



High-efficiency oxygen evolution by photosystem II oxygen-evolving complex containing 3Mn per reaction center

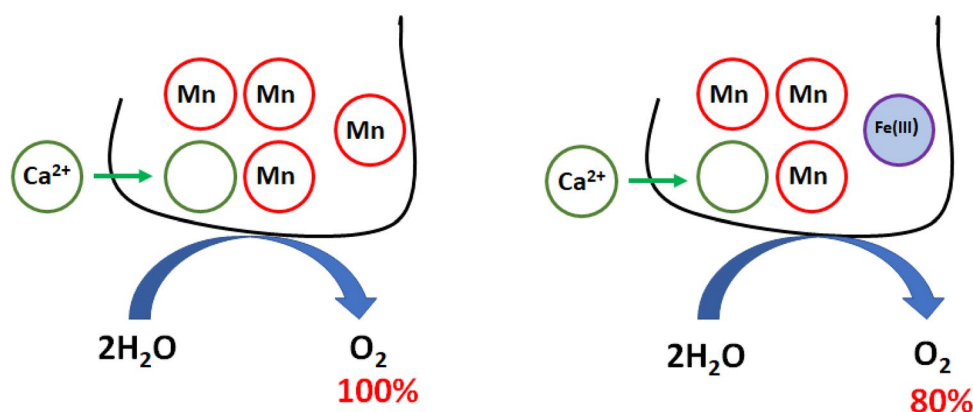
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Abstract

Ca-depleted photosystem II membranes obtained by treatment with acidic buffer do not contain Ca^{2+} in the Mn_4CaO_5 cluster but contain all extrinsic proteins protecting this cluster (PSII(-Ca/low pH)). However, unlike native photosystem II, Mn cluster in PSII(-Ca/low pH) samples is available for small-sized reductants. Using this property, we investigated the substitution possibility of Mn cation(s) with Fe cation(s) to obtain a chimeric cluster in PSII(-Ca/low pH) samples containing extrinsic proteins. We found that Fe(II) cation replaces Mn cation at pH 6.5, however, PSII(-Ca/low pH) membranes with the 3Mn1Fe chimeric cluster in the oxygen-evolving complex evolve O_2 with high intensity in the presence of exogenous Ca^{2+} . The O_2 evolution rate is about 80% of the same rate in PSII(-Ca/low pH) membranes.

Graphical abstract



Keywords Photosystem II · Oxygen-evolving complex · Manganese · Ferrous iron · Extrinsic proteins · Calcium

Abbreviations

Chl	Chlorophyll	MES	2-(N-morpholino)-ethanesulfonic acid
DCBQ	2,6-Dichloro-1,4-benzoquinone	OEC	Oxygen-evolving complex
HA	High-affinity Mn-binding site	PSII	Photosystem II
H_2Q	Hydroquinone	PSII(-Ca)	Ca-depleted PSII
		PSII(-Ca/low pH)	Ca-depleted PSII membranes using low pH treatment
		PSII(-Ca/NaCl)	Ca-depleted PSII membranes using NaCl treatment
		PSII(-Ca/low pH/3Mn1Fe)	Low pH-treated PSII(-Ca) membranes with chimeric cluster 3Mn1Fe

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PSII(-Mn)	Mn-depleted PSII membranes
RC	Reaction center
TMB	3,3',5,5'-Tetramethylbenzidine

Introduction

Photosystem II (PSII) in oxygenic photosynthetic organisms has a catalytic complex that oxidizes water under illumination and synthesizes the molecular oxygen. The main component of the oxygen-evolving complex (OEC) is a cluster Mn_4CaO_5 formed by 4 manganese cations and one calcium cation interconnected by oxygen bridges [1]. Mn/Ca cluster can be extracted from PSII using various methods, including its treatment by NH_2OH , Tris free base, lipophilic chelator, N,N,N',N'-tetrapropionato-1,3-bis(aminomethyl)benzene (TPDBA) [2]. Mn-depleted PSII samples (PSII(-Mn)) have the high-affinity (HA) Mn-binding site [3]. Possibly there is a second Mn-binding site in such preparations [4].

HA site in the PSII(-Mn) samples binds with high-affinity Mn(II) cation, then bound Mn(II) cation is oxidized by Y_Z [5]. This reaction is a primary step in the photoactivation process [6]. During the study of the possibility of interaction with the HA site the cations of other metals, we found highly effective binding of iron cations to this site [7]. Binding is observed at very low concentration of Fe(II) cations comparable to the binding Mn(II) concentration – 2 to 4 μM [7, 8]. Such a large similarity in binding to the HA site between Mn(II) and Fe(II) cations can be due to at least two factors: (1) the ionic radii are very close: 0.80/0.66 Å and 0.74/0.64 Å for Mn(II)/Mn(III) and Fe(II)/Fe(III), respectively; (2) Mn and Fe cations have the possibility of redox transformation at physiological pH utilized in many enzymes. In subsequent studies [8], we found that Fe cations irreversibly bind to the HA site (the bound cation cannot be removed by washing with buffer but can be extracted by citrate treatment at the acidic pH [9]). Irreversible binding needs illumination by weak light (like Mn(II) binding during photoactivation process) providing the oxidation of Fe(II) cation on the HA site [8]. Fe(III) cation bound to the HA site prevent the binding of Mn(II) cation to this site and its oxidation. In fact, Fe(III) cation blocks the HA site. According to kinetic measurements, blocking of the HA site is determined not by one iron cation, but by two or more [7, 8]. Measurement of the Fe(II) concentration in the medium during blocking showed that oxidation of 5 Fe(II) cations is necessary for this effect [10]. As in the case of photoactivation a dark interval is needed when blocking occurs under pulsed light [11]. The carboxyl-containing amino acid involved in binding the Mn(II) cation at the HA site is involved in binding the blocking iron cation [9]. High

similarity in the interaction of Mn(II) and Fe(II) with the donor side of Mn-depleted PSII membranes is supported by the fact that Fe(II) cation binding is accompanied by reconstruction of the low barrier hydrogen bond between Y_Z and D1-His190 [12]. However, despite the great similarity between Mn(II) and Fe(II) cations in the mechanism of their interaction with PSII(-Mn) membranes, neither oxygen release nor electron transport was found in the PSII(-Mn) preparations containing iron cations.

The high binding efficiency of Fe(III) cation to the Mn-binding site and its significantly low redox potential (about 0 V for Fe(II)/Fe(OH)₃ at pH 7.0 [13]), characterizing the Fe(II) cation as a strong reducing agent, made it possible to obtain preparations of PSII with chimeric clusters in the OEC consisting of manganese and iron cations. To carry out such substitution, we incubated Ca-depleted PSII membranes (PSII(-Ca)) without extrinsic proteins PsbP and PsbQ with Fe(II) cations (to get access for cations to the manganese cluster) in the dark in buffer with pH 6.5. We found that Fe(II) cations substitute two of the four Mn ions in PSII(-Ca) [14]. No O₂ evolution is observed, but the heteronuclear metal cluster in PSII(2Mn,2Fe) samples is still able to supply electrons for reduction of the exogenous electron acceptor by photooxidizing water and producing H₂O₂ [14]. The decrease in the pH of the incubation buffer up to pH 5.7 is accompanied by an increase in the resistance of one of the Mn cations in the cluster to the action of reducing agents (Fe(II), hydroquinone) [15]. Incubation of the PSII(-Ca) membranes obtained by the NaCl treatment (PSII loose PsbP and PsbQ proteins after such treatment) preparation with Fe(II) cations at pH 5.7 is accompanied by substitution not 2 Mn cations, but only one [16]. NaCl-treated PSII(-Ca) membranes with chimeric cluster 3Mn1Fe are able to reduce the exogenous electron acceptors oxidizing water to H₂O₂. However, these preparations are able also to produce O₂ in the presence of exogenous Ca²⁺ [16]. The rate of O₂ generation is about 27% in relation to native membranes (PSII).

It should be noted that we did not use native PSII membranes for substitution, but Ca-depleted PSII membranes that did not contain two extrinsic proteins – PsbP and PsbQ and Ca²⁺ cation in the OEC. In this regard, in the proposed work, we investigated the possibility of preparing a PSII samples with a chimeric cluster in OEC but containing extrinsic proteins PsbP and PsbQ. To this end, we used PSII preparations obtained by treating of PSII membranes by citrates in acid buffer [17]. Such PSII membranes do not contain a Ca²⁺ in the OEC but do contain all extrinsic proteins. But, crucially, unlike the native PSII membranes in citrate-treated PSII (-Ca) membranes, the Mn cluster is available for small-sized reducing agents [18, 19]. Therefore, we used such citrate-treated PSII(-Ca) preparations to replace the manganese cation with iron cations and found that at pH 6.5, one Mn cation in OEC is substituted, but the preparations with the

chimeric cluster 3Mn1Fe in the presence of exogenic Ca^{2+} release oxygen at a high rate, which is about 80% of the control (PSII(-Ca/low pH)).

Materials and methods

Preparation of PSII-enriched membranes

Membranes (BBY type) were prepared from market spinach according to Ghanotakis and Babcock [20]. Samples were stored at $-80\text{ }^{\circ}\text{C}$ in buffer A (50 mM 2-(N-morpholino)-ethanesulfonic acid (MES) at pH 6.5, 15 mM NaCl, and 400 mM sucrose). The rates of O_2 evolution ($450\text{--}550\text{ }\mu\text{mol O}_2\text{ mg chlorophyll (Chl)}^{-1}\text{ h}^{-1}$) were measured polarographically using a Clark-type oxygen electrode under continuous, saturating white light illumination (through a water filter) at $25\text{ }^{\circ}\text{C}$ with 0.2 mM 2,6-dichloro-1,4-benzoquinone (DCBQ) as the exogenous electron acceptor. Concentration of PSII during the measurement of oxygen evolution was $10\text{ }\mu\text{g Chl/ml}$. Chlorophyll concentrations were determined in 80% acetone, according to the method of Porra et al. [21].

Preparation of Ca-depleted PSII membranes (PSII(-Ca))

Two types PSII(-Ca) membranes were prepared. 1. PSII(-Ca) membranes without extrinsic proteins PsbP and PsbQ (PSII(-Ca/NaCl)) were prepared according to [22]. PSII membranes (0.5 mg/ml Chl) were incubated in the buffer 2 M NaCl, 0.4 M sucrose, and 25 mM MES (pH 6.5) for 15 min at room temperature under low illumination ($4\text{--}5\text{ }\mu\text{E m}^{-2}\text{ s}^{-1}$, room fluorescent light). The resulting material was washed twice and resuspended in a buffer A. Besides Ca^{2+} cation PSII(-Ca/NaCl) membranes lack the PsbQ and PsbP extrinsic proteins, which prevent exogenous reducing agents from attacking the Mn/Ca cluster. 2. PSII(-Ca) membranes containing PsbQ and PsbP extrinsic proteins (PSII(-Ca/low pH)) were prepared according to [17]. PSII membrane preparations in a buffer A were centrifuged, and the pellets resuspended in citrate buffer (400 mM sucrose, 20 mM NaCl, 10 mM sodium citrate, pH 3.4) at 2 mg Chl ml^{-1} . Further, the membranes were incubated for 5 min on ice in the dark. The samples were finally diluted (1:3 v/v) with buffer A (adjusting pH to 6.5) and incubated for 20 min at $4\text{ }^{\circ}\text{C}$ in the dark. The membranes were then pelleted, washed once with buffer A, and subsequently resuspended in buffer A.

Determination of Mn content in the PSII membranes

Mn concentrations in different samples were measured as described in a previous publication [23] with minor modifications [24]. Samples ($100\text{ }\mu\text{g Chl}$) were suspended in 1 ml

of buffer A and then incubated with 50 mM CaCl_2 for 2 min in the dark at $5\text{ }^{\circ}\text{C}$. After incubation, the samples were pelleted. The pellet was resuspended in $90\text{ }\mu\text{l}$ of 0.6 N HCl to solubilize the functional Mn remaining in the pellet. Next, 0.9 ml of glass-distilled water was added to the membrane suspension, and finally, the microfuge tube was centrifuged for 3 min at $15,000\times g$ ($22\text{ }^{\circ}\text{C}$). The supernatant (0.9 ml) was filtered through a 13-mm Acrodisc syringe filter containing a $0.2\text{ }\mu\text{m}$ nylon membrane (Pall Life Sciences, Ann Arbor, USA). The filtrate (in a 1-ml glass cuvette) was mixed consecutively according to Serrat [25] with $40\text{ }\mu\text{l}$ of 2 M NaOH, $40\text{ }\mu\text{l}$ of a stock solution of 3,3',5,5'-tetramethylbenzidine (TMB) (100 mg TMB in 100 ml of 0.1 M hydrochloric acid), and $40\text{ }\mu\text{l}$ of 5.3 M phosphoric acid. The absorbance at 450 nm was used to calculate Mn(II) concentrations (extinction coefficient of $34\text{ mM}^{-1}\text{ cm}^{-1}$) in the samples [25]. PSII reaction center (RC) concentrations were calculated in μM using 250 molecules of Chl/RC in the PSII samples [26, 27].

Substitution of Mn ions in PSII(-Ca/low pH) membranes with Fe cations

PSII(-Ca/low pH) membranes ($100\text{ }\mu\text{g Chl/ml}$) were incubated with Fe(II) for 120 min in the dark at $4\text{ }^{\circ}\text{C}$ in buffer A (pH 6.5). After incubation, the membranes were pelleted, washed, and resuspended in buffer A.

Determination of protein profiles of protein composition in spinach PSII membranes

One-dimensional SDS-polyacrylamide gel electrophoresis was performed in polyacrylamide gel by the Laemmli method [28] using 6% concentrating gel in 1.0 M Tris-HCl (pH 6.8), 0.4% SDS and 15% separating gel in 1.0 M Tris-HCl (pH 8.8). The protein samples for electrophoresis were prepared in a fourfold buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 40% sucrose, 0.05% bromophenol blue, 5% β -mercaptoethanol) and incubated for 5 min at $95\text{ }^{\circ}\text{C}$. Electrophoresis was performed in Mini-Protean III cell (Bio-Rad, USA). When the bromophenol blue has run to the bottom of the gel the electrophoresis was terminated by 1-h incubation at room temperature in solution containing 25% isopropanol and 10% acetic acid. Then, proteins on the gel were stained for 1 h with Coomassie R-250 (Bio-Rad) and incubated overnight with a solution, containing 25% ethanol and 7.5% acetic acid. Then, gel was washed two times in the distilled water.

Results and discussion

Exposure of PSII to high ionic strengths ($\geq 1\text{ M NaCl}$) is used often to extract Ca^{2+} from the OEC [22, 29, 30]. Addition of Ca^{2+} to such preparation reactivate the

oxygen-evolving function, however, such membranes don't contain extrinsic PsbP and PsbQ polypeptides. Ono and Inoue [17] suggested another method of Ca^{2+} extraction from the OEC. They used citrate treatment of PSII membranes in acidic pH buffer followed by pH adjustment (using MES buffer) to normal pH value (6.5). This procedure provides the reconstruction of all extrinsic proteins, i.e., the preparation of Ca-depleted PSII membranes containing all extrinsic proteins. Rebinding of the PsbP and PsbQ polypeptides to PSII in the absence of added Ca^{2+} does not restore activity, whereas addition of exogenous Ca^{2+} recover upon long-term incubation the O_2 evolution reaction [17] which indicates the possibility of metal cation access to the Mn cluster through the protective layer of extrinsic proteins. Indeed, Yocum with coworkers [18, 19] found that Mn cluster in the OEC of the low pH-treated PSII preparation is available for small type reductant like NH_2OH but not too large like hydroquinone (H_2Q). The reason for this may be the channel between the solvent and the Mn cluster, that is normally occluded by a bound Ca^{2+} cation [18].

Availability of Mn cluster for H_2Q in the low pH-treated PSII(-Ca) membranes

We used this method to prepare PSII membranes that do not contain Ca^{2+} but contain all extrinsic proteins: PSII membranes were incubated for 5 min in the dark in citrate buffer (pH 3.4) then the suspension was diluted with buffer A (MES buffer), adjusting the pH to 6.5. After dilution, the buffer contained 25% citrate buffer and 75% buffer A. However, before incubation of the obtained PSII(-Ca) preparations with Fe(II) cations in order to replace Mn cation(s) in the OEC, it was necessary to remove citrate from the buffer, since citrate can bind iron cations and first stability constants ($\log K_1$) of Fe(II) and Fe(III) cations chelates with citric acid is equal to 3.2 and 11.85, respectively [31]. Therefore, we removed citrate from the medium after dilution by transferring/centrifugation of the sample using buffer A. As we mentioned before, extrinsic proteins do not pass the large reductants like hydroquinone to the manganese cluster in low pH-treated PSII samples [18]. This is an important feature of low pH-treated PSII membranes and therefore, we tested the presence of such property in our preparations. Results of experiments are given in Table 1. Obtained data clearly show that H_2Q cannot extract Mn cations from the low pH-treated PSII(-Ca) membranes, while Mn cluster is available for H_2Q in the NaCl-treated PSII(-Ca) samples. These data are consistent with the results obtained by Vander Meulen et al. [18] and indicate that the Mn cluster in the low pH-treated PSII(-Ca) membranes is protected by extrinsic proteins.

Table 1 Effect of hydroquinone on the O_2 evolution rate in NaCl or low pH-treated PSII(-Ca) samples

Sample	^a Treatment by H_2Q	^b O_2 evolution rate	Mn/RC	References
PSII(-Ca) obtained by NaCl treatment	–	341 ± 19 ^c (100%)	4.0 ± 0.2	[16]
	+	55 ± 4 (16%)	1.0 ± 0.2	[15]
PSII(-Ca) obtained by low pH treatment	–	301 ± 10 (88%)	4.0 ± 0.2	Present work
	+	200 ± 12 (59%)	3.8 ± 0.2	Present work

^aTreatment by H_2Q : PSII(-Ca) membranes (20 μg Chl/ml) were incubated with H_2Q (200 μM) for 20 min in dark at 4 °C

^bOxygen-evolution rate was measured in the presence of 30 mM Ca^{2+}

^c100%—the rate of oxygen evolution in the NaCl-treated PSII(-Ca) membranes

Effect of Fe(II) cations on the Mn content in low pH-treated PSII(-Ca) membranes and on their oxygen-evolving activity

Unlike large reductant H_2Q small reductant Fe(II) cations can reach Mn cluster in PSII(-Ca/low pH) samples and interact with Mn cations. The incubation of the low pH-treated PSII(-Ca) membranes with Fe(II) is accompanied by a decrease in Mn cation content in the OEC per reaction center with time incubation (Fig. 1). The degree of Mn extraction reaches a saturation value 1Mn/RC after about 80 min incubation in the dark at 4 °C and Fe(II) concentration 80 μM . The kinetics of the Mn cation displacement (presence of saturation) by the iron cation indicate that the pool of Mn cations available for reduction is exhausted and, in the area binding the Mn cations, only cations unavailable for reduction remain. According to results presented in Fig. 1 the number of remaining Mn cations per RC is 3, i.e., it is not a mixture of RC with different Mn content giving an average of 3. Similar (in nature) effect occurs during extraction of Mn cations by hydroquinone [15, 32] and by Fe(II). It was found that Fe(II) extract 2Mn cations at pH 6.5 [14] and 1 Mn/RC at pH 5.7 [16]. We suggest that it takes place the formation of a cluster consisting of 3 Mn cations and possibly 1 Fe (III) cation. In favor of the latter suggestion, it was demonstrated very high efficiency of the Fe cation binding to the free Mn-binding site [7, 8]. The vacant Mn-binding site appears as result of the Mn cation reduction by Fe(II) cation and release of Mn(II) cation from binding site. This is evidenced by the fact that (a) binding takes place only in samples where Mn is available for Fe(II) cations (there is

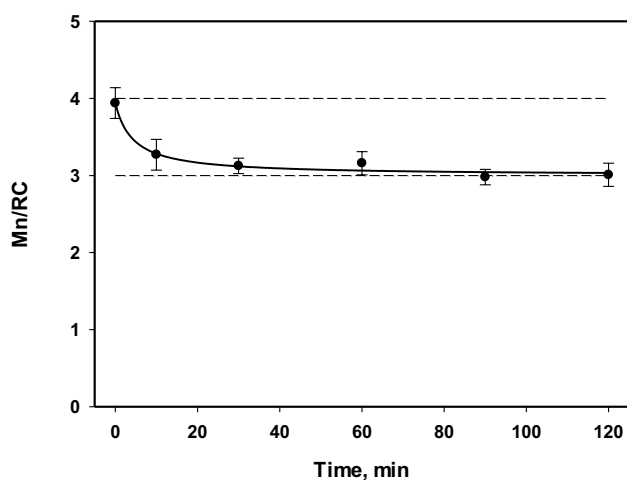


Fig. 1 Content of Mn cations in the low pH-treated PSII(-Ca) preparations after incubation with Fe(II) cations. Membranes (100 μg Chl/ml) were incubated with Fe(II) for 120 min in the dark at 4 °C in buffer A (pH 6.5)

no binding in intact PSII) [14, 16]; (b) binding is accompanied by removal of Mn cation from OEC. The presence of the Fe(III) cation in the mixed Mn/Fe cluster is indicated by an increase in the resistance of the Mn cations in such clusters to the action of H_2Q [16]. Figure 2 shows concentration dependence of substitution of Mn cation in the low pH-treated PSII(-Ca) membranes with Fe(II). An important circumstance should be noted here. Fe(II) cation replaces one manganese cation in the low pH-treated PSII(-Ca) membranes (all extrinsic proteins are present in the membrane) whereas it replaces 2 Mn cations in the NaCl-treated PSII(-Ca) preparation (PsbP and PsbQ are absent) although the pH of the incubation buffer was the same—6.5. This fact indicates a significant effect of PsbP and PsbQ extrinsic proteins on the properties of the Mn cluster (possibly on the redox potential value of the Mn cation(s) in the OEC).

Confirmation of this comment is the effect of Fe cation on the oxygen evolution in the low pH-treated PSII(-Ca) with the chimeric cluster 3Mn1Fe (PSII(-Ca/low pH/3Mn1Fe)) (Table 2). In the process of the O_2 evolution rate measuring in the PSII(-Ca/low pH/3Mn1Fe) membranes we discovered that such samples release oxygen at a high rate (Table 2). If we take the oxygen-evolving activity of PSII(-Ca) membranes (obtained by low pH treatment) as 100% in the presence of 30 mM exogenous Ca^{2+} (this value is equal to 248 μmol O_2/mg Chl h and corresponds to the level of reactivation of similar preparations measured by Vander Meulen et al. [18]), then after replacing one of the Mn cations with an iron cation, the activity of the preparation (in the presence of exogenous Ca^{2+}) decreases, but slightly—by about 20% (Table 2). That is, PSII(-Ca) membranes containing in

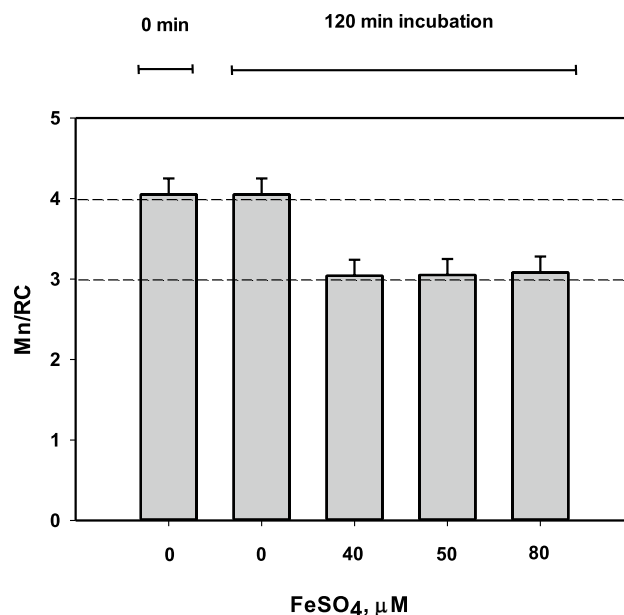


Fig. 2 Substitution of Mn cation in the OEC of low pH-treated PSII(-Ca) preparations with Fe(II) cation as a function of time incubation without Fe(II) cations (1st and 2d column) and as a function of Fe(II) concentration (columns 3–5)

the catalytic center not 4 Mn cations, but 3 together with 1 Fe cation oxidize water to molecular oxygen with rather high activity.

Effect of extrinsic proteins on the oxygen evolution in PSII(-Ca/low pH/3Mn1Fe) membranes

A feature of the PSII(-Ca/low pH/3Mn1Fe) membranes is the presence of all extrinsic proteins. And it can be assumed that this may be the reason for the high catalytic activity of the chimeric cluster. To test this assumption, we used the standard method of extrinsic proteins PsbP and PsbQ extraction by high concentration NaCl. To this end, PSII(-Ca/low pH/3Mn1Fe) membranes were pelleted, then the pellet was resuspended in buffer containing 2 M NaCl, 25 mM MES, 400 mM sucrose (pH 6.5), 0.5 mg Chl/ml. Membranes were incubated for 15 min at room temperature and illumination. Then membranes were washed twice by buffer A. Obtained results showed that washing of membranes PSII(-Ca/low pH/3Mn1Fe) with NaCl solution removes only 1 extrinsic protein (PsbQ), whereas washing intact PSII membranes removes 2 extrinsic proteins (PsbP and PsbQ) (Fig. 3, lanes 5 and 2, respectively). Washing of Ca-depleted membranes obtained by treatment with citrate buffer as in our work also extracts PsbP and PsbQ proteins [17]. The lack of 2 M NaCl effect on extrinsic protein PsbP is unexpected. This effect is evidence of stronger protein binding to the reaction center in membranes with chimeric

Table 2 The rate of oxygen evolution in the low pH-treated PSII(-Ca) membranes containing a chimeric cluster 3Mn1Fe and all extrinsic proteins

Sample	Presence of Ca ²⁺ in the buffer when measuring O ₂	Incubation of sample for 120 min at 4 °C in the dark	Rate of O ₂ evolution (μmol O ₂ /mg Chl h)
PSII(-Ca/low pH)	–	–	80 ± 4 (32%)
	+	–	248 ± 3 (100%)
	–	+	78 ± 1 (31%)
	+	+	234 ± 16 (94%)
PSII(-Ca/low pH) + 80 μM Fe(II)	–	–	86 ± 2 (35%)
	+	–	223 ± 13 (90%)
PSII(-Ca/low pH/3Mn1Fe)*	–	+	69 ± 6 (28%)
	+	+	198 ± 4 (80%)
PSII(-Ca/low pH/3Mn1Fe)**	–	+	74 ± 3 (30%)
	+	+	167 ± 20 (67%)

Conditions: low pH-treated PSII(-Ca) membranes (100 μg Chl/ml) were incubated in buffer A (pH 6.5) for 120 min at 4 °C in dark with or without FeSO₄. Determination of the O₂ evolution rate: the sample (20 μg Chl/ml) was incubated in buffer A (pH 6.5) for 10 min at 4 °C in dark with or without CaCl₂ (30 mM), then the rate of oxygen evolution was measured after DCBQ addition. Activity of the native PSII membranes was 490 μmol O₂/mg Chl h

*after incubation with 40 μM Fe(II)

**after incubation with 80 μM Fe(II)

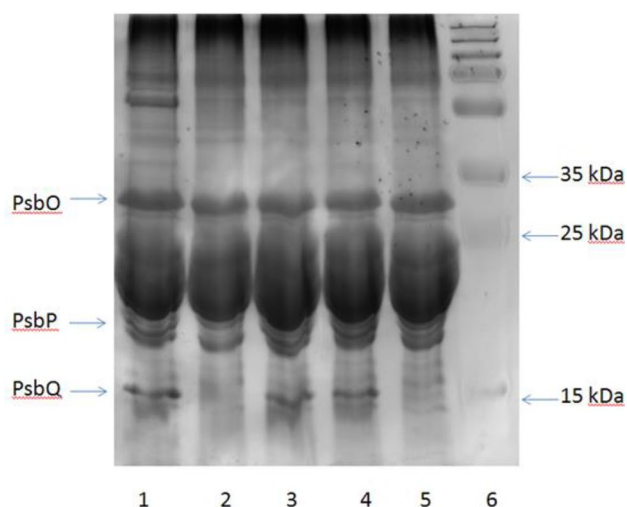


Fig. 3 Polyacrylamide gel electrophoretogram of PSII membranes fragments. Lane 1, intact PSII membranes; lane 2, NaCl-washed PSII(-Ca) membranes without PsbP and PsbQ extrinsic proteins; lane 3, low pH-treated PSII(-Ca) membranes; lane 4, low pH-treated PSII(-Ca) membranes after incubation with FeSO₄; lane 5, low pH-treated PSII(-Ca) membranes with chimeric cluster 3Mn1Fe in the OEC after 2 M NaCl treatment; lane 6, protein standards. The positions of the PSII extrinsic proteins (PsbO, PsbP, and PsbQ) are indicated by arrows on the left

cluster than in native membranes. Perhaps, stronger binding of this protein is carried out due to the participation of the amino acid residue of the PsbP protein in the coordination of the iron cation. In this regard, it should be noted that the PsbP protein has 2 Mn-binding sites and 1 Zn-binding site [see for review 33].

At the same time 2 M NaCl washing does not affect the activity of preparations: activity of PSII(-Ca/low pH/3Mn1Fe) membranes in the presence of exogenic Ca²⁺ (30 mM) without NaCl treatment was 189 μmol O₂/mg Chl (Mn/RC = 3.1) and after treatment activity was 202 μmol O₂/mg Chl (Mn/RC = 3.0). It should be noted that even in native PSII membranes, from which extrinsic proteins PsbP and PsbQ are extracted by a medium with high ionic strength (Ca²⁺ is simultaneously removed from OEC) high concentrations of calcium can support reasonably high rates of oxygen evolution even in the absence of the PsbP and PsbQ components [34]. These proteins are not involved in the binding of Mn and Ca²⁺ cations forming the catalytic center of OEC but serve to stabilize the Mn₄CaO₅ cluster and optimize oxygen evolution at physiological calcium and chloride concentrations and shield the catalytic center [33]. Therefore, the absence of NaCl effect on the rate of oxygen evolution in PSII(-Ca/low pH/3Mn1Fe) preparation is not surprising, since the oxygen evolution activity was measured in the presence of a large concentration of Ca²⁺. Moreover, the main extrinsic protein PsbP involved in stabilization of Ca²⁺ and Cl⁻ is not extracted (Fig. 3, lane 5) while the extracted protein PsbQ is much less effective in maintaining the function of OEC [33].

The question remains—why the substitution of Mn cation by Fe cation in the presence of PsbP and PsbQ proteins leads to significantly greater oxygen-evolving activity of the chimeric cluster 3Mn1Fe than substitution in their absence? It is not yet clear. We can suggest that the PsbP changes the conformation of the protein around the Mn cluster, which makes it more optimal for coordination of Fe or/and Mn.

For example, FTIR studies demonstrated that the binding of PsbP to PS II induced protein conformational changes in the vicinity of the Mn_4CaO_5 cluster [35]. Another hypothetical option is that a PsbP protein is involved in the coordination of the bound Fe cation, which increases the efficiency of the catalytic center.

Effect of citrate on PSII(-Ca/low pH/3Mn1Fe) membranes

We have previously shown that Fe(III) cation(s) blocking the HA Mn-binding site in the Mn-depleted PSII membranes can be extracted with citrate both at an acidic pH 3.0 and at a neutral pH 6.5 [9]. We used the same technique to extract the cation Fe(III) from the chimeric cluster (3Mn1Fe) in the NaCl-treated PSII(-Ca) membranes after substitution of 1Mn cation with iron cation at pH 5.7 [16]. Indirect results suggest that the Fe cation is extracted [16]. However, in the experiment conducted in the present work, we did not find extraction of iron cation by citrate from the chimeric cluster in the low pH-treated PSII(-Ca) membranes. This assumption is based on the fact that oxygen activity did not change and the amount of Mn cations in the cluster did not change.

Extrinsic proteins in the low pH-treated PSII(-Ca) membranes before and after substitution of manganese cation in the OEC with iron cation

The obtained results demonstrate that chimeric (3Mn1Fe) OEC in the PSII(-Ca) preparations with (present work) or without [16] PsbP and PsbQ extrinsic proteins are very different in its efficiency to release oxygen. Therefore, we examined the content of extrinsic proteins in the PSII(-Ca) preparations after various treatments (Fig. 3). To this end, we used polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate–urea. As controls, the electrophoretograms for intact PSII (Fig. 3, lane 1) and NaCl-treated PSII(-Ca) membranes (Fig. 3, lane 2) have been done. Control sample PSII lane (Fig. 3, lane 1) demonstrate the presence of all extrinsic proteins whereas NaCl-treated PSII(-Ca) membranes contain only one extrinsic protein—PsbO (Fig. 3, lane 2). Electrophoretogram of low pH-treated PSII(-Ca) membranes (Fig. 3, lane 3) has shown the presence all extrinsic proteins that is in line with results of [17]. After incubation of this sample with Fe(II) cations for substitution we did not observe the changes in the composition of extrinsic proteins (Fig. 3, lane 4). It is interesting that washing of PSII(-Ca/low pH/3Mn1Fe) membranes with NaCl solution (standard procedure for removal of PsbP and PsbQ extrinsic proteins) does not affect the oxygen-evolving activity (Table 2) and remove only one extrinsic protein – PsbQ (Fig. 3, lane 5). This result indicates that extrinsic proteins PsbP and PsbQ have significant effect on the substitution

of Mn cation with Fe cation and the properties of chimeric catalytic center in the OEC.

Conclusion

The most interesting result in our opinion is the high oxygen-evolving activity of the low pH-treated PSII(-Ca/3Mn1Fe) preparations since chimeric cluster has only 3 Mn cations in catalytic center. We have previously shown that in the NaCl-treated PSII(-Ca/2Mn2Fe) membranes one of Fe cations bound to the HA Mn-binding site [14]. The HA site in the native PSII is occupied by cation Mn4 (numbering according to work [1]), connected to an irregular cubane of 3 manganese cations and Ca^{2+} cation. We can suggest that Fe cation also associated with the HA Mn-binding site in the chimeric clusters 3Mn1Fe, i.e., the Fe cation possibly replaces the Mn4. It is assumed that Mn4 participates in the initiation and organization of the photoactivation process [3, 36, 37]. Mn4 has 2 water molecules W1 and W2 as ligands [1] and this can be important if one (or both) of these molecules is substrate water but currently there is no convincing evidence of this. In this regard, it should be noted that Mn4 is connected to the oxygen bridge O5 [1]. Currently, O5 (slowly exchanging water Ws binds as an OH^- in the S0 state being deprotonating during the oxidation to S1) is considered as the most likely oxygen atom involved in the synthesis O_2 [38]. Other ligands coordinating Mn4 are carboxyl-containing amino acid residues D1-Asp170 and D1-Glu333 [1]. Thus, the binding site Mn4 contains ligands typical of the Fe(III) coordination sphere and if the Fe(III) cation is bound to this site, then it can participate in the oxidation of one of the substrate molecules of water. A second substrate water may be the water molecule bound to the Mn1 [38, 39]. This substrate water enters the reaction as OH^- (O6—fast exchanging water Wf) [38]. Ca^{2+} -bound water molecules (W3 and W4) are also considered as sources of oxygen atoms [38, 39].

It should be noted that the Fe cation is in a trivalent state in the chimeric OEC, since substitution of the Mn cation is carried out by its reduction with an iron cation. The physiological valence states of the Fe cation 2 and 3, i.e., Fe(III) cation is not able to oxidize water molecules bound to cation.

However, 4-stroke Kok cycle (water oxidation and O_2 evolution) can be carried out by a chimeric cluster according to the low valent scheme (manganese cluster is in valent states II/III/III/III (S0 state) [40]. Perhaps the iron cation bound in the OEC plays a stabilizing role. In this regard, it should be noted that the cations Mn in the OEC are more resistant to the action of H_2Q if the chimeric cluster contains 1 or 2 bonded iron cations [16]. Thus, we have shown that a chimeric cluster based on manganese and iron cations can oxidize water, releasing molecular oxygen. In this regard,

it is interesting to note, that Dismukes with coworkers [41] recently synthesized OEC in apo PSII using Co^{2+} cations instead of $\text{Mn}(\text{II})$ cations during photoactivation. Catalytic center consisting of 4 cations of Co^{2+} and Ca^{2+} cation can oxidize water to O_2 although with much less efficiency than manganese OEC (~25% of the activity with $\text{Mn}(\text{II})$). The summary of these results may be of interest for studies of the mechanism of water oxidation, the evolutionary origin of OEC and the development of artificial water splitting systems [42].

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Data availability The data that support the findings of this study are available from the corresponding author, [BS], upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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