INACTIVATION OF THE O_2 EVOLVING MECHANISM BY EXOGENOUS Mn^{2+} IN Cl^--DEPLETED CHLOROPLASTS

A. MUALLEM[+] and S. IZAWA*
Department of Biological Sciences, Wayne State University, Detroit, MI 48202, USA

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1. Introduction

Photosynthetic electron transport in chloroplasts is known to require Cl^- [1] at the level of water oxidation [2], but the action mechanism of Cl^- remains to be elucidated. The water proton and ^{19}F relaxation measurements in [3] produced results which may be interpreted to suggest an interaction between Cl^- and the Mn of O_2 evolving centers [4]. Cl^- removal from chloroplasts reversibly enhances the susceptibility of the O_2 evolving mechanism to NH_2OH, suggesting a role for Cl^- as a structural as well as catalytic component of the water oxidizing enzyme [5].

We report here findings which provide further clues to the role of Cl^- in O_2 evolution: a Cl^--sensitive inhibition of O_2 evolution by exogenous Mn^{2+}. This is an inhibition which only occurs when Cl^--deficient chloroplasts are incubated with Mn^{2+} in a Cl^--free medium and which is completely prevented (though not reversed) when the treatment medium contains as low as 1 mM Cl^-. The light-sensitivity of this Mn^{2+} inhibition provided the first indication that the non-functional state of the O_2 evolving centers in Cl^--depleted chloroplasts can store at least part of the oxidizing equivalents deposited by photoact II.

2. Materials and methods

All of these experiments were conducted using EDTA-uncoupled, Cl^--deficient chloroplasts prepared from commercial spinach (*Spinacia oleracea* L.) essentially as in [2]. After EDTA-wash at pH 8, chloroplasts were washed a further 2–3-times with an EDTA-free medium containing 0.2 M sucrose, 10 mM MOPS–NaOH buffer (pH 7.4) and stored in the same medium. The chloroplasts used were functionally 80–90% Cl^--depleted, i.e., their residual Hill activity in a Cl^--free reaction medium was 10–20% of maximally Cl^--stimulated rates. To eliminate the possibility of thylakoid-trapped EDTA influencing the mode of Mn^{2+} inhibition, we tested chloroplasts prepared with a medium in which EDTA was replaced by polygalacturonic acid (0.4%), a membrane-impermeant swelling agent [6] which we found effective in inducing both uncoupling and Cl^--deficiency. No difference was observed, however, in the pattern of Mn^{2+} inhibition between EDTA-washed and polyanion-washed chloroplasts.

Dark pretreatment of chloroplasts with Mn^{2+} was carried out at 0°C in a sealed, black-taped culture tube using chloroplasts dark-adapted for ≥1 h. The treatment mixture consisted of 0.1 M sucrose, 10 mM MOPS buffer (pH 7.4), 1–5 mM $MnSO_4$ and chloroplasts equivalent to 200 μg chl/ml. For Hill activity assay, a 0.1 ml aliquot was taken at appropriate intervals and diluted with 1.9 ml Cl^--sufficient reaction medium consisting of 0.1 M sucrose, 30 mM MOPS buffer (pH 7.1), 25 mM NaCl and 30 μM DCIP. This operation was done in near darkness (<1 Lux). The reaction mixture thus completed was incubated for 2.5 min in the dark at 21°C (reaction temperature) to allow the chloroplasts to equilibrate with Cl^-, then illuminated with a near rate-saturating red light (640–700 nm, 400 W/m^2). Photoreduction of DCIP was continuously monitored at 585 nm (slightly off peak) on a strip-chart recorder. The reaction time was 10–30 s depending on the rate.

Abbreviations: chl, chlorophyll; DCIP, 2,6-dichlorophenol-indophenol; MOPS, *N*-(2-morpholino)propanesulfonic acid

[+] Present address: Service de Biophysique, Département de Biologie, Centre d'Etudes Nucléaires de Saclay, BP 2, 91190 Gif-Sur-Yvette, France

* To whom reprint requests should be addressed

3. Results

When Cl^--depleted chloroplasts are incubated with 1–5 mM $MnSO_4$ in complete darkness at 0°C in a Cl^--free medium, their Hill activity (measured with excess Cl^-) decreases to ~ 50% of the original level in 1–2 h. The inhibition does not progress any further (fig.1). A striking feature of this partial inhibition is that it is completely or almost completely prevented when Cl^- or Br^- (e.g., 25 mM as sodium salts) is present in the treatment medium (fig.2A). These two anions are the most effective of several anions which are known to be capable of acting as cofactors of O_2 evolution [1,3]. Acetate and sulfate ions, which are ineffective as the cofactor, show no such preventive action at all. Mn^{2+} inhibition can also be prevented by EDTA (fig.2B) but in this case undoubtedly the prevention is due to removal of free Mn^{2+} (aquo complex) by chelation and is unrelated to the effect of Cl^- and Br^-, anions which do not complex Mn^{2+} in dilute aqueous solutions to any significant extent. However, neither EDTA nor Cl^- reverses Mn^{2+} inhibition, nor does washing of inhibited chloroplasts. So far, all attempts to reverse the inhibition have been unsuccessful.

In fig.3, curve A represents the maximum extent of inhibition attainable by dark Mn^{2+} treatment as a function of $[Cl^-]$ added to the treatment medium. (Hill activity was assayed with excess Cl^- as in fig.1,2.) As the data show, Cl^- at <1 mM is sufficient to prevent Mn^{2+} inhibition almost completely (half-maximum effect at 0.1 mM Cl^-). Curve B shows that the Hill activity of control chloroplasts saturates in about the same range of $[Cl^-]$ (84% rate-saturation at 1 mM Cl^-). The extrapolation of curve A to the 'true Cl^- = 0' point (the point obtained by extrapolating curve B to zero activity) suggests that Mn^{2+} inhibition may be >50% but still remains partial (<70%) if the chloroplast preparation used were absolutely Cl^--free.

In the above experiments, Mn^{2+} treatment was carried out in complete darkness (see section 2). We found that exposure of chloroplasts to weak red light (650 nm; 0.5 W/m²) during Mn^{2+} treatment markedly accelerates the development of Mn^{2+} inhibition (fig.4). The inhibition now readily exceeds 50% and approaches 100%. The red light had little effect by

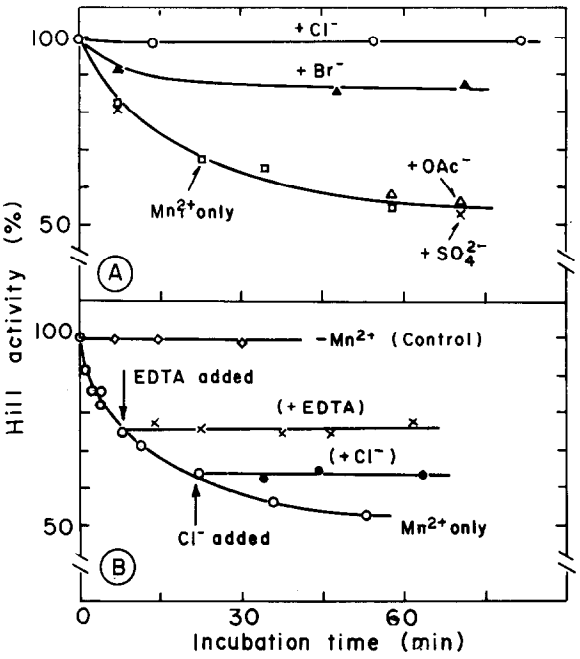

Fig.2. Prevention of Mn^{2+} inhibition in dark by Cl^-, Br^- or EDTA, and the lack of effect of acetate and sulfate ions. In (A), anions (25 mM as sodium salts) were added to the treatment medium (with 1 mM $MnSO_4$) prior to addition of chloroplasts. In (B), 25 mM Cl^- or 2 mM EDTA was added to the complete mixture where Mn^{2+} inhibition was already in progress. The rate of DCIP reduction at t = 0 was 720 μequiv./h . mg chl. For basic procedures and conditions, see section 2.

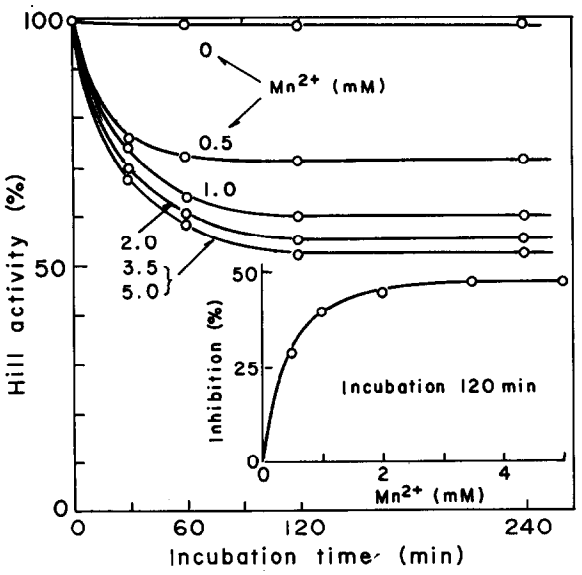

Fig.1. Time course of Hill activity decay during Mn^{2+} treatment in dark at 0°C. For experimental conditions and procedures, see section 2. The rate of DCIP reduction at t = 0 was 810 μequiv./h . mg chl.

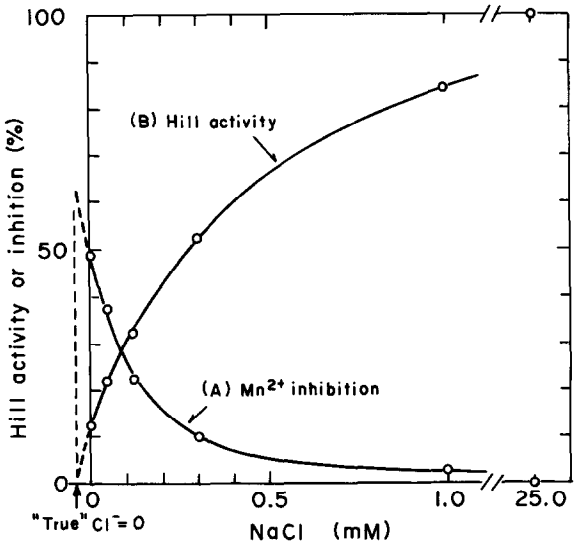

Fig.3. Concentration effects of Cl^- on Mn^{2+} inhibition and on Hill activity. Curve A: chloroplasts were preincubated for 3 h at 0°C in darkness with 2 mM $MnSO_4$ plus the indicated concentrations of Cl^- (NaCl) then assayed for Hill activity with excess Cl^- (see section 2). Curve B: Hill activity of control chloroplasts was assayed directly in the reaction mixture containing the indicated [NaCl]. The Cl^--saturated control rate of DCIP reduction was 650 μequiv./h . mg chl.

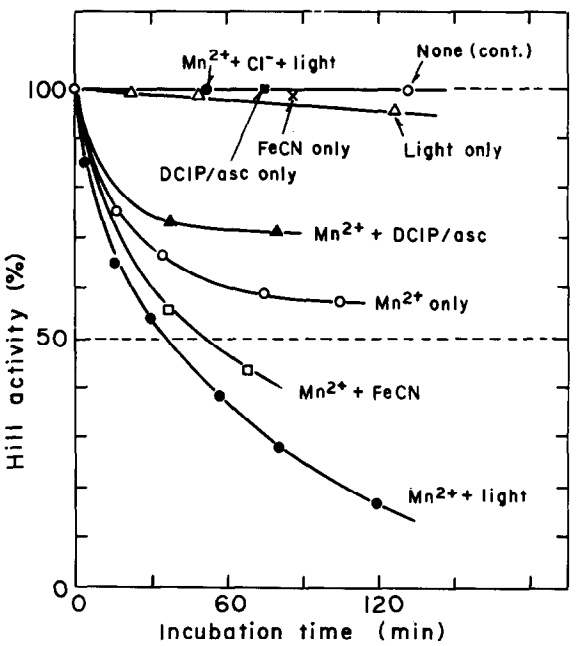

Fig.4. Effects of weak 650 nm light and of redox agents on the time course and extent of Mn^{2+} inhibition. Chloroplasts were incubated with 1 mM $MnSO_4$ at 0°C under the conditions indicated, and their Hill activity was followed with the incubation time as in section 2. Mn^{2+} treatment in 650 nm light (from a Bausch and Lomb monochromator) was carried out in a small test tube (i.d. 5 mm). At the surface of the test tube light was 0.5 W/m^2. In experiments involving ferricyanide (0.1 mM) or DCIP (0.1 mM) plus ascorbate (5 mM), 2 ml samples taken from the incubation mixture (10 ml) were quickly diluted with 10 vol. cold buffer (0.1 M sucrose plus 5 mM MOPS buffer, pH 7.4) and centrifuged at 2000 × *g* for 5 min. The sedimented chloroplasts were washed and resuspended in 2 ml same buffer, then subjected to Hill activity assay. The rates thus obtained were corrected for the small amount of chloroplasts (chl) lost during the washing procedure. The DCIP reduction rate at $t = 0$ was 860 μequiv. h . mg chl.

itself (negligible photodamage) as expected from the fact that the intensity used was sufficiently low so that it could not even support DCIP reduction at 1% of light-saturated rates. The Mn^{2+} inhibition is also significantly enhanced when the weak illumination is replaced by the addition of ferricyanide (0.1 mM). Reduced DCIP (DCIP plus ascorbate) exhibits an opposite effect: it tends to alleviate the inhibition. Preliminary experiments (not shown) indicate that an enhancement of inhibition can also be brought about by exposing chloroplasts to a single Xenon flash immediately before or during Mn^{2+} treatment.

Chloroplasts which had been severely inhibited by Mn^{2+} plus red light were found capable of high rates of DCIP reduction using various artificial electron donors to photosystem II (including added Mn^{2+}) as shown in table 1. In this experiment we used chloroplasts which had been thoroughly washed with EDTA after Mn^{2+} treatment in order to eliminate the possibility of Mn^{2+}-mediated donor reactions. As is clear from table 1, the rates of donor reactions in Mn^{2+} inhibited chloroplasts are similar to those found in NH_2OH- or Tris-treated [7] chloroplasts. Furthermore, they are totally independent of the presence or absence of Cl^- in the reaction mixture (plus-Cl^- data not shown for brevity). These results indicate that:

(i) Mn^{2+} inhibits the O_2 evolving mechanism without affecting the reaction center of photosystem II;
(ii) The Mn^{2+}-sensitive step and the Cl^--requiring step are closely related to each other.

It is highly unlikely the P680, the reported site of electron donation by exogenous Mn^{2+} [8] (but see [9]), represents the site of Mn^{2+} inhibition in Cl^--depleted chloroplasts.

None of the 4 other cations tested, Zn^{2+}, Cu^{2+}, Ca^{2+} and Mg^{2+}, behaved like Mn^{2+}. The first two ions were definitely inhibitory at 1 mM but their effects

Table 1
Photosystem II donor reactions in chloroplasts whose Hill activity was abolished by Mn^{2+} treatment in 650 nm light

Chloroplast treatment	Electron donor	DCIP reduction (μequiv./h . mg chl)
None	H_2O	805[a]
Mn^{2+} + red light	H_2O	10
	Mn^{2+}	242
	DPC	284
	NH_2OH	480
NH_2OH	NH_2OH	475
Tris	Mn^{2+}	255
	DPC	310

[a] The reaction mixture contained 25 mM Cl^- (NaCl) to activate O_2 evolving centers. All the other reactions were totally independent of the presence or absence of Cl^-

Chloroplasts were treated with Mn^{2+} in weak 650 nm light (as in fig.4), washed once with a medium containing 0.1 M sucrose, 5 mM MOPS buffer (pH 7.4) and 0.5 mM EDTA, and then subjected to donor reaction assay in a medium consisting of 0.1 M sucrose, 30 mM MOPS buffer (pH 7.1), 40 μM DCIP and one of the following donors: 1 mM Mn^{2+} (as sulfate), 0.5 mM DPC (diphenylcarbazide), and 1 mM NH_2OH (as sulfate). The mixture had 10 μg chl/ml. Data from experiments with NH_2OH-treated or Tris-treated [7] chloroplasts are given for comparison. All of the reactions shown were inhibited by 1 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea

were completely independent of the presence or absence of Cl^-; hence no further study. Mg^{2+} and Ca^{2+} had no or little effect at 5 mM. Fe^{2+} was not tested because of its autoxidizability at neutral pH. Cu^{2+} has been reported to inhibit the water oxidizing side of photosystem II in an unknown manner [10].

4. Discussion

These results revealed a new property of the O_2 evolving enzyme complex in chloroplasts: susceptibility to inhibition by exogenous Mn^{2+} under Cl^--deficient conditions. The inhibition is effectively prevented by the same low concentrations of Cl^- as required for the activation of the O_2 evolving enzyme (half-maximum effect at 0.1–0.3 mM), suggesting a common action mechanism of Cl^-. These low Cl^- concentration requirements are in sharp contrast to the cation requirement of photosystem II reaction centers reported for pea chloroplasts (half-maximum effect with 2 mM Mg^{2+} or 75 mM Na^+) [11]. In our chloroplast preparations, this cation requirement was apparently satisfied by 15 mM Na^+ which was always present in the reaction mixture as a buffer salt.

The high Cl^- sensitivity noted above almost certainly implies a direct binding between Cl^- and a key enzyme (E) involved in O_2 evolution:

$$E_f + Cl^- \rightleftharpoons E \cdot Cl^-$$

Because Mn^{2+} inhibits only Cl^--deficient chloroplasts, we may assume that Mn^{2+} attacks only the Cl^--free, temporarily non-functional form E_f. The Cl^--complexed functional form $E \cdot Cl^-$ is not attacked. However, even in severely (90%) Cl^--depleted chloroplasts, the inhibition is ⩽50% (fig.1) unless the chloroplasts are exposed to weak light or to ferricyanide during Mn^{2+} treatment (fig.4). This suggests that:

(i) E_f can exist at least in two different oxidation states, $E_{f.red}$ and $E_{f.ox}$;
(ii) Only the more oxidized form $E_{f.ox}$, which comprises ~50% of total E in dark-adapted (Cl^--depleted) chloroplasts, is sensitive to Mn^{2+}.

Weak red light enhances Mn^{2+} inhibition by converting $E_{f.red}$ to $E_{f.ox}$ through photoact II. The ferricyanide effect suggests that the standard redox potential of E_f may not be much higher than +0.5 V.

The model presented above is obviously oversimplified, for instance totally ignoring the kinetic implications of the results (exploration of which must await further experiments). We believe, however, that the results are clear enough to permit the conclusion that the Cl^--depleted, non-functional state of O_2-evolving centers can store, practically indefinitely, at least part of the oxidizing equivalents deposited by photoact II and that in this 'charged' state the Cl^--free O_2 centers are inactivated by Mn^{2+}. The slow development of inhibition ($t_{1/2}$ 10–20 min at 0°C in dark) is presumably due, at least in part, to the slow trans-membrane diffusion of Mn^{2+} [12]. Although we used EDTA-uncoupled chloroplasts here, the permeability of thylakoid membranes to divalent cations (e.g., Ca^{2+}) is not significantly altered by EDTA uncoupling [13]. As for the inhibition mechanism, we can only assume that the oxidizing equivalents stored in a Cl^--free O_2 center are in such a form that they induce Mn^{2+} (a reductant) to interact with the center in some irreversible manner. Interestingly, Mn^{2+} has been shown to act as a powerful inhibitor of bush bean lipoxygenase and here again,

a redox interaction between Mn^{2+} and the enzyme is suspected [14].

Weak red light or brief flashes are known to enhance the inhibition of O_2 evolution by such agents as NH_3 [15], Tris [16] and OH^- (high pH) [17]. In all of these cases the light effect has been attributed to the high reactivity of the 'S_2 state' of O_2 evolving centers. Investigations are underway to find out how the Mn^{2+}-sensitive, charged form of non-functional O_2 centers in Cl^--depleted chloroplasts is related to the S states of functional O_2 centers in Cl^--sufficient chloroplasts.

Finally, these results suggest that, in various inhibitory treatments of chloroplasts, the Mn^{2+} released endogenously from the denatured O_2 centers [18] could cause complications. Although under these conditions Mn^{2+} seemed quite harmless in the presence of Cl^-, there may be conditions other than Cl^- deficiency where O_2 centers (native or modified) are affected by Mn^{2+} in some manner.

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