Effect of the Ca^{2+} channel activator CGP 28392 on reactivation of oxygen evolution of Ca^{2+}-depleted photosystem II

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Abstract The effect of the Calcium channel activator, CGP 28392, on the reactivation of oxygen evolution in Ca^{2+}-depleted Photosystem II (PS II) particles has been investigated. Ca^{2+}-binding is associated with a functional water splitting complex of PS II. In the presence of the activator, a low affinity site of Ca^{2+}-binding is converted into a high affinity binding site. Following removal of the extrinsic proteins (17 and 23 kDa), any effect of the activator is no longer observed. Ca^{2+} channel inhibitors can inhibit the Ca^{2+}-dependent reactivation of oxygen evolution. The activator partially protects against this type of inhibition. It is suggested that the extrinsic proteins form a Ca^{2+} channel-like structure at the donor side of PS II.

Key words: Calcium channel activator; Oxygen evolution; Photosystem II

1. Introduction

The oxidation of water and the resultant oxygen evolution by photosystem II (PS II) involves a manganese cluster linked to the reaction centre. During the process of water oxidation, the manganese cluster cycles through five oxidation states called S_0 to S_4. Oxygen is evolved during the transition S_4 to S_0 (for review see [1]). Three extrinsic proteins, with the molecular mass of 17, 23 and 33 kDa, are bound to the donor side of PS II. The 33 kDa protein is the most tenaciously bound and stabilises the Mn cluster. The 17 kDa protein has been reported to enhance the binding of Cl^- [2], whilst in the absence of the 23 kDa protein a specific requirement for Ca^{2+} is observed [3,4]. It has been proposed that the extrinsic proteins, especially the 23 kDa protein, build a diffusion barrier that prevents rapid equilibration of Ca^{2+} with the external aqueous phase. Although Ca^{2+} and Cl^- are thought to be obligatory cofactors for photosynthetic oxygen evolution, their exact function is still unknown (for review see [5,6]).

One possibility for studying the function of Ca^{2+} is first to remove it and then to investigate its binding behaviour by following reactivation of oxygen evolution in the presence of $CaCl_2$. Two methods have frequently been used to remove one Ca^{2+} per reaction centre: washing of PS II enriched membranes with 1–2 M NaCl, which removes additionally the 17 and 23 kDa proteins, e.g. [2] or incubation of PS II enriched membranes at pH 3.0 [7]. Incubation at low pH does not lead to a loss of the extrinsic proteins. In the light, Ca^{2+} is released under less acidic conditions. The pK-value for this process is 4.7 [8].

After Ca^{2+}-depletion the S_3 to S_0 transition of the Mn cluster is blocked, oxygen evolution is inhibited and a signal of the S_3 state is found in EPR spectroscopy [9]. Under such condition the oxidation of Tyr_Z, the electron donor to P_{680}, is also inhibited and 'normal' photochemistry no longer occurs in PS II [10].

The binding affinity of Ca^{2+} to Ca^{2+}-depleted PS II has been studied by several groups [7,11,12]. Different K_m-values for reactivation by Ca^{2+} have been reported: Boussac et al. [11] found a high affinity site with a K_m-value of 50–100 μM in 70% of the reaction centres and a low affinity site, K_m = 1–2 mM, for the remaining 30% of reaction centres. Homann [12] obtained the same K_m values and showed that the high and low affinity site are interconvertible, depending on pH. He concluded that the two Ca^{2+}-affinities represent different states of the same binding site at the donor side of PS II. Kalosaka et al. [13] observed a high affinity site with a K_m = 4 μM.

Calcium channel blockers, calmodulin antagonists and Ca^{2+} channel activators active in mammalian tissue have been used to inhibit electron transport activity in PS II [14–16]. Active, Ca^{2+}-containing thylakoid membranes and PS II enriched membranes have been used in these studies at neutral pH. It was suggested that inhibition by the calcium channel blockers occurs at the level of the water splitting system at the site of Ca^{2+} binding [15]. It was reported that a Ca^{2+} channel activator, CGP 28392, had no effect on electron transport activity, at the used concentration, while Ca^{2+} channel inhibitors like verapamil, a phenylalkylalmine, and derivatives of 1.4-dihydropyridine like nifedipine caused inhibition [16].

The Ca^{2+} channel activator CGP 28392 acts on dihydropyridine sensitive sites in mammalian tissue by shifting the Ca^{2+} dose-response curves to lower concentrations. It interacts competitively with Ca^{2+} channel blockers (e.g. [17]).

In the present study, the effect of the activator CGP 28392 on the activity of oxygen evolution and the binding of Ca^{2+} to PS II is investigated and related to the presence of the extrinsic proteins.

2. Materials and methods

Photosystem II enriched membrane fragments (BBY particles) were prepared from spinach according to the method of Berthold et al. [18] with the modifications described in ref. [19].

pH treatment was performed by incubation of samples at room temperature for 5 min in light (10–12 μmol quanta $m^{-2} \cdot s^{-1}$) in a buffer containing 300 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 30 mM succinic acid pH 4.5 to 5.5. The same buffer containing 80 mM MES (pH 6.5) instead of succinic acid was added after the incubation time to adjust

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Abbreviations: CGP 28392, 4-[2-(Difluormethoxy)phenyl]-1,4,5,7-tetrahydro-2-methyl-5-oxo-furo[3, 4-b]pyridine-3-carboxylic acid ethylester; Chl, chlorophyll; EGTA, ethylenglycol bis(β-aminoethylether)-*N,N,N',N'*-tetra-acetate; MES, 4-morpholino ethanesulfonic acid; PS II, photosystem II; P_{680}, reaction centre chlorophyll in photosystem II; Tyr, tyrosine

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the pH to 6.5 and oxygen evolution was measured. The final chlorophyll concentration was 50 $\mu g \cdot ml^{-1}$.

NaCl washing was performed as described in ref. [9]. The PS II enriched membranes were incubated in room light at 4°C in 300 mM sucrose, 1.2 M NaCl and 25 mM MES, pH 6.5 at a chlorophyll concentration of 0.5 mg Chl $\cdot ml^{-1}$. After 30 min incubation 50 μM EGTA was added. The NaCl-washed particles were pelleted by centrifugation at 40,000 × g, washed once in 30 mM NaCl, 25 mM MES, pH 6.5, and 50 μM EGTA, pelleted again and resuspended in the same medium. Activity measurements were performed in the same medium.

Oxygen evolution was measured with a Hansatech oxygen electrode using 0.5 mM p-phenylbenzoquinone and 1 mM potassium ferricyanide as electron acceptors at saturating light intensity (2,000 μmol quanta $\cdot m^{-2} \cdot s^{-1}$).

4-[2-(Difluormethoxy)phenyl]-1,4,5,7-tetrahydro-2-methyl-5-oxo-furo [3,4-b]pyridine-3-carboxylic acid ethylester (CGP 28392) was obtained from Ciba-Geigy, Switzerland.

3. Results

Fig. 1 shows the Ca^{2+}-dependence of oxygen evolution activity of low pH-treated PS II enriched membrane fragments in the presence and absence of CGP 28392. In the absence of CGP 28392 maximal activity of oxygen evolution was observed at 20 mM $CaCl_2$. In the presence of the activator, the maximal activity was already obtained at 1000 times lower $CaCl_2$ concentrations. The apparent K_m-values for Ca^{2+} rebinding were around 10 mM in the absence and around 6 μM or even lower in the presence of CGP 28392. The K_m-value in the presence of the activator is difficult to estimate, due to Ca^{2+} contamination of the medium, which was about 6 μM as determined by the Ca^{2+} indicator tetramethylmurexide. The affinity of the donor side of PS II for Ca^{2+} seems to be so much increased in the presence of the activator that, even without any external addition of $CaCl_2$, the activity is partially restored. The maximal activity at saturating $CaCl_2$ concentrations was the same in the presence and absence of the activator but still somewhat lower (20%) than in control samples.

Similar effects of CGP 28392 were found after treatment at different pH-values in the range pH 4.0 to pH 5.0 (data not shown).

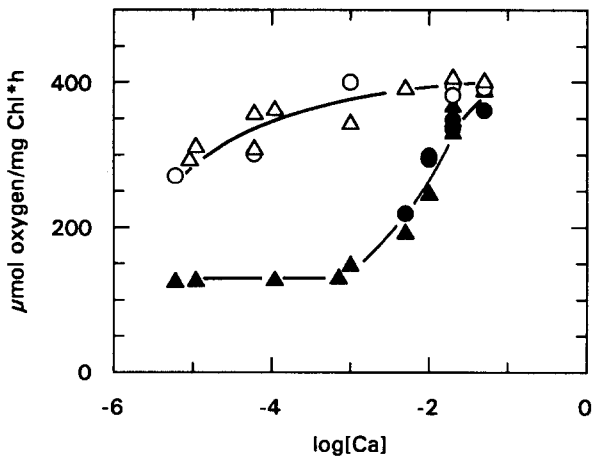

Fig. 1. Reactivation of oxygen evolution by addition of $CaCl_2$ to pH-treated PS II particles in the presence (open symbols) or absence of 5 μM CGP 28392 (closed symbols). Circles and triangles represent two different sets of measurements with different PS II particles preparations. PS II particles were incubated for 5 min at pH 4.5, CGP 28392 was added at the final pH of 6.5 and incubated for 5 min, $CaCl_2$ was added immediately before the measurement. The activity of control samples was between 480 and 520 μmol $O_2 \cdot mg\ chl^{-1} \cdot h^{-1}$.

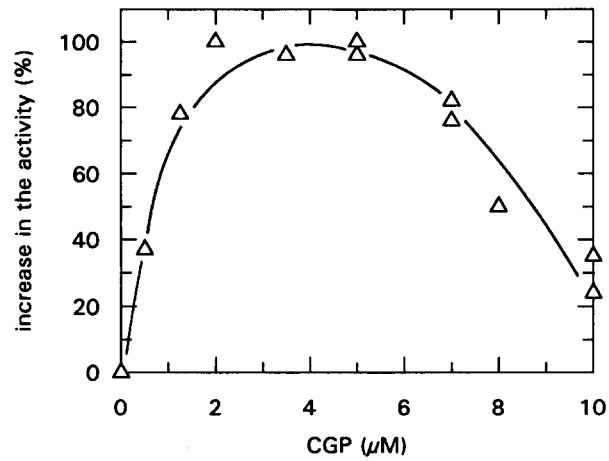

Fig. 2. Dependence of the stimulation of the activity of oxygen evolution on the CGP 28392 concentration. PS II particles were incubated at pH 4.5 for 5 min. CGP 28392 was added at the final pH of 6.5. The sample was incubated for 5 min before measuring oxygen evolution. The Ca^{2+}-concentration in the medium was about 6 μM.

The maximal stimulatory effect of CGP 28392 on oxygen evolution was obtained after 5 min incubation at a concentration of 2–5 μM (Fig. 2). Higher concentrations of the activator or longer incubation times (not shown) led to an inhibition of oxygen evolution. In control samples (not incubated at low pH), 5 μM CGP 28392 inhibited oxygen evolution activity by 30% after an incubation time of 5 min (not shown), indicating that the activator stimulated oxygen evolution activity only after previous Ca^{2+}-depletion.

Fig. 3 shows the Ca^{2+}-dependence of oxygen evolution of salt-washed PS II particles, where the extrinsic 23 and 17 kDa proteins are absent. Salt-washing inhibited oxygen evolving activity by 90%, addition of $CaCl_2$ (20 mM) restored it to 80% of the maximal value (data not shown).

It can be seen clearly in Fig. 3 that following salt washing CGP 28392 no longer has any effect on the K_m-value for Ca^{2+}-binding and restoration of oxygen evolution activity. Independent of the presence of the activator, the K_m-value is 1 mM, a small part of PS II reaction centres (ca. 15%) is already reactivated at low Ca^{2+} concentrations (high affinity site).

Oxygen-evolution activity can be inhibited independent of pH by addition of Ca^{2+} channel blockers such as nifedipine (1,4-dihydropyridine type) or verapamil [15,16]. It has been described previously [20] that, in thylakoids, inhibition of oxygen evolution by a high proton gradient across the thylakoid membrane can be influenced by Ca^{2+} channel inhibitors. Addition of inhibitors prior to the proton gradient protected oxygen evolution against inhibition whilst addition of inhibitors after formation of a proton gradient suppressed the restoration of oxygen evolution after uncoupling.

Here, the effect of a combination of Ca^{2+} channel inhibitors and the activator CGP 28392 on Ca^{2+}-dependent reactivation of oxygen evolution was investigated. At inhibitor concentrations where no inhibition of control samples was observed, inhibition of the activity of pH-treated PS II-enriched membranes was found (Table 1). Addition of CGP 28392 prior to

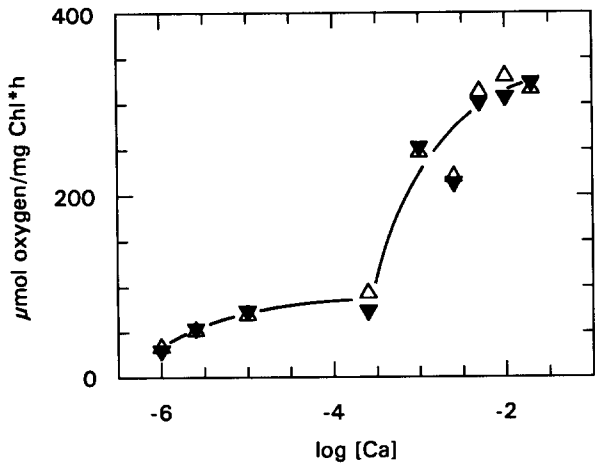

Fig. 3. Reactivation of oxygen evolution by addition of $CaCl_2$ to salt-washed PS II particles in the presence (open triangles) and absence (closed triangles) of 5 μM CGP 28392. The samples were incubated with CGP 28392 for 5 min at pH 6.5 before starting the measurement.

incubation at low pH and addition of the inhibitor, partially protected the oxygen evolving system against inhibition. The activity in the presence of the activator was 1.4 to 1.5 times higher than in its absence independent of whether verapamil or nifedipine was used as inhibitor. Nifedipine caused a stronger inhibition of oxygen evolution but the percentage of protection by CGP 28392 was the same. Addition of the activator after the addition (and binding) of the inhibitor did not lead to any restoration of activity (not shown).

4. Discussion

In the present paper it is shown that the low affinity site for Ca^{2+}-binding at the donor side of PS II, found normally after low pH treatment at least in a part of the reaction centres, is changed to a high affinity site by addition of the Ca^{2+} channel activator CGP 28392 (Fig. 1). The Ca^{2+}-binding affinity of PS II and also the effect of the Ca^{2+} channel activator are modulated by the 17 and 23 kDa proteins (Fig. 3).

The extrinsic proteins, especially the 23 kDa protein, seem on one hand to be required for retention of Ca^{2+} at its site in PS II [21], on the other hand they seem to facilitate after depletion the rebinding of Ca^{2+}. In the presence of the calcium channel activator and the extrinsic proteins, the binding affinity of the functional Ca^{2+}-binding site is obviously high. A very high affinity Ca^{2+}-binding site (K_m = 1–4 μM) has been observed by Kalosaka et al. [13] for PS II core complexes, which were Ca^{2+}-depleted by a combination of salt washing and low pH treatment.

Ca^{2+} channel blockers (e.g. nifedipine) and the Ca^{2+} channel activator CGP 28392 are known to interact competitively at dihydropyridine-sensitive sites of Ca^{2+} channels [17]. They clearly influence the Ca^{2+}-affinity of PS II, suggesting that a Ca^{2+} channel-like binding site is formed between the extrinsic proteins and the PS II reaction centre. Another possibility might be that either the 17 or the 23 kDa protein function itself as Ca^{2+} channel. The extrinsic proteins themselves seem not be the place of the active Ca^{2+}-binding site. The exact site of Ca^{2+}-binding in PS II is unknown, but good evidence exists that it might be close to the Mn cluster, which is probably located at the D1 protein [22]. A close Mn-Ca interaction has been shown by the stabilisation of the g = 4.1 EPR signal of the S_2 state after Ca^{2+}-depletion [9]. From EXAFS studies, a molecular distance between Mn and Ca of 4.3 Å has been given [23].

In PS II the reactivation of oxygen evolution after pH treatment can be inhibited by Ca^{2+} channel blockers, as shown in Table 1. Much higher concentrations were used than those effective in animal tissue, where verapamil was used in concentration of 200 nM to 2 μM (as a racemic mixture) and nifedipine 10 to 35 nM, depending on their site of action (e.g. [24]).

The calcium channel activator seems to bind specifically. In these experiments, 50 μg Chl/ml were used. Assuming a chlorophyll to reaction centre ratio of 200 to 250:1, the concentration of reaction centres is about 200 nM. A concentration of 500 nM CGP 28392 stimulates oxygen evolution by 50% (Fig. 2).

It is difficult to judge if the Ca^{2+} channel inhibitors act still specifically on channels or channel-like structures at the high concentrations used in the measurements shown in Table 1. One has to be careful with extrapolating knowledge on the effects of inhibitors and activators in animal to plants. Some studies with nifedipine, verapamil and other Ca^{2+} channel blockers have been performed in various plant tissues (e.g. [25,26]). The concentration needed to obtain 50% inhibition were often higher (μM) and non-specific effects have been observed (e.g. [27]). A recent study has shown that verapamil and nifedipine (IC_{50} = 5 μM) inhibit K^+ fluxes in Nicotiana protoplasts [28]. The authors concluded that studies using these inhibitors to demonstrate the involvement of Ca^{2+} channels in plant physiology should be regarded with caution. Nevertheless, concerning the present study, it is known that oxygen evolution is exclusively activated by Ca^{2+}, which can partially be replaced by Sr^{2+} but not by other cations [9]. The specific effects of the Ca^{2+} channel activator (effective at low concentrations) and inhibitors on reactivation of oxygen evolution imply that a Ca^{2+} channel-like structure might exist at the donor side of PS II.

The effect of CGP 28392 on photosynthetic electron transport has already been investigated at neutral pH without observing any effect [16]. In that study the activator was used in such high concentrations (100 μM) that inhibition of oxygen evolution should already be expected (see Fig. 2).

Table 1
The effect of the Ca^{2+} channel blockers verapamil and nifedipine and the Ca^{2+} channel activator CGP 28392 on pH-treated PS II enriched membranes

		Control	Preincubated at pH 4.7	+ 5 μM CGP, preincubated at pH 4.7
		450	350	432
100 μM Verapamil,	5 min	445	267	381
	10 min		226	350
100 μM Nifedipine,	5 min	441	100	147
	10 min		62	88

The Ca^{2+} channel activator (5 μM) was added at pH 6.5, incubated for 5 min, and then the sample was incubated for 5 min at pH 4.7. The inhibitors were added, incubated for 5 or 10 min, then the pH was returned to pH 6.5 and oxygen evolution was measured, using 1 mM ferricyanide and 0.5 mM *p*-phenylbenzoquinone as electron acceptors. The control samples were directly incubated at pH 6.5 with the inhibitor. The activity is given in μmol oxygen/mg chl·h.

It has been suggested that pH-dependent Ca^{2+}-release and rebinding may play an important role in the down regulation of PS II under high light stress conditions [8]. In the presence of a high proton gradient across the thylakoid membrane oxygen evolution is reversibly inhibited. Ca^{2+} channel inhibitors are able to influence this down regulation of PS II [20]. Unfortunately, CGP 28392 can not be used to study this down regulation of PS II in thylakoid membranes, because it uncouples already at low concentrations the proton gradient (data not shown).

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