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Slow release of chloride from ^{36}Cl-labeled photosystem II membranes

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Photosystem II (PS II) membrane fragments isolated from spinach cultured on a medium containing $^{36}Cl^-$ retain a significant amount of radioactive chloride corresponding to at least one Cl^- per PS II unit. The release of chloride from these sites occurs with halftimes of several hours. Treatments which cause the release of the manganese and/or the extrinsic proteins also result in the loss of chloride from these sites, indicating a possible role for this chloride in photosynthetic oxygen evolution.

Chloride binding; Oxygen evolution; Photosystem II; Photosynthesis

1. INTRODUCTION

Chloride ions play an important role in the photosynthetic water oxidation process. Recent studies have focused on the role of the 33-, 24- and 16-kDa extrinsic proteins since the removal of these proteins from Photosystem II lowers the O_2 evolution activity and raises the requirement of Cl^- several-fold (for recent reviews, see [1-4]). However, questions concerning the number of Cl^- ions required for function and their location still remain to be answered.

One reason for the ambiguities is that in most studies only the effects due to changes in the Cl^- concentration or affinity have been monitored, rather than the Cl^- binding itself. In a different approach, by growing spinach on a medium containing $Na^{36}Cl$, we have studied the exchange of Cl^- in ^{36}Cl-labeled PS II membranes and shown that there exists a slowly exchangeable pool of Cl^-. Since this pool is sensitive to the removal of Mn and/or extrinsic proteins, it may be important in the photosynthetic water-oxidation process.

2. MATERIALS AND METHODS

2.1. *Photosystem II preparation from spinach grown on $Na^{36}Cl$*

One week old spinach seedlings were grown on a nutrient medium containing 0.5 mM Cl^- (2.5 μM $Na^{36}Cl$). PS II membrane fragments were prepared from 6-8 week old plants as in [5] except that after the detergent treatment the membrane fragments were washed only twice with 20 mM MES (pH 6.3), 5 mM $MgCl_2$, 15 mM NaCl and 400 mM sucrose and centrifuged for 20 and 10 min, respectively. The total preparation time was about 4 h. The ^{36}Cl-labeled PS II membranes, which gave an O_2 evolution activity of 600-700 μmol O_2 per mg Chl and per hour, were stored in liquid nitrogen at 6-7 mg Chl/ml. In one case, ^{36}Cl-labeled PS II membranes were prepared as in [5], with a total preparation time of about 6 h.

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Abbreviations: Chl, chlorophyll; MES, 2-(*N*-morpholino)ethanesulfonic acid; PS II, photosystem II

2.2. *Incubation of ^{36}Cl-labeled PS II membranes and measurement of bound ^{36}Cl*

The PS II membranes were diluted to 1.5 mg Chl/ml in 20 mM MES/NaOH (pH 6.3), 5 mM $MgSO_4$, 5 mM NaCl and 400 mM sucrose and incubated in room light, with stirring at 0°C. The O_2 evolution activity remained unaffected for several hours. Samples of 1 ml were taken out at various times, centrifuged at 5200 × *g* for 1.5 min, diluted immediately to 35 ml in the same buffer medium and centrifuged again at 27 200 × *g* for 2.5 min.

The membrane pellets were subsequently transferred to 20 ml scintillation vials and the amount of chlorophyll determined. The samples were bleached by the addition of an equal volume of H_2O_2 (30%) [6], followed by incubation at 60°C for 4-5 h. A control sample of PS II membranes prepared in the same way from plants grown without $Na^{36}Cl$ was treated identically. After cooling to room temperature, the samples were adjusted to pH 7 with NaOH. To remove the excess H_2O_2 (H_2O_2 interferes in scintillation counting), 0.2 μg catalase was added, and the samples were left at room temperature overnight. Another 0.1-0.2 μg catalase was added to remove any residual traces of the H_2O_2. The samples were then mixed with 15 ml of scintillation liquid (Optiphase Highsafe II, LKB), and, to avoid chemiluminescence, left in the dark for 1-2 h at 4°C before counting. Each sample was counted twice, for 30 min.

2.3. *Selective removal of Mn and/or proteins from ^{36}Cl-labeled PS II membranes*

Release of manganese and/or the extrinsic proteins from PS II was carried out as described by Ono and Inoue [7] with only minor modifications. The PS II membranes were diluted in a medium consisting of 20 mM MES/NaOH (pH 6.3), 300 mM sorbitol and 10 mM NaCl to 0.5 mg Chl/ml and immediately centrifuged at 27 200 × *g* for 2.5 min. These samples were then resuspended to 0.5 mg Chl/ml and incubated in darkness for 20 min at 0°C in one of the following media: (i) the buffer medium only (control), (ii) the buffer medium plus 1 M NaCl, (iii) the buffer medium plus 1 M $CaCl_2$, or (iv) 0.8 M Tris/HCl (pH 8.3). After the incubation, the samples were diluted

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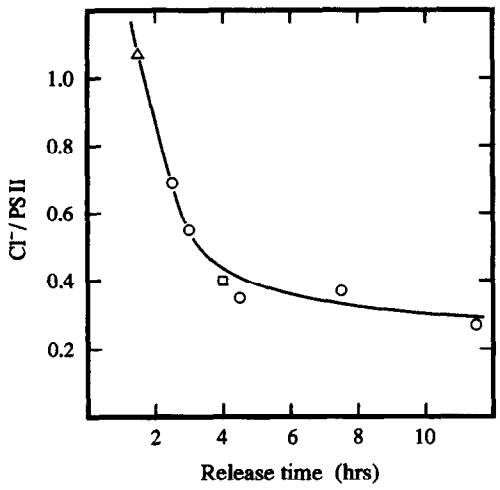

Fig. 1. Time course for the release of chloride from ^{36}Cl-labeled PS II membranes. Cl content in PS II membranes after the second resuspension following the Triton X-100 treatment (△), in PS II membranes incubated in buffer (○), in PS II membranes after a regular PS II preparation (◇).

(×12.5) with the buffer medium and centrifuged. The amount of the ^{36}Cl-bound to the PS II membrane pellet was measured as described above.

3. RESULTS

Fig. 1 shows the amount of $^{36}Cl^-$ that remains associated with the labeled PS II membranes as a function of incubation time. The zero time is taken to be when the thylakoids were first exposed to Triton X-100. At the end of a regular preparation (about 4 h after the start of the detergent treatment), the samples contained 0.4 Cl^-/PS II. In the modified, faster, preparation procedure 1.1 Cl^-/PS II remained after the second wash step (about 2 h after the start of the detergent treatment). There was no measureable amount of $^{36}Cl^-$ left in the supernatant in either case. It is obvious from Fig. 1 that there exists a very slowly exchangeable pool of Cl^- in PS II.

Table I shows the effects of alkaline Tris, mono- and divalent high-salt washes on the labeled PS II membranes. Only about 0.1 Cl^-/PS II remained, compared with 0.55 Cl^-/PS II in the control.

Table I

Amount of Cl^- remaining in PS II membranes after various washing treatments

Incubation/treatment[a]	Cl^-/PS II[b]
Untreated	0.51
Buffer	0.55
0.8 M Tris	0.07[c]
1 M $CaCl_2$	0.09[c]
1 M NaCl	0.10[c]

[a] See Materials and Methods
[b] Assuming 235 chlorophylls/PS II
[c] Contribution from Cl^- in the supernatant subtracted

4. DISCUSSION

The results in this communication show that a small, slowly exchangeable pool of Cl^- exists in PS II. Significant amounts of $^{36}Cl^-$, in which the plants were grown, remain bound even after many hours during which the membrane samples were exposed to extraction with detergent and extensive washing with the buffer medium.

Fig. 1 indicates that at least one Cl^- ion per PS II unit is in the slowly exchangeable pool. Presently, we are not able to give an upper limit to the number of Cl^- ions bound due to the uncertainty involved in setting the zero time point for the release of this Cl^-. In Fig. 1 the beginning of the detergent treatment of the thylakoids was taken as the zero time point since, prior to this treatment, the vesicular nature of the samples may have entrapped unbound pools of Cl^- which would obscure the results.

Several earlier attempts to determine the number of bound Cl^- and their rate of exchange were based on Cl^--sensitive electron transport activities, such as O_2 evolution or fluorescence. Halftimes for the release and rebinding of Cl^- from seconds to several minutes were obtained [6,8,9]. However, most of these studies were based on treatments at high pH [10] and/or the addition of high concentrations of other anions, such as sulfate [8], which are believed to displace Cl^- from PS II. Whether these treatments remove all tightly bound Cl^- from PS II is not known.

On the other hand, full recovery of the O_2 evolution activity after the usual Cl^- depletion treatments is rarely achieved (e.g., see [8]), indicating that irreversible alterations in PS II may well occur [11]. A detailed study of activity losses simply induced by the absence of Cl^- in the suspension medium [12], suggests that reversible and irreversible secondary structural changes affecting the oxygen-evolving complex strongly influence the Cl^- dependence of the O_2 evolution activity. Experiments with samples subjected to the usual Cl^- depletion treatments should, consequently, be interpreted with caution.

In contrast, the experiments presented here were performed with samples that were never exposed to low Cl^- nor to the usual Cl^- depletion treatments. The pool of slowly exchangeable Cl^- that we observe, therefore, is likely to reflect properties of a population of Cl^--binding sites as they would appear under close to native conditions.

Chloride NMR, although restricted to measurements of very rapid exchange rates ($>> 10^2\ s^{-1}$), also provides a non-destructive method to monitor Cl^- binding. This technique has pointed to correlations between Cl^-

binding and the O_2 evolution activity [1,13]. In addition, a very complex interaction between Cl^- and PS II samples, subjected to the usual Cl^- depletion treatments [4,14], has been reported. However, in recent reassessments of the NMR method it is concluded that instrumental irregularities (unpublished observations) and paramagnetic impurities [12,15] may severely interfere with the NMR measurements. It does appear, through, that the NMR can monitor another small pool of rapidly exchangeable Cl^--binding sites in PS II (manuscript in preparation).

The diversity of chloride effects on PS II suggests the existence of many different interaction sites in PS II which may influence the water oxidation process. The evidence for the association of the slowly exchangeable pool, reported here, with the water oxidizing function is only circumstantial. However, the sensitivity of this Cl^- to alkaline Tris (removing Mn and the 16, 24 and 33 kDa extrinsic proteins) and $CaCl_2$ or NaCl washes (releasing the 33, 24 and 16 kDa or the 24 and 16 kDa proteins, respectively) is certainly consistent with such a role, since these treatments increase the demand for chloride in O_2 evolution [2]. It may be that the role of the extrinsic proteins is to create a pocket at the oxygen-evolving complex in which Cl^- is trapped. Removal of these proteins would therefore allow a rapid equilibration with the surrounding medium. It is also conceivable the Cl^- directly ligated to manganese [1, 16] exchanges slowly. However, attempts to demonstrate direct Cl^- binding to the manganese by spectroscopic methods have so far been unsuccessful [17–19].

Preliminary experiments with intact isolated PS II membranes in our laboratory suggest that the binding of Cl^- to a site with very slow release occurs with a halftime in the order of hours. Whether this site is identical with that incorporating Cl^- during growth is currently under investigation.

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