

Modification of the pheophytin midpoint potential in photosystem II: Modulation of the quantum yield of charge separation and of charge recombination pathways

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Received 18th May 2004, Accepted 3rd August 2004

First published as an Advance Article on the web 18th August 2004

We investigated the dependence of both the quantum yield of charge separation and pathways of charge recombination on the free energy level of the radical pair state P^+Ph^- in photosystem II. This was done by comparing the basal (F_0) fluorescence yield and the recombination rate of the $S_2Q_A^-$ state in various strains of *Chlamydomonas reinhardtii* in which the strength of a H-bond to the pheophytin bound to the D1 subunit has been modified by site-directed mutagenesis. In agreement with previous results with homologous mutants in the cyanobacterium *Synechocystis*, the quantum yield decreased and the recombination of $S_2Q_A^-$ was slowed down when the energy level of the radical pair was increased. The decreased quantum yield is analyzed in terms of a modified equilibrium between exciton and radical pair. The effects on the recombination rate confirm that in the wild type the process involves the P^+Ph^- state. Analysis of these results shows that, as in the case of *Synechocystis*, the energy level of the P^+Ph^- is less negative than currently thought by about 250 meV. An important consequence is a similar upward revision of the potential of the P^+/P couple.

Introduction

Photosynthesis is driven by light-induced charge separation processes, which ultimately result in ATP synthesis and production of reducing power. In photosystem II, the stabilization of the charge separated state is associated with the following sequence of electron transfer reactions: the primary radical pair state, P^+Ph^- ,[†] is formed in tens of ps (see ref. 1 for a review), then the reduced pheophytin is quickly oxidized by the primary quinone Q_A (half time of about 300 ps²). Subsequently, P^+ is reduced by a tyrosine (Y_Z) in some tens of ns and Y_Z^{ox} is in turn reduced by an electron extracted from the Mn cluster of the water splitting enzyme, yielding the $S_2Y_ZP_{680}PhQ_A^-$ state (noted $S_2Q_A^-$, in the following) (see ref. 3 for a review). Charge recombination by direct electron tunneling may occur at the level of the P^+Ph^- or $P^+Q_A^-$ states. In the case of the *Rhodospira rubra* bacterial reaction center, direct electron tunneling between Q_A^- and P^+ is largely predominant⁴ whereas in *Rhodospseudomonas viridis* charge recombination occurs essentially via an "indirect pathway" involving the thermally activated repopulation of the P^+Ph^- state.⁵ The

relative weight of these two pathways can be modified by modulating the energy levels of either the P^+Ph^- or $P^+Q_A^-$ states. This is expected to affect the rate of the indirect pathway, but it should have little consequence on the rate of the direct one, which is essentially activationless. We have recently followed this approach and studied various *Synechocystis* sp. PCC 6803 strains in which the redox potential of the pheophytin, $E_m(Ph)$, had been changed by site directed-mutation of a residue involved in a H-bond with the 9-keto group of the chlorine ring.⁶ The recombination rate of $S_2Q_A^-$ was markedly modified in the mutants, evidencing the predominance of the indirect pathway in the WT,⁶ in agreement with the previous analysis of van Gorkom.⁷ Moreover, we could, based on the current knowledge of rates of the various electron transfer reactions involved in the overall charge recombination process, redraw the energetic picture in photosystem II and especially reevaluate the midpoint potential of the P^+/P couple. This is of particular importance with regard to the function of photosystem II, for it determines the available driving force for water oxidation.⁶ A possible weakness in this analysis was the compilation of literature data that were obtained with photosystem II either from higher plants or cyanobacteria. It was thus of interest to extend such studies to an eucaryotic organism: *Chlamydomonas reinhardtii*.

Several spectroscopic studies suggested that the pheophytin bound by the D1 subunit of photosystem II is involved in a hydrogen bond with a neighboring residue^{8,9} at position 130 of the D1 subunit. It is a glutamic acid in *Chlamydomonas reinhardtii* as well as in higher plants but a glutamine in cyanobacteria. According to the recent crystallographic

[†] Abbreviations: D1, the D1 polypeptide of the PSII reaction center; DCMU: diuron, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; HEPES: N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); P: the primary donor (P-680) of photosystem II; Ph: pheophytin, primary electron acceptor in photosystem II; PSII: photosystem II; Q_A : primary quinone acceptor in photosystem II; Y_Z : D1-Tyr161, the redox active tyrosine of PSII on the D1 polypeptide, E_m the midpoint potential; LHC, the light harvesting complex; RC the reaction center; RP, the radical pair state, WT the wild type strain; ΔG and ΔH the free energy and enthalpy changes.

structure of *Thermosynechococcus elongatus* photosystem II, the distance between the oxygen atom of this keto group and the nitrogen of D₁Gln130 is 3.2 Å, consistent with a weak H-bond.¹⁰ High field EPR studies of the pheophytin anion radical in WT *Chlamydomonas reinhardtii* and in the D₁Glu130Gln or D₁Glu130Leu mutants showed a shift of the g_x value, consistent with a weakening of the hydrogen bond in the D₁Glu130Gln mutant compared to the WT, and a loss of this bond in the D₁Glu130Leu mutant.¹¹ A weakening or loss of the hydrogen bond to the pheophytin is expected to destabilize Ph⁻ and thus make its redox potential more negative. This was shown in *Synechocystis* by measurements of the equilibrium constant between the excited singlet state P* and the P⁺Ph⁻ radical pair state and of the quantum yield for nanosecond stabilization of the radical pair.^{12,13} It was estimated that mutating Gln for Glu shifts the pheophytin potential by +33 mV and mutating Gln for Leu shifts it by -56 mV. In *Chlamydomonas reinhardtii*, the mutations of the D₁130 residue have been characterized with regard to the strength of the H-bond to the pheophytin, but their functional consequences have not yet been analyzed. In this paper we show that the mutation-induced changes in redox potential of the pheophytin strongly affect the lifetime of the S₂Q_A⁻ state, consistent with previous findings in *Synechocystis*. Lowering the midpoint potential of Ph also affects the quantum yield for charge separation, it is decreased in the D₁Glu130Leu mutant when compared to the WT. Consequently, the revision of the energetic picture that we derived previously also applies to higher plants photosystem II.

Material and methods

A. Biological material

Three *Chlamydomonas reinhardtii* strains were studied: a wild type one, and two site-directed mutants of the *psbA* gene which codes for the D1 subunit of photosystem II: D₁Glu130Leu and D₁Glu130Gln (see ref. 11 for a detailed description of the mutagenesis). The algae were grown under light in TAP medium. Unless stated otherwise, the cells were treated with *p*-benzoquinone, in order to inhibit the cell metabolism that maintains in the dark the partial reduction of the plastoquinone pool and a permanent transmembrane electrochemical potential.¹⁴ Benzoquinone (300 μM) was added to the cells in their culture medium. They were then immediately harvested by centrifugation at 4000 g for 5 minutes, washed once and finally resuspended in Hepes (20 mM) at pH 7.2 with Ficoll (30% w/w), to increase the viscosity of the medium and to slow down sedimentation of the cells during the experiment. The electron transfer between Q_A and Q_B was inhibited either by DCMU (10 μM) or bromoxynil (10 μM), a kind gift of A.W. Rutherford.

B. Measurement of the S₂Q_A⁻ decay

The S₂Q_A⁻ decay was followed by monitoring the time course of the flash-induced fluorescence yield changes with a Joliot-type spectrophotometer.¹⁵ The actinic flash is provided by a xenon lamp with a broad band blue glass filter (BG39 Schott). The fluorescence emission is sampled by weak monochromatic xenon flashes (450 nm). A combination of red filters (2 Wratten 2192 + RG 665) is used to cut off both the actinic and detecting flashes. Experiments at variable temperature were done in a similar way, using the setup described in ref. 16.

It has been known for long that, due to antenna connectivity, the photochemical efficiency of open reaction centers increases along with the closing of neighboring centers. The exciton visiting a closed center has a significant probability to continue its random walk and to eventually hit an open trap.¹⁷⁻¹⁹ A consequence is that the fluorescence yield F depends on the fraction of closed centers c in a hyperbolic, rather than linear

way, according to:

$$F(c) = \frac{c}{1 + J - Jc} \quad (1)$$

The parameter J expresses the antenna connectivity ($J + 1$ is the average number of photosynthetic units visited by an exciton when the reaction centers are closed). The slope dF/dc is thus quite significantly (typically 10-fold) increased from the low to high c range; a noteworthy consequence is that the fluorescence decay $F(t)$ during the recombination process following a saturating flash is distorted and appears several-fold faster than the true $c(t)$ kinetics. This effect can be corrected from a calibration of the $F(c)$ relationship, where c may be estimated from the integration of the fluorescence induction curve (see refs. 19 and 20) or from absorption changes monitoring the amount of Q_A⁻.²¹ We present here an alternative and simpler method that allows one to obtain directly the $c(t)$ kinetics from fluorescence. Fig. 1A shows the theoretical relationship of eqn. (1), where a moderate value of 2 was taken for the connection parameter J . Panel B shows how the $c(t)$ decay, assumed for simplicity to be mono-exponential (curve 1), appears when monitored as $F(t)$ (curve 2): the apparent half-time is more than halved and the time course

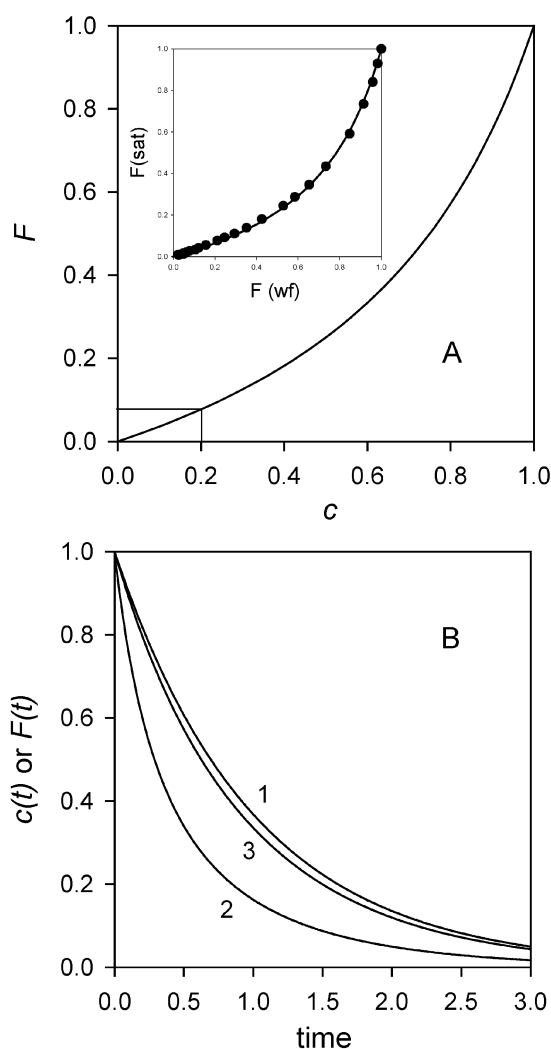


Fig. 1 A, a theoretical plot of the relative variable fluorescence yield as a function of the fraction of closed centers, using eqn. (1), with $J = 2$. B, curve 1 shows the $c(t)$ decay, assumed to be mono-exponential. Curve 2 is the corresponding $F_{sat}(t)$ kinetics induced by a saturating flash, obtained from the $F(c)$ relationship. Curve 3 is the normalized $F_{wf}(t)$ kinetics induced by a weak flash assumed to hit one fifth of the centers (bottom left rectangle in A). The inset in A is a plot of experimental data (benzoquinone-treated WT cells, 20 °C) for $F_{sat}(t)$ vs. $F_{wf}(t)$ with time as a parameter; the line is a fit, with $J = 2.6$.

Table 1 Compilation of the results obtained for the WT and D₁Glu130Leu strains^a

	$t_{1/2}$ of S ₂ Q _A ⁻ charge recombination at 20 °C			Fluorescence parameters					
	DCMU	BQ + DCMU	BQ + Bx	F_0	F_{\max}	ΔF	$\Delta\Delta H_{\text{PhQ}}$	Φ	K_{cs}
WT	0.7 s	2.4 s	0.8 s	1 ^b	3.3	0.041	—	0.85–0.9 ^c	5.3–6.5
D ₁ Glu130Leu	20.5 s	41 s	17 s	2.1	3.6	0.072	+70 meV	0.63–0.75	0.34–0.42

^a The decay of the fluorescence yield was measured with the two strains in the presence of DCMU (10 μM) omitting the benzoquinone treatment (DCMU), or after benzoquinone treatment in the presence of DCMU or bromoxynil (10 μM) (BQ + DCMU and BQ + Bx, respectively). F_0 and F_{\max} are the basal and maximum fluorescence yield, ΔF is the relative fluorescence change induced by a subsaturating flash. Φ is the quantum yield of charge separation, $\Delta\Delta H_{\text{PhQ}}$ the change induced by the mutation in the enthalpy difference between the P⁺Ph⁻ and P⁺Q_A⁻ states and K_{cs} the equilibrium constant for charge separation. ^b For the WT the F_0 level was 1. ^c Assumed range of values for the WT.

appears multiphasic (it is well-fitted as a sum of two exponentials). Let us consider now the fluorescence kinetics following a weak subsaturating flash that hits, for instance, one fifth of the centers. The region of the $F(c)$ curve concerned is the rectangle (bottom left) in Fig. 1A. The relative fluorescence amplitude is 0.077. Over this small section of the hyperbola, the curvature is much less pronounced, so that the normalized plot of $F_{\text{wf}}(t)$ (wf for weak flash), curve 3, is only slightly distorted and can be taken as an acceptable approximation of the true $c(t)$ decay. In practice we generally used a weak flash such that the relative fluorescence amplitude was about 0.045. The inset in panel A shows a plot of experimental results for the normalized variable fluorescence induced by a saturating flash (F_{sat}) vs. F_{wf} , with time as a parameter. Within a good approximation, this is equivalent to the $F(c)$ function; the line is a fit using eqn. (1), with $J = 2.6$.

Results

A. Recombination rates in the WT and mutant strains

From the finding that substituting the D₁Glu130 residue for Gln or Leu results in a weakening or loss, respectively, of the H-bond to the pheophytin,¹¹ one expects $E_{\text{m}}(\text{Ph})$ to be more negative in the D₁Glu130Gln mutant than in the WT, and still more negative in the D₁Glu130Leu. This change in redox potential should increase the free energy gap between the S₂Q_A⁻ and S₂Ph⁻ states and should slow down the indirect recombination process going through P⁺Ph⁻. It is thus expected to induce an increase of the S₂Q_A⁻ lifetime inasmuch as the indirect route has a significant relative weight. Fig. 2 shows the fluorescence decay after a weak actinic flash in the three strains. The lifetime of S₂Q_A⁻ increased as the strength of

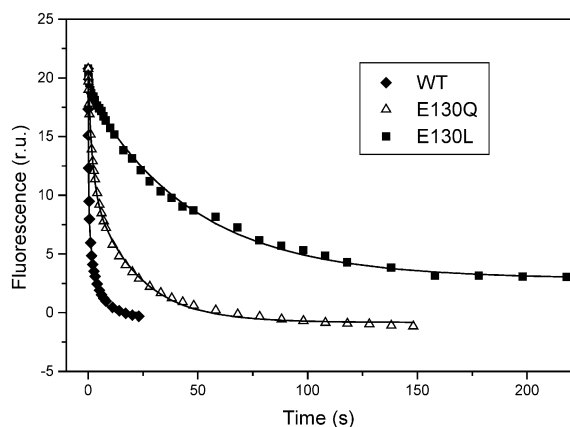


Fig. 2 Decay of the fluorescence yield after a weak actinic flash in the presence of DCMU in benzoquinone treated cells of *Chlamydomonas reinhardtii*, WT (diamonds), D₁Glu130Gln (open triangles) and D₁Glu130Leu (squares) at 20 °C. The lines are the best fit of the data with two exponentials.

H-bond to the pheophytin decreased, consistent with the data obtained with *Synechocystis*.⁶

B. Effect of bromoxynil and membrane potential

One may resort to other tools than site-directed mutagenesis to modulate the free energy gap between the S₂Ph⁻ and S₂Q_A⁻ states. As shown by Krieger *et al.*, the redox potential of Q_A may be decreased by the use of phenolic herbicides rather than urea herbicides.²² When using bromoxynil rather than DCMU the lifetime of S₂Q_A⁻ was decreased in both the WT and D₁Glu130Leu strains (the acceleration factor with respect to DCMU was 3 in the WT and 2.4 in the D₁Glu130Leu strain, see Table 1).

It has been known for some time that the lifetime of the S₂Q_A⁻ state is decreased in the presence of a transmembrane electrical potential.^{14,23} In the present framework, this effect is likely to reflect the sensitivity to a transmembrane electric field of the free energy change associated with electron transfer between P⁺Ph⁻ and P⁺Q_A⁻ which is known to be electrogenic. Although this is not a quantitative method, it is convenient to compare untreated (living) algae, where the metabolism maintains a significant membrane potential in the dark¹⁴ with benzoquinone treated cells where this potential is collapsed. We found faster kinetics in the untreated cells, both for the WT and mutants (Table 1). Therefore, both the effects of bromoxynil and of the membrane potential indicate that the indirect route is important in the three strains.

C. Temperature dependence of the charge recombination rate

In order to estimate the change in ΔH_{PhQ} resulting from the mutations, we studied the temperature dependence of the S₂Q_A⁻ recombination rate. As shown in Fig. 3, for the WT and D₁Glu130Gln strain, the temperature dependence could be well fitted by a linear Arrhenius function with $\Delta H = 625$ meV and 656 meV, respectively. For the D₁Glu130Leu mutant the dependence of the rate on $1/T$ was not linear and less steep than in the WT.

It is well known that the recombination of the S₂Q_A⁻ state does not follow a simple first order time course.²⁰ We found that the recombination kinetics are well fitted by a sum of two exponentials,[‡] for the WT as well as for the D₁Glu130Gln or D₁Glu130Leu mutants at room temperature. In the WT, the fast phase accounts typically for 35% of the amplitude, with $t_{1/2} \approx 1$ s and the slow phase is about five times slower. As discussed in more details elsewhere (Rappaport *et al.*, submitted), this feature is not due to a progressive stabilization of

[‡] This applies to the “true” kinetics induced by a weak flash, where the fluorescence kinetics reflect in a linear way the recombination process (see section Methods). This should not be confused with the fact that a mono-exponential process, when distorted by the hyperbolic dependence of eqn. (1), would appear as bi-exponential (a point made earlier about Fig. 1).

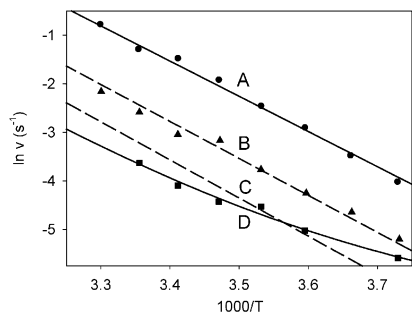


Fig. 3 Arrhenius plots for the recombination in the WT (circles), D1Glu130Gln (triangles) and D1Glu130Leu (squares). The rates v were estimated from the effective half-lives, as $v = \ln 2/t_{1/2}$. Curve A, best fit of the WT data with a single Arrhenius component, yielding $\Delta H_{\text{ind}} = 625$ meV and a pre-exponential factor $s_{\text{ind}} = 1.07 \cdot 10^{10} \text{ s}^{-1}$. Curves B and C (dashed) are best fits of the Gln et Leu mutants, for a single Arrhenius component, imposing the same pre-exponential factor as in A: this yields $\Delta H_{\text{ind}} = 656$ meV and 676 meV, respectively. Curve D is a simulation for mixed contributions of both the indirect and direct routes. The ΔH_{ind} was taken equal to 695 meV (the medium value of the range estimated in the text) and the other parameters were determined for a best fit of the data, yielding $\Delta H_{\text{dir}} = 200$ meV and $s_{\text{dir}} = 623 \text{ s}^{-1}$ (s_{ind} is kept fixed as previously). For these particular parameters v_{ind} accounts for about 66% of the rate at 293 K and 24% at 268 K; the temperature for which $v_{\text{dir}} = v_{\text{ind}}$ is 283 K.

the charge-separated state competing with recombination, but reflects a heterogeneity (on the recombination time scale) between two, or possibly more, conformations with different recombination rates. The biphasic character was present over the whole explored temperature range (-5 °C to 35 °C) for the WT. Unfortunately, the accuracy of the decomposition was not sufficient to obtain reliable temperature dependencies for the amplitudes and rates of the individual phases. Therefore we only showed in Fig. 3 the Arrhenius plot using the rate deduced from the overall half-life of the kinetics. In contrast with the WT, the biphasic character disappeared at low temperature (e.g. -5 °C) in the D₁Glu130Leu. As shown in Fig. 4, the kinetic heterogeneity was clearly present at room temperature but was lost at -5 °C where the fitting procedure converged towards a single exponential component.

D. Fluorescence levels in the WT and mutant strains

We determined the (basal) F_0 and (maximal) F_m fluorescence levels of the D₁Glu130Leu mutant with respect to the WT. This was done at equal transmittance (50%) of the algal samples at 450 nm, the wavelength of the measurement flashes. The data

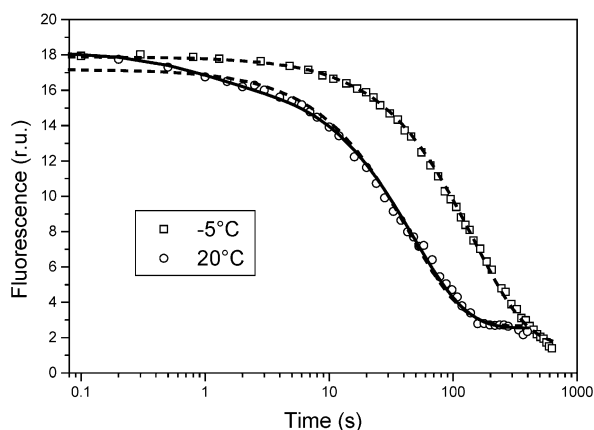


Fig. 4 Decay of the fluorescence yield after a weak actinic flash in the presence of DCMU in Benzoquinone-treated cells of the D₁Glu130Leu mutant at 20 °C (open circles) and -5 °C (squares) on a semi-logarithmic time scale. The lines are the best fits of the data with one (dashed line) or two exponential (solid line) plus an offset.

were carefully corrected for the offset due either to excitation light transmitted through the fluorescence filters or to fluorescence from the filters. The measured levels must, however, include a significant (but fixed) contribution of fluorescence emitted from PS I, that was estimated as about 25% of the apparent F_0 .¹⁹ Taking the F_0 (WT) as a unit, we obtained F_m (WT) = 3.3, F_0 (L) = 2.1 and F_m (L) = 3.6 (Table 1). The relative amplitude of the fluorescence change induced by a weak flash, ΔF_{wf} , was 4.1% (with respect to the maximal variable fluorescence) in the WT and 7.2% in the D₁Glu130-Leu strain (Table 1).

Discussion

A. Recombination pathways in the WT and mutant strains

In agreement with our previous findings, the present results show that the lifetime of the $S_2Q_A^-$ state depends on the free energy gap between the $S_2Q_A^-$ and S_2Ph^- states. This implies that, at least in the WT (i.e. in the strain with the fastest recombination rate), the indirect route for charge recombination is significantly faster than the direct one and thus predominant. Noting v_{ind} and v_{dir} the rates of the indirect and direct routes, the weight of each pathway is $v_{\text{ind}}/(v_{\text{ind}} + v_{\text{dir}})$ and $v_{\text{dir}}/(v_{\text{ind}} + v_{\text{dir}})$, respectively. If v_{ind} is decreased far below v_{dir} the recombination is dominated by the direct pathway. In this case the recombination rate is expected to be little dependent on the midpoint potential of Q_A and Ph (no differential effect of bromoxynil vs. DCMU is expected) and to show little stimulation by the membrane potential. In our previous work with *Synechocystis*, bromoxynil caused no significant acceleration of the recombination rate in the D₁Gln130Leu mutant,⁶ indicating that the slowing down of the indirect route was such that the direct route had become predominant. In the homologous strain of *Chlamydomonas*, bromoxynil still accelerates the recombination, suggesting that the indirect pathway remains important at room temperature. The acceleration caused by the membrane potential is another indication that such is the case.

B. Temperature dependence of the $S_2Q_A^-$ charge recombination

Whereas the effects discussed above implies that the indirect route is still important in the D1Glu130Leu mutant, the temperature dependence (Fig. 3) shows that the direct route also participates in the overall recombination process in this mutant. The activation energy of the direct route is the enthalpy difference between the S_2P and S_1P^+ states (ΔH_{SP}). In addition to this donor side enthalpy the activation energy for the indirect route also includes the enthalpy change between P^+Ph^- and $P^+Q_A^-$ ($\Delta H_{\text{ind}} = \Delta H_{\text{SP}} + \Delta H_{\text{QPh}}$). Considering a purely indirect recombination process, the increased ΔG_{QPh} between Q_A and Ph in the mutant strains is expected to increase the activation energy keeping the preexponential factor unmodified.

It is clear that the recombination in the WT must be predominantly indirect (because it is more than ten times faster than that of the D₁Glu130Leu mutant: which, as noticed above, must still be contributed by the indirect route). The data points are satisfactorily fitted as a single Arrhenius function, with $\Delta H_{\text{ind}} = 625$ meV (somewhat larger than the 578 meV obtained by ref. 24) and a pre-exponential factor of about 10^{10} s^{-1} . If the recombination process in the mutants is still essentially indirect, their Arrhenius slope should increase, with a fixed pre-exponential factor. The dashed lines, B and C are the best fits of the D₁Glu130Gln and D₁Glu130Leu data points based on this assumption. While line B is still an acceptable fit for the Gln mutant, this is clearly not the case any more for the Leu mutant (line C). Indeed, the data points are not arranged along a straight line and also, their

dependence on $1/T$ is much less pronounced than in the WT. In this model, the increase in ΔH required to account for the slower rates would, on the contrary, predict a steeper dependence (e.g. $\Delta H_{\text{ind}} = 675$ meV for curve C). On the other hand, a decreased slope is expected if the observed process includes a significant contribution of the direct pathway, with a smaller ΔH_{SP} . Our interpretation is that this experiment shows the transition region between the low temperature range where the direct route (smaller Arrhenius slope) is fastest, and the high temperature range where the indirect route (steeper slope) is predominant. The limited explored temperature range is not sufficient to allow a fully accurate determination of the three parameters of interest (the ΔH for the indirect and direct routes and the pre-exponential factor for the latter): a Levenberg–Marquardt fitting procedure gives $\Delta H_{\text{ind}} = 699 \pm 17$ meV and $\Delta H_{\text{SP}} = 254 \pm 200$ meV, thus a large uncertainty on the latter quantity. One may also estimate a range for ΔH_{ind} , based on qualitative arguments. The lower limit is that of curve C (675 meV), required to account for the slower kinetics when assuming a negligible contribution of the direct route throughout. An upper limit is found by assuming that at room temperature the indirect route must contribute at least to about 1/3 (taken as the “significant” contribution required to account for the bromoxynil and membrane potential effects), i.e. $\Delta H_{\text{L}} \leq 715$ meV. Therefore, $\Delta H_{\text{ind}}^{\text{L}} = 695 \pm 20$ meV (in agreement with the fitting result) and the increase in ΔH brought about by the Glu to Leu mutation is $695 - 625 = 70 \pm 20$ meV. This may be compared with the 89 mV estimated by Merry *et al.* for the shift towards more negative values of the $E_{\text{m}}(\text{Ph})$ for the same amino acid change in *Synechocystis*.¹³ Curve D is a simulation assuming $\Delta H_{\text{ind}} = 695$ meV and obtaining the other parameters (see legend) from a fitting procedure.

The above estimates imply that in the Leu mutant, the predominant recombination pathway switches from indirect at room temperature to direct at low temperature. The evolution in this strain from a biphasic recombination process at room temperature to monophasic kinetics at -5 °C is probably a consequence of this increased weight of the direct process. This would be the case if the heterogeneity responsible for the biphasic kinetics is specific to the indirect pathway (for instance it may be due to slightly different ΔH_{QPh} values). The disappearance of the biphasic behavior would then reflect the predominance of the direct pathway at -5 °C.

C. The energetic picture of Photosystem II

The expressions for the direct and indirect recombination rates are (see ref. 6):

$$v_{\text{dir}} = \frac{k_{\text{QP}}}{K_{\text{SP}}} \quad v_{\text{ind}} = \frac{k'_{\text{PhP}}}{K_{\text{SP}}K_{\text{QPh}}} \quad (2)$$

Here, k_{QP} is the rate constant for the direct electron transfer from Q_A^- to P^+ , k'_{PhP} is the rate constant for the electron transfer from Ph^- to P^+ (with a correction due to the fact that the electron transfer equilibrium between Q_A and Ph is not very fast with respect to the $\text{Ph}^- \rightarrow \text{P}^+$ reaction, see ref. 6); K_{SP} and K_{QPh} are the equilibrium constants on the donor and acceptor side, respectively.

Incorporating in eqn. (2) the estimates $k_{\text{PQ}} \approx 410 \text{ s}^{-1}$ ²⁵ and $k'_{\text{PhP}} \approx 1.6 \times 10^9 \text{ s}^{-1}$ (from literature data, as explained in ref. 6), one obtains, from $v_{\text{ind}} = 0.23 \text{ s}^{-1}$ (the WT rate at 293 K): $(K_{\text{SP}}K_{\text{QPh}}) \approx 7 \times 10^9$, or 570 meV for the free energy difference between states (P^+Ph^-) and ($\text{S}_2\text{Q}_\text{A}^-$). This is close to but somewhat below the $\Delta H_{\text{ind}} = 625$ meV value obtained from the Arrhenius plot of the WT (see also the 578 meV estimated by ref. 24), suggesting a negative ΔS contribution. We cannot determine the respective contributions of the acceptor and donor side stabilizations in this total, but an upper limit may be estimated for the ΔG on the donor side. We have noted that

the indirect route is still quite significant in the $\text{D}_1\text{Glu130Leu}$ mutant at 293 K. Therefore, $v_{\text{dir}} \leq 0.0167 \text{ s}^{-1}$ (the recombination rate of this strain) and $K_{\text{SP}} \geq 410/0.0167 = 24\,550$ or $\Delta G_{\text{SP}} \geq 255$ meV (notice that v_{dir} is assumed to be independent on the Ph ligands, hence the same in the $\text{D}_1\text{Glu130Leu}$ strain). Accordingly, $\Delta G_{\text{QPh}} \leq 570 - 255 = 315$ meV. This is comparable to the 360 meV value that we estimated before in *Synechocystis*⁶ or to the 340 meV estimated by van Gorkom.⁷

The present analysis supports our previous conclusions that the free energy gap between Q_A and Ph is markedly smaller than estimated from redox titrations of Ph (-640 mV^{26,27}) and Q_A (-30 mV^{28,29}). These would predict 610 meV, exceeding the above estimate by more than 295 meV. We argued that the faulty E_{m} was in all likelihood that of Ph , because of the much greater technical difficulties involved in this low potential range. Yet, although this point is easily overlooked, the $E_{\text{m}}(\text{Ph})$ happens to play a pivotal role in the current picture for the energetics of the PS II donor side. This is because the redox titrations of these high potential carriers (P , Y_Z , S states) have not been possible thus far, so that all our knowledge comes from laying end to end successive energy differences, starting from $E_{\text{m}}(\text{Ph})$: i.e. the gap between Ph and P^* , then the 1.8 eV between P^* and P , then the gaps between P and Y_Z or S state couples. Therefore an *aggiornamento* on $E_{\text{m}}(\text{Ph})$ has repercussions on the estimates of the midpoint potentials of the donor side carriers. We do not repeat here the detailed analysis developed in our previous paper: its conclusion was that these midpoint potentials must be more positive by about 150 mV than the currently accepted values. Thus, the E_{m} of the P^+/P couple is about +1260 mV and the oxidative thrust for driving water oxidation becomes much more comfortable than previously thought.

D. Photochemical and fluorescence yields

If we assume that the supramolecular arrangement and stoichiometry of the light harvesting complexes and reaction centers are not modified by the mutation, the increased basal fluorescence yield of the $\text{D}_1\text{Glu130Leu}$ mutant (Table 1) is indicative of a diminished photochemical efficiency, as expected from the shallower trap resulting from the more negative $E_{\text{m}}(\text{Ph})$. The relationship between the photochemical efficiency Φ and the F_0 (corrected for its PS I contribution) can be obtained as follows. One has:

$$F_0 = \frac{f}{d+p} \quad (3)$$

where p is the rate constant for excitation decay through photochemistry (all centers open), d for non photochemical de-excitation and f for fluorescence (included in d). Similarly, the photochemical yield is:

$$\Phi = \frac{p}{d+p} \quad (4)$$

Therefore:

$$F_0 = \frac{f}{d}(1 - \Phi) \quad (5)$$

Thus, assuming that f and d are not affected by the mutation,

$$\frac{F_0^{\text{L}}}{F_0^{\text{WT}}} = \frac{1 - \Phi_{\text{L}}}{1 - \Phi_{\text{WT}}} \quad (6)$$

using subscript L for the parameters obtained with $\text{D}_1\text{Glu130Leu}$ strain.

This ratio, after subtraction of the 0.25 contribution of PS I, is $(2.1 - 0.25)/(1 - 0.25) = 2.47$. The photochemical quantum yield of PS II, Φ_{WT} is most probably in the range 0.85–0.90 (see the rate constants estimated by ref. 30). One thus obtains for Φ_{L} a range of 0.63 (for $\Phi_{\text{WT}} = 0.85$) to 0.75 (for $\Phi_{\text{WT}} = 0.90$). If

the small increase in F_m observed for the D₁Glu130Leu mutant is significant, this suggests that there is still some trapping by the “closed” centers of the WT (involving formation of the P⁺Ph⁻Q_A⁻ state), in line with the findings of van Mieghem *et al.*,³¹ and that the more negative $E_m(\text{Ph})$ in the mutant lowers the probability of this pathway.

In their work on the *Synechocystis* mutants, Merry *et al.* have determined two quantities: the equilibrium constant (denoted K_{CS} above) between the exciton and the radical pair P⁺Ph⁻ at 60 ps and the “nanosecond quantum yield of charge separation”.¹³ The latter is not directly comparable with the quantum yield for P⁺Q_A⁻ formation (the present Φ), for two reasons. Firstly, the material used by these authors was “D1-D2-b₅₅₉” PS II reaction centers, devoid of antennae, so that the exciton/(radical pair) equilibrium should be displaced towards the RP with respect to antenna-containing material (see ref. 32). Secondly, Q_A is absent in this material, so that the observed charge separation and its yield concern a relaxed state of the RP. In our case, the (300 ps)⁻¹ electron transfer towards Q_A provides the major route for stabilization of the charge separation. Before discussing the relationship between Φ and K_{CS} and the quantitative consequences of the Glu to Leu mutations on these parameters, we will now discuss the puzzling finding that the fluorescence change induced by a weak flash was larger in amplitude in the D₁Glu130Leu mutant than in the WT.

Because of the impaired photochemical efficiency of the D₁Glu130Leu mutant, we expected at first thought a diminished relative amplitude of the fluorescence change induced by a weak flash, compared with the WT. This proved to be false: the same weak flash inducing a fluorescence change of *e.g.*, 4% of the total amplitude in the WT, caused a *larger* relative change in the mutant (by a factor of about 1.8). The reason for this lies in the distortion brought about by the $F(c)$ relationship. The increased fluorescence yield in the mutant, reflecting a longer exciton lifetime and mean path, results in a steeper slope of this function. Assuming that the supramolecular organization of the antennae and RC complexes is not modified in the mutant, its $F(c)$ dependence can be deduced from that of the WT, just by renormalizing the hyperbola section pertaining to the variable fluorescence of the mutant. This procedure is illustrated in Fig. 5. The solid line shows the $F(c)$

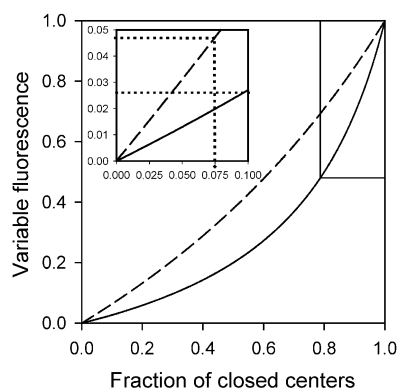


Fig. 5 A theoretical description of the $F(c)$ relationship for the WT (solid line) and D₁Glu130Leu mutant (dashed line). The solid line is a plot of eqn. (1), assuming $J = 3$. The top-right rectangle shows the variable fluorescence range for the mutant, from $F_0(L) = (2.1 - 1)/(3.3 - 1) = 0.48$ to 1 [ignoring the higher $F_m(L)$]. We denote as $c_L (=0.79)$ the value of c for which the WT function equals 0.48, *i.e.* $F_{WT}(c_L) = F_0(L)$. The $F_L(c)$ function (dashed) is the renormalized $F_{WT}(c)$ section in the top-right rectangle. It has the form of eqn. (1), with $J_L = J_{WT}(1 - c_L)$, thus $J_L = 0.63$. The inset is a zoom on the bottom left corner. A weak flash hitting 10% of the centers in the WT induces a relative fluorescence change of 2.5%. In the D₁Glu130Leu mutant, assuming its photochemical efficiency is 75% that of the WT, 7.5% of the centers will be hit, but due to the larger slope of the dashed line, the relative fluorescence change is 4.7%, thus 1.74-fold larger than in the WT.

dependence of the WT. A value of $J = 3$ was assumed, in the range that we obtained for this parameter (see the inset of Fig. 1). The rectangle in the top-right corner shows the fluorescence range of the D₁Glu130Leu mutant (assuming the F_m is unchanged). The $F(c)$ relationship for the mutant is obtained by expanding this section of the WT curve to normalized horizontal and vertical scales. This yields the dashed line, with $J_L = 0.63$ (computed as explained in the legend). In agreement with this analysis, the plot of the “saturating vs. weak flash” fluorescence kinetics (as in the inset of Fig. 1) for the D₁Glu130Leu mutant was close to the diagonal (not shown), indicating $J < 1$ (an accurate value could not be determined due to the drifts affecting the tails of these slow kinetics). The initial slope $dF(0)/dc$ is $1/(1 + J)$, thus 2.4 times larger for the mutant than for the WT. The relative amplitude of the fluorescence change caused by a weak flash is the product of this sensitivity factor (enhanced in the mutant) by the photochemical yield (decreased in the mutant). The inset of Fig. 5 illustrates the case where Φ_L is decreased by 25% with respect to the WT (as estimated if $\Phi_{WT} = 0.85$, see above): the increased sensitivity factor predominates and the predicted enhancement of the amplitude is 1.74, similar to the observed value.

A broadly accepted picture for PS II trapping is that a rapid equilibrium is established between the exciton (singlet excited state in the pigment domain, including LHCs and RC) and the radical pair (RP = P⁺Ph⁻). The kinetic scheme for the charge separation process is thus:



K_{CS} is the equilibrium constant (RP/exciton), k_{PhQ} is the rate constant for the electron transfer from Ph to Q_A. The exciton may also decay through a non-photochemical route, with rate constant d . If the RP/exciton equilibrium is rapid with respect to k_{PhQ} , the photochemical rate is:

$$p = \frac{K_{CS}}{1 + K_{CS}} k_{\text{PhQ}} \quad (7)$$

The $K_{CS}/(1 + K_{CS})$ factor is the fraction of RP. The equilibrium constant K_{CS} depends on the midpoint potential of Ph, here denoted $E_m(\text{Ph})$ in the following way:

$$K = K_0 10^{E_m(\text{Ph})/58} \quad (8)$$

(approximating $2.3RT/F$ as 58 meV). The factor K_0 does not depend on $E_m(\text{Ph})$, but is determined by the midpoint potential of the P⁺/P couple, the energy of the Chl* state and the entropic stabilization caused by the antenna size. The effect on p of a given shift of $E_m(\text{Ph})$ will depend on K_{CS} . Let us assume, for instance, that the $E_m(\text{Ph})$ of the D₁Glu130Leu mutant is 70 mV more negative than that of the WT (this is the estimate obtained above for the ΔH : we suppose that the mutation does not affect ΔS so that $\Delta\Delta G = \Delta\Delta H$). Noting that $10^{70/58} \approx 16$ and using eqns. (7) and (8), one obtains:

$$\frac{p_{WT}}{p_L} = \frac{16 + K_{CS}}{1 + K_{CS}} \quad (9)$$

where K_{CS} is the equilibrium constant for the WT. Thus, if $K_{CS} \gg 16$, the effect of the modified $E_m(\text{Ph})$ is negligible, whereas if $K_{CS} \ll 1$ the photochemical rate constant is decreased 16-fold in the mutant.

From the increased fluorescence of the D₁Glu130Leu strain, we have gained some useful information on the ratio p_{WT}/p_L , which can be computed when the photochemical yield of the WT is known. For instance, let us assume that this yield is $\Phi_{WT} = 0.85$. Incorporating the estimated fluorescence ratio (≈ 2.47) in eqn. (6) and using eqn. (4), one obtains $p_{WT}/p_L = 3.3$. From eqn. (8), the K_{CS} value associated with this ratio is $K_{CS} = 5.3$ ($\Delta G \approx -40$ meV), suggesting that the equilibrium is

moderately in favor of the radical pair in the WT and becomes in favor of the exciton ($K_{CS}^L = 0.34$) in the mutant. Qualitatively similar features are found when assuming a higher Φ_{WT} . For instance, if $\Phi_{WT} = 0.9$, $p_{WT}/p_L = 2.9$ and $K_{CS} = 6.5$ ($\Delta G_{CS} \approx -50$ meV). Such values of K_{CS} are larger than estimated by Barter *et al.* in PS II particles with 250 chlorophylls, *i.e.* $K_{CS} \approx 0.54$ at 250 ps (or 0.25 at 150 ps).³² The measurement time is important here because the RP undergoes a rapid stabilization process, so that K_{CS} increases with time: a significant complication that was ignored in our simple scheme. Thus, our estimates pertain to some averaged effective value of K_{CS} during the whole charge separation towards Q_A and the discrepancy with the value estimated at 250 ps by Barter *et al.* is probably not significant. The moderate sensitivity found here for the dependence of the photochemical yield on the $E_m(\text{Ph})$ confirms that, in a physiological context, the radical pair state constitutes a “shallow trap”, only 40–50 meV below the energy level of the chlorophyll excited state.

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