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Characterization of fluoride inhibition in photosystem II lacking extrinsic PsbP and PsbQ subunits

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ABSTRACT

Photosynthetic oxygen evolution occurs through the oxidation of water at a catalytic Mn_4CaO_5 cluster in photosystem II and is promoted by chloride, which binds at two sites near the Mn_4CaO_5 cluster. Fluoride is a competitive inhibitor of chloride activation, but study of its effects is complicated by the possibility that it may form an insoluble CaF_2 complex. In this study, the effects of fluoride were studied using PSII lacking the PsbP and PsbQ subunits, which help to regulate the requirements for the inorganic cofactors Ca^{2+} and Cl^-. In this preparation, which allows easy exchange of ions, it was found that F^- does not directly remove Ca^{2+} even when catalytic turnovers take place, suggesting that fluoride is not able to access the inner coordination sphere of Ca^{2+}. By monitoring the loss in O_2 evolution activity, the dissociation constant of F^- was estimated to be about 1 mM in intact PSII, consistent with previous studies, and about 77 mM in PSII lacking the extrinsic subunits. The significantly higher value for PSII lacking PsbP and PsbQ is consistent with results for other ions. The effects of F^- on electron transfer to Tyr Z was also studied and found to show similar trends in PSII with and without the two extrinsic subunits, but with a more pronounced effect in PSII lacking the extrinsic subunits. These results indicate that in PSII lacking PsbP and PsbQ, fluoride does not directly interact with or remove Ca^{2+} and inhibits O_2 evolution in a manner comparable to PSII with the extrinsic subunits intact.

1. Introduction

Photosystem II (PSII), the light-absorbing water oxidase of higher plants, algae and cyanobacteria, produces oxygen at a Mn_4CaO_5 cluster in an oxidation state cycle referred to as the S-state or Kok cycle [1–4]. The states, designated S_0 through S_4, increase one oxidation step at a time as an electron from the Mn_4CaO_5 cluster enters the electron transfer chain driven by light absorption at the PSII reaction center, P680. Once the transient S_4 state is reached, molecular oxygen is produced and the catalytic site returns to the S_0 state. The immediate electron acceptor that the Mn_4CaO_5 cluster donates to is a redox active tyrosine residue, referred to as Tyr Z, located about 7 Å from the cluster.

The Mn_4CaO_5 cluster is at the heart of the oxygen evolving complex (OEC), which includes amino acid residues, cofactors, and other protein structural features that assist in the conversion of H_2O to O_2. Chloride is an ion cofactor that is well known to be a requirement for oxygen evolution [5–7]. It has been shown that in its absence the S-state cycle is unable to proceed past the S_2 state. A single high affinity site for Cl^- was demonstrated for higher plant PSII using ^{36}Cl to track binding [8,9]. Similarly, using EXAFS a single site about 5 Å from the Mn_4CaO_5 cluster was found in spinach PSII when Cl^- was replaced with Br^-, which is more detectable in X-ray absorption or diffraction studies [10]. X-ray diffraction studies of PSII from thermophilic cyanobacteria have shown the presence of two binding sites for chloride located 7.4 and 6.7 Å from the Mn_4CaO_5 cluster [11–13]. While both Cl^- sites show ligation from amino acid backbone amide nitrogens, one of these (termed Site 1) is also ligated to a positively charged lysine residue side chain and is therefore of significantly higher affinity.

Fluoride, an inhibitor of oxygen evolution, is competitive with Cl^- activation with K_i values in the 2–8 mM range [14–16]. Fluoride also shows a relatively weak effect as an uncompetitive inhibitor with respect to Cl^- activation, with K_i' of 54–78 mM [15,16]. The secondary site of inhibition has recently been found to be associated with inhibition of electron transfer on the acceptor side of PSII [16]. F^- does not support the formation of the normal S_2 state multiline electron paramagnetic resonance (EPR) signal and under the same conditions enhances and alters the appearance of the S_2 state $g = 4.1$ EPR signal [17–21]. F^- treatment of PSII has also been found to lead to the formation of a broad radical signal at $g = 2$ [18,20,22], which indicates blockage of electron transfer from the Mn cluster to Tyr Z, the next site in normal electron transfer. The signal is very similar to the $S_2Y_Z^\bullet$ EPR signal observed in Ca^{2+}-depleted PSII [23–26], in which relatively

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labile Ca^{2+} is lost from the Mn_4CaO_5 cluster facilitated by removal of extrinsic PsbP and PsbQ subunits. However, whether F^- treatment results in loss of Ca^{2+} or prevents electron transfer through another mechanism has not been previously shown.

In higher plant PSII, the access and retention of Ca^{2+} and Cl^- at the OEC is regulated by several extrinsic subunits. Removal of two of these, the PsbP (23 kDa) and PsbQ (17 kDa) subunits, allows rapid exchange of chloride and facilitates study of chloride activation using enzyme kinetics methods. Their removal also introduces a partial or full Ca^{2+} dependence of oxygen evolution activity [27,28]. Because of the Ca^{2+} requirement, study of F^- inhibition in this preparation is potentially complicated by the possible formation of the insoluble CaF_2 compound which has $K_{sp} = 3.45 \times 10^{-11}$ at 25 °C [29]. For this reason, previous studies of F^- inhibition of PSII have been carried out using PSII with the extrinsic subunits bound ("intact PSII"). Because the PsbP and PsbQ subunits present a barrier for ion diffusion, this limits the types of experiments that can be performed to understand the nature of F^- inhibition in PSII. Since F^- is one of the few inhibitors that act almost completely competitively with respect to Cl^- activation, this has been an unfortunate hindrance to using F^- to better understand the site of Cl^- activation in PSII.

In the experiments presented here, the effects of fluoride on oxygen evolution have been explored in NaCl-washed PSII, which lacks the PsbP and PsbQ subunits. We first address the question of whether F^- directly removes Ca^{2+} from the OEC in PSII lacking the PsbP and PsbQ subunits (NaCl-washed PSII). Results indicate that it does not, even when catalytic turnovers take place, indicating that fluoride is not able to access the inner coordination sphere of Ca^{2+}. Next, the binding of inhibitory F^- to PSII lacking PsbP and PsbQ in the absence of added Ca^{2+} or Cl^- is quantified by monitoring oxygen evolution activity, with the finding that the affinity for F^- is lower in the absence of the extrinsic subunits. Finally, the properties of the Tyr Z radical induced by F^- treatment are characterized as a means to understand the interaction between the Tyr Z radical and the Mn cluster. These studies show that the effect of F^- on PSII lacking the extrinsic subunits is very similar to its effect on intact PSII, suggesting that F^- does not directly interfere with the function of Ca^{2+}.

2. Materials and Methods

2.1. Preparation of PSII Samples

PSII-enriched thylakoid membranes (intact PSII) were prepared from fresh market spinach by extraction with Triton X-100 as described previously [30] and modified by others [31,32]. The final preparation was stored in liquid nitrogen in buffer containing 20 mM MES-NaOH, pH 6.3, 0.4 M sucrose, and 15 mM NaCl.

NaCl-washed PSII, which lacks the PsbP (23 kDa) and PsbQ (17 kDa) subunits, was prepared essentially as described previously [33]. Intact PSII was incubated in buffer containing 1.5 M NaCl in 20 mM MES-NaOH, pH 6.3, and 0.40 M sucrose for 30 min on ice in the dark. After centrifuging for 10 min at 17,400 ×*g* in a Beckman Avanti J-25 high speed centrifuge, the PSII pellet was washed twice by centrifugation in buffer containing 20 mM MES-NaOH, pH 6.3, and 0.40 M sucrose. For experiments testing the loss of activity due to NaF treatment during illumination, 1 mM NaCl was also added to the wash buffer.

Rates of O_2 evolution activity were measured at 25 °C using a Clark-type O_2 electrode (Yellow Springs Instruments, model 5331) in the presence of 1 mM phenyl-*p*-benzoquinone (PPBQ) as electron acceptor, as described previously [34]. Intact PSII generally showed control rates of 600–800 μmol O_2 $mgChl^{-1}\,h^{-1}$ and NaCl-washed PSII showed control rates of 200–600 μmol O_2 $mgChl^{-1}\,h^{-1}$, depending on the preparation. Rates given represent the averages of three or more separate measurements.

2.2. Tests for Activity Loss Due to NaF During Illumination

For experiments to test the effect of F^- treatment during catalytic turnovers, intact PSII or NaCl-washed PSII was microcentrifuged to pellet and resuspended to 1.0–1.2 mgChl mL^{-1} in buffer containing 20 mM MES-NaOH, pH 6.3, and 0.40 M sucrose (SM buffer) with the addition of 25 mM NaF or NaCl for NaCl-washed PSII and 20 mM NaF or NaCl for intact PSII. Samples were illuminated using a 150 W halogen lamp while on ice for up to 4 min in the presence or absence of 2–4 mM PPBQ (added from a 50 mM stock solution in DMSO), as specified; at the sample position the illuminance was about 11×10^3 lx, which corresponds to an irradiance of about 16 W m^{-2} using a wavelength of 555 nm for energy conversion. Samples were then incubated in the dark on ice for 1–1.5 h before washing by microcentrifugation with SM buffer containing 25 mM NaCl to remove the illumination buffer. A control sample was prepared by incubating on ice in the matching NaCl-containing buffer in the dark. O_2 evolution assays were carried out in SM buffer in the presence of 25 mM NaCl or 12.5 mM $CaCl_2$ for NaCl-washed PSII and 20 mM NaCl or 10 mM $CaCl_2$ for intact PSII. Calcium dependence was calculated by comparing the activity in the presence of NaCl versus $CaCl_2$.

2.3. Fluoride Binding/Cl^- Displacement Study

To find the binding affinity of F^- for PSII, the loss in activity was tracked as F^- was added in the presence of very little or no added Ca^{2+} and Cl^-. Because the activity of NaCl-washed PSII is generally low in the absence of added Ca^{2+} or Cl^-, various pretreatments were carried out to increase the initial activity for the Cl^- displacement/F^- binding studies. Parallel pretreatments were carried out for intact PSII samples, which were studied for comparison. NaCl-washed or intact PSII (about 1 mgChl mL^{-1}) was thawed from storage, microcentrifuged to pellet and brought back to the original volume using SM buffer containing either 10 mM $CaCl_2$ or 20 mM NaCl. Where indicated, samples were illuminated for 2 min as described in the last section at an illuminance of about 4000 lx (or about 6 W/m^2 for 555 nm light). Samples were then incubated on ice in the dark for 30 min before O_2 evolution assays were begun. The effects of the pretreatments were assessed by measuring O_2 evolution activity in the absence of added Ca^{2+} or Cl^- (initial activity) and in the presence of 10 mM $CaCl_2$ (control activity). For NaCl-washed PSII, the initial activity in the absence of Ca^{2+} or Cl^- was 26–28% of the control for all four of the samples used, where the control activity was 190–260 μmoles O_2 $mgChl^{-1}\,h^{-1}$. The pretreatments were found to approximately double the initial activity of the NaCl-washed PSII samples, probably due to carry-over of Cl^- from the pretreatment buffer. For intact PSII, the initial activity in the absence of Ca^{2+} or Cl^- was 91–100% of the control, where the control activity was 640–790 μmoles O_2 $mgChl^{-1}\,h^{-1}$.

Using the intact and NaCl-washed PSII samples described above, the binding of F^- was assessed by monitoring the loss of activity in the presence of increasing amounts of NaF up to 20 mM. The concentration of Cl^- present during the assay was 0.4 mM due to carry over from the pretreatment buffer; the concentration of Ca^{2+} was 0.2 mM initially for those samples pretreated with $CaCl_2$, but this amount was assumed to be removed as CaF_2 by the first addition of NaF ($K_{sp} = 3.45 \times 10^{-11}$ at 25 °C). Assays were carried out at 25 °C in SM buffer containing the indicated amounts of NaF, but no added Ca^{2+} or Cl^-. The initial activity was taken as full activity and the amount of active PSII as F^- was added was then expressed as a fraction of the full activity. This removed from the calculation a need to know the true enzyme or PSII concentration, which was generally around 20 μgChl mL^{-1} or 0.09 μM.

2.4. EPR Spectroscopy of Tyrosine Radical

Intact PSII samples were prepared for EPR spectroscopy by washing by centrifugation in SM buffer containing the indicated amount of NaCl