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ORIGINAL PAPER

The effect of hydration on protein flexibility in photosystem II of green plants studied by quasielastic neutron scattering

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Abstract The effect of hydration on protein dynamics in photosystem II (PS II) membrane fragments from spinach has been investigated by using the method of quasielastic neutron scattering (QENS) at room temperature. The QENS data obtained indicate that the protein dynamics is strongly dependent on the extent of hydration. In particular, the hydration-induced activation of localized diffusive protein motions and Q_A^- reoxidation by Q_B in PS II appear to be correlated in their onset at a hydration value of about 45% relative humidity (r.h.). These findings underline the crucial functional relevance of localized diffusive protein motions on the picosecond-timescale for the reactions of light-induced photosynthetic water splitting under formation of plastoquinol and molecular oxygen in PS II of green plants.

Keywords Protein dynamics · Photosystem II · Electron transfer · Pigment–protein complex · Photosynthesis

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Introduction

The key steps of photosynthetic water splitting take place in a multimeric protein complex referred to as photosystem II (PS II), which is embedded into the thylakoid membrane and functions as a light-driven water–plastoquinone oxidoreductase (Ke 2001). Electronic excitation of a special chlorophyll a complex (P680) in PS II leads to formation of the ion radical pair $P680^{\bullet+}$ $Pheo^{\bullet-}$. This step is followed by the stabilization of the light-induced primary charge separation via electron transfer from $Pheo^{\bullet-}$ to a permanently and non-covalently bound plastoquinone 9 (PQ-9) molecule Q_A that acts as a one-electron redox group under physiological conditions (Renger and Holzwarth 2005 and references therein). The "stabilized" radical pair $P680^{\bullet+}$ $Q_A^{\bullet-}$ is formed with a time constant of about 300 ps (Bernarding et al. 1994) and provides the driving force for plastoquinol (PQH_2) formation and oxidative water splitting. A second PQ-9 molecule transiently bound in a special pocket, the Q_B binding site, becomes reduced at the acceptor side of PS II via two sequential one-electron steps with $Q_A^{\bullet-}$ acting as a reductant. This process is coupled with proton uptake reactions leading to the formation of plastoquinol and subsequent replacement of PQH_2 by another PQ9 molecule (for a review, see Petrouleas and Crofts 2005). On the PS II donor side, $P680^{\bullet+}$ is the oxidant for water splitting into molecular oxygen and four protons that takes place at a manganese containing water oxidizing complex (WOC) through a four step reaction sequence referred to as Kok-cycle (Kok et al. 1970). A heterodimeric protein matrix consisting of polypeptides D1 and D2 binds all cofactors participating in these reaction sequences. Their spatial arrangement was unraveled by recent X-ray crystallographic studies at resolutions of 3.5 Å (Ferreira et al. 2004) and 3.0 Å (Loll et al. 2005).

Remarkably different temperature dependences are observed for individual electron transfer steps in the PS II reaction center. The light-induced $P680^{\bullet+}$ $Q_A^{\bullet-}$ formation remains active at very low temperatures (Hughes et al. 2006), while the subsequent reactions of plastoquinol formation and oxidative water splitting are inhibited below characteristic threshold temperatures. On the acceptor side, the reoxidation of $Q_A^{\bullet-}$ by Q_B becomes completely blocked below 200 K (Joliot and Joliot 1973; Renger et al. 1993; Garbers et al. 1998). Surprisingly, the second electron transfer step from $Q_A^{\bullet-}$ to $Q_B^{\bullet-}$ exhibits a markedly different temperature dependence as found in experiments using samples frozen after preillumination with one laser flash at room temperature (Reifahrt and Renger 1998). In addition to thermal "freezing", the extent of $Q_A^{\bullet-}$ reoxidation was also shown to be strongly dependent on the level of sample hydration (Kaminskaya et al. 2003). Analogously, the reactions on the donor side are also blocked by lowering the temperature (for a review, see Renger 2001) or the hydration level (Noguchi and Sugiura 2002) depending on the redox state of the WOC.

Protein flexibility has been intensively studied using quasielastic neutron scattering (QENS) taking advantage of the large incoherent scattering cross-section of the proton and of their almost homogeneous distribution in protein molecules (Smith 1991; Fitter et al. 1999; Gabel et al. 2002; Kneller 2005). The molecular motions accessible by QENS typically encompass internal stochastic structural fluctuations such as reorientations of methyl groups, protons in hydrogen bonds or other small polypeptide side chains (Fitter et al. 1996). A number of QENS studies have revealed that proteins undergo a drastic change in their flexibility with the onset of localized diffusive protein motions on the picosecond timescale at temperatures of about 230 K in aqueous solution, which is referred to as the "dynamical transition" (Doster et al. 1989). Similar effects have also been observed for the purple membrane of *Halobacterium salinarum* (Ferrand et al. 1993; Fitter et al. 1999), lipid-bilayers as well as lipid–protein systems (Natali et al. 2004). In addition, both transition temperature and extent of the induced protein flexibility vary with external factors like hydration (Ferrand et al. 1993; Lehnert et al. 1998; Fitter 1999), lipid/protein ratio of the membrane (Fitter et al. 1998), or solvent viscosity (Cordone et al. 1999; Paciaroni et al. 2003; Marconi et al. 2005).

Protein dynamics in PS II membrane fragments from spinach has recently been investigated by QENS in the temperature range between 5 and 300 K (Pieper et al. 2007). The protein dynamics of samples hydrated at 90% r.h. was found to be dominated by harmonic vibrational motions below 120 K, while the onset of diffusive protein motions with characteristic relaxation times in the picosecond range takes place at two distinct transition temperatures of 120 and 240 K, respectively. The hydration-independent transition at 120 K is usually identified with the onset of methyl group rotations (Curtis et al. 2004; Roh et al. 2005). The "dynamical transition" in hydrated PSII membrane fragments is observed at 240 K and was shown to be strictly correlated with the temperature-dependent increase of the electron transport efficiency from $Q_A^{\bullet-}$ to Q_B (Pieper et al. 2007). In marked contrast, the "dynamical transition" was strongly suppressed in vacuum-dried PS II membrane fragments indicating that—in agreement with studies on other systems (see above)—the protein flexibility exhibits a remarkable hydration dependence. The latter phenomenon may thus be responsible for the blockage of $Q_A^{\bullet-}$ reoxidation by Q_B upon dehydration of PS II membrane fragments below a threshold value of about 45% relative humidity (r.h.) (Kaminskaya et al. 2003). So far, however, a detailed investigation of the hydration-dependence of protein dynamics in PS II membrane fragments by QENS is lacking.

To address this point, QENS spectra of PS II membrane fragments were measured at room temperature as a function of hydration level. The QENS data are compared to results gathered from optical spectroscopy revealing correlations between protein flexibility and electron transport in PS II. The results of the present study provide further support for a crucial role of the hydration level in sustaining protein flexibility that is indispensable for the functional competence of PS II.

Materials and methods

Sample preparation

PS II membrane fragments were isolated from spinach (*Spinacea oleracea*) following the procedure described by Berthold et al. (1981) with modifications according to Völker et al. (1985). All samples were washed 3× in a buffer solution containing D_2O, 50 mM MES (pD 6.5), 0.4 M sucrose, 15 mM NaCl, and 10 mM $CaCl_2$. Finally, the sample material was equilibrated using D_2O vapors of different r.h. values. The water content of the sample set hydrated at 90% r.h. was ~45% of its dry mass. One sample set was vacuum-dried for comparison. Buffer samples were prepared under identical conditions for separate QENS measurements. The buffer spectra were subtracted from the QENS spectra as described before (Pieper et al. 2007).

Measurements of flash-induced oxygen evolution (Christen et al. 1999) revealed that D_2O-hydrated PS II membrane fragments remain functionally competent. Laser flash induced absorption changes at 830 nm (Christen and Renger 1999) and fluorescence quantum yield measurements (Renger et al. 1995) prove that both, the reduction of $P680^{\bullet+}$

by Y_Z and the reoxidation of $Q_A^{\bullet -}$ by Q_B, remain fully active upon D_2O-hydration.

QENS measurements

QENS experiments were carried out using the time-of-flight spectrometer NEAT (Lechner et al. 1996) at BENSC (Hahn-Meitner Institute, Berlin, Germany). The measurements were performed with an incident neutron wavelength of 5.1 Å (~3.2 meV) corresponding to an elastic Q range of 0.3–2.3 $Å^{-1}$. The elastic energy resolution determined by vanadium standard runs was $\Delta E = 0.093$ meV corresponding to an observation time window from ~0.2 to ~20 ps. The data were corrected for empty cell contribution, detector efficiency, and sample-geometry dependent attenuation, normalized to the integrated vanadium intensity, and converted to energy transfer scale using the program package FITMO-4 (Rufflè 2000). The sample transmission was typically 96% so that multiple scattering effects should be negligible in the QENS data. For each separate measurement, the sample cell was placed carefully at the same position relative to the incident neutron beam in order to avoid normalization errors in buffer subtraction. Before subtraction, the buffer spectra were normalized by weight to determine their contribution to the total sample scattering.

Theoretical background

In an incoherent QENS experiment, the measured quantity is the double-differential cross section (Lechner and Riekel 1983; Bee 1988), which describes the number of neutrons scattered into a solid angle element $\delta\Omega$ and an energy transfer element $\delta\omega$

$$\frac{\delta^2\sigma}{\delta\Omega\delta\omega} = \frac{|\mathbf{k_1}|}{|\mathbf{k_0}|}\left[b_{\mathrm{inc}}^2 S_{\mathrm{inc}}(\mathbf{Q},\omega)\right], \tag{1}$$

where $\mathbf{k_0}$ and $\mathbf{k_1}$ are the wave vectors of incident and scattered neutrons, respectively; b_{inc} is the incoherent scattering length; $S_{\mathrm{inc}}(\mathbf{Q},\omega)$ is the incoherent scattering function with $\mathbf{Q}$ being the momentum transfer defined by $\mathbf{Q} = \mathbf{k_1}–\mathbf{k_0}$ and $\hbar\omega$ reflects the energy transfer. The function $S_{\mathrm{inc}}(\mathbf{Q},\omega)$ is related to the Van Hove self-correlation function $G_S(\mathbf{r},t)$ (see e.g. Lechner and Riekel (1983) and Bee (1988))

$$S_{\mathrm{inc}}(\mathbf{Q},\omega) = \frac{1}{2\pi}\int_{-\infty}^{\infty} \mathrm{e}^{-i\omega t}\int_{-\infty}^{\infty} \mathrm{e}^{i\mathbf{Qr}} G(\mathbf{r},t)\mathrm{d}\mathbf{r}\mathrm{d}t, \tag{2}$$

where, in the classical approximation, $G_S(\mathbf{r},t)$ is the average time-dependent probability density distribution of the hydrogen atoms in the studied sample. In practice, $S_{\mathrm{inc}}(\mathbf{Q},\omega)$ needs to be replaced by an experimental scattering function $S_{\mathrm{exp}}(\mathbf{Q},\omega)$ with

$$S_{\mathrm{exp}}(\mathbf{Q},\omega) = F_{\mathrm{N}} \exp\left(-\frac{\hbar\omega}{2kT}\right) R(\mathbf{Q},\omega) \otimes S_{\mathrm{theo}}(\mathbf{Q},\omega), \tag{3}$$

which is composed of a normalization factor F_N, the detailed balance factor $\exp\left[-\frac{\hbar\omega}{2kT}\right]$, and the convolution of an experimentally obtained resolution function $R(\mathbf{Q},\omega)$ with a theoretical model function $S_{\mathrm{theo}}(\mathbf{Q},\omega)$ describing the dynamics of the sample system:

$$S_{\mathrm{theo}}(\mathbf{Q},\omega) = \mathrm{e}^{-\langle u^2\rangle Q^2} \left\{A_0(\mathbf{Q})\delta(\omega) + \sum_n A_n(\mathbf{Q}) L_n(H_n,\omega) + S_{\mathrm{in}}(\mathbf{Q},\omega)\right\}. \tag{4}$$

In this phenomenological description, the scattered intensity consists of three contributions, a $\delta(\omega)$-shaped elastic component, a sum of quasielastic Lorentzian-shaped components $L_n(H_n,\omega)$ with half-width at half maximum (HWHM) H_n, and an inelastic part $S_{\mathrm{in}}(\mathbf{Q},\omega)$ describing low-frequency, moderately damped vibrational motions. The fractional intensities of the elastic and quasielastic contributions are given by the elastic and quasielastic incoherent structure factors (EISF and QISF), $A_0(Q)$ and $A_n(Q)$, respectively. The term $\mathrm{e}^{-\langle u^2\rangle Q^2}$ is the Debye–Waller factor characterized by the "global" vibrational mean square displacement $\langle u^2\rangle$.

The Q-dependence of the EISF $A_0(Q)$ has been fitted using the model of an isotropic rotation on the surface of a sphere according to Sears (1967)

$$A_0(Q) = \frac{\sin^2(QR)}{(QR)^2}, \tag{5}$$

where R is the radius of the sphere and Q is the momentum transfer.

Results and discussion

QENS spectra of PS II membrane fragments were measured for several relative hydration (r.h.) values at room temperature. The r.h.-values employed span the whole range between the two limiting cases of vacuum-dry and hydrated (90% r.h.) samples studied before in temperature-dependent QENS experiments (Pieper et al. 2007). Typical hydration-dependent QENS spectra are shown in Fig. 1 for three representative r.h.-values. The QENS spectrum for PS II membrane fragments hydrated at 44% r.h. appears to be dominated by elastic scattering. For hydration values of 66 and 90% r.h., however, the quasielastic contribution apparently increases with increasing r.h.-value, concomitant with a decrease in elastic scattering. A more detailed

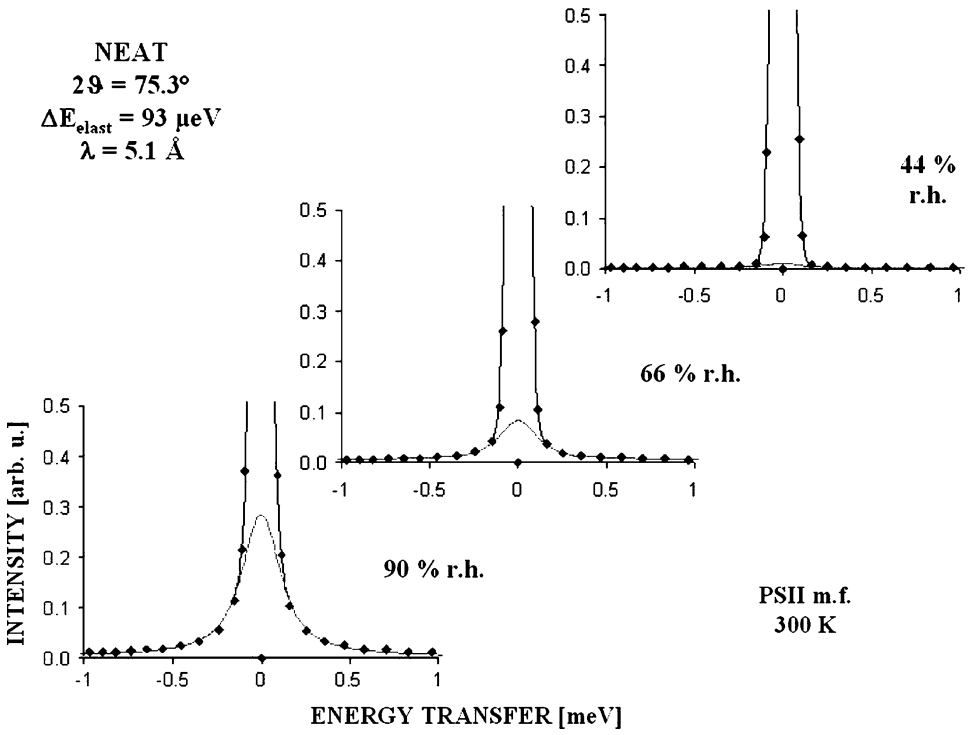

Fig. 1 QENS spectra (*diamonds*) of PS II membrane fragments hydrated in D_2O vapours of 44, 66, and 90% r.h., respectively, obtained with an incident neutron wavelength of 5.1 Å and an elastic resolution of 0.093 meV at a scattering angle of 75.3° and at a temperature of 300 K. *Black full lines* represent phenomenologic fits with one Lorentzian component (*grey full lines*); see text for details

analysis of the QENS spectra of PS II membrane fragments is achieved by employing a phenomenological model according to Eq. 4. Satisfactory fits require a model scattering function comprising a single quasielastic Lorentzian component and a flat background. The Lorentzian width (HWHM) of 0.1135 meV was found to be independent of the momentum transfer Q. It is important to note that a Q-independent linewidth is the signature of localized diffusive proton motions in the protein–lipid system under study.

The width (HWHM) of 0.1135 meV is the same as that of the "slow" protein relaxation (5.8 ps) reported before from fits of temperature-dependent QENS spectra of PS II membrane fragments (Pieper et al. 2007). In this regard it has to be kept in mind that a broad and widely continuous distribution of linewidths (and corresponding relaxation times) would be expected in a complex protein–lipid system (Fitter et al. 1999; Kneller 2005; Doster and Settles 2005) so that the correlation time of 5.8 ps has to be understood as a mean value of the former distribution. Since the present study is restricted to a single energy resolution and, thus, to a limited observation time window from $\sim$0.2 to $\sim$20 ps, the Lorentzian width (HWHM) of 0.1135 meV (5.8 ps) was kept constant in the following analysis, while only the amplitude factor EISF was treated as a free parameter (see Eq. 4).

The elastic intensities (EISF) approximated by the phenomenological fits described above are shown in Fig. 2 as a function of momentum transfer Q. Generally, the decrease of the EISF with increasing momentum transfer Q becomes more pronounced with increasing hydration level. This finding clearly proves that the membrane flexibility in PS II membrane fragments is hydration-dependent and that the average amplitude of motion increases with increasing hydration level. In order to describe the effect of hydration water on membrane flexibility in a quantitative way by a single variable parameter, the Q-dependence of the EISF was fitted with the well-known model function for a rotation on a sphere of radius R at each hydration level according to Eq. 5. The corresponding radii of rotation R are shown in Fig. 3 as a function of hydration (r.h.) level.

The hydration dependence of the radius of rotation R of PS II membrane fragments at room temperature exhibits mainly two different ranges: (a) at r.h.-levels below 45%, R is relatively small corresponding to only some residual membrane flexibility and (b) above 45% r.h., the R-values exhibit a strong increase with increasing hydration level. This indicates the onset of localized diffusive protein motions at a hydration level of about 45% r.h. An analogous hydration dependence of the protein mobility has been observed before for the less complex purple membrane of *H. salinarum*, revealing that the interaction with hydration water has a plasticising effect on the protein flexibility (Fitter et al. 1999). This similarity suggests that a first layer of hydration water molecules is completed only above 45% r.h. in the case of PS II membrane fragments, while the same effect occurs at about 86% r.h. in the case of the purple membrane (Fitter et al. 1999). The observation of very different r.h.-levels for the onset of protein flexibility in PS II membrane fragments and purple

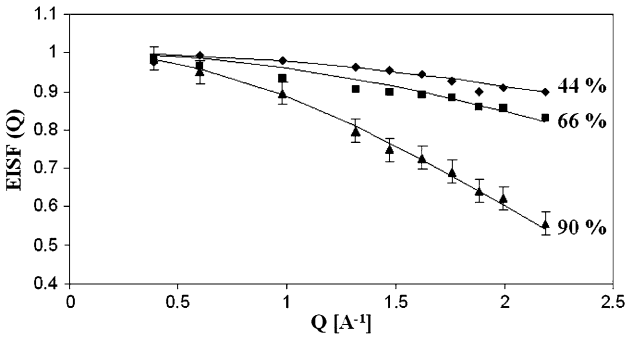

Fig. 2 EISF obtained from phenomenologic fits of QENS spectra of PS II membrane fragments hydrated in D_2O vapours of 44 (*diamonds*), 66 (*squares*), and 90% r.h. (*triangles*), respectively, as a function of momentum transfer *Q*. The *black full lines* are fits using the model function for an isotropic rotation on the surface of a sphere (see text). Typical *error bars* are shown for the *lower* data set

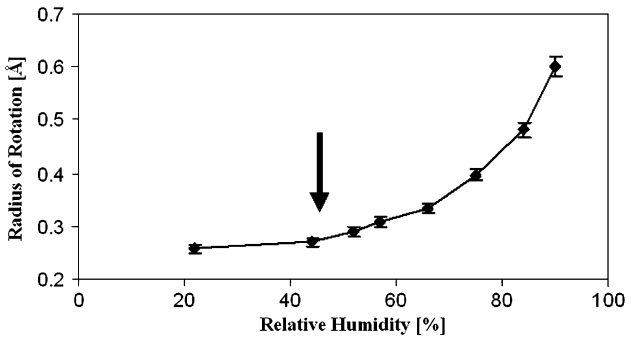

Fig. 3 Hydration dependence of the radius of rotation obtained from the fits shown in Fig. 2 (*diamonds*). The onset of Q_A^- reoxidation by Q_B according to Kaminskaya et al. (2003) is indicated by an *arrow*. *Error bars* indicate the uncertainty of *R*

membrane, respectively, implies that both systems are well adapted to the different environmental conditions encountered by green plants and halobacteria, respectively.

Furthermore, the observed phenomenon is in agreement with our previous finding of a hydration-dependent "dynamical transition" in PS II membrane fragments (Pieper et al. 2007). Here, the dynamical transition at 240 K was shown to be absent in dry PS II membrane fragments as reflected by both the temperature dependence of the total mean square displacement and the significantly smaller quasielastic contributions to the QENS spectra of PS II membrane fragments at room temperature. The latter finding is the most striking difference observed between dry and hydrated samples in the former study. Thus, dehydration of PS II membrane fragments leads to a considerable "freezing" of protein flexibility, that is, dehydration has an analogous effect on protein dynamics as temperature decreases.

In addition to the presence of water, the buffer solution of the PS II membrane fragments used in this study contained sucrose as a cryoprotectant (see "Materials and methods" section). As pointed out in the "Introduction" section, it is generally known that solvents of different viscosity affect both transition temperature and the extent of protein flexibility above the dynamical transition. This effect was investigated in detail, for example, for lysozyme (Marconi et al. 2005). In the present study, however, all experiments were performed at a constant sucrose concentration, so that the temperature and hydration-dependent effects observed here are virtually free from contributions due to a variation of the sucrose concentration. On the other hand, the sucrose concentration has to be taken into account when the results presented here are compared to those of other studies. Therefore, it is important to note that the sucrose concentration of 0.4 M is identical to that used by Kaminskaya et al. (2003) in investigations of the hydration-dependence of $Q_A^{\bullet-}$ reoxidation by Q_B in PS II membrane fragments.

In summary, protein dynamics of PS II membrane fragments exhibits a dependence on hydration, which is quite typical for protein systems in general. However, it is remarkable that the activation of protein dynamics occurs at the same r.h.-value as the hydration-dependent onset of $Q_A^{\bullet-}$ reoxidation by Q_B (Kaminskaya et al. 2003). This similarity suggests that protein flexibility plays an important role for the electron transport from $Q_A^{\bullet-}$ to Q_B in PS II. Analogously, the thermally activated onset of protein mobility at 240 K was shown to be correlated with the temperature-dependence of $Q_A^{\bullet-}$ reoxidation by Q_B (Pieper et al. 2007).

There are several lines of evidence for a similar correlation between protein flexibility and the efficiency of electron transport from $Q_A^{\bullet-}$ to Q_B in the bacterial reaction center (Parak et al. 1980), whose structure appears to be highly conserved in PS II (Ferreira et al. 2004; Loll et al. 2005). Based on X-ray crystallography, a drastic reorientation of the quinone headgroup of Q_B with a shift by about 5 Å and a rotation of 180° was observed upon the light-induced formation of the semiquinone state $Q_B^{\bullet-}$ in isolated reaction centers of *Rhodobacter sphaeroides* (Stowell et al. 1997). However, the idea of this structural change to be the origin of a conformational gating mechanism of $Q_A^{\bullet-}$ reoxidation by Q_B has been questioned (Xu et al. 2002) and is not supported by recent FTIR measurements (Remy and Gerwert 2003; Breton 2004) as well as a time-resolved X-ray crystallography study (Baxter et al. 2004). It seems more likely that the triggering conformational changes required for Q_B reduction by $Q_A^{\bullet-}$ comprise the rearrangement of hydrogen bonds, most prominently the reorientation of the Ser223 of the L-subunit (Mulkidjanian et al. 2005; Lancaster 2007). Based on the similarity of the acceptor side reactions in anoxygenic purple bacteria and in PS II, it seems most likely that an analogous conformational triggering of $Q_A^{\bullet-}$ reoxidation by Q_B exists in both

systems (for a review, see Renger 2007). The assumption of a conformational triggering in PS II is also in line with the finding that $Q_A^{\bullet-}$ reoxidation can still occur at much lower temperatures than 240 K once the semiquinone state $Q_B^{\bullet-}$ is populated by preillumination before freezing (Reifahrt and Renger 1998). With respect to the PS II reaction pattern, it is important to note that the functional role of protein flexibility is not restricted to acceptor side reactions, but also observed for distinct redox steps of the oxidative water splitting under release of O_2 in the WOC (for a review, see Renger 2001). This conclusion is also supported by the freezing temperatures reported for the $S_2 \rightarrow S_3$ and $S_3 \rightarrow\rightarrow S_0 + O_2$ reactions (Koike and Inoue 1987; Styring and Rutherford 1988) which resemble that of the $Q_A^{\bullet-}$ reoxidation by Q_B (Pieper et al. 2007). Likewise, these redox transitions in the WOC are also inhibited upon dehydration of PS II core complexes from the thermophilic cyanobacterium *T. elongatus* (Noguchi and Sugiura 2002). In conclusion, these findings underline that protein dynamics on the picosecond timescale plays a key role for the reactions of oxidative water splitting and plastoquinol formation that are energetically driven by the radical ion pair $P680^{\bullet+}$ $Q_A^{\bullet-}$ generated via light-induced charge separation in PS II.

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