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Photosynthetic water oxidation in *Synechocystis* sp. PCC6803: mutations D1-E189K, R and Q are without influence on electron transfer at the donor side of photosystem II

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Abstract

The oxygen-evolving manganese cluster (OEC) of photosynthesis is oxidised by the photochemically generated primary oxidant ($P_{680}^{+\bullet}$) of photosystem II via a tyrosine residue (Y_Z, Tyr161 on the D1 subunit of *Synechocystis* sp. PCC6803). The redox span between these components is rather small and probably tuned by protonic equilibria. The very efficient electron transfer from Y_Z to $P_{680}^{+\bullet}$ in nanoseconds requires the intactness of a hydrogen bonded network involving Y_Z, D1-His190, and presumably D1-Glu189. We studied photosystem II core particles from photoautotrophic mutants where the residue D1-E189 was replaced by glutamine, arginine and lysine which were expected to electrostatically differ from the glutamate in the wild-type (WT). Surprisingly, the rates of electron transfer from Y_Z to $P_{680}^{+\bullet}$ as well as from the OEC to Y_Z^{ox} were the same as in the WT. With the generally assumed proximity between D1-His190 (and thus D1-Glu189) and Y_Z, the lack of any influence on the electron transfer around Y_Z straightforwardly implies a strongly hydrophobic environment forcing Glu (acid) and Lys, Arg (basic) at position D1-189 into electro-neutrality. As one alternative, D1-Glu189 could be located at such a large distance from the OEC, Y_Z and $P_{680}^{+\bullet}$ that a charge on D1-189X does not influence the electron transfer. This seems less likely in the light of the drastic influence of its direct neighbour, D1-His190, on Y_Z function. Another alternative is that D1-Glu189 is negatively charged, but is located in a cluster of acid/base groups that compensates for an alteration of charge at position 189, leaving the overall net charge unchanged in the Gln, Lys, and Arg mutants.

Keywords: Photosynthesis; Water oxidation; Proton coupled electron transfer; Tyrosine; Photosystem II

Abbreviations: β-DM, *n*-dodecyl β-D-maltoside; chl, chlorophyll; D1, D2, core subunits of photosystem II; DCBQ, 2,5-dichlorobenzoquinone; FWHM, full width at half-maximum; MES, 2-(*N*-morpholino)ethanesulphonic acid; OEC, oxygen-evolving complex; P_{680}, primary electron donor of PS II; PS II, photosystem II; Q_A, Q_B, bound quinone acceptors in PS II; S_i, ($i=0$–4), redox states of the catalytic centre; Y_D, redox-active tyrosine-160 of D2 with auxiliary function; Y_Z, redox-active tyrosine-161 of D1

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1. Introduction

Photosystem II (PS II) of green plants and cyanobacteria produces dioxygen from two water molecules at the expense of four quanta of light (see [1–8] for review). Absorption of light induces electron transfer from a chlorophyll *a* moiety, P_{680}, to a bound plastoquinone, Q_A. The $Q_A^{-\bullet}$ radical reduces a secondary quinone, Q_B, while $P_{680}^{+\bullet}$ is reduced by Y_Z (Tyr161 on the D1 subunit of PS II). The Y_Z^{ox} radical is reduced by the catalytic Mn cluster (a tetramer) which is thereby stepped forward from lower to higher oxidation state, from S_0 to S_4. The S_4 state spontaneously decays to the S_0 state concomitant with the release of oxygen from water. In darkness, S_1 is the most stable state, whereas S_2 and S_3 are slowly reduced by Y_D (Tyr160 on the D2 subunit) and $Q_B^{-\bullet}$ to yield S_1.

The roles of other amino acids on the donor side of the D1 polypeptide of PS II are under active investigation. Recent studies suggest that the oxidation of Y_Z by $P_{680}^{+\bullet}$ requires the deprotonation of Y_Z by D1-His190 [9–12], that D1-His332 almost certainly ligates the assembled Mn cluster [13], and that D1-Asp170 either ligates the assembled Mn cluster or is coupled to it through a hydrogen bond [14] (for review, see [15,16]). A number of studies have focused on D1-Glu189. At least 17 mutations have been constructed: Gln, Asp, Asn, His, Ser, Thr, Ala, Gly, Cys, Lys, Arg, Leu, Ile, Val, Met, Phe, and Tyr [17–20]. Of these only the Gln, Lys, Arg, Leu, and Ile mutants are photoautotrophic. When normalised to the apparent PS II contents of the mutant cells, the PS II reaction centres in the Lys, Arg, and Ile mutants evolved O_2 with rates comparable to that of wild-type PS II reaction centres, whereas the PS II reaction centres in the Gln and Leu mutants evolved O_2 with rates that were about 70% of the rate of wild-type PS II reaction centres [17,20]. None of the other 12 D1-Glu189 mutants evolved O_2 to any significant extent. This points out the importance of a long side chain at position D1-189. The influence of most of the 17 mutations on the assembly or stability of the Mn complex in vivo was minor, so D1-Glu189 was not proposed to ligate Mn [17,18,20]. On the basis of an early study of the Gln, Asp, and Asn mutants, it was proposed that D1-Glu189 participates in a network of hydrogen bonds that positions a group that participates in proton release [17]. More recently, D1-Glu189 has been postulated to accept a proton from D1-His190 either directly or by positioning a water molecule or other group that acts as the proton acceptor [7,21,22]. Several authors have proposed that a hydrogen bond connects D1-Glu189 and D1-His190 [7,18,21–23], with D1-Glu189 either serving to optimally orient [18] or to shift the pK_a value [23] of D1-His190. Recently, the EPR and electron transfer properties of PS II core particles isolated from the Gln, Leu, Asp, Asn, His, Gly, and Ser mutants were examined [20]. Intact, O_2-evolving PS II core particles isolated from the Gln mutant (D1-E189Q) exhibited normal S_1 and S_2 state multiline EPR signals, whereas PS II core particles isolated from D1-Glu189 mutants that evolved no O_2 were unable to advance beyond an altered $S_2Y_Z^{ox}$ state [20]. Charge recombination between $Q_A^{-\bullet}$ and Y_Z^{ox} was also accelerated in these mutants, showing that the mutations alter the redox properties of Y_Z in addition to those of the Mn cluster [20]. On the basis of these results, it was proposed that D1-Glu189 participates in a network of hydrogen bonds that optimises the $(Mn)_4$-Y_Z configuration for rapid electron/proton transfer from the Mn cluster to $Y_Z^{\bullet}$ and for rapid proton transfer from Y_Z to D1-His190 [20]. It was also proposed that D1-Glu189 neither accepts nor donates protons directly, but rather helps to position a group that accepts a proton from D1-His190 in response to the oxidation of Y_Z [20].

To further define the role of D1-Glu189 in determining the properties of Y_Z and the Mn cluster, we have measured the rates of electron transfer from the Mn cluster to Y_Z^{ox} and from Y_Z to $P_{680}^{+\bullet}$ in PS II core particles isolated from the photoautotrophic mutants D1-E189Q, D1-E189R and D1-E189K, where we expected to detect electrostatic effects if the side chain was varied between Glu (hypothesised to be negatively charged), Gln (neutral) and Arg, Lys (hypothesised to be positively charged).

2. Materials and methods

Cells of the unmodified wild-type (WT), modified 'wild-type' (WT*) [12] and the E189Q, R and E189K mutants of *Synechocystis* sp. PCC6803 were culti-

vated as described in [24] but in an atmosphere enriched with 4% of CO_2 under illumination with (cool-white) fluorescent light at 0.8–1.4 mW cm^{-2}. The mutants were constructed in a strain of *Synechocystis* that lacks PS I and *apc*E function [25]. The medium was supplemented with 15 mM glucose. Oxygen-evolving PS II core particles were prepared as described in [12] with some minor modifications. The detergent extracted thylakoid membranes were applied to a 40 ml DEAE-Toyopearl 650s column. All buffers, except the equilibration buffer, contained 1 M glycine betaine. The equilibration buffer and the elution buffer contained 5 and 50 mM $MgSO_4$, respectively. The eluate was concentrated with Centriprep 50 centrifugal concentrators (Amicon) for 3–4 h at 4°C until a chlorophyll (chl) concentration of 200–400 μM was reached.

Oxygen evolution of PS II core particles with continuous saturating illumination was measured with a Clark-type electrode at 21°C. Frozen and thawed PS II particles (1.5 μM chl) were suspended in 50 mM MES pH 6.6, 1 M sucrose, 25 mM $CaCl_2$, 10 mM NaCl, 1 M glycine betaine, with 2,5-dichlorobenzoquinone (DCBQ; 1 mM) added as electron acceptor. Typical rates were 2000–3000 μmol of O_2/(mg of chl·h) for WT* (depending on the light conditions, see Section 3.2), 1300 μmol of O_2/(mg of chl·h) for D1-E189Q, 1900–2100 μmol of O_2/(mg of chl·h) for D1-E189R and 1900–2200 μmol of O_2/(mg of chl·h) for D1-E189K. The experiments were carried out with six preparations of WT*, one of D1-E189Q, three of D1-E189K and three of D1-E189R, which were all prepared after the procedure described above and eight preparations of WT (prepared after [26]) and five preparations of D1-E189Q (prepared after [26]). Cells of core particles that were prepared after [26] did not lack PS I and *apc*E function.

Depletion of manganese was performed by incubation of PS II core particles at pH 9.5 (5 min) in total darkness as described [27,28]. This procedure diminished the rate of O_2 evolution under continuous illumination to less than 10% of the rate of untreated samples.

Flash-induced release of O_2 was measured polarographically with a bare platinum electrode. Cells were suspended at 25 μM chlorophyll in 20 mM HEPES pH 7.2, 20 mM NaCl, and 1 mM $CaCl_2$, and then dark-adapted for 10 min. To increase the time resolution of the electrode, aliquots of 20 μl were deposited on the electrode, and the cells were pelleted upon the metal by centrifugation ($1000 \times g$, 10 min, 8°C). The cathode (Pt) was negatively polarised with −800 mV against the anode (Ag/AgCl). Polarographic transients were digitised and recorded with a time resolution of 50 μs/address (Xenon-flash lamp, Schott RG610, 1 s between flashes). Two to five recordings were averaged. All experiments were carried out at room temperature (approx. 20°C).

For flash-spectrophotometric measurements [29], PS II core particles were suspended in 50 mM MES pH 6.6, 1 M sucrose, 25 mM $CaCl_2$, 10 mM NaCl, 1 M glycine betaine, 0.06% *n*-dodecyl β-D-maltoside (β-DM; w/v). PS II core particles prepared after [26] were suspended in 20 mM MES pH 6.35, 20 mM $CaCl_2$, 5 mM $MgCl_2$, 0.06% β-DM (w/v). 200 μM DCBQ was used as electron acceptor. Electron transfer from the Mn cluster to Y_Z^{ox} was recorded at a wavelength of 360 nm under excitation with five saturating flashes (100 ms between flashes). We used repetitively dark-adapted samples as detailed elsewhere (8 μM chl, 2 min dark adaptation) [30]. The samples were excited by a Nd:YAG laser (532 nm, full width at half-maximum (FWHM) 6 ns, 100 ms between flashes). The optical pathlength was 1 cm. The time resolution was 10 μs/address. The reduction of $P_{680}^{+\bullet}$ was measured at 827 nm (optical pathlength 5 cm) as described elsewhere [31]. The PS II core particles (15–30 μM chlorophyll) were suspended in buffer with DCBQ (1 mM) added as electron acceptor. Electron transfer from Y_Z to $P_{680}^{+\bullet}$ in O_2-evolving PS II core particles was measured under repetitive saturating excitation (Nd:YAG laser, FWHM 3 ns, 532 nm, 100 ms between flashes) at a time resolution of 4 ns/address. The recombination between $Q_A^{-\bullet}$ and $P_{680}^{+\bullet}$ in Mn-depleted PS II core particles (prepared after [26]) was assayed with a saturating flash sequence (Nd:YAG laser, FWHM 3 ns, 532 nm) of two flashes (20 ms between flashes) that were repetitively given to the sample every 500 ms. This flash regime maximised the extent of the recombination between $Q_A^{-\bullet}$ and $P_{680}^{+\bullet}$ on the second flash. The recombination rate was recorded at a time resolution of 4 μs/address. Photometric transients were digitised on a Nicolet Pro30 or a Tektronix DSA602 recorder and up to 400 signals were averaged. Data were fitted

by standard routines of the program Origin (Microcal).

3. Results

3.1. The half-times of S-state transitions in D1-E189Q, D1-E189K, D1-E189R and wild-type* PS II core particles

Fig. 1 shows flash-induced absorption transients at 360 nm on the first two flashes given to repetitively dark-adapted PS II core particles of the mutants D1-E189R, D1-E189K and wild-type*. The data were fitted with two exponentially rising phases (solid lines). These transients are composites from the following events: the rapid rise ($t_{1/2} \ll 20$ μs), here unresolved, is attributable to the reduction of the primary quinone acceptor (Q_A) (on the first and the second flash). The slow phase is indicative of the oxidation of the Mn cluster. In D1-E189K PS II core particles, the slow phase was characterised by half-rise times of $t_{1/2} = 35 \pm 10$ μs on the first flash (Fig. 1A) and of 90 ± 15 μs on the second flash (Fig. 1B). In D1-E189R PS II core particles, the slow phase was characterised by half-rise times of 49 ± 10 μs on the first flash (Fig. 1C) and of 104 ± 20 μs on the second flash (Fig. 1D). Because of the accumulation of S_1 in the dark, these slow phases resulted primarily from the transition

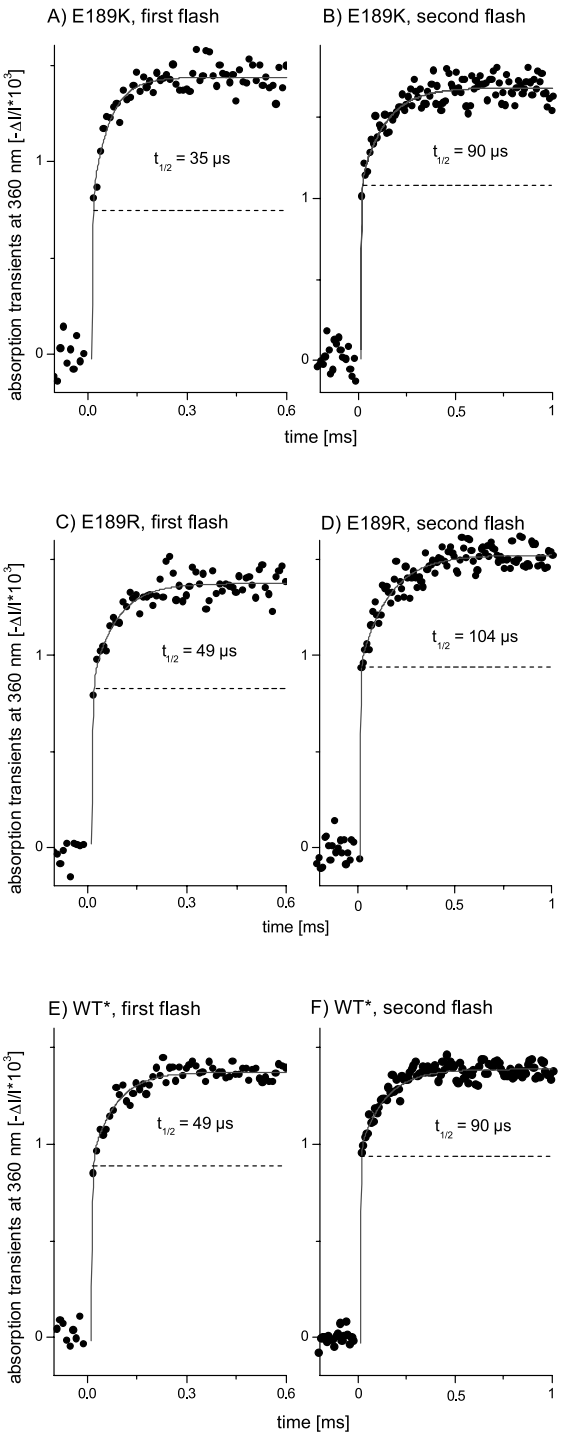

Fig. 1. Absorption transients representing the electron transfer from OEC to Y_Z^{ox} observed at 360 nm on the first flash (A,C,E), and on the second flash (B,D,F) in repetitively dark-adapted D1-E189K (A,B), D1-E189R (C,D) and wild-type* PS II core particles (E,F). Transients of mutants were comparable to those reported and measured for wild-type (see Section 3.1 and E,F). The slow phases (indicated by the dashed line) on the first and second flash, representing mainly the transitions $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$, respectively, were fitted by exponentials (solid lines) with respective half-times $t_{1/2} = 35 \pm 10$ μs and 90 ± 15 μs for D1-E189K (Table 1). The D1-E189R PS II core particles exhibited half-rise times of 49 ± 10 μs ($S_1 \rightarrow S_2$) and 104 ± 20 μs ($S_2 \rightarrow S_3$) and the wild-type* core particles exhibited half-rise times of 49 ± 10 μs ($S_1 \rightarrow S_2$) and 90 ± 10 μs ($S_2 \rightarrow S_3$) (Table 1). DCBQ (200 μM) was used as electron acceptor; the time resolution was limited by the address setting of the signal averager, 10 μs/address, and the concentration of chl was 8 μM. Signals were averaged over 80 events (A,B), 90 events (C,D) and 190 events (E,F), respectively.

$S_1 \rightarrow S_2$ on the first flash and $S_2 \rightarrow S_3$ on the second flash. This was confirmed by the oscillation pattern during a train of nine flashes under repetitive conditions (Fig. 2). In D1-E189K and D1-E189R PS II

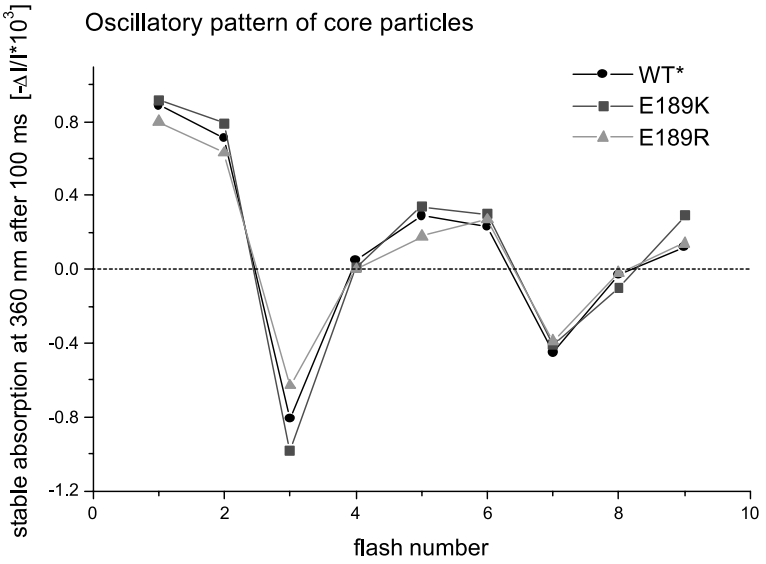

Fig. 2. The extent of the 'stable' component of absorption transients at 360 nm taken 100 ms after each exciting flash as function of the flash number. Oscillations with period four were clearly expressed in core particles of D1-E189K (square), D1-E189R (triangle) and wild-type (circle). Repetitively dark-adapted samples.

core particles, the half-rise times of transitions $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$ were almost the same as in wild-type* PS II core particles from *Synechocystis* sp. PCC6803 (49 ± 10 μs and 90 ± 10 μs, Fig. 1E,F). Further experiments in this line were performed. They are summarised in Table 1. Within error limits the respective rise times were identical with those reported for core complexes of the thermophilic cyanobacterium *Synechococcus* sp. (with 40 μs for $S_1 \rightarrow S_2$ and 100 μs for $S_2 \rightarrow S_3$ [32]). They were similar to those in core complexes from higher plants ($t_{1/2} = 55$–110 μs for $S_1 \rightarrow S_2$ and 130–380 μs for $S_2 \rightarrow S_3$ [33–36] and references therein) and from *Synechocystis* sp. PCC6803, prepared after a different protocol [26] (100 μs and 220 μs for $S_1 \rightarrow S_2$ and $S_2 \rightarrow S_3$, respectively). Core complexes of the mutant D1-E189Q that were also prepared after the latter procedure [26] revealed unchanged half-rise times for $S_1 \rightarrow S_2$ (100 ± 50 μs) and $S_2 \rightarrow S_3$ (210 ± 50 μs) (data not shown).

The rise time of the O_2-evolving transition $S_3 \rightarrow S_0$ was determined with two independent methods. The results are shown in Fig. 3. Time resolved polarographic measurements of O_2 release after the third flash (Fig. 3A) showed the same rate with wild-type cells (closed circles) as with the mutant D1-E189Q (open triangles) (Fig. 3A). We assumed that the rising transient with an effective half-rise time of $t_{1/2} = 3 \pm 0.5$ ms (wild-type and D1-E189Q) resulted from two consecutive reactions: (1) the production of O_2 ($t_{1/2} \approx 1.5$ ms, Table 1) and (2) the diffusion of O_2 to the electrode ($t_{1/2} \approx 1.5$ ms) (for details see [26]).

The half-time of the reduction of the Mn cluster in $S_3 \rightarrow S_0$ was detected via flash-induced absorption transients at 360 nm with repetitively dark-adapted, O_2-evolving PS II core particles of D1-E189Q and wild-type in the absence of glycerol. Both samples were prepared after [26]. Oxidation/reduction reactions of the Mn cluster and of the acceptor side (Q_A) both contribute to absorption transients at 360 nm. To correct for the acceptor side contribution, which is the same on every flash, we analysed the difference of absorption transients after the third flash ($S_3 \rightarrow S_0$ plus $Q_A^{-\bullet}$ oxidation) minus the fifth flash ($S_1 \rightarrow S_2$ plus $Q_A^{-\bullet}$ oxidation) (Fig. 3B). The data (closed circles) were fitted (solid line) with one exponentially decaying phase ($S_3 \rightarrow S_0$) and an unresolved negative jump ($S_1 \rightarrow S_2$) (time resolution 100 μs/address). The resulting half-decay time with D1-E189Q PS II core particles of $t_{1/2} = 4.7 \pm 0.5$ ms for $S_3 \rightarrow S_0$ (Fig. 3B) was similar to the one detected with PS II core particles from wild-type *Synechocystis* PS II core particles in the absence of glycerol ($t_{1/2} = 4.0$–

Table 1
Half-times of electron transfer reactions in cells and PS II core particles from different mutants compared to wild-type

Reaction	WT	E189Q	E189K	E189R
$S_1 \rightarrow S_2$	46 ± 5 μs, $n = 190$	55 ± 40[b,d] μs, $n = 20$	44 ± 7 μs, $n = 80$	41 ± 7 μs, $n = 90$
$S_2 \rightarrow S_3$	90 ± 5 μs, $n = 190$	90 ± 50[b,d] μs, $n = 20$	99 ± 7 μs, $n = 80$	96 ± 7 μs, $n = 90$
$S_3 \rightarrow S_0$ (cores, −G)	4.5 ± 0.4 ms[a,b]	4.7 ± 0.5[b,c] ms, $n = 80$	n.d.	n.d.
$S_3 \rightarrow S_0$ (cores, +G)	1.5 ± 0.1 ms[a,b]	n.d.	n.d.	n.d.
$S_3 \rightarrow S_0$ (cells)	(1.5 ± 0.5 ms[b,e]), $n = 5$	(1.5 ± 0.5 ms[b,e]), $n = 5$	n.d.	n.d.
Reduction of $P680^+$:				
oxygen-evolving PS II	30 ± 3 ns (36%), $n = 1950$	38 ± 2 ns[b] (24%), $n = 800$	27 ± 0 ns (48%), $n = 1800$	30 ± 1 ns (41%), $n = 1750$
	253 ± 21 ns (20%), $n = 1950$	259 ± 10[b] ns (21%), $n = 800$	251 ± 6 ns (21%), $n = 1800$	304 ± 21 ns (24%), $n = 1750$
	> 2 μs (45%), $n = 1950$	> 2 μs (55%), $n = 800$	> 2 μs (31%), $n = 1800$	> 2 μs (35%), $n = 1750$
Mn-depleted PS II	780 ± 10 μs[b,c] (87%), $n = 300$	650 ± 20 μs[b,c] (96%), $n = 150$	n.d.	n.d.
	> 2 ms (13%), $n = 300$	> 2 ms (4%), $n = 150$		

Mean values with standard deviations. +/−G, presence or absence of glycerol; *n*, number of measurements.
[a]Data from [37].
[b]Error calculated from the fit routine.
[c]Core particles prepared after [26].
[d]Data not shown in Section 3.1.
[e]Relaxation times in parentheses have been calculated under correction for the diffusion of O_2 to the electrode as specified in Section 3.1.

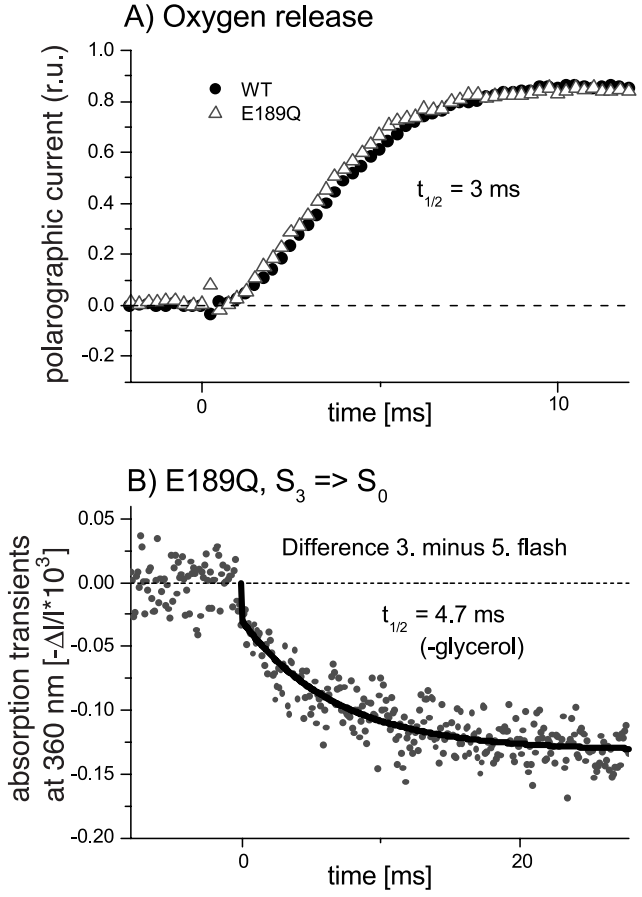

Fig. 3. Polarographic (A) and absorption transients (B) representing the terminal and oxygen producing electron transfer, $S_3 \rightarrow S_0$, in D1-E189Q and wild-type cells (A) and PS II core particles (B). (A) Flash-induced oxygen release on the third flash was measured polarographically with dark-adapted cells. Wild-type (closed circles) and D1-E189Q (open triangles) cells were centrifuged onto a bare platinum electrode. The digital time per address was 50 μs, five measurements were averaged, and every fifth data point is shown. The transients revealed a similar effective half-rise time of oxygen liberation in 3 ms for both the wild-type and D1-E189Q cells (the intrinsic half-rise time was about 1.5 ms (see Section 3.1)). (B) The absorption transient at 360 nm with D1-E189Q PS II core particles (prepared after [26], minus glycerol, difference third minus fifth flash, see Section 3.1) was fitted (solid line) with a half-decay time of $t_{1/2} = 4.7 \pm 0.5$ ms for $S_3 \rightarrow S_0$ (time resolution 100 μs/address), comparable to the half-time of wild-type PS II core particles under these conditions (see Section 3.1). DCBQ (200 μM) was used as electron acceptor, 80 transients were averaged.

4.5 ms [37,38]). The 'normal' half-time of 1.5 ms that has been observed in whole cells was only observed in PS II core particles in the presence of glycerol [37]. However, in the presence of glycerol, the slow relaxation of the S-states in darkness prevents 'repetitive dark adaptation' of samples, prepared after [26]. Consequently, in the presence of glycerol, large amounts of material are required that are difficult to isolate from the mutants (see [37,30]).

3.2. The half-times of the reduction of $P_{680}^{+\bullet}$ by Y_Z and $Q_B^{-\bullet}$ in wild-type*, D1-E189K, D1-E189R and D1-E189Q PS II core particles

The reduction of $P_{680}^{+\bullet}$ was analysed by absorption transients at 827 nm. The reduction of $P_{680}^{+\bullet}$ by Y_Z was measured in O_2-evolving PS II core particles (Fig. 4). The O_2-evolving samples were repetitively pre-excited, so that the populations of the S-states were equalised. The reduction of $P_{680}^{+\bullet}$ by $Q_A^{-\bullet}$ was measured in Mn-depleted PS II core particles (Fig. 5), that were prepared after [26]. The Mn-depleted samples were excited by a pair of flashes (20 ms spacing) given repetitively to the sample every 500 ms. Fig. 5 shows the result of the second flash, mainly representing the recombination between $Q_A^{-\bullet}$ and $P_{680}^{+\bullet}$. The decay of transients in O_2-evolving wild-type* PS II core particles (corresponding to the reduction of $P_{680}^{+\bullet}$ by Y_Z) (Fig. 4A) was fitted (solid line) with two exponentially decaying phases with half-decay times of 30 ± 2 ns (38%), 260 ± 25 ns (14%), and an offset of a μs phase (48%, $t_{1/2} > 2$ μs). Similar half-times (20–40 ns (50%), 100–300 ns (20%), and > 2 μs (30%)) have been reported previously for chloroplasts and PS II preparations from spinach [39] and for PS II core particles from *Synechococcus elongatus* [40]. The data fit obtained with O_2-evolving core particles of the mutants revealed the following results: For D1-E189K 27 ± 1 ns (49%), 247 ± 10 ns (16%), > 2 μs (35%); for D1-E189R, 30 ± 1 ns (43%), 310 ± 15 ns (19%), > 2 μs (38%); for D1-E189Q, 38 ± 2 ns (24%), 259 ± 10 ns (21%), > 2 μs (55%). Further experiments in this line were performed and the data and their statistics are summarised in Table 1. These data show that the half-times of the ns components were almost unchanged between wild-type* and mutant PS II core particles. Only the extent of the slow components ($t_{1/2} > 2$ μs) differed between the preparations. This deserves comment: the relative extent of slow reduction of $P_{680}^{+\bullet}$ ($t_{1/2} > 2$μs) has been assessed as a measure for the *upper limit* of the proportion of inactive reaction centres [26,41–43]. An upper limit, because

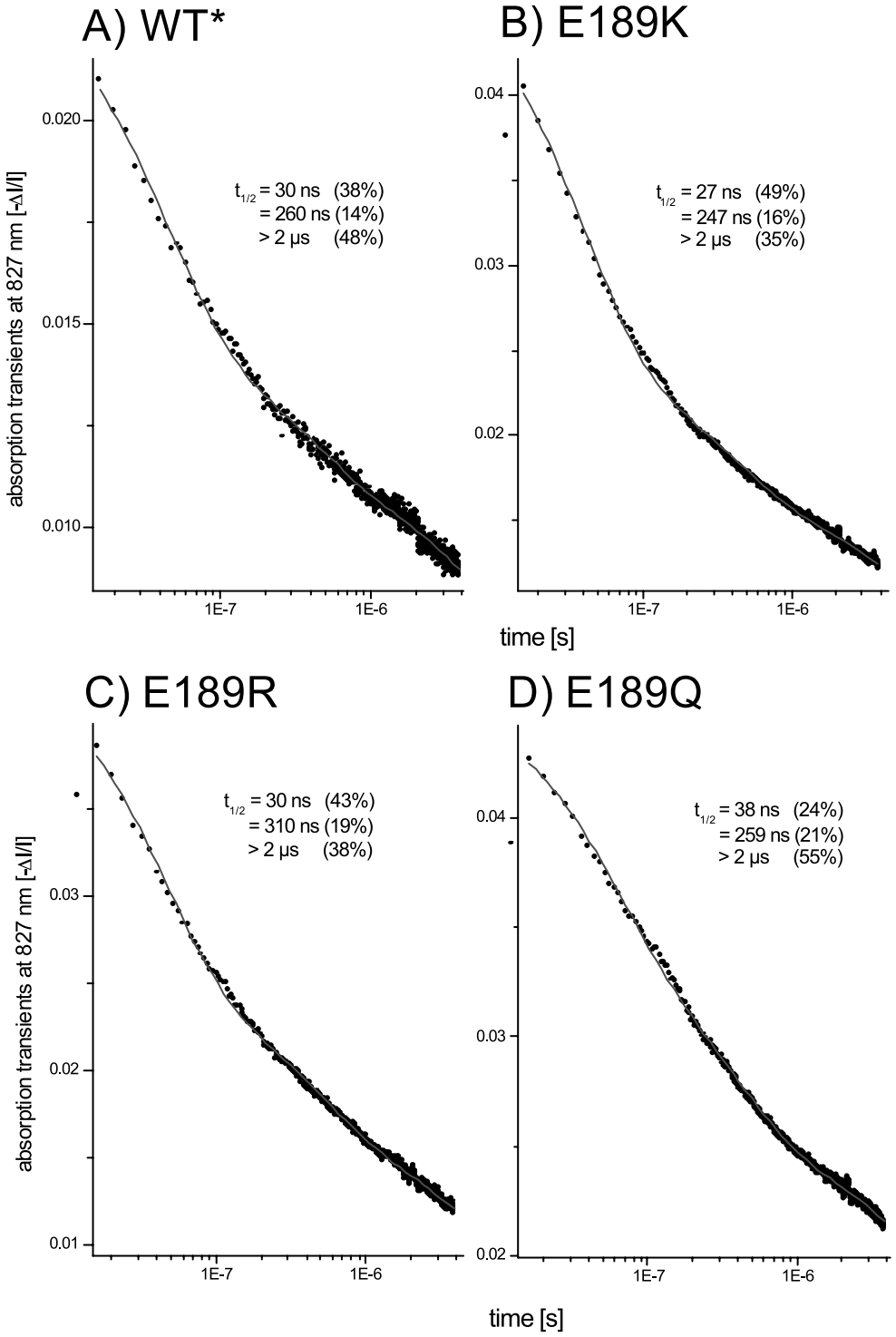

Fig. 4. The reduction of $P_{680}^{+\bullet}$ as measured at 827 nm in wild-type (A), D1-E189K (B), D1-E189R (C) and D1-E189Q (D) PS II core particles. DCBQ (1 mM) served as electron acceptor. The rate of electron transfer from Y_Z to $P_{680}^{+\bullet}$ was measured with a time resolution of 4 ns/address with O_2-evolving PS II core particles that were repetitively excited (100 ms between flashes). The number of transients averaged were 800 (wild-type*, 15 µM), 1000 (D1-E189K, 29.8 µM), 950 (D1-E189R, 30 µM) or 800 (D1-E189Q, 30 µM). The data (closed circles) were fitted (solid lines) with half-times of 30 ± 2 ns (38%), 260 ± 25 ns (14%), and > 2 µs (48%) for WT*, 27 ± 1 ns (49%), 247 ± 10 ns (16%), and > 2 µs (35%) for D1-E189K, 30 ± 1 ns (43%), 310 ± 15 ns (19%) and > 2 µs (38%) for D1-E189R, and 38 ± 2 ns (24%), 259 ns (21%) and > 2 µs (55%) for D1-E189Q.

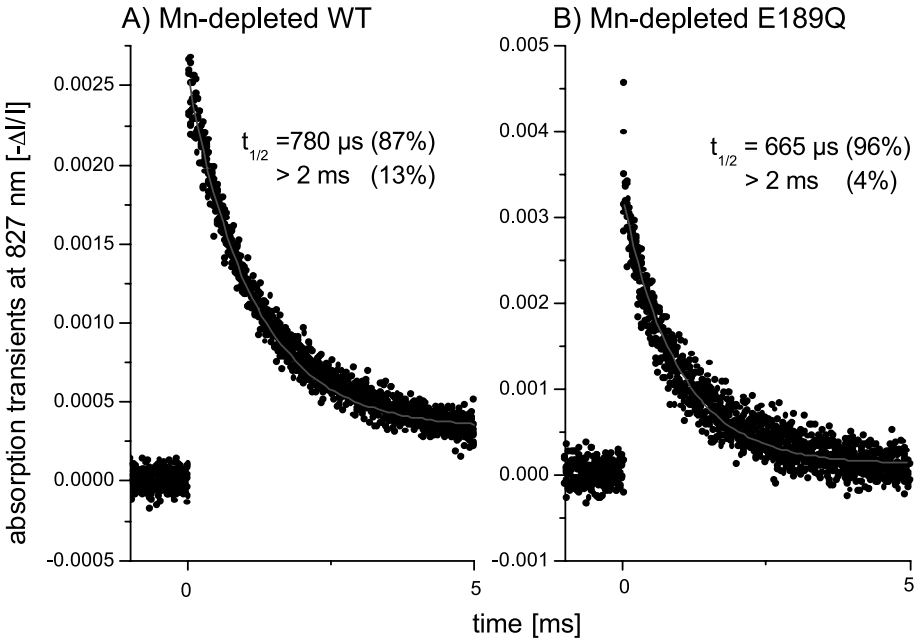

Fig. 5. The rate of charge recombination between $Q_A^{-\bullet}$ and $P_{680}^{+\bullet}$ was measured with Mn-depleted PS II core particles with a pair of saturating flashes (20 ms between them) that were given every 500 ms to the sample. This flash regime maximised the proportion of recombination between $Q_A^{-\bullet}$ and $P_{680}^{+\bullet}$ on the second flash, which is shown in the figure (time resolution 4 µs/address). The fits (solid lines) revealed half-times of 780 ± 10 µs (87%) and > 2 ms (13%) for wild-type and 665 ± 20 µs (83%) and > 2 ms (17%) for D1-E189Q. The number of transients averaged was 300 (wild-type) or 150 (D1-E189Q).

there are minor slow components in active centres as well [42–44]. In core complexes of D1-E189K and D1-E189R the relative extent of the slow phase was around 30% (Table 1). Core particles from wild-type* cells grown under the same light conditions as these mutants expressed a comparable amount of slow phase (34%, data not shown). However, for this work wild-type* core particles were prepared from cells that were grown under conditions of higher light intensities. This resulted in a higher percentage of inactive centres and therefore in a higher extent of the slow reduction of $P_{680}^{+\bullet}$ (45%, see Table 1).

The decay of transients with Mn-depleted wild-type PS II core particles (corresponding to reduction of $P_{680}^{+\bullet}$ by $Q_A^{-\bullet}$) (Fig. 5A) was fitted (solid line) with two exponentially decaying phases with half-decay times of 780 ± 10 µs (87%) and > 2 ms (13%) (see also [45]). Comparable half-times of 800–900 µs have been reported for charge recombination between $Q_A^{-\bullet}$ and $P_{680}^{+\bullet}$ in the cyanobacteria *Phormidium laminosum* [46] and *Synechocystis* sp. PCC6803 [9,47,48], although in the thermophilic cyanobacterium *S. elongatus* the recombination was multiphasic, with half-times of 170 µs (60%), 800 µs (25%), and 6 ms (15%) [49]. In Mn-depleted D1-E189Q PS II core particles (Fig. 5B), the decay was fitted with half-times of 665 ± 20 µs (96%) and > 2 ms (4%), parameters that resembled those of wild-type *Synechocystis* PS II core particles (Table 1), leaving the charge recombination between $Q_A^{-\bullet}$ and $P_{680}^{+\bullet}$ unaffected by the D1-E189Q mutation.

4. Discussion

The stepped progression of water oxidation is characterised by rather small driving forces for electron transfer between the catalytic Mn cluster (the oxygen-evolving complex (OEC)), the intermediate electron carrier (Y_Z), and $P_{680}^{+\bullet}$. Current estimates for the respective midpoint potentials at pH 6 range around 0.90–0.95 V (S_2/S_1, S_3/S_2), 0.95–0.99 V (Y_Z^{ox}/Y_Z) and 1.1 V ($P_{680}^{+\bullet}/P_{680}$), respectively [50]. It has been proposed that the net driving forces for forward electron transfer are tuned by liberating protons from the water-derived ligands of the Mn cluster

[7,22,51–58] or by internally displacing protons around Y_Z [28,59,60]. The rapid and efficient oxidation of Y_Z in nanoseconds requires the intactness of the hydrogen bonded network around Y_Z [12,28,59], including the proton acceptor for Y_Z (D1-Y161), which is proposed to be D1-His190 [9–12]. The same hydrogen bonded network has been proposed to optimise the $(Mn)_4$-Y_Z configuration for rapid electron/proton transfer from the Mn cluster to Y_Z^{ox} [7,51,54,61]. On the basis of a recent study of cells from 17 D1-Glu189 mutants and PS II core particles from seven D1-Glu189 mutants [20], it was proposed that D1-Glu189 is part of this hydrogen bonded network. We hypothesised that replacing D1-Glu189 with neutral (Gln) and positively charged (Lys, Arg) residues would influence the rates of electron transfer from the Mn cluster to Y_Z^{ox} and from Y_Z to $P_{680}^{+\bullet}$ either electrostatically or by altering the hydrogen bonded network. Contrary to our expectations, we found that the O_2-evolving core particles of the mutants exhibited normal rates for electron transfer reactions to and from Y_Z. In the absence of a high-resolution structure of PS II, there are at least four different interpretations of these data.

(1) The environment of D1-Glu189 is strongly hydrophobic, forcing any side chain at position D1-189 to be uncharged, in particular Glu in the wild-type and Arg and Lys in the mutants. However, because D1-Glu189 is assumed to be close to Y_Z and the Mn cluster (see below), this possibility is difficult to reconcile with modelling studies that predict the environment of Y_Z to be hydrophilic [62,63] and with spectroscopic studies that show the Mn cluster to be readily accessible to alcohols [64] and Y_Z to be readily accessible to solvent [28,65] and reductants [66,67], at least in the absence of the Mn cluster.

(2) The absence of any significant effect of mutations of D1-Glu189 on the electron transfer might be caused by a location of D1-Glu189 which is so far away from Y_Z that electrostatic effects on the OEC, Y_Z and $P_{680}^{+\bullet}$ are negligible. However, this interpretation is difficult to reconcile with a wealth of indirect evidence for placing D1-His190, the direct neighbour of D1-E189, in close vicinity of Y_Z. This has been inferred from structural modelling based on the bacterial reaction centre [62,63,68], on studies that show that D2-His189 is the proton acceptor for Y_D [69–72] and on experiments with site-directed mutagenesis that reveal an impaired electron transport from Y_Z to P_{680} in D1-His190 mutants [9–12,17,73–75]. The slowed electron transfer from Y_Z to P_{680} in D1-His190 mutants is accelerated after addition of imidazole or other small organic bases [9,12] or by raising the pH [10,12]. According to modelling studies [62,63] D1-Glu189 and D1-His190 are located on the intersection of the two helices CD and D of the D1 subunit. In the crystal structure of photosystem II at 3.8 Å resolution [76] this intersection seems to lay at a distance from the postulated location of Y_Z on the C-helix. Unfortunately, a firm estimate of the distance between Glu189/His190 and Y_Z must await a crystal structure obtained at higher resolution.

(3) A third, though pretty far fetched possibility is that electrostatically active mutations at position D1-189 affected the redox midpoint potentials of the Mn cluster, Y_Z and P_{680} in parallel fashion. Especially because we found that the electron back-transfer between $Q_A^{-\bullet}$ and $P_{680}^{+\bullet}$ was not affected by the mutation D1-E189Q in Mn-depleted core particles (Table 1), we consider this possibility unlikely.

(4) Another possibility is that a cluster of acid/base groups around D1-Glu189 compensates the alteration of the charge at this residue, so that the properties of its direct neighbour, D1-His190, are virtually unchanged. This possibility would be compatible with the assumption that D1-Glu189 participates in a network of hydrogen bonds involving D1-His190 and Y_Z [16,17,20].

In conclusion, our data imply that residue D1-X189, the direct neighbour of D1-His190 which seems to directly interact with Y_Z, could either be forcedly electroneutral (strongly hydrophobic environment!), remote from Y_Z, or located in a cluster of acids and bases that compensates the charge at position D1-189. The first two interpretations call for a revision of current concepts. A discrimination between these possibilities has to wait for the assignment of amino acids to the crystal structure. Our data suggest that D1-Glu189 does not play a *special* role as an acid/base or as an electrostatic modulator in photosystem II.

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