not imply any assumptions. It demonstrates that the molecular rate constants of primary charge separation in the singly and doubly reduced state are comparable.

To determine the sets of molecular rate constants for the three redox states of QA we used two mathematical approaches (Appendix 2), both leading essentially to the same results (Table 3; compare results in sets 1 and 2 with those in set 3). As even with the simplified scheme, an attempt to determine precise values of the molecular rate constants would necessitate too many assumptions we rather restricted the analysis to the determination of the ranges of their possible values.

Most of the molecular rate constants are sufficiently well defined to allow drawing conclusions about the effect of the redox state of QA. Inspection of the values determined for $k_1$ and $k_{-1}$ (in all three sets in Table 3) reveals the interesting result that the rate of RP recombination, $k_{-1}$, is strongly dependent on the redox state of QA, being similar for QA and QAH2 but more than one order of magnitude higher for QA. On the other hand, the molecular rate constant for primary charge separation, $k_1$, is rather independent of the redox state of QA. This result contrasts with the result of kinetic analysis of time-resolved fluorescence (and transient absorption changes) from the literature. On a PS II preparation from Synechococcus sp. with 80 Chl/RC, the single reduction of QA was found to affect $k_1$ much more than $k_{-1}$ (Schatz et al., 1988), and a similar result has been obtained in other works using fluorescence techniques (Roelofs et al., 1992; Vass et al., 1993). A possible reason for this difference may be the heterogeneity of PS II. In a very detailed study of picosecond chlorophyll fluorescence from pea chloroplasts, global target analysis allowed detection of a distinctly different behavior of β-centers compared with
TABLE 3 Sets of parameters calculated from photovoltage data for the three redox states of QA on the basis of Scheme 1

<table>
<thead>
<tr>
<th></th>
<th>$Q_A$</th>
<th>$Q_A^-$</th>
<th>$Q_AH_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_3 = 1.0 \text{ ns}^{-1}$</td>
<td>$k_1 (\text{ns}^{-1})$</td>
<td>3.0 (2.6–3.4)</td>
<td>3.2 (2.0–4.4)</td>
</tr>
<tr>
<td></td>
<td>$k_{-1} (\text{ns}^{-1})$</td>
<td>0.52 (0.42–0.62)</td>
<td>20 (10–30)</td>
</tr>
<tr>
<td></td>
<td>$k_2 (\text{ns}^{-1})$</td>
<td>1.75 (1.7–1.8)</td>
<td>&lt;2.3</td>
</tr>
<tr>
<td></td>
<td>$RP_{\text{max}} %$</td>
<td>10 (7–13)</td>
<td>62 (58–66)</td>
</tr>
<tr>
<td></td>
<td>$a_2^0$</td>
<td>0.22 (0.21–0.23)</td>
<td>0.87 (0.84–0.90)</td>
</tr>
<tr>
<td></td>
<td>$\Phi_0$</td>
<td>0.30 (0.27–0.33)</td>
<td>0.88 (0.80–0.96)</td>
</tr>
<tr>
<td></td>
<td>$Y_{CS} %$</td>
<td>70 (68–72)</td>
<td>-44 (&lt;–49–&lt;41)</td>
</tr>
<tr>
<td></td>
<td>$\Delta G^0 (\text{meV})$</td>
<td>-44 (&lt;–49–&lt;41)</td>
<td>-40 (&lt;–60–&lt;38)</td>
</tr>
<tr>
<td>$k_3 = 0.4 \text{ ns}^{-1}$</td>
<td>$k_1 (\text{ns}^{-1})$</td>
<td>3.8 (3.4–4.2)</td>
<td>4.0 (2.5–5.5)</td>
</tr>
<tr>
<td></td>
<td>$k_{-1} (\text{ns}^{-1})$</td>
<td>0.3 (0.2–0.4)</td>
<td>16 (7–25)</td>
</tr>
<tr>
<td></td>
<td>$k_2 (\text{ns}^{-1})$</td>
<td>1.75 (1.70–1.80)</td>
<td>4.9 (3.6–6.2)</td>
</tr>
<tr>
<td></td>
<td>$RP_{\text{max}} %$</td>
<td>13 (11–15)</td>
<td>70 (65–75)</td>
</tr>
<tr>
<td></td>
<td>$a_2^0$</td>
<td>0.14 (0.13–0.15)</td>
<td>0.86 (0.82–0.90)</td>
</tr>
<tr>
<td></td>
<td>$\Phi_0$</td>
<td>0.28 (0.25–0.31)</td>
<td>0.88 (0.80–0.96)</td>
</tr>
<tr>
<td></td>
<td>$Y_{CS} %$</td>
<td>89 (87–91)</td>
<td>+38 (+30–+46)</td>
</tr>
<tr>
<td></td>
<td>$\Delta G^0 (\text{meV})$</td>
<td>-65 (&lt;–68–&lt;63)</td>
<td>+38 (+30–+46)</td>
</tr>
<tr>
<td>3. Weak constraints</td>
<td>$k_1 (\text{ns}^{-1})$</td>
<td>2.8 (2.2–3.4)</td>
<td>3.3 (2.8–3.8)</td>
</tr>
<tr>
<td></td>
<td>$k_{-1} (\text{ns}^{-1})$</td>
<td>0.7 (0.4–1.0)</td>
<td>15 (12–17)</td>
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<tr>
<td></td>
<td>$k_2 (\text{ns}^{-1})$</td>
<td>1.75 (1.50–2.00)</td>
<td>1.0 (0.4–1.6)</td>
</tr>
<tr>
<td></td>
<td>$k_3 (\text{ns}^{-1})$</td>
<td>1.0 (0.4–1.6)</td>
<td>14 (12–16)</td>
</tr>
<tr>
<td></td>
<td>$RP_{\text{max}} %$</td>
<td>&lt;5</td>
<td>&lt;5</td>
</tr>
<tr>
<td></td>
<td>$\Delta G^0 (\text{meV})$</td>
<td>-36 (&lt;–52–&lt;20)</td>
<td>+37 (+29–+45)</td>
</tr>
</tbody>
</table>

$k_i$, molecular rate constants; $RP_{\text{max}}$, maximum transient concentration of the primary radical pair; $a_2^0$, fluorescence amplitude of slower decay component; $\Phi_0$, fluorescence yield; $Y_{\text{CS}}$, the yield of charge stabilization; $\Delta G^0$, standard free energy difference between the states P680 $^\bullet$Pheo $^\bullet$ and (Chl$P680^*$). See text for further details.

that of α-centers (Roelofs et al., 1992). Upon single reduction of $Q_A$, in PS IIβ, $k_1$ decreased by a factor of 3, but $k_{-1}$ increased by a factor of 5. A previous study based on photovoltage (and fluorescence) kinetics on destacked PS II membranes revealed a similar change of the values of $k_1$ and $k_{-1}$ upon single reduction of $Q_A$ with a reduction of $k_1$ by a factor of 3 and an increase of $k_{-1}$ by about a factor of 8 (Leibl et al., 1989). This resembles a behavior proposed for PS IIβ although the membranes had been prepared from PS IIa containing grana fragments. In thylakoids, PS IIa is located in the stacked grana region of the thylakoids, whereas PS IIβ is located in the unstacked stroma regions, and evidence for a reversible conversion of PS IIa to PS IIβ has been reported, probably connected with migration of PS II from grana to stroma region (Sundby et al., 1986; Guenther and Melis, 1990). It is possible that such a conversion takes place upon destacking of the grana membranes. It should be noted that in the photovoltage study mentioned above (Leibl et al., 1989), double reduction of $Q_A$ had neither been studied nor considered. It is therefore likely that part of the difference between the results of this work and those reported by Leibl et al. (1989) can be attributed to the contribution of some doubly reduced $Q_A$ in the presumed $Q_A$ state in the earlier study.

The rate $k_2$ is not very well defined in the state $Q_A^-$(Table 3). This is mainly due to the fact that $k_{-1}$ in this state is very high and that both reactions (described by $k_2$ and $k_{-1}$) compete in the depopulation of the radical pair state. However, all three sets show that in the state $Q_AH_2$, $k_3$ is smaller than 0.2 ns$^{-1}$. This may serve as an estimation of the upper limit for the recombination rate in open RCs (state $Q_A$) of the primary radical pair directly or via the triplet state of P680 to the ground state. The relatively small value of 0.2 ns$^{-1}$, compared with $k_2$ ($Q_A^- = 1.75$ ns$^{-1}$, is in line with an efficient charge stabilization in open RCs (Kramer and Mathis, 1980; Thienel and van Gorkom, 1981; Schatz et al., 1988).

The rate constant for nonphotochemical decay, $k_3$ (Table 3, set 3) also shows a large range of values, which are compatible with the measured quantities and the constraints. This rate, like $k_2$ in closed RCs, describes a wasteful loss reaction, and variation of this rate has only relatively weak effects on the other molecular rate constants. The most significant effect of an increase of $k_3$ is a drop of the quantum yield of charge stabilization in open RCs. The range for $k_3$ determined in set 3 covers the two values used for calculation of sets 1 and 2.

To demonstrate the influence of the determined molecular rate constants on other observable quantities, some additional values are given in Table 3. The calculated maximum transient concentrations of the primary radical pair, $RP_{\text{max}}$ (Eq. A7), in the states $Q_A^{-}$ and $Q_AH_2$ are expressed in percentages of the initially absorbed photons per RC and may be compared to the RP yields from the literature (see...
Introduction). For sets 1 and 2 also theoretical values for relative fluorescence amplitudes, \( a_2^0 \), and fluorescence yields, \( \Phi^0 \), are calculated according to Eq. A12. These fluorescence quantities might be compared with the constraints related to fluorescence data that were used to calculate set 3 (see Appendix 2) showing that the constraints are justified. Only \( a_2^0(Q_A) = 0.14 \) in set 2 is significantly lower than the corresponding constraint (0.25). However, as calculations show, a decrease of the lower limit for \( a_2^0(Q_A) \) to 0 affects significantly only the ranges of \( k_{-1}(Q_A) \) in set 3 (0–1.6 ns) and has no influence on the main conclusions. Finally, also the yields of charge stabilization in open RCs, \( Y_{CS} \), calculated in sets 1 and 2 (Eq. A8) are in accordance with the corresponding constraint used for the calculation of set 3 \( (Y_{CS} > 0.5) \) and with literature data (Kramer and Mathis, 1980; Thielen and van Gorkom, 1981; Schatz et al., 1988).

The standard free energy of the primary charge separation

Knowledge of the values of the molecular rate constants for the forward and back reactions allows us to obtain information on the energetics of the charge separation reaction. The values of \( k_1 \) and \( k_{-1} \) define the free energy difference, \( \Delta G^o \), between the states \( P680^+ \text{Pheo}^- \) and \( (\text{Chl}_h\text{P680})^+ \) in the intact photosystem:

\[
\Delta G^o = -k_B T \ln(k_1/k_{-1}),
\]

where \( k_B \) is the Boltzmann constant and \( T \) is the absolute temperature. \( \Delta G^o \) is negative and of similar magnitude for the states \( Q_A \) and \( Q_A^+ \text{H}_2 \), whereas for the state \( Q_A^- \) it is positive (Table 3), indicating a significant shift of the energetic equilibrium toward the (equilibrated) excited state. The latter result is consistent with the idea that a negative charge on \( Q_A \) leads to a destabilization of the state \( P680^+ \text{Pheo}^- \) due to repulsive electrostatic interaction. On the other hand, a similar driving force observed for charge separation in the oxidized and doubly reduced state indicates that the electrostatic repulsion has disappeared. This result confirms that upon double reduction the charges on \( Q_A \) are neutralized by double protonation (Vass et al., 1992; van Mieghem et al., 1992, 1994, 1995; Liu et al., 1993). It therefore appears that the state \( Q_A^- \) can be used to provide an internal modulation of the standard free energy of primary charge separation when compared with the oxidized or fully reduced state.

In the framework of the exciton/radical pair equilibrium model, the molecular rate of charge separation from the equilibrated excited state, \( k_1 \), is linearly related to the intrinsic molecular rate, \( k_{int}^1 \), which describes electron transfer within the RC from the excited primary donor, \( P680^+ \), to the primary acceptor:

\[
k_{int}^1 = k_1 N_{eff},
\]

where \( N_{eff} \) is an entropy factor representing the effective number of chlorophylls over which the excitation is equilibrated. If all pigments are isoenergetic, \( N_{eff} \) equals the actual antenna size (in our case \( N \approx 250 \)). By taking into account a slight difference in the wavelength of the maximum absorption between the primary donor \( P680^+ (680 \text{ nm}) \) and the antenna chlorophylls (673 nm) and assuming a Boltzmann distribution of the excited states, \( N_{eff} \) is reduced to \( \approx 125 \) (see, e.g., Schatz et al., 1988). With the values for \( k_1 \) given in Table 3, \( k_{int}^1 \) can be estimated at \((2–3 \text{ ps})^{-1} \), in accordance with values reported for isolated RCs (Wasielewski et al., 1989; Roelofs et al., 1991; Chang et al., 1994; Schelvis et al., 1994; Müller et al., 1996; Greenfield et al., 1997). The standard free energy difference between the primary radical pair and \( P680^+ \), \( \Delta G^{o, int} \), is given by

\[
\Delta G^{o, int} = -k_B T \ln(k_{int}^1/k_{-1}) = \Delta G^o - k_B T \ln N_{eff},
\]

where for \( N_{eff} = 125 \) and at room temperature the last entropy term adds \( \approx -120 \text{ meV} \) to the free energy gap. The diagram in Fig. 4 presents, for the three redox states of \( Q_A \), the calculated free energy differences for charge separation from the excited states \( (\text{Chl}_h\text{P680})^+ \) and \( P680^+ \). Fig. 5 is a logarithmic plot of the intrinsic molecular rate constants for the three redox states of \( Q_A \) \( (k_{int}^1 \text{ and } k_{-1}, \text{ Table 3}) \) against the corresponding values of \( -\Delta G^{o, int} \).

It should be emphasized that the values of \( \Delta G^o \) and \( \Delta G^{o, int} \), as well as for all molecular rate constants, are calculated based on the simple exciton/radical pair equilibrium model (Scheme 1). This model accounts neither for a possible intermediate in electron transfer between \( P680^+ \) and \( \text{Pheo}^- \) nor for energetic relaxation of the primary radical pair. These phenomena exist in bacterial RCs (Woodbury and Parson, 1984; Peloquin et al., 1994; Holzapfel et al., 1989, 1990; Holzwarth and Müller, 1996), and the strong struc-
tural and functional similarity of PS II and purple bacterial RCs suggests that they may also exist in PS II, although this has not been established. Controversial interpretations about the existence of relaxation of the RP were given in papers describing primary reactions in PS II membranes (Roelofs and Holzwarth, 1990; Vass et al., 1993, Yruela et al., 1996) and in isolated RCs (Booth et al., 1991; Schelvis et al., 1994; Müller et al., 1996). During relaxation the free energy level of the primary radical pair decreases with time, most probably due to the dielectric response of the protein, and \(-\Delta G^{\text{int}}\) increases. In this case the \(-\Delta G^{\text{int}}\) for the forward and backward electron transfer would be different. However, if there is no intermediate, and relaxation for times shorter than several nanoseconds may be neglected, one could assume the reorganization energy \(\lambda\) and \([\Delta G^{\text{int}}]\) to be the same for the forward and backward reaction and fit the points in Fig. 5 with a Marcus parabola (Marcus, 1956; Marcus and Sutin, 1985). Such a fit would give a value for the reorganization energy \(\lambda\) of \(\sim 120\) meV. In the framework of this analysis the weak dependence of the charge separation rate \(k^{\text{int}}\) on the redox state of \(Q_A\) (Fig. 5) is consistent with the activationless character of this reaction occurring with a rate near its optimum value (\(\lambda = -\Delta G^{\text{int}}\)). This is typical of primary charge separation reactions in photosynthetic RCs (Krishtalik, 1989).

**CONCLUSIONS**

The results of this work show that in intact PS II the yield of charge separation is strongly diminished when the primary quinone acceptor is singly reduced. This behavior might have a physiological role. Under high light conditions it minimizes formation of \(P680^-\) and \(^3P680\) (triplet form of \(P680\)) by fast charge recombination to the excited state. Fast recombination to the ground state is probably not a possible alternative for reactions within the RC, as the latter has to be optimized for high quantum yield of charge separation and stabilization under normal conditions. However, highly efficient, pH-dependent quenching processes based on fast nonradiative dissipation of excitation energy exist in the antenna (Mullineaux et al., 1993). The prevention of \(P680^-\) and \(^3P680\) formation is essential in PS II because of the high oxidizing power of \(P680^-\) and the risk of formation of reactive oxygen species by reaction with \(^3P680\), both potentially leading to severe damage of the protein. A reduced yield of RP formation is therefore a protection mechanism. Even with this and other protection mechanisms, PS II becomes degraded and permanently has to be rebuilt as apparent from the rapid turnover of the D1 protein (for a review see Andersson and Barber, 1996). On the other hand, double reduction of \(Q_A\) results in a high yield of RP formation. It probably occurs only under extreme conditions and might be a precursor state for photoinhibition and degradation of PS II (e.g., Vass et al., 1992).

A comparison of the RP yield in the different redox states of \(Q_A\) reveals the effect of electrostatic interactions within the RC protein. Due to a relatively low dielectric constant, the Coulomb interaction can amount to \(\sim 100\) meV for a distance on the order of 15 Å. The change in the free energy is large enough to cause significant modifications of the electron transfer rates, except those that are kinetically optimized. This internal modulation of \(\Delta G^o\) gives, in principle, access to the reorganization energy in a way analogous to the application of an external electric field (Feher et al., 1988; Franzen et al., 1990; Dau et al., 1992) with the advantage that it is experimentally much easier to perform. Still another possibility to modify \(\Delta G^o\) would be to introduce in a controlled way charges in the protein by site-directed mutagenesis. This approach has been used successfully to modulate the midpoint potentials of cofactors and even the electron transfer pathways in RCs from purple bacteria (for a review see Woodbury and Allen, 1995).

**APPENDIX 1**

The analytical solutions for the time dependencies of the concentrations of the states B and C (Scheme 1) are, respectively

\[
B(t) = \left[ k_1 \tau_2/\left( \tau_2 - \tau_1 \right) \right] \left[ \exp\left( -t/\tau_1 \right) + \exp\left( -t/\tau_2 \right) \right]
\]

(A1)

and

\[
C(t) = \left[ k_2 \tau_1/\left( \tau_2 - \tau_1 \right) \right] \cdot \left[ \tau_1 \exp\left( -t/\tau_1 \right) - \tau_2 \exp\left( -t/\tau_2 \right) + \tau_2 - \tau_1 \right],
\]

(A2)
where $\tau_1$ and $\tau_2$ are functions of all molecular rate constants $k_i$ (see below and Leibl et al., 1989). The photovoltage $PV(t)$ is the sum of the concentrations $B(t)$ and $C(t)$, weighted with their electrogenericity factors $e_1$ and $e_2$:

$$PV(t) = e_1B(t) + e_2C(t)$$

$$\propto (1 + A_2^{pp}) - \exp(-t/\tau_1) - A_2^{pp}\exp(-t/\tau_2),$$

(A3)

where

$$A_2^{pp} = [(e_2/e_1)k_2\tau_2 - 1][1 - k_2\tau_1(e_2/e_1)].$$

(A4)

Note that $PV(t)$ contains only two exponential terms. Thus, it may also be expressed by only three apparent parameters, two exponential time constants $\tau_1$ and $\tau_2$ and one relative amplitude $A_2^{pp}$, which can easily be determined by fitting the experimental traces.

Useful for calculations of molecular rate constants are the relations

$$(1/\tau_1) + (1/\tau_2) = k_1 + k_{-1} + k_2 + k_3$$

(A5)

$$(1/\tau_1\tau_2) = k_{-1}k_2 + k_1k_3 + k_1k_2$$

(A6)

and the expression for the maximum transient concentration of the RP, $RP_{max}$:

$$RP_{max} = [k_1/(1/\tau_1 - 1/\tau_2)][[(\tau_2/\tau_1)\tau_1^{(\tau_2/\tau_1 - \tau_2)}] - (\tau_2/\tau_1)\tau_1^{(\tau_2/\tau_1 - \tau_1)}].$$

(A7)

The yield of charge stabilization, $Y_C$, calculated as the concentration of the state C for open RCs at $t\to\infty$, $C(\infty)$, is given by the equation

$$Y_C = k_1k_2[k_3(k_2 + k_{-1})].$$

(A8)

According to Scheme 1, the time dependence of the concentration of the excited state, $A(t)$ (and consequently of fluorescence) is given by

$$A(t) = a_1^0\exp(-t/\tau_1) + a_2^0\exp(-t/\tau_2),$$

(A9)

with the amplitude factors

$$a_1^0 = 1 - a_2^0$$

(A10)

$$a_2^0 = [(k_2 + k_{-1}) - (1/\tau_2)][(1/\tau_1) - (1/\tau_2)].$$

(A11)

The fluorescence yield, $\Phi^0$, is calculated as

$$\Phi^0 = \int_0^\infty A(t)dt = (k_2 + k_{-1})\tau_1\tau_2.$$  

(A12)

**APPENDIX 2**

In the first approach (Table 3, sets 1 and 2) the input data for the calculations (Eqs. A4–A7) are the fit parameters of photovoltage traces and their relative peak amplitudes with the experimental errors of these quantities (Table 2). The only assumption made is that the value of $k_i$ is the same for all three redox states of QA. The relative electrogenericity of charge stabilization in the state QA, $e_2/e_1 = 1.85$ was taken from the literature (Leibl et al., 1989; Pokorny, 1994). Values of $k_i$ were fixed either to 1 ns$^{-1}$ (set 1) or to 0.4 ns$^{-1}$ (set 2). This range corresponds to the observed fluorescence lifetimes of isolated Chl antenna complexes and covers the range of values proposed for $k_i$ in the literature (Eadts et al., 1987; Ide et al., 1987; Roelofs and Holzwarth, 1990; Roelofs et al., 1992; Mullineaux et al., 1993). It turns out that the value of $k_i$ has only a minor influence on the determination of the other molecular rate constants (for an extensive discussion see Roelofs and Holzwarth, 1990; see also Roelofs et al., 1992, Dau and Sauer, 1992).

In the second approach (Table 3, set 3) the input data for calculations (Eqs. A5–A7) are the time constants, $\tau$, and the relative peak amplitudes of the photovoltage (Table 2). Additionally we applied some constraints, a part of them being based on fluorescence data. Besides trivial constraints (no negative rate constants, no negative fluorescence amplitudes) only the following very conservative constraints were used: 1) the yield of charge stabilization in open PS II, $Y_C$ (Eq. A8), is higher than 0.5 (Kramer and Mathis, 1980; Thielen and van Gorkom, 1981; Schatz et al., 1988); 2) the fluorescence yield, $\Phi^0$ (Eq. A12), in the states QA and QAH2 is at least twice as high as in the state QA (compare Table 1); 3) the fluorescence yield in the state QAH is not lower than in the state QAH2 (compare Table 1); 4) the amplitude of the slower fluorescence phase in the state QA, $a_2^0(QA)$ (Eq. A11), is limited to the range 0.25 $< a_2^0(QA)$ $< 0.7$ (compare $a_2^0(QA)$ in Table 1); 5) the rate of nonphotochemical decay of the excited state, $k_i$, is the same in both reduced states of QA; and 6) this rate is not higher than in open RCs ($k_i(QAH) \geq k_i(QA)$) (and $k_i(QAH) \approx k_i(QAH_2)$, account for a possible quenching of excited states by the oxidized plastoquinone pool (Delosme, 1967; Vermot et al., 1979; Pokorny, 1994). In this second approach we use an iteration procedure to narrow down the ranges of possible values for the molecular rate constants.

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