Rate of exchange of Cl^- between the aqueous phase and its action site in the O_2 evolving reaction of photosystem II studied by rapid, ionic-jump-induced Cl^- depletion

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Rates of release and binding of Cl^- from/to its action site in the O_2 evolving reaction in photosystem II particles derived from spinach chloroplasts were estimated by measuring the suppression of O_2 evolution by salt addition (ionic-jump) and its recovery by the readdition of Cl^-. It was estimated that depletion and rebinding of Cl^- were completed within a few seconds. These results suggest that the Cl^--action site is located in a space which is almost freely accessible to various ions in the outer medium, with no barrier to ion movements. These results can be explained by electrostatic attraction of Cl^- to its action site, as was proposed in a study of anion effects on O_2 evolution [(1986) Plant Cell Physiol. 27, in press].

Cl^- effect *Oxygen evolution* *Photosystem II* *Salt effect* *PS II particle*

1. INTRODUCTION

The photosynthetic O_2 evolving reaction requires Cl^- as a cofactor [1,2]. Methods for depleting thylakoid membranes of Cl^- have been extensively studied by Izawa and his colleagues [1,2]. When O_2 evolving activity was measured, full Cl^- depletion was shown to be completed within 20 min at 0°C under optimal conditions (washing thylakoids in Cl^--free medium at pH 9.3 in the presence of EDTA and uncouplers) [2]. When the fluorescence increase, which reflects electron donation to PS II, was measured, full Cl^- depletion occurred within 10 min at room temperature in thylakoid membranes suspended in Cl^--free medium at pH 7.8 which contained 0.1 M Na_2SO_4 but no EDTA or uncouplers [3]. This time scale seems to be too long for a simple ion exchange between the outer aqueous phase and the membrane surface, and rather suggests the existence of a barrier against Cl^- exchange. This barrier may simply represent the slow permeation of Cl^- (or other competitive ions) from the site of action on the inner surface of thylakoids to the outer medium through the membrane, or may also reflect the existence of an additional physical permeation barrier in the proximity of the Cl^--action site. To explain the effects of uncouplers on the depletion of Cl^-, Theg and Homann [4] proposed that the barrier against the release of H^+ from the oxygen evolving system may work to limit the release of Cl^- from its action site.

Recent studies using O_2 evolving PS II particles showed that the Cl^- requirement for O_2 evolution is regulated by 3 proteins (with molecular masses of 33, 24 and 18 kDa) attached to the inner surface of the thylakoids [5–8]. Miyao and Murata [5] showed that depletion of the 33 kDa protein from PS II preparations increases the optimal concentration of Cl^- for O_2 evolution from a few to 200 mM [5]. Kuwabara et al. [8] showed that reconstitution of the 33 kDa protein restores the high affinity for Cl^-, indicating that, in addition to its effect on the stabilization of manganese ions in

Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; PS II, photosystem II

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water oxidation, this 33 kDa protein plays a major role in causing the high affinity for Cl^- in the oxygen evolving process. Depletion of the 24 and 18 kDa proteins also increases the Cl^- requirement for O_2 evolution [7,9], but to a lesser extent than following depletion of the 33 kDa protein. Based on the observation that in PS II particles with these 3 proteins intact O_2 evolution can proceed almost normally, even under a very low Cl^- concentration, Miyao and Murata [9] proposed that the 24 kDa protein works as a 'barrier' for the release of Cl^- from the active site. However, a ^{35}Cl NMR study [10] suggests a relatively fast exchange rate for Cl^-, although this Cl^- is probably nonspecifically bound to thylakoids.

In this study an ionic-jump (rapid addition of various salts) at pH 7.5 was shown to induce very rapid and almost complete Cl^- depletion from the PS II particles. The time required for the complete exchange of Cl^- was estimated to be a few seconds. This suggests that Cl^- is electrostatically attracted to its action site, which exists in a space almost freely accessible to various ions.

2. MATERIALS AND METHODS

PS II particles were prepared from spinach leaves using Triton X-100 according to Kuwabara and Murata [11] and were stored at 77 K in liquid nitrogen until use, in a medium containing 0.3 M sucrose, 0.025 M Mes buffer (pH 6.5), 0.025 M NaCl and 30% glycerol. O_2 evolution was measured by a Clark type electrode (Yellow Springs) in a vessel (2.5 ml) thermostatted by circulating water. The reaction medium was stirred by a magnetic stirrer. Excitation light (white, saturating intensity) was obtained from a projector (Cabin Mmulti, with a 650 W tungsten iodine lamp) in conjunction with a 10 cm heat cut water layer.

3. RESULTS

Fig.1 shows the effects of the addition of various salts on the O_2 evolution of PS II particles at pH 7.5. A high rate of O_2 evolution, equal to 90% of that measured in the presence of a high enough concentration of Cl^- (e.g. 20 mM), was observed in the low ionic medium even without added Cl^- (at 20 μM Cl^-) (cf. broken lines in traces a–e to

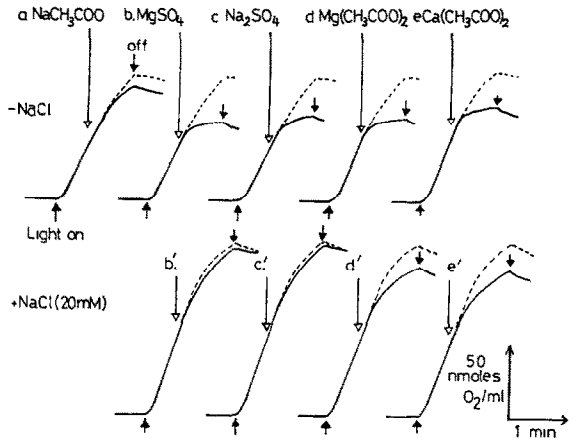

Fig.1. Effects of addition of various salts on O_2 evolution of PS II particles in the presence and absence of added Cl^-. (a–e) 20–30 μl solution of salt was added to the reaction mixture as indicated to give a final concentration of 20 mM in each case. Basal low ionic reaction medium contained 0.3 M sucrose, 0.3 mM *p*-phenylquinone, 5 mM Mops buffer (pH 7.5) and PS II particles (15 μg chlorophyll/ml). (b′–e′) Similar to b–e except that 20 mM NaCl was added to the reaction mixture about 3 min before each measurement. Concentration of Cl^- in a–e was about 20 μM.

those in b′–e′). This confirms the report of Itoh and Uwano [12] under similar conditions and of Miyao and Murata [9] at pH 6.5. However, this high rate was depressed almost instantaneously after the addition of various salts in the light (fig.1, solid lines in traces a–e). All the salts tested, except those containing Cl^- or Br^-, which can functionally replace Cl^- [1] (not shown), were highly suppressive. Salts of multivalent ions (cations as well as anions) were more effective than those of monovalent ones. For example, concentrations required to suppress the rate of O_2 evolution by 50% were 0.5 mM for $(CH_3COO)_2Ca$ and 10 mM for CH_3COONa (not shown). The time for suppression was similar to the response time of the O_2 electrode. However, the loss of activity was smaller when salts were added either in the presence of a high enough concentration Cl^- (traces b′–e′) or at pH 6.5 (not shown, see fig.2), at which pH the affinity of the Cl^- for its action site is about 10-times higher [12]. Even with 20 mM Cl^- or at pH 6.5, suppression of O_2 evolution was observed when higher concentrations of these salts were added (not shown), as was previously shown with

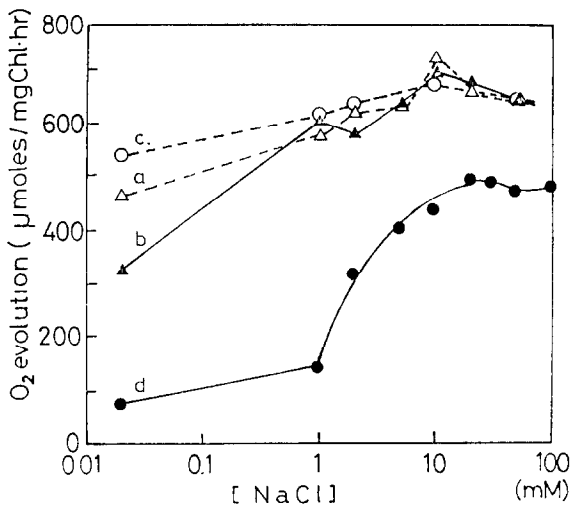

Fig.2. Dependence of O_2 evolving activity of PS II particles on the Cl^- concentration. a and b, pH 6.5. c and d, pH 7.5. In b and d, 25 mM Na_2SO_4 was added to the basal low ionic medium containing 0.3 M sucrose, 0.3 mM *p*-phenylquinone, 5 mM Mops buffer and PS II particles (15 μg chlorophyll/ml). NaCl was added to the reaction medium in the dark about 3 min before each measurement.

multivalent anions [12]. These results suggest competition between these salts and Cl^-.

The Cl^- requirement for O_2 evolution was highly dependent on the ionic conditions as well as the pH of the medium (fig.2). In the higher ionic medium, more Cl^- was required at the higher pH. The suppressive effects of salts were reduced as the Cl^- concentration increased and as the pH decreased. The requirement for added Cl^- at pH 7.5 with 25 mM Na_2SO_4 (fig.2, curve d) was similar to that at pH 6.5 with a higher concentration (e.g. 100 mM) of Na_2SO_4 (not shown). This seems to reflect the electrostatic nature of the interaction of Cl^- with its action site. It is assumed that Cl^- is electrostatically attracted to the action site, and that other anions are required to displace Cl^- from the site. This was proposed in the previous study on the effect of multivalent anions, such as ferricyanide, ferrocyanide, succinate, sulfate, etc. on the Cl^- affinity of oxygen evolution in PS II particles [12]. The salt-induced suppression of O_2 evolution is probably due to the exchange of Cl^- with added anions, which seems to be completed within a few seconds.

Fig.3 shows the effect of readdition of Cl^- to PS II particles preincubated in low-Cl^- medium containing 20 mM $MgSO_4$. The suppression by $MgSO_4$ was almost completely reversed if a sufficiently high concentration (20 mM) of NaCl was readded in the dark (traces b–d). However, if added after a short period of illumination, almost no reactivation was observed, indicating irreversible damage of the O_2 evolving reaction (traces e and f). This irreversible light-induced inactivation may be similar to that observed in Cl^--depleted thylakoids [2], although the inactivation in thylakoids re-

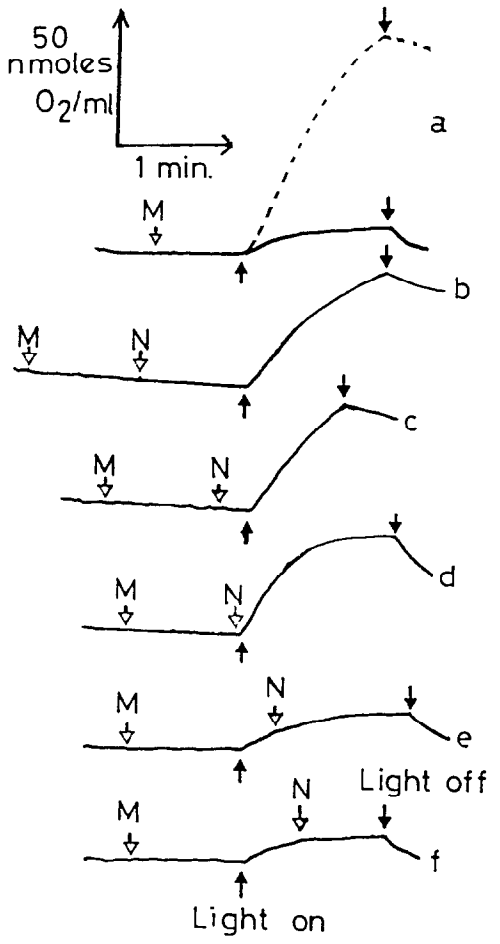

Fig.3. Recovery of O_2 evolution by readdition of Cl^- to $MgSO_4$-inhibited PS II particles. $MgSO_4$ (M) or NaCl (N) was added to the basal low ionic reaction medium used in fig.1 (at pH 7.5) to give a final concentration of 20 mM. Broken line, control without addition of these salts. Other conditions were similar to those in fig.1.

quired a longer (more than 10 min) illumination time. The mechanism of this inactivation is now under investigation including such different aspects as manganese release, protein integrity, etc. and is not discussed further here. The time between the addition of NaCl and the start of illumination was varied to determine the time required for the reactivation by Cl^-. Even when NaCl was added 1 s before the onset of illumination, a significant recovery of O_2 evolving activity was observed. This result indicates that reactivating and/or light-inhibition-protecting action(s) of Cl^- take place in a very short time, probably in a time range similar to the time resolution of the present system (a few seconds).

4. DISCUSSION

This study has shown that the Cl^- requirement for O_2 evolution in untreated PS II particles is mainly determined by 2 factors: (i) ionic (especially multivalent ion) concentration and (ii) medium pH. More Cl^- is required at higher ionic (and OH^-) concentration. Thus, Cl^- seems to be depleted from the active site only in exchange with other ions (probably including OH^-). However, to confirm this, the amount of Cl^- bound to the action site should be measured directly. Until recently, the significance of the ionic condition of the medium during Cl^- depletion was not recognized [12]. These characteristics of the Cl^- requirement can be explained by postulating electrostatic attraction between Cl^- (and other anions) and the positive charges in the vicinity of action site, as proposed in [12]. This is consistent with the fact that, in previous studies [1,2,4] efficient Cl^- depletion by washing membranes with Cl^--free low ionic medium (i.e. without exchanging ions) has only been accomplished at alkaline pH (i.e. at a high OH^- concentration).

Both the salt-induced inhibition of O_2 evolution under low Cl^- conditions and the reactivation by Cl^- were completed within a few seconds of the addition of salts or Cl^-. This suggests that in the O_2 evolving reaction in PS II particles, the exchange of Cl^- between its action site and the outer medium is completed within a very short time. This clearly indicates that there is no barrier to diffusion of Cl^- in the untreated PS II particles which have a fully active O_2 evolving system with 3 peripheral proteins. The barrier-like action of the 24 and 18 kDa proteins observed by Miyao and Murata [9] and this study under low ionic conditions can be explained by assuming that these proteins carry positive charges which work to increase the electrostatic attraction of Cl^- to its action site (i.e. to increase the affinity for Cl^-). This effect should be more marked in low ionic medium in which the positive charges are not screened by other ions [12]. The suppression of O_2 evolution by calcium salts confirms that the suppression is not due to the salt-induced release of 18 or 24 kDa proteins from PS II particles; this occurs at higher concentrations of salts [5–9,13], but calcium is known to replace functionally these proteins [6,14,15]. It seems likely that the Cl^- binding site is located on the inner thylakoid surface in a local domain to which various ions have almost free access, and that the surface in the vicinity of the Cl^--action site carries positive charges which attract Cl^-. Positive charges on the surfaces of the 33, 24 and 18 kDa proteins facing the Cl^--action site may be arranged along a 3-dimensional concave which is open to the outer bulk medium. Depletion of the 24 kDa protein as well as the 33 kDa protein would then decrease the affinity for Cl^-. The positive charges on the 33 kDa protein seem to be the most important in attracting Cl^- since a higher concentration of Cl^- (about 200 mM) is required for optimal O_2 evolution after depletion of this protein [5,8].

The above discussion was based on the assumption that, in the dark, the depletion of Cl^- from the active site induces some reversible effect before the measurement of O_2 evolution, and that irreversible damage occurs after illumination in the absence of Cl^-. However, the light-induced irreversible inhibition observed in fig.3 can also be explained by assuming that actual Cl^- depletion (i.e. exchange) does not occur until the start of O_2 measurement, and that irreversible Cl^- depletion from the active site occurs following the onset of illumination. Direct measurement of the number of Cl^- bound to the active site is necessary to distinguish between these possibilities. However, it does seem significant that, without any specific inhibitors of O_2 evolution such as Tris, Mn^{2+}, NH_3, NH_2OH etc. illumination in the absence of Cl^- leads to rapid irreversible damage. Inactive intermediates (probably inactive S_2 state) may be

formed as proposed by Izawa et al. [2] when, in the absence of Cl^-, the S_1 state is oxidized by donating an electron to the secondary electron donor Z [3,4]. A re-examination of previously reported Cl^--depletion studies, noting the ionic and pH conditions used, may provide new information on the O_2 evolving mechanism. For example, the results of Izawa et al. [2] using thylakoids, which gave new information on the role of Cl^- by showing irreversible light-induced damage of O_2 evolution by manganese or by a low concentration of Tris under low Cl^- and low ionic conditions may, at least partially, be explained by the ionic-effect shown in this study. Washing membranes in high ionic medium containing these ions would be expected to enhance the Cl^- exchange. The Cl^- concentration at the active site seems to be in equilibrium with that in the bulk outer medium, but, under physiological conditions, seems to be higher than the latter due to the electrostatic interaction between Cl^- and charges in the vicinity of the action site. The local Cl^- concentration at the active site seems to vary depending on the ionic (including pH) condition of the medium and on the binding state of the peripheral proteins, even when the amount of Cl^- in the reaction medium does not change.

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