ELSEVIER

Biochimica et Biophysica Acta 1276 (1996) 35–44

BBA Biochimica et Biophysica Acta

# On the role of exchangeable hydrogen bonds for the kinetics of $P680^{+\cdot}$ $Q_A^{-\cdot}$ formation and $P680^{+\cdot}$ $Pheo^{-\cdot}$ recombination in photosystem II

S. Vasil'ev [a,1], A. Bergmann [b], H. Redlin [c], H.-J. Eichler [b], G. Renger [d,*]

[a] *Faculty of Biology, Moscow State University, Moscow 119899, Russia*
[b] *Optical Institute, Technical University, Berlin, D-10623 Berlin, Germany*
[c] *Institute of Physics, Humboldt University, Berlin, D-10099 Berlin, Germany*
[d] *Max-Volmer-Institute, Technical University, Berlin, D-10623 Berlin, Germany*

Received 27 February 1996; accepted 20 March 1996

**Abstract**

Possible effects of the hydrogen bond network on the reactions leading to the stabilized ion-radical pair $P680^{+\cdot}$ $Q_A^{-\cdot}$ formation and $P680^{+\cdot}$ $Pheo^{-\cdot}$ recombination in PS II were analyzed by introducing modifications due to replacement of $H_2O$ by $D_2O$ or the addition of cryoprotectants. The rate constants of primary charge separation and subsequent stabilization were derived from measurements of time resolved fluorescence decay curves in PS II membrane fragments with reaction centers kept in the open state by addition of $K_3[Fe(CN)_6]$. The numerical analysis of the experimental data was performed on the basis of the exciton radical pair equilibrium model proposed by Holzwarth and coworkers (Schatz, G. et al. (1988) Biophys. J. 54, 397–405). Recombination kinetics of the radical pair $P680^{+\cdot}$ $Pheo^{-\cdot}$ were measured using D1-D2-cyt *b*559 preparations resuspensed in either $H_2O$ or $D_2O$. It was found that: (a) the molecular rate constants $k_{PC}$ of the primary charge separation and that of the subsequent stabilization step, $k_{stab}$, were about $(3\ ps)^{-1}$ and $(300\ ps)^{-1}$, respectively; (b) these rate constants were only slightly affected by $D_2O$ or cryoprotectants; (c) small H/D isotope exchange effects were also found in PS II core complexes and Tris-washed PS II membrane fragments; (d) the recombination kinetics of the $P680^{+\cdot}$ $Pheo^{-\cdot}$ radical pair in D1-D2-cyt *b*559 preparations is almost invariant to replacement of $H_2O$ by $D_2O$. The results obtained reveal that in PS II the charge separation and recombination processes are only weakly dependent on the network of hydrogen bridges with exchangeable protons, in contrast to pronounced effects observed in anoxygenic purple bacteria (Paschenko et al. (1987) FEBS Lett. 214, 28–34). The measured differences between both types of reaction centers are discussed.

*Keywords:* Photosystem II; Hydrogen bond; Cryoprotectant; LHC II; Charge separation

## 1. Introduction

The key steps of photosynthetic water cleavage into dioxygen and metabolically bound hydrogen take place within a multimeric protein complex referred to as Photosystem II (PS II) that acts as a light driven water-plastoquinone oxidoreductase. The trapping and transformation of light energy in PS II comprises three different types of reaction sequences: (a) generation of an electronically excited state at a special photoactive chlorophyll (Chl-a), P680, and transformation into an electron–hole pair of sufficient stability and redox power, (b) water oxidation to $O_2$ by cooperative reaction of four holes, and (c) plastoquinone reduction to plastoquinol via cooperative reaction of the two electrons.

The first reaction sequence includes not only the photochemical reactions leading to charge separation but also excited state energy transfer processes between antenna pigments that permit an optimal adaptation of photosynthesizing organisms to different illumination conditions (for a review see [1]). An excited singlet state created by light absorption at an antenna pigment is rapidly delivered to P680 where an electron becomes ejected from its lowest excited singlet state, $^1P680^*$, and transferred to pheophytin (Pheo) thus forming the primary radical pair $P680^{+\cdot}Pheo^{-\cdot}$. In order to permit water cleavage, the

Abbreviations: Chl, chlorophyll; DMSO, dimethylsulfoxide; LHC II, light harvesting complex II; Pheo, pheophytin; PS II, photosystem II; $Q_A$, primary plastoquinone; Tris, Tris(hydroxymethyl)aminoethane.

* Corresponding author. Fax: +49 30 31421122.
[1] Present address: Max-Volmer-Institute, Technical University, Berlin, Germany.


*PII* S0005-2728(96)00027-8

primary charge separation has to be stabilized by rapid reoxidation of $Pheo^{-\cdot}$ with a special plastoquinone $Q_A$ acting as acceptor [2]. For kinetic reasons and owing to rather small spectral differences of the chlorophyll molecules in the antenna, the lowest excited singlet states are assumed to attain a quasi Boltzmann equilibrium among the levels of these pigments [3,4] and P680 before charge separation takes place. Accordingly, the transformation of light within PS II is a trap limited process and the overall kinetics are dependent on the number and spectral properties of the antenna chlorophylls [5,6]. The functional redox groups P680, Pheo and $Q_A$ are incorporated into a heterodimeric protein matrix consisting of polypeptides D1 and D2 (for a review see [1]). General considerations of the mechanism of electron transfer processes in biological systems [7,8] revealed that electrostatic properties and dynamics of proteins are important regulatory parameters. Recent analyses on the efficiency of electron transfer processes in the reaction center of anoxygenic photosynthetic purple bacteria led to the conclusion that relaxation processes, presumably protein dielectric polarisation in close proximity of the redox active groups are of functional relevance [9,10]. Experimental evidence for effects on electron transfer kinetics owing to modification of the protein matrix has been presented previously. It was shown that the electron transfer from $BPheo^{-\cdot}$ to $Q_A$ in reaction centers of anoxygenic purple bacteria becomes slowed down by a factor of 2–4 after incubation in either $D_2O$ or solvents that affect the protein dynamics by interfering with hydrogen bonds [11]. A latest report presents results which are indicative of an analogous modulation of the primary charge separation [12]. The heterodimeric protein matrix (subunits L and M) of these reaction centres exhibits striking similarities to the D1/D2 heterodimer of PS II [13,14]. Futhermore, the rate constants of the electron transfer steps leading to a stable charge separation are quite similar in both types of reaction centers (see [6] and references therein). Therefore one might expect that analogous phenomena are exerted by $D_2O$ and cryoprotectants also in PS II. On the other hand, there exist marked differences between the reaction centers of purple bacteria and PS II as reflected by the presence of the H-subunit in the former and of cytochrome *b*559 and the *psbI* gene product in the latter type. As a consequence, the modulation of the reaction rates could be different. This is really the case as shown for the effect of removal of the non heme iron center. In isolated reaction centers of purple bacteria the electron transfer from $BPheo^{-\cdot}$ to $Q_A$ is markedly retarded [15,16] after iron extraction while that in PS II membrane fragments remains virtually unaffected [17].

The present study is an attempt to analyse effects caused either by $D_2O/H_2O$ exchange or addition of molecules that affect the hydrogen bond network on processes of charge separation in PS II preparations containing $Q_A$. Likewise, H/D isotope exchange effect on the $P680^{+\cdot}Pheo^{-\cdot}$ recombination are investigated in D1-D2-cyt *b*559 preparations

## 2. Materials and methods

PS II membrane fragments were prepared from spinach according to the procedure of Berthold et al. [18] with some modifications as outlined by Völker et al. [19]. Tris washing was performed by incubation of PS II membrane particles in 0.8 M Tris (pH 8.0) for 1 h at 4°C in the dark. Core complexes were obtained as outlined in Haag et al.

Table 1
Fluorescence lifetimes and normalized amplitudes of PS II membrane fragments after $H_2O$–$D_2O$ exchange and in the presence of cryoprotectants

| Sample | $A_1$ (%) | $\tau_1$ (in ps) | $A_2$ (%) | $\tau_2$ (in ps) | $A_3$ (%) | $\tau_3$ (in ns) | $\tau_{av}$ (in ps) | $\chi 2$ |
|---|---|---|---|---|---|---|---|---|
| Control (lyophilised) | 62 | 135 | 38 | 398 | 0.2 | 1.73 | 239 | 0.86 |
| | (±3) | (±6) | (±2) | (±34) | (±0.06) | (±0.28) | (±17) | |
| $D_2O$ | 59 | 148 | 40 | 435 | 0.4 | 1.52 | 269 | 0.95 |
| | (±3) | (±7) | (±2) | (±36) | (±0.1) | (±0.26) | (±19) | |
| Tris-washed $H_2O$ | 65 | 133 | 35 | 350 | 0.2 | 1.85 | 230 | 1.06 |
| | (±3) | (±7) | (±2) | (±30) | (±0.1) | (±0.25) | (±17) | |
| Tris-washed $D_2O$ | 67 | 140 | 33 | 370 | 0.2 | 1.7 | 245 | 1.01 |
| | (±3) | (±6) | (±2) | (±35) | (±0.1) | (±0.3) | (±15) | |
| Control * | 70 | 131 | 30 | 316 | 0.3 | 2.17 | 221 | 0.94 |
| | (±4) | (±7) | (±2) | (±28) | (±0.09) | (±0.39) | (±15) | |
| 50% Ethyleneglycol | 57 | 138 | 43 | 325 | 0.2 | 1.79 | 222 | 1.03 |
| | (±3) | (±7) | (±2) | (±30) | (±0.06) | (±0.3) | (±15) | |
| 60% Glycerol | 55 | 141 | 45 | 348 | 0.3 | 1.59 | 238 | 0.95 |
| | (±3) | (±7) | (±2) | (±35) | (±0.1) | (±0.27) | (±18) | |
| 50% Propyleneglycol | 56 | 148 | 44 | 359 | 0.3 | 1.84 | 245 | 1.06 |
| | (±3) | (±7) | (±2) | (±33) | (±0.1) | (±0.33) | (±17) | |
| 50% DMSO | 60 | 177 | 39 | 441 | 1.0 | 2.27 | 301 | 1.01 |
| | (±3) | (±9) | (±2) | (±42) | (±0.25) | (±0.39) | (±20) | |

The preexponential factors $A_i$ are normalized to give a sum of 100. The average lifetime $\tau_{av}$ was calculated by $\tau_{av} = \Sigma A_i \tau_i / \Sigma A_i$.
* Control for cryoprotectants studies (not lyophilised).

Table 2
Rate constants $k_{PC}^{trap}$, $k^*$ and $k_{stab}$ derived from a numerical fit of the fluorescence life times $\tau_1$ and $\tau_2$ within the framework of the simplified exciton-radical pair equilibrium model described by Eq. (1)

| Sample | $(k_{PC}^{trap})^{-1}$, ns | $(k^*)^{-1}$, ns | $(k_{stab})^{-1}$, ns | $(k_{PC})^{-1}$, ps |
|---|---|---|---|---|
| Control $H_2O$ | $0.19 \pm 0.01$ | $1.23 \pm 0.25$ | $0.29 \pm 0.02$ | $1.9 \pm 0.1$ |
| $D_2O$ | $0.21 \pm 0.01$ | $1.33 \pm 0.3$ | $0.31 \pm 0.03$ | $2.1 \pm 0.1$ |
| Control | $0.19 \pm 0.08$ | $1.04 \pm 0.2$ | $0.24 \pm 0.02$ | $2.0 \pm 0.1$ |
| 50% Ethyleneglycol | $0.26 \pm 0.01$ | $1.09 \pm 0.2$ | $0.25 \pm 0.025$ | $2.9 \pm 0.15$ |
| 60% Glycerol | $0.28 \pm 0.02$ | $1.00 \pm 0.25$ | $0.27 \pm 0.025$ | $3.2 \pm 0.15$ |
| 50% Propyleneglycol | $0.29 \pm 0.02$ | $1.09 \pm 0.3$ | $0.28 \pm 0.03$ | $3.3 \pm 0.15$ |
| 50% DMSO | $0.33 \pm 0.025$ | $1.30 \pm 0.26$ | $0.35 \pm 0.03$ | $3.9 \pm 0.2$ |

The last column compiles $k_{PC}$ values calculated using Eq. (2).

[20]. D1-D2-cyt *b*559 preparations were isolated using a modified protocol reported by Seibert et al. [21]. For $H_2O$ exchange by $D_2O$ samples were lyophilized and resuspensed in either $H_2O$ or $D_2O$. Lyophilized PS II membrane fragments resuspended in $H_2O$ were always used as control samples in $H_2O/D_2O$ exchange studies. Investigations of effects induced by cryoprotectants were performed with unlyophilized preparations.

Steady state absorption measurements were carried out with an absorption spectrometer (Perkin Elmer, Lambda 17) at room temperature. Decomposition analyses of the absorption spectra in terms of Gaussian bands have been performed using a simplex fitting algorithm.

Fluorescence decay measurements were performed using a single photon counting apparatus outlined in [22] with some modifications as described in [17]. The samples were excited at a repetition rate of 0.8 MHz by 650 nm pulses (FWHM 15 ps), derived from a cavity-dumped dye laser system (Spectra Physics). Fluorescence light at 684 nm was selected using an interference filter (8 nm bandwidth) and detected by a R1645 U-01 MCP-photomultiplier (Hamamatsu). Resolution of the time-to-amplitude converter (Ortec 457) was 12 ps/channel. The FWHM of the system response function in this configuration was about 60 ps, as measured with a Latex scattering solution. Decays were recordered with 15000 counts in the peak channel. Results of three measurements were then summarised to give the final value of $\approx 45000$ counts. Fluorescence decay kinetics of D1-D2-cyt *b*559 particles were measured in time windows of 12.5, 125 and 250 ns. All decay curves were fitted simultaneously with lifetimes linked across the whole data set. The error ranges for the optimal set of fit parameters were found in the exhaustive search procedure, as described in [23]. Results, summarised in Tables 1–3 represent the average values, measured with the samples from three preparations.

The composition of the sample suspensions are given in the figure captions. Samples for the fluorescence decay measurements were diluted to 10 μg Chl/ml and kept near 2°C by a thermoelectric cooler.

## 3. Results and discussion

### *3.1. Effects of $H_2O$-$D_2O$ exchange and addition of cryoprotectants on the fluorescence decay kinetics of PS II membrane fragments*

Information on the primary processes of exciton trapping in PS II can be obtained by measuring time resolved fluorescence decay curves. Fig. 1 shows traces obtained in PS II membrane fragments with open reaction centers suspended in buffer solutions containing either $H_2O$ or $D_2O$. An inspection of the data readily shows that the overall relaxation kinetics are somewhat slower in samples with $D_2O$. This finding suggests that the exciton trapping might be retarded by substitution of exchangeable protons to deuterium in the protein matrix and/or hydrogen bridges to functional redox groups. A significant retardation by a factor of about two has been observed for the oxidation of the reduced bacteriopheophytin ($BPheo^{-\cdot}$) acceptor by $Q_A$ in isolated reaction centers of purple bacteria after substitution of $H_2O$ by $D_2O$ [11]. An analogous effect is also induced by addition of cryoprotectants [11]. Therefore, it is

Table 3
Effects of $H_2O$–$D_2O$ exchange on the fluorescence decay lifetimes and normalized amplitudes in isolated reaction center complexes

| Sample | $\tau_1$ ($A_1$) ns, (%) | $\tau_2$ ($A_2$) ns, (%) | $\tau_3$ ($A_3$) ns, (%) | $\tau_4$ ($A_4$) ns, (%) | $\tau_5$ ($A_5$) ns, (%) |
|---|---|---|---|---|---|
| Control $H_2O$ | $0.02 \pm 0.016$ | $1.1 \pm 0.25$ | $5.1 \pm 0.35$ | $16.9 \pm 3$ | $52.5 \pm 3$ |
| | (75.3) | (7.9) | (14.3) | (1.9) | (0.7) |
| $D_2O$ | $0.02 \pm 0.016$ | $1.2 \pm 0.25$ | $5.4 \pm 0.4$ | $18.9 \pm 3.5$ | $57.3 \pm 4$ |
| | (75.8) | (8.2) | (13.3) | (2) | (0.7) |

Error for amplitudes about $\pm 10\%$.

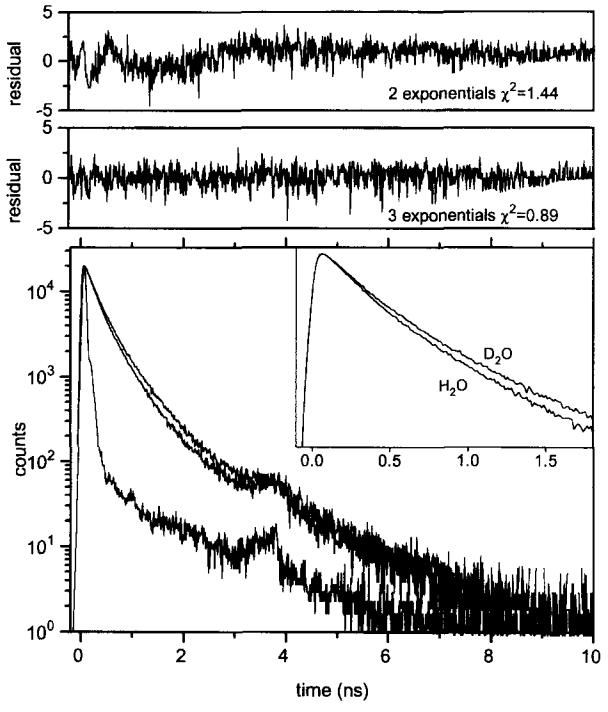


Fig. 1. System responce function and fluorescence decay traces from PS II membrane fragments (10 μM chlorophyll) in buffer solutions (20 mM MES + 10 mM NaCl pH 6.5) containing $H_2O$ or $D_2O$. The upper parts of the figure show typical weighted residuals for the three-exponential and two-exponential fits.

worth analyzing the effect of these organic solvents on exciton trapping in PS II. Fig. 2 presents fluorescence decay curves measured in aqueous suspensions of PS II membranes in the absence or presence of 50% v/v of different solvents. A pronounced retardation effect is observed. The potential of these cryoprotectants to slow down the fluorescence decay increases in the order ethyleneglycol < glycerol < propyleneglycol < DMSO. This phenomenon qualitatively resembles that observed in isolated reaction centers of purple bacteria [11].

In order to gather from these experimental data information on the rate constants of the light induced charge separation, a deconvolution was performed into exponential kinetics according to $F(t) = \sum_i a_i \exp(-t/\tau_i)$. It was shown that a satisfactory fit could be achieved by three components. In some cases the best fit requires a fourth component with $\tau_i$ of about 30 ps and very small amplitude. It probably originates — at least in part — from contaminations by PS I that are always monitored in PS II membrane fragment preparations when using sensitive detection methods like silver staining (Irrgang, K.-D. and Renger, G., unpublished results). Therefore, this component was omitted and the remaining three kinetics obtained by a numerical fit of the data presented in Fig. 1 and Fig. 2 are summarized in Table 1.

An inspection of these results reveals that the overall decay is dominated by two kinetics with τ-values of 130–180 ps and 320–450 ps, respectively. The slowest component with a lifetime of the order of 2 ns exhibits rather small amplitudes and is ascribed to a very minor fraction of PS II membrane fragments that contain closed reaction centers. The alternative possibility that the 2 ns kinetics reflects a relaxation process of the primary radical pair due to reorientation of the protein environment appears to be unlikely because in open reaction centers $Pheo^{-\cdot}$ becomes reoxidized by $Q_A$ with kinetics of about 300 ps (see [17] and results of the present study). Accordingly, these 2 ns kinetics will not be considered for further analyses of the processes taking place in open reaction centers.

A biexponential fluorescence decay can be easily reconciled with the model of exciton radical pair equilibration in PS II as described by Schatz et al. [24]. A simplified kinetic pattern is summarized in Eq. (1):

$$[Chl_N P680]^* PheoQ_A \underset{k^*}{\overset{k_{PC}^{trap}}{\rightleftharpoons}} Chl_N P680^{+\cdot} Pheo^{-\cdot} Q_A \overset{k_{stab}}{\rightarrow} Chl_N P680^{+\cdot} PheoQ_A^{-\cdot}$$

$$h\nu \uparrow\downarrow k_A$$

$$Chl_N P680\, Pheo\, Q_A \qquad (1)$$

where $[Chl_N P680]^* PheoQ_A$ symbolizes the exciton equilibration among N antenna chlorophyll molecules and P680 and the rate constants describe excitation decay from the antenna ($k_A$), the primary charge separation ($k_{PC}^{trap}$), its reversal ($k^*$) and stabilization by rapid electron transfer from $Pheo^{-\cdot}$ to $Q_A$ ($k_{stab}$). For the sake of simplicity and for kinetic reasons the recombination of radical pairs into the ground state and triplet state formation are omitted. Furthermore, relaxation processes of the primary radical pair are not explicitly taken into account.

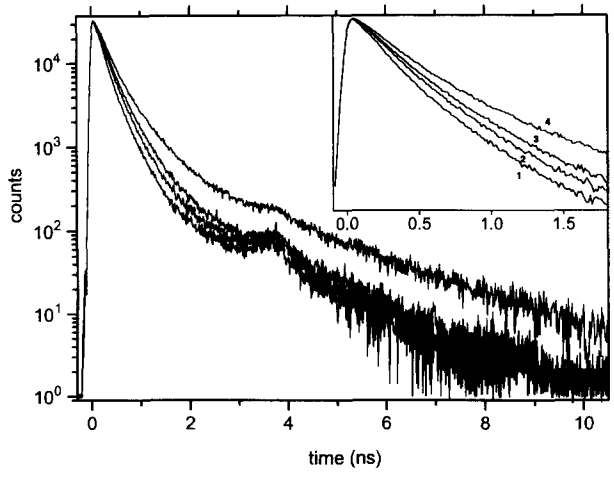


Fig. 2. Fluorescence decay traces from PS II membrane fragments (10 μM chlorophyll) in buffer solutions (20 mM MES + 10 mM NaCl pH 6.5) without additions (1) and with 50% (v/v) of different cryoprotectants: ethyleneglycol (2), propyleneglycol (3), DMSO (4).

### 3.2. Determination of the rate constants of exciton trapping in PS II by a model based on evaluation of the fluorescence decay curves in samples with open reaction centers

The rate constants $k_{PC}^{trap}$, $k^*$ and $k_{stab}$ of open reaction centers in PS II membrane fragments were determined by a numerical fit of the fluorescence life times $\tau_1$ and $\tau_2$ and normalized amplitudes $A_1$ and $A_2$ compiled in Table 1 within the framework of the simplified exciton radical pair equilibrium model described by Eq. (1). This procedure comprises also the rate constant $k_A$ that can depend on changes of the protein hydrogen bond network owing to $H_2O/D_2O$ substitution and/or addition of cryoprotectants. In an attempt to estimate possible changes of $k_A$, experiments were performed with isolated LHC II complexes. Substitution of $H_2O$ by $D_2O$ was found to effect only slightly the exciton dynamics in isolated LHC II, whereas addition of cryoprotectants accelerated the singlet state decay by a factor of 2–3 (data not shown). In the natural membrane environment pigment–protein complexes should be less accessible to the cryoprotectants and therefore their effect is expected to be less pronounced. To illustrate the possible relevance of $k_A$ variations for the determination of the rate constants $k_{PC}^{trap}$, $k^*$ and $k_{stab}$, model calculations were performed with different $k_A$ values. The results presented in Fig. 3 reveal that the system is rather robust to variations of $k_A$. As the LHCII complex represents a large part of the PS II antenna system, the average life time of isolated LHCII (4 ns) was used as $k_A$ value.

The rate constants $k_{PC}^{trap}$, $k^*$ and $k_{stab}$ obtained are compiled in Table 2. In control samples the rate constant of the photochemical trapping ($k_{PC}^{trap}$) is shown to be of the order of $(200\ ps)^{-1}$ and that of the subsequent electron transfer from $Pheo^{-\cdot}$ to $Q_A$ ($k_{stab}$) covers a range of $(250–300\ ps)^{-1}$. These values are in good correspondence with data gathered from fluorescence life time measurements (see [23] and references therein) or for $k_{stab}$ obtained by monitoring time resolved UV absorption changes [25]. A somewhat larger rate constant was obtained for radical pair recombination into $^1P680^*$. The $k^*$ values of $(1–1.3\ ns)^{-1}$ are about twice as large as those reported recently for the same type of samples [6]. The origin of this difference has not been analyzed systematically because it was found that this parameter is most sensitive to the numerical fit (see error bars in Fig. 3). Therefore, it seems likely that this difference is not relevant.

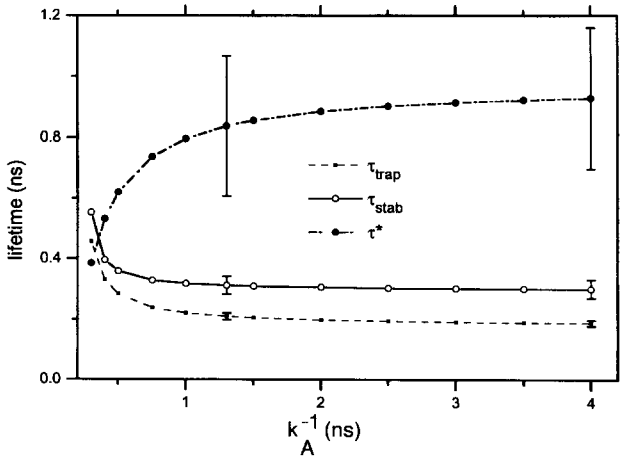


Fig. 3. Values of $\tau_{trap}$, $\tau_{stab}$ and $\tau^*$, calculated according to the exciton radical pair equilibrium model as a function of $k_A$.

A closer inspection of the data reveals that both, the rate constants $k_{PC}^{trap}$ and $k_{stab}$ are slightly retarded by $D_2O$ and cryoprotectants. The recombination rate ($k^*$) remained virtually unaffected, but this conclusion is meaningless because of the large uncertainty of the $k^*$ values (see Fig. 3). The general feature of slowing down the electron transfer from $Pheo^{-\cdot}$ to $Q_A$ and in particular the order of the effect exerted by different cryoprotectants qualitatively corresponds with effects observed for the kinetics of $BPheo^{-\cdot}$ reoxidation by $Q_A$ in isolated reaction centers of purple bacteria [11] but the quantitative differences are quite substantial. A comparison of the extent of modification in both types of organisms shows that the effects are much smaller in PS II than in isolated reaction centers of anoxygenic purple bacteria: in PS II $k_{stab}$ is retarded by at most 45% (in the case of DMSO) as shown in Table 2, whereas in purple bacteria the analogous process is slowed down by a factor of up to four [11]. The conclusion of a negligibly small effect of an $H_2O/D_2O$ exchange on the rate constant $k_{stab}$, gathered from a model based numerical evaluation of fluorescence decay kinetics is in perfect agreement with recent measurement of time resolved UV-absorption changes at 325 nm [26].

Before discussing in more detail the rather weak kinetic modifications that are caused in PS II by H/D isotope exchange and cryoprotectants, it remains to be shown that the trivial effect of a limited substitution by drastically reduced accessibility of solvent molecules can be excluded. Unfortunately, directly comparable measurements are impossible with PS II preparations of a protein composition analogous to that of reaction centers isolated from purple bacteria. D1-D2-cyt *b*559 preparations have been isolated from PS II that resemble in several aspects purple bacteria reaction center [27,28]. However, all D1-D2-cyt *b*559 preparations known so far are lacking $Q_A$ and therefore the electron transfer from $Pheo^{-\cdot}$ to $Q_A$ cannot be analysed in these samples. Therefore, as the best compromise experiments were performed with PS II preparations that are deprived of some polypeptides (with respect to PS II membrane fragments) but still retain $Q_A$. Furthermore, in a complementary study the recombination kinetics of the ion-radical pair $P680^{+\cdot}Pheo^{-\cdot}$ in D1-D2-cyt *b*559 preparations was investigated. In order to achieve the highest possible extent of exchangeability all experiments were performed with lyophilized samples that were dissolved in either $H_2O$ as a control or $D_2O$.

### 3.3. Effects of $H_2O$-$D_2O$ exchange on the fluorescence decay kinetics of Tris-washed PS II membrane fragments and PS II core complexes

Tris-washed PS II membrane fragments are deprived of the three extrinsic proteins of 18, 23 and 33 kDa mol. wt. and have lost most of the manganese that is an indispensible constituent of a functionally competent water oxidizing complex [29,30]. Also the reaction coordinate of the electron transfer from $Y_Z$ to $P680^{+\cdot}$ is markedly changed as reflected by an increase of the activation energy by a factor of about 4 [31,32]. Furthermore, the reaction exhibits a pronounced kinetic H/D isotope exchange effect of 2.7 in Tris-washed PS II membrane fragments (Christen, G. and Renger, G. unpublished results) in contrast to a virtually negligible effect on the ns-kinetics in untreated samples [33]. Opposite to this particular effect, the fluorescence decay curves of Tris-washed samples exhibit an even less pronounced effect (the results obtained by deconvolution are compiled in Table 1.) than untreated PS II membrane fragments. These findings show that the extrinsic proteins do not modify effects of $D_2O/H_2O$ exchange on the reactions leading to $P680^{+\cdot}Q_A^{-\cdot}$ formation. It is therefore concluded that the modulation of the kinetics of $P680^{+\cdot}$ reduction by $Y_Z$ is a rather specific effect probably related to the loss of the tetranuclear manganese cluster of the water-oxidising complex.

In contrast to Tris-washed PS II membrane fragments, core-complexes isolated with β-dodecylmaltoside have lost the pigment–protein compexes of the peripheral antenna but retain the manganese cluster and the extrinsic 33 kDa protein and exhibit high oxygen evolution capacity [20,34]. The fluorescence decay curves measured in PS II core complexes with open reaction centers (not shown) were dominated by the kinetics with lifetimes of 40–80 ps and 170–320 ps. These data are in close correspondence with those reported previously [35]. A significant difference of the kinetics could not be found between lyophilized samples dissolved in $H_2O$ and $D_2O$, respectively. The rate constants $k_{PC}^{trap}$ and $k_{stab}$ gathered by a numerical evaluation were virtually the same within the experimental error, i.e., the possible retardation in $D_2O$ dissolved samples cannot exceed 10 and 20%, respectively.

The results of the above mentioned investigations clearly show that neither the removal of the manganese cluster together with the extrinsic proteins (Tris-washed membrane fragments) nor the preparative separation of the peripheral antenna complexes (PS II core complexes) affect the kinetic H/D isotope exchange effect on the rate constants of the reactions that lead to $P680^{+\cdot}Q_A^{-\cdot}$ formation. This finding together with the rather strong exchange treatment (lyophilization and resuspension in $D_2O$ or $H_2O$) highly support the idea that the measured effects are not limited by proteinous barriers to H/D exchange.

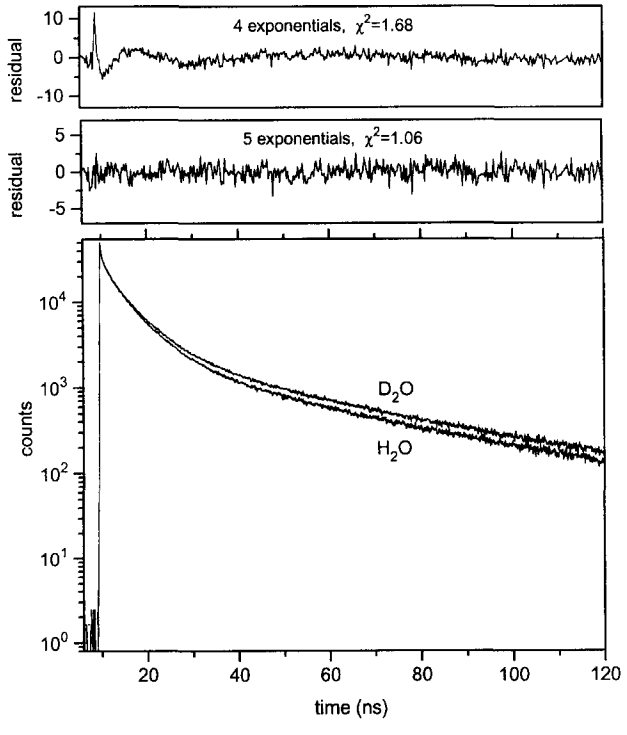


Fig. 4. Fluorescence decay traces of D1-D2-Cyt *b559* particles (10 μM chlorophyll) resuspensed in $H_2O$ or $D_2O$. The upper parts of the figure show typical weighted residuals for the four-exponential and five-exponential fits.

### 3.4. Charge recombination in D1-D1-cyt b559-particles

Fig. 4 shows the decay curves measured in lyophilized D1-D2-cyt *b*559 preparations dissolved either in $D_2O$ or $H_2O$. A rather small retardation is observed in $D_2O$. The residuals of data evaluation depicted on the top reveal that a satisfying fit of the data requires five exponentials. The lifetimes and normalized amplitudes are compiled in Table 3. The very fast kinetics are ascribed to the charge separation and energy transfer from accessory Chl to P680 [36], the origin of the 1 ns component is not yet quite clear while the 5 ns kinetics is assumed to reflect singlet state decay of a functionally 'disconnected' Chl *a* [36–38]. The slow phases with τ-values of 15–20 ns and 50–60 ns as well as a fraction of the 5 ns phase are attributed to ion radical pair, $P680^{+\cdot}Q_A^{-\cdot}$, recombination ([36] and references therein). An inspection of the data in Table 3 readily shows that the kinetic H/D isotope exchange effects are rather small.

### 3.5. Effects on electron transfer from $Pheo^{-\cdot}$ to $Q_A$

Therefore, based on the results of this study and of the previous data presented in [26] the kinetics of electron transfer from $Pheo^{-\cdot}$ to $Q_A$ are inferred to be much less affected by the network of deuterium exchangeable hydrogen bonds than the rate of $BPheo^{-\cdot}$ reoxidation by $Q_A$ in anoxygenic purple bacteria. This different fine tuning of the reaction coordinate could originate either directly from

the mode of hydrogen bonding to the functional redox groups or from particular effects owing to the dielectric relaxation of the protein matrix.

Recently, the formation of hydrogen bridges to $Pheo^{-\cdot}$ and $Q_A^{-\cdot}$ was analyzed by EPR and ENDOR investigations. It was shown that $BPheo^{-\cdot}$ and $Pheo^{-\cdot}$ exhibit a similar pattern of hydrogen bonding between a Glu-residue and the $C_9$ keto group [39]. Although its strength is significant it seems unlikely that this hydrogen bond accounts for the quantitative differences between purple bacteria and PS II with respect to the effect of an $D_2O/H_2O$ exchange on the kinetics of $(B)Pheo^{-\cdot}$ reoxidation by $Q_A$. This conclusion is mainly based on the finding that a complete removal of this hydrogen bond by genetically induced substitution of Glu-104 in the L-subunit of purple bacteria caused only minor kinetic effects (less than 50%; see [40]) while suspension of the isolated reaction centers in $D_2O$ or addition of cryoprotectants leads to marked retardation of electron transfer (factor 3–4, see [11]).

The nature of hydrogen bridges to $Q_A^{-\cdot}$ was investigated in iron depleted PS II membrane fragments [41]. This study revealed that the plastosemiquinone anion radical $Q_A^{-\cdot}$ in PS II exhibits a similar asymmetric hydrogen bond pattern as the anion radical ubisemiquinone $Q_A^{-\cdot}$ in bacterial reaction centers but in general the strength of the hydrogen bonds is weaker in PS II. Accordingly, the latter effect might be responsible for the significant quantitative differences in modulating of $k_{stab}$ by $D_2O$/cryoprotectants in PS II and reaction centers of purple bacteria. This idea would imply the conclusion that the hydrogen bonding around $Q_A$ is the dominating factor for the dependence of rate constant $k_{stab}$ on $D_2O/H_2O$ exchange and/or addition of cryoprotectants.

However, it has to be emphasized that with respect to $Q_A$ there exist additional differences between PS II and the reaction centers of purple bacteria. It has been found that removal of the non heme iron from bacterial reaction centers retaining the H-type subunit markedly retards electron transfer from $BPheo^{-\cdot}$ to $Q_A$ [15,16] while in PS II the kinetics remains virtually invariant to iron depletion [17]. Interestingly, after depletion of the H-type subunit from bacterial reaction centers the kinetics of $BPheo^{-\cdot}$ reoxidation by $Q_A$ is almost invariant to the absence of the iron center [16]. PS II does not contain an H-type subunit. Therefore, it appears attractive to speculate that the significant effects due to $D_2O$ and/or cryopotectant in purple bacterial reaction centers are related to the H-type subunit rather than to the mode of hydrogen bond interaction with $Q_A^{-\cdot}$. Experiments with purple bacteria reaction centers deprived of the H-type subunit will be performed to check this idea.

### 3.6. Excited singlet state equilibration between antenna and P680

The data presented in Table 2 reveal that $D_2O/H_2O$ exchange and/or addition of cryoprotectants affects not only the rate constant $k_{stab}$ but also the overall photochemical trapping reflected by $k_{PC}^{trap}$. As in the case of $k_{stab}$ the effects are comparatively small. At first glance, this result can be taken as evidence that the molecular rate constant of the primary charge separation, $k_{PC}$, is only moderately affected by changes in the hydrogen bond network. However, for thorough considerations the composite nature of $k_{PC}^{trap}$ has to be taken into account. Within the framework of the exciton radical pair model [24] the molecular rate constant $k_{PC}$ is given by:

$$k_{PC} = \frac{k_{PC}^{trap}}{\boldsymbol{P}_{P680}} \tag{2}$$

where $\boldsymbol{P}_{P680}$ is the population probability of state $^1P680^*$ in the presence of one singlet exciton within PS II.

Both parameters, $k_{PC}$ and $\boldsymbol{P}_{P680}$, could be affected by $D_2O$ and/or cryoprotectants. Accordingly, it appears worth analyzing possible effects on $\boldsymbol{P}_{P680}$. The value of the probability factor $\boldsymbol{P}_{P680}$ can be calculated by the assumption of a very rapid Boltzmann-equilibration of the excited state among the chlorophyll molecules of the antenna and P680 [24]. This idea implies that the number of pigments participating in this process and their spectral properties determine the ensemble of energy levels for excited singlet state distribution. An exact calculation of $\boldsymbol{P}_{P680}$ is impossible for several reasons: the pigment levels are dependent on the strength of excitonic coupling between pigments and their interaction with the surrounding protein matrix. The latter effect gives rise to inhomogenous line broadening. Furthermore it is not precisely known to what extent the condition is satisfied that the excited state equilibration is sufficiently rapid [4]. Therefore, only approximate estimations can be presented. If one describes the spectral properties of the antenna pigments (that are involved in the fast equilibration) by a Boltzmann weighted average wavelength $\lambda_{eq}$ the probability $\boldsymbol{P}_{P680}$ is given by:

$$\boldsymbol{P}_{P680} = \frac{1}{N} \cdot \exp\left[\frac{hc}{k_B T}\left(\frac{1}{\lambda_{eq}} - \frac{1}{\lambda_{P680}}\right)\right] \tag{3}$$

where $k_B$ is Boltzmann's constant.

In the case of discrete energy levels characterized by wavelength $\lambda_i$ and abundance $n_i$, the wavelength $\lambda_{eq}$ can be calculated by:

$$\lambda_{eq} = \frac{hc}{k_B T}\left[\ln \sum_i \frac{n_i}{N} \cdot \exp\left(\frac{hc}{k_B T} \cdot \frac{1}{\lambda_i}\right)\right]^{-1} \tag{4a}$$

The averaged wavelength $\lambda_{eq}$ can be estimated from the absorption spectra measured under the same conditions as fluorescence decay kinetics. A deconvolution into Gaussians provides an idea about the structure of the energy level ensemble assuming equal transition matrix elements for all pigment pools represented by a Gaussian.

Fig. 5 shows absorption spectra of PS II membrane fragments measured at room temperature in normal buffer

and in the presence of $D_2O$ and cryoprotectants. A direct comparison of the spectra reveals that some minor spectral changes are induced by these treatments. A decomposition into Gaussian bands for samples in normal buffer and in presence of 50% DMSO is given in Fig. 6. Decompositions of absorption spectra 4 in presence of other cryoprotectants and $D_2O$ are not shown because the differences between these samples and the spectrum of non-treated control were less pronounced. In a rough approximation $\lambda_j$ and $n_j$ can be substantiated as the peak wavelength and oscillator strength, respectively, of these subbands. In this case one obtains:

$$\lambda_{eq} = \frac{hc}{k_B T}\left[\ln \sum_j A_j \cdot \exp\left(\frac{hc}{k_B T} \cdot \frac{1}{\lambda_j}\right)\right]^{-1} \quad (4b)$$

where $A_j$ is the normalized area and $\lambda_j$ the peak maximum of the $j$th Gaussian subband.

Although limitations of this procedure are obvious (the excitonic coupling, the existence of 'allowed' and 'forbidden' transitions and line broadening effects are not taken into account) the analysis shows that ethyleneglycol does not markedly affect $\lambda_{eq}$. Similar features are obtained with other cryoprotectants (data not shown).

The parameter $P_{P680}$ can be interpreted as a measure of the Gibbs energy loss due to distribution of the excited singlet state among P680 and the antenna pigments. This contribution is given by

$$\Delta G^{Ant} = k_B T \cdot \ln P_{P680} \quad (5)$$

Calculation of $\lambda_{eq}$ values leads to $\Delta G^{Ant}$ value of $-108$ meV for the control samples. $H_2O/D_2O$ exchange was virtually without effect on $\Delta G^{Ant}$, the maximal variation ($-105$ meV) was observed in the presence of 50% DMSO (data not shown).

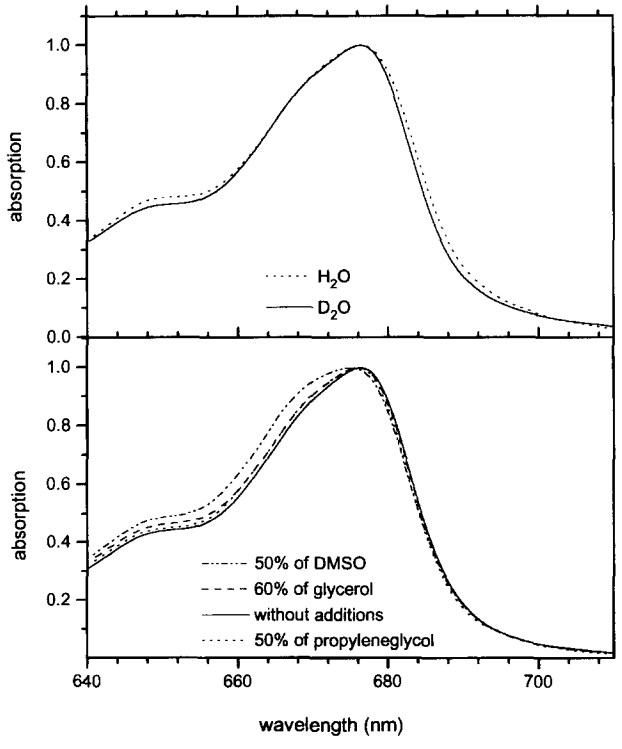


Fig. 5. Absorption spectra of PS II membrane fragments (100 μM chlorophyll) in buffer solutions (20 mM MES + 10 mM NaCl pH 6.5) containing different cryoprotectants.

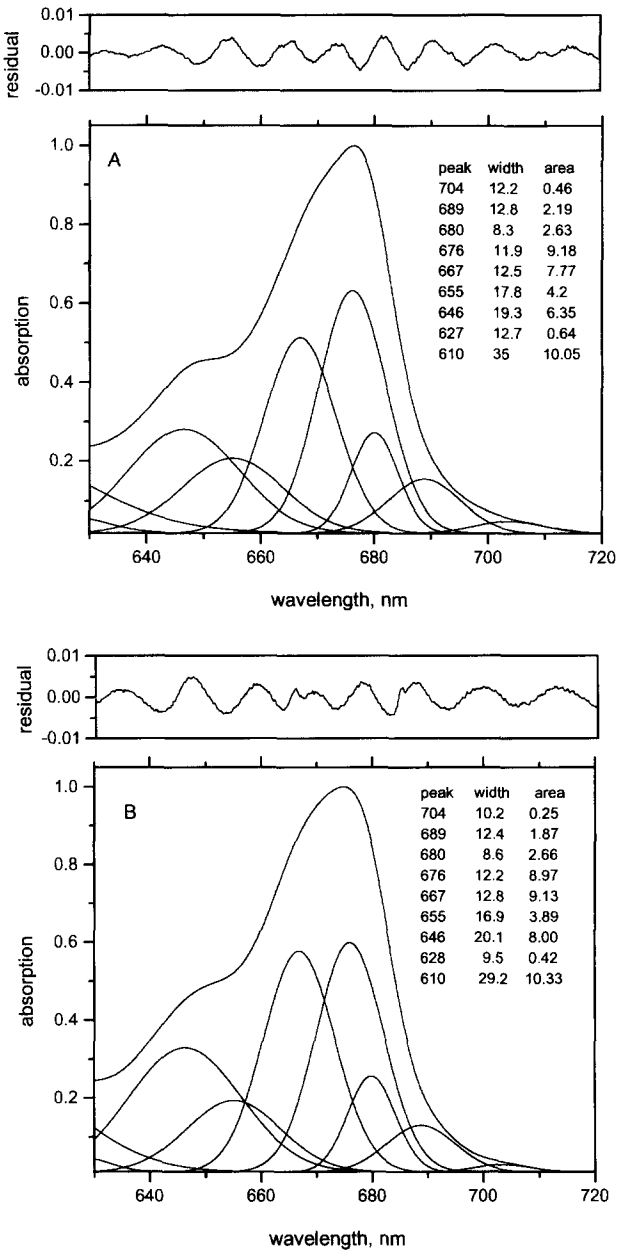


Fig. 6. Decomposition of the room temperature absorption spectra of PSII membrane fragments into Gaussian components. (A) Sample in normal (20 mM MES + 10 mM NaCl pH 6.5) buffer without additions, (B) In the same buffer with 50% DMSO. Residuals shown in the upper part were used to judge the quality of the fits.

$\Delta G^{Ant}$ values of about −100 mV would be also obtained for an idealized system consisting of about 50 antenna chlorophylls that are isoenergetic with P680 and satisfy the condition of perfect equilibration due to sufficiently fast excitation energy transfer. This indicates that the spectral properties and/or incomplete equilibration markedly modify the 'effective' antenna size of PS II membrane fragments because the average number of chlorophyll molecules per PS II are of the order of 220 as deduced from the experimentally determined average oxygen yield per flash in the PS II membrane fragments used.

### 3.7. Molecular rate constant of primary charge separation

The molecular rate constants $k_{PC}$ obtained by using Eq. (2), Eq. (3) and Eq. (4b) are compiled in Table 2 (last column). These $k_{PC}$ values calculated for control samples and our recent data [6] are in close correspondence with those obtained by analogous analyses performed by Holzwarth's group (for a review, see [24] and references therein) and by time resolved spectroscopic measurements in D1-D2-cyt *b*559 preparations [42–44]. A comparison of these results shows that the maximal variation of $k_{PC}$ does not exceed about 50%

Knowledge of the molecular rate constants $k_{PC}$ and $k^*$ also permits the calculation of Gibbs energy difference between $^1P680^*$ and the primary radical pair $P680^{+\cdot}Q_A^{-\cdot}$:

$$\Delta G^{\circ}_{PC} = -k_B T \ln \frac{k_{PC}}{k^*} \quad (6)$$

Using the rate constants derived from the time resolved fluorescence measurements of this study and Eq. (6) leads to $\Delta G^{\circ}_{PC}$ values of the order of −150 meV. Replacement of $H_2O$ by $D_2O$ or addition of cryoprotectants changes this value by no more than 20%.

### 3.8. Concluding remarks

The results of this study reveal that the rate constants of the electron transfer steps leading to a light induced stable charge separation (formation of the ion-radical pair $P680^{+\cdot}Q_A^{-\cdot}$) in PS II are only slightly affected by changes of the hydrogen bond network. Likewise, in D1-D2-cyt *b*559 preparations that are lacking $Q_A$, recombination kinetics of the ion radical pair $P680^{+\cdot}Pheo^{-\cdot}$ also exhibits a rather small H/D isotope exchange effect. Surprisingly, in $Q_A$ containing PS II preparations the rate constant $k_{stab}$ for the stabilization step of the primary charge separation, the reoxidation of $Pheo^{-\cdot}$ by $Q_A$, was found to be markedly less sensitive to replacement of $H_2O$ by $D_2O$ or addition of cryoprotectants than the corresponding electron transfer from $BPheo^{-\cdot}$ to $Q_A$ in isolated reaction centers of anoxygenic purple bacteria [11]. Latest experiments indicate that in PS II preparations that are fully competent in water cleavage the kinetics of $P680^{+\cdot}$ reduction by $Y_Z$ remains also virtually invariant to $H_2O/D_2O$ exchange [33], whereas the subsequent reactions in the water oxidizing complex and $Q_A^{-\cdot}$ reoxidation by $Q_B$ exhibit more pronounced H/D isotope exchange effects (Ref. [33] and [6], respectively). These findings indicate that the protein matrix of the redox groups leading to formation of the radical pair $Y_Z^{OX}P680Q_A^{-\cdot}$ in PS II seems to be rather 'stiff'. This idea is supported by the observation that allosteric effects between the donor and acceptor side are almost absent [45]. The strikingly different extent of $H_2O/D_2O$ exchange and cryoprotectant-induced modulation of the $k_{PC}$ and $k_{stab}$ in reaction centers of anoxygenic purple bacteria and PS II might be related to functional differences, especially the generation of strongly oxidizing redox equivalents at $P680^{+\cdot}$ in PS II. In this respect it is interesting to note that the efficiency of cryoprotectants in decreasing $k_{stab}$ exhibits the same order among the agents analysed but the absolute effect is much more pronounced in purple bacteria. This finding would suggest that the underlying mechanism is rather similar in both sample types but the flexibility of the protein matrix is different. This problems will be addressed in forthcoming studies.

## Acknowledgements

The authors would like to thank J. Kurreck for providing the D1-D2-cyt *b*559 preparations and B. Lange for skillful technical assistance. The supports by Deutsche Forschungs-gemeinschaft, the Alexander von Humboldt Foundation (to S.V.) and Fonds der Chemischen Industrie (to G.R.) are gratefully acknowledged.

## References

[1] Renger, G. (1992) in The Photosystems: Structure, Function and Molecular Biology (Barber, J., ed.), pp. 45–99, Elsevier, Amsterdam.
[2] Renger, G. and Eckert, H.-J. (1980) Bioelectrochem. Bioenerg. 7, 101–124.
[3] Mc Cauley, S.W., Bittersmann, E. and Holzwarth, A.R. (1989) FEBS. Lett. 249, 285–288.
[4] Kehrberg, G., Voigt, J., Schrötter, T. and Renger,G. (1995) Biochim. Biophys. Acta 1231, 147–156.
[5] Dau, H. (1994) Photochem. Photobiol. 60, 1–23.
[6] Renger, G., Eckert, H.-J., Bergmann, A., Bernarding, J., Liu, B., Napiwotzki, A., Reifarth, F. and Eichler, H.J. (1995) Aust. J. Plant Physiol. 22, 167–181.
[7] Warshel, A. and Aqvist, J. (1991) Annu. Rev. Biophys. Chem. 20, 267–298.
[8] Parson, W.W., Chu, Z.-T. and Warshel, A. (1990) Biochim. Biophys. Acta 1017, 251–272.
[9] Xu, D. and Schulten, K. (1994) Chem. Phys. 182, 91–117.
[10] Krishtalik, L.I. (1995) Biochim. Biophys. Acta 1228, 58–66.
[11] Paschenko, V.Z., Korvatovsky, B.N., Logunov, S.L., Kononenko, A.A., Knox, P.P., Zakharova, N.I., Grishanova, N.P. and Rubin, A.B. (1987) FEBS Lett. 214, 28–33.
[12] Paschenko V.Z., Vasil'ev S.S., Gorokhov V.V., Knox, P.P. and Rubin, A.B (1995). in Photosynthesis: from Light to Biosphera (Mathis, P., ed.), vol.1, pp. 491–494, Kluwer Academic, Dordrecht.

[13] Michel, H. and Deisenhofer, J. (1988) Biochemistry 27, 1–7.
[14] Trebst, A. (1986) Z. Naturforsch. 41c, 240–245.
[15] Kirmaier, C., Holten, D., Debus, R.J., Feher, G and Okamura, H. (1986) Proc. Natl. Acad. Sci. USA 83, 6407–6411.
[16] Liu, P., Van Kan, P.J.M. and Hoff, A.J. (1991) FEBS Lett. 289, 23–28.
[17] Bernarding, J., Eckert, H.-J., Eichler, H.J., Napiwotski, A. and Renger, G. (1994) Photochem. Photobiol. 59, 566–573.
[18] Berthold, D.A., Babcock, G.T. and Yocum, C.F. (1981) FEBS Lett. 134, 231–234.
[19] Völker, M., Ono, T., Inoue, Y. and Renger, G. (1985) Biochim. Biophys. Acta 806, 25–34.
[20] Haag, E., Irrgang, K.-D., Boekema, E.J. and Renger, G. (1990) Eur. J. Biochem. 189, 47–53.
[21] Seibert, M., Picorel, R., Rubin, A. and Connolly, J.S. (1988) Plant Physiol. 87, 303–306.
[22] Liu, B., Napiwotzki, A., Eckert, H.-J., Eichler, H.J. and Renger, G. (1993) Biochim. Biophys. Acta 1142, 129–138.
[23] Roelofs, T.A., Lee, C.-H. and Holzwarth, A.R. (1992) Biophys. J. 61, 1147–1163.
[24] Schatz, G.H., Brock, H. and Holzwarth, A.R. (1988) Biophys. J. 54, 397–405.
[25] Eckert, H.-J., Wiese, N., Bernarding, J., Eichler, H.J., and Renger, G. (1988) FEBS Lett. 240, 153–158.
[26] Bernarding, J., Wiese, N., Eckert, H.-J., Eichler, H.J., Haag, E. and Renger, G. (1991) In Proc. Int. Conf. Lasers (Harris, G.D. ed.), pp. 162–167, STS Press, McLean, VA.
[27] Nanba, O. and Satoh, K. (1987) Proc. Natl. Acad. Sci. USA 84, 109–112.
[28] Chapman, D.J., Gounaris, K., and Barber, J. (1988) Biochim. Biophys. Acta 933, 423–431.
[29] Yamamoto, Y., Doi, M., Tamura, N., and Nisimura, M. (1981) FEBS Lett. 133, 265–268.
[30] Yamashita, T. and Tomita, G. (1976) Plant Cell Physiol. 17, 571–582.
[31] Reinman, S. and Mathis, P. (1981) Biochim. Biophys. Acta 635, 249–258.
[32] Eckert, H.-J. and Renger, G. (1988) FEBS Lett. 236, 425–431.
[33] Karge, M., Irrgang, K.-D., Sellin.S., Feinängle, R., Liu,B., Eckert, H.-J., Eichler, H.-J. and Renger, G. (1996) FEBS Lett. 378, 140–144.
[34] Gleiter, H.M., Haag, E., Inoue, Y. and Renger, G. (1993) Photosynth. Res. 35, 41–53.
[35] Hodges, M. and Moya, I. (1988) Biochim. Biophys. Acta 935, 41–52.
[36] Roelofs, T.A., Kwa, S.L.S., van Grondelle, R., Dekker, J.P., and Holzwarth, A.R. (1993) Biochim. Biophys. Acta 1143, 147–157.
[37] Freiberg, A., Timpmann, K., Moskalenko, A.A., and Kuznetzova, N.Y. (1994) Biochim. Biophys. Acta 1184, 45–53.
[38] Volk, M., Gilbert, M., Rousseau, G., Richter, M., Ogrodnik, A. and Michel-Beyerle, M.-E.(1993) FEBS Lett. 336, 357–362.
[39] Lubitz, W., Isaacson, R.A., Okamura, M.Y., Abresh, E.C., Plato, M. and Feher, G. (1989) Biochim. Biophys. Acta 977, 227–232.
[40] Bylina, E.J., Kirmeier, Ch., McDowell, L., Holten, D., Youvan, D.C. (1988) Nature 336, 182–184.
[41] MacMillan, F., Lendzian, F., Renger, G. and Lubitz, W. (1995) Biochemistry 34, 8144–8156.
[42] Wasielewski, M.R., Johnson, D.G., Seibert, M. and Govindjee, (1989) Proc. Natl. Acad. Sci. USA 86, 524–528.
[43] Schelvis, J.P.M., Van Noort, D.I., Aartsma, T.J. and Van Gorkom, H.J. (1994) Biochim. Biophys. Acta. 1184, 242–250.
[44] Wiederrecht, G.P., Seibert, M., Govindjee and Wasielewski, M.R. (1994) Proc. Natl. Acad. Sci. USA 91, 8999–9003.
[45] Renger, G., Eckert, H.-J. and Völker, M. (1989) Photosynth. Res. 22, 247–256.