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The rate of reduction of oxidized redox-active tyrosine, Z^+, by exogenous Mn^{2+} is slowed in a site-directed mutant, at aspartate 170 of polypeptide D1 of Photosystem II, inactive for photosynthetic oxygen evolution

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Eleven different site-directed mutants at Asp-170 of Photosystem II polypeptide D1 of *Synechocystis* PCC 6803 show varying degrees of activity for light-driven water oxidation depending on the nature of the substitution (Nixon, P.J. and Diner, B.A. (1992) Biochemistry 31, 942–948). These range from the most active, glutamate, with near wild-type rates of oxygen evolution to the totally inactive serine and alanine. Photosystem II core complexes lacking the tetranuclear Mn cluster responsible for water oxidation and isolated from wild-type and D1-Asp170Ser, were compared with respect to the kinetics of reduction by Mn^{2+} of the photooxidized redox-active tyrosine, Z^+. The dependence of the rate of reduction of Z^+ on the Mn^{2+} concentration shows mixed first and second-order behavior in the case of the wild-type and second-order behavior alone in the case of the mutant. The second-order rate constants for the wild-type and mutant complexes were $3.1 \cdot 10^7$ M^{-1} s^{-1} and $5.5 \cdot 10^5$ M^{-1} s^{-1}, respectively. The ratio of these rate constants is consistent with the K_m values determined earlier for the blockage of charge recombination by Mn^{2+}. D1-Aspartate 170 is therefore implicated in a facilitated pathway for the oxidation of Mn^{2+}, which most likely includes participation in a high-affinity metal binding site (Mn^{2+} and/or Mn^{3+}) in the Photosystem II reaction center.

Introduction

We have recently shown [1] in *Synechocystis* 6803 that mutations at Asp-170 of polypeptide D1 of the Photosystem II (PS II) reaction center impair the ability of the mutant cells to photooxidize water to molecular oxygen. Of the eleven mutants isolated, two (Ala and Ser) have lost such activity entirely, while still retaining near-wild-type levels of PS II reaction centers. The site of lesion of this inactivation of the oxygen-evolving activity was localized to the water oxidation site. Photooxidized redox-active tyrosine, Z^+, could no longer be reduced except by charge recombination with the reduced primary quinone electron acceptor, Q_A^-. We showed that the more impaired the mutants were for water oxidation, the higher the concentration of exogenous Mn^{2+} was required to block charge recombination between Z^+ and Q_A^- in isolated PS II core complexes following a single saturating light flash. These core complexes, including those of wild-type (WT), had lost, during isolation, the secondary quinone electron acceptor, Q_B, and the tetranuclear manganese cluster responsible for water oxidation. In agreement with the observations of Hoganson et al. [2] the site of reduction by exogenous Mn^{2+} was found to be Z^+. In our earlier publication [1] we had determined the K_m for the blockage of charge recombination by measuring, following a single saturating light flash, how much Q_A^- was stabilized. A range of K_m

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Abbreviations: Chl, chlorophyll *a*; D1-Asp170Ser, a site-directed mutant of *Synechocystis* 6803 in which aspartate 170 of the D1 polypeptide of PS II has been replaced by serine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; P680, primary electron donor, a monomer or dimer of chlorophyll *a*; PS II, Photosystem II; Q_A, primary plastoquinone electron acceptor; TC31, a wild-type strain of *Synechocystis* 6803 in which the D1 polypeptide is encoded only by *psbA*3, the first two copies of *psbA* having been deleted; WT, wild-type, referring here to strain TC31; Z, secondary electron donor, D1-Tyr161.

(half blockage of charge recombination) was observed for the set of mutants, ranging from 1 μM for wild-type and 2 μM for the most conservative amino acid replacement (Glu) to 50–60 μM for the mutants most impaired in oxygen evolution (Asp170 to Asn, Ala and Ser). Experiments providing direct evidence for reduction of Z^+, while alluded to earlier [1] were not shown. These data are presented here and correspond to a direct spectroscopic detection of Z^+ in the blue region of the visible spectrum where Z^+-Z shows a bleaching peaking at 429 nm. This region of the spectrum is, however, complicated by interference from $P680^+$-P680 and Q_A^--Q_A. Careful selection of wavelengths was required to deconvolute the contribution specific to Z^+-Z.

Materials and Methods

The contribution of $P680^+$ in the 430 nm region was determined by measuring a flash-induced difference spectrum in core complexes isolated according to Rögner et al [3] from the D1-Tyr161Phe mutant of *Synechocystis* 6803 that has lost the redox active tyrosine, Z [4,5]. The flash-induced absorbance changes measured at 50 μs following a saturating actinic flash are composed exclusively of $P680^+$-P680 plus Q_A^--Q_A. The difference spectrum of Q_A^--Q_A (see below, Fig. 1) was subtracted and the remaining spectrum of $P680^+$-P680 is shown in Fig. 1. A minimum is observed at 433 nm. A flash-induced difference spectrum composed of Z^+-Z and Q_A^--Q_A was measured at 500 μs after a saturating actinic flash in core complexes isolated from the wild-type strain TC31, which contains only one (*psbA3*) of three copies of the gene encoding polypeptide D1 [1]. This spectrum was also corrected for the contribution of Q_A^--Q_A and is shown as Z^+-Z alone in Fig. 1. The spectrum of Q_A^--Q_A alone was measured between 270 ms and 1s using 20 μM benzidine to rapidly reduce Z^+ as described in Metz et al. [4]. This spectrum, also shown in Fig. 1, was normalized to its amplitude at 500 μs using the measured Q_A^- relaxation kinetics at 325 nm where Z^+-Z does not contribute. To measure the reduction of Z^+ specifically following a saturating light flash, measurements were made at 429 and 436 nm and the difference taken. This difference removes the contribution of $P680^+$-P680 (see Fig. 1). Q_A^--Q_A contributes about 35% to the 429–436 nm difference measured between 100–500 μs. The relaxation kinetics of Q_A^--Q_A alone were determined at 325 nm, corrected for the difference in extinction coefficient at 325 nm ($\epsilon = 13$ mM^{-1} cm^{-1} [6]) and 429–436 nm ($\epsilon = 1.6$ mM^{-1} cm^{-1}) and subtracted from the relaxation kinetics measured at 429–436 nm at each concentration of $MnCl_2$. The only remaining component is Z^+-Z.

All spectroscopic measurements were performed at room temperature using a flash detection spectrophotometer based on a design by Joliot et al. [7].

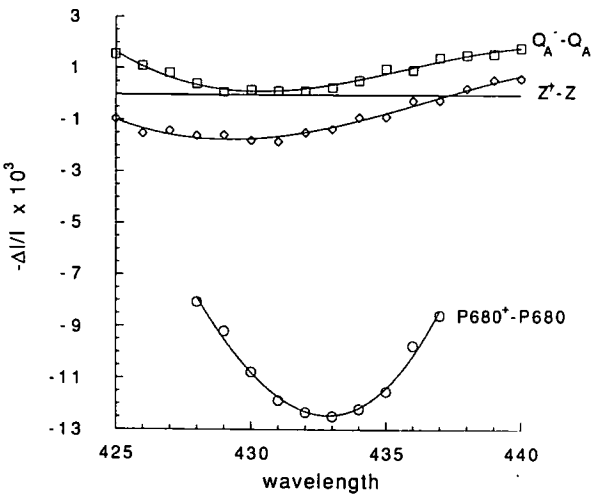


Fig. 1. Flash-induced absorbance changes in the 430 nm region. (○) Absorbance changes arising from $P680^+$-P680 measured 50 μs after a single saturating actinic flash in core complexes from mutant D1-Tyr161Phe suspended in 20 mM Mes (pH 5.7) containing 2 μM $K_3Fe(CN)_6$. The contribution of Q_A^--Q_A (see below) at 50 μs was subtracted from the overall absorbance changes to yeidl $P680^+$-P680 alone. (□), Absorbance changes in WT core complexes associated with Q_A^--Q_A at 0.5 ms after a single saturating actinic flash in the presence of 10 μM $K_3Fe(CN)_6$, 20 μM benzidine in 20 mM Hepes (pH 7.5). Measured as in Ref. 10. (◇) Absorbance changes in WT core complexes associated with Z^+-Z at 0.5 ms after a single saturating actinic flash in the presence of 1 μM $K_3Fe(CN)_6$ in 20 mM Hepes (pH 7.5). Measured as in Ref. 10. All core complexes were at 10 μM chlorophyll.

Core complexes were isolated [3] from wild-type strain TC31 and from mutant D1-Asp170Ser [1]. Saturating flash-induced absorbance changes were followed at 429–436 nm in core complexes suspended at 10 μM Chl in 20 mM Mes (pH 5.7), 1 mM $CaCl_2$, 1 μM $K_3Fe(CN)_6$ and at the indicated concentrations of $MnCl_2$. Corrections for the contribution of Q_A^--Q_A were made as indicated above under the same conditions.

Results and Discussion

The rates of reduction of Z^+ at various concentrations of $MnCl_2$ are indicated in Fig. 2A and B for WT (TC31) and Asp170Ser, respectively. The points were fit by a least-squares method to the sum of two variable exponential components for the mutant and the sum of two variable exponential components plus a fixed component (see below) using Kaleidograph 2.1 for Macintosh (Synergy Software). The kinetics of the slowest phase (4-40 s^{-1}) indicate it to correspond primarily to Z^+-Q_A^- charge recombination in those centers where Z^+ is not reduced by Mn^{2+}. Fig. 3 shows the pseudo-first-order rate constants for the faster of the two

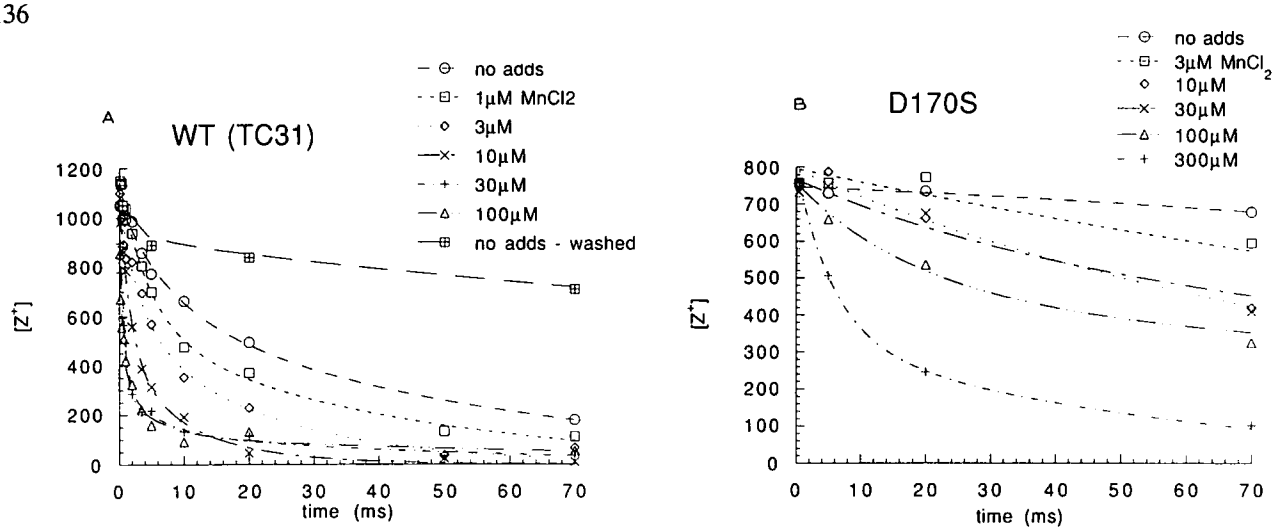


Fig. 2. Kinetics of reduction of Z^+ (expressed as $\Delta I/I \times 10^6$) following a single saturating actinic flash in core complexes at the indicated concentrations of $MnCl_2$. Measurements at 429–436 nm were corrected for the contribution of Q_A^--Q_A by measuring the latter at 325 nm and normalizing the Q_A^- relaxation kinetics to their contribution at 429–436 nm (see Materials and Methods). All core complexes were suspended at 10 μM chlorophyll in 20 mM Mes (pH 5.7), 1 mM $CaCl_2$, 1 μM $K_3Fe(CN)_6$. (A) Wild-type (TC31); (B) D1-Asp170Ser mutant.

variable phases plotted as a function of the Mn^{2+} concentration. In both mutant and wild-type the pseudo-first-order rate constants showed a linear dependence on Mn^{2+} concentration, apparently saturating in WT at ≥ 30 μM and remaining linear at least up to 300 μM in the Asp170Ser mutant. The second-order rate constants determined from the slopes of Figs. 3A and B were $(4.0–4.8) \cdot 10^7$ and $5.5 \cdot 10^5$ M^{-1} s^{-1} for the WT and mutant, respectively. The ratio of these rate constants is consistent with the relative K_m (1 and 60 μM, respectively) determined for the blockage by Mn^{2+} of Z^+-Q_A^- recombination in similar core complexes [1]. Another difference between the dependencies of the first-order rate constants on $[Mn^{2+}]$ is that the WT points extrapolate to an intercept on the ordinate axis well above the origin. One likely interpretation of this behavior is that there is a high-affinity binding site that saturates at ≤ 1 μM $MnCl_2$. This component would be expected to show first-order behavior characteristic of prebound Mn^{2+}. It is apparent in only 20–30% of the centers at pH 5.7 and would constitute a pathway for Z^+ reduction parallel to the second-order process. The presence of a tight binding component is also consistent with our inability to eliminate a fast component to Z^+ reduction ($k = 175$ s^{-1}) except by extensive washing or EDTA treatment of WT core complexes that slows the reduction to $k = 3–10$ s^{-1}, depending on pH. This is why two points are

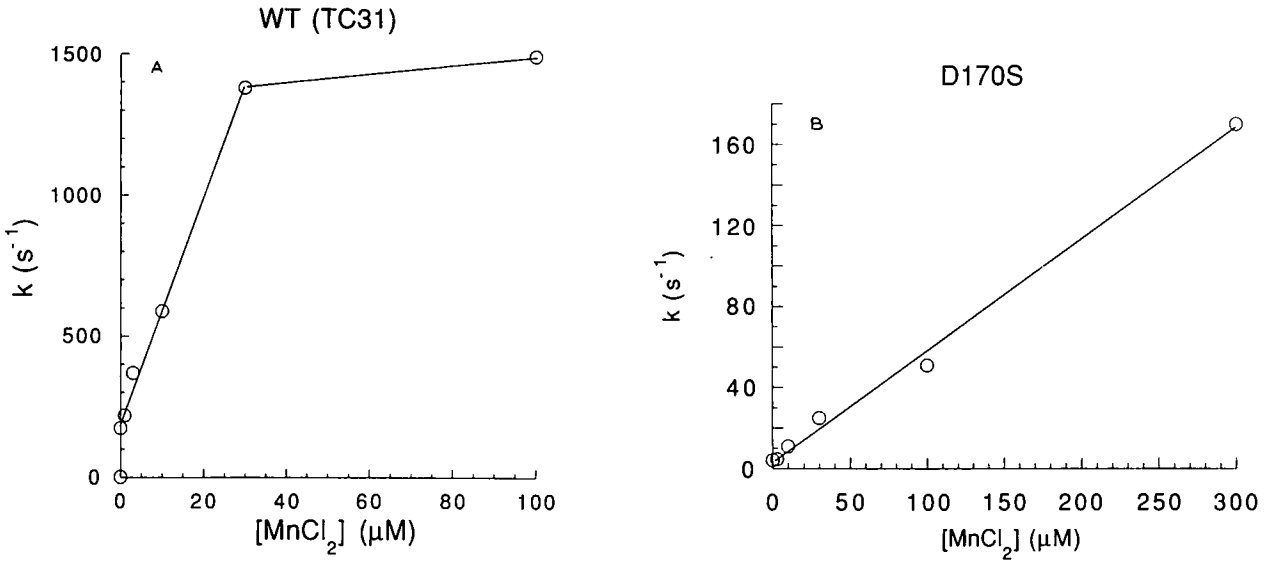


Fig. 3. Pseudo-first-order rate constants for the faster of two variable exponential components of Z^+ reduction as a function of $MnCl_2$ concentration. The rate constants were determined by biexponential fit of the data of Fig. 2 (see text) except in WT, where a third fixed exponential component ($k = 175$ s^{-1}) contributing 20–30% of the total relaxation was also present. (A) Wild-type (TC31); (B)-D1-Asp170Ser mutant.

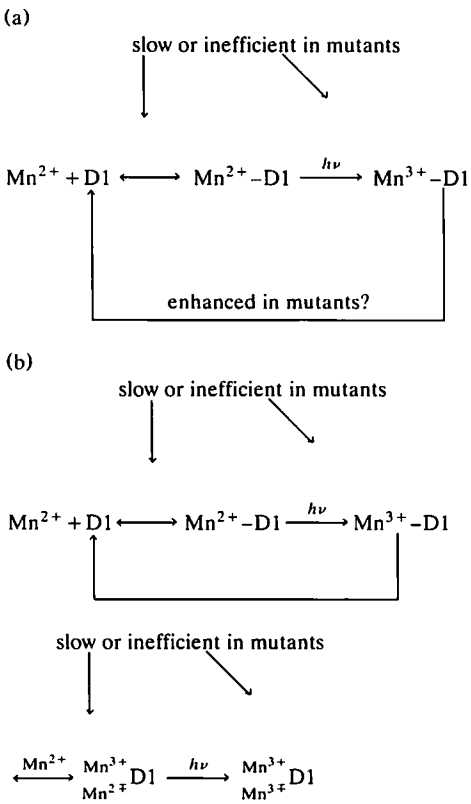
indicated for 0 μM $MnCl_2$ in Fig. 3A. The fitting of the WT data (Fig. 2A and 3A) was performed including, in addition to the two freely varying exponential components, a third constant first-order component ranging from 20–30% of the total with a first-order rate constant of 175 s^{-1}. The range of 20–30% contribution for the fixed component leads to the range of (4.0 to 4.8) · 10^7 M^{-1} s^{-1} for the second-order rate constant for Mn^{2+} reduction of Z^+ in WT. This component was often observed in WT core complexes in the absence of added Mn^{2+} but was never observed in the mutant core complexes. While in our earlier work [1] we could not see the second-order component below 10 μM, it is clear in this more detailed study that we can detect second-order behavior down to 1 μM.

The kinetic behavior we report here for WT core complexes resembles that of Hoganson et al. [2] in that there is apparently some inhibitory process or saturation that sets in at > 30 μM $MnCl_2$. The second-order rate constant measured here at pH 5.7 in the presence of 1 mM $CaCl_2$ is, however, 7-fold higher that that reported by these authors at pH 6.0 at the same $CaCl_2$ concentration. Among the possible explanations for this difference are that these authors used a different material, BBY membranes, but also repetitive measurements (200–500 experiments at 0.25 Hz) during which undissociated Mn^{3+} might have weakened the binding of fresh Mn^{2+} at a binding site near Z^+. Other steady-state measurements of inhibition by Mn^{2+} of diphenylcarbazide oxidation have been used tc estimate a binding constant for Mn^{2+} [8,9]. Such measurements actually yield an inhibition constant that is a function not only of the second-order rate constant measured here, but of the dark limiting steps that allow recovery of a reaction center from charge separation in saturating light and of the rate of dissociation of Mn^{3+}.

The observations reported here indicate that the mutation Asp170Ser results in impairment of a Mn^{2+} oxidation site present in WT. This observation is in agreement with the 60-fold higher K_m observed in the mutant as compared to the wild-type for the blockage of charge recombination by Mn^{2+} also in isolated core complexes. Together, they show that this blockage is caused by the reduction of Z^+ and not by intrinsic effects of Mn^{2+} on the kinetics of charge recombination. While the rates of Z^+ reduction were not directly measured in the other Asp170 mutants, the increase in the K_m for Mn^{2+} blockage of Z^+-Q_A^- charge recombination with respect to WT would suggest that in these mutants a pathway for Mn^{2+} binding and oxidation is impaired. The loss of a high-affinity Mn^{2+} binding site appears to account for at least part of the difference with WT.

We point out that all strains, despite their differing K_m are all capable of showing Mn^{2+} oxidation by Z^+. We have interpreted [1] the decreased oxygen-evolving activity as reflecting a reduced number of intact oxygen-evolving complexes. Mutants showing intermediate activity for oxygen evolution also showed near normal centers (with regard to S_2-state lifetimes and oscillation of a period four of oxygen flash yields) mixed with those unable to reduce Z^+ [1]. We propose that the photoactivation of the tetranuclear Mn cluster (photooxidation of bound manganese leading to assembly of the oxygen-evolving complex) is slowed resulting from the reduced probability of oxidizing Mn^{2+} during the lifetime of the charge-separated state. However, if a mutant can assemble intact oxygen-evolving complexes in a fraction of centers, why not in all of them?

An additional process must be occurring that reverses the photoactivation process, resulting, before there is further assembly of the Mn cluster, in a rereduction of Mn^{3+} back to Mn^{2+} followed by its release or, less likely, a dissociation of Mn^{3+}. Either (a) this loss reaction competes with the slowed manganese photoactivation in the mutants and may even be enhanced in these strains or (b) a second binding and oxidation step necessary for the stabilization of the first bound Mn^{2+} is also impaired in the mutants becoming less competitive with the loss reaction. These schemes are illustrated below:



Both schemes would result in a lowered steady-state concentration of the oxygen-evolving complex in the mutants as compared with wild-type.

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References

1 Nixon, P.J. and Diner, B.A. (1992) Biochemistry 31, 942–947.
2 Hoganson, C.W., Ghanotakis, D.F., Babcock, G.T. and Yocum, C.F. (1989) Photosyn. Res. 22, 285–293.
3 Rögner, M., Nixon, P.J. and Diner, B.A. (1990) J. Biol. Chem. 265, 6189–6196.
4 Metz, J.G., Nixon, P.J., Rögner, M., Brudvig, G.W. and Diner, B.A. (1989) Biochemistry 28, 6960–6969.
5 Debus, R.J., Barry, B.A., Sithole, I., Babcock, G.T. and McIntosh, L. (1988) Biochemistry 27, 9071–9074.
6 Van Gorkom, H.J. (1974) Biochim. Biophys. Acta 347, 439–442.
7 Joliot, P., Béal, D. and Frilley, B. (1980) J. Chim. Phys. 77, 209–216.
8 Hsu, B.-D., Lee, J.-Y. and Pan, R.L. (1987) Biochim. Biophys. Acta 890, 89–96.
9 Seibert, M., Tamura, N. and Inoue, Y. (1989) Biochim. Biophys. Acta 974, 185–191.
10 Diner, B.A. and De Vitry, C. (1984) Advances in Photosynthesis Research (Sybesma, C., ed.), pp. 407–411, Martinus Nijhoff/Dr. W. Junk, The Hague.