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The effect of chloride on the thermal inactivation of oxygen evolution

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Abstract. The effect of Cl^- depletion on the sensitivity of the oxygen-evolving complex of Photosystem II (PS II) to heat treatment was examined by a parallel study of the Hill activity ($H_2O \rightarrow$ 2,6-dichlorophenolindophenol), Cl^- binding (by ^{35}Cl-NMR) and Mn release (by EPR). The extent of thermal inactivation in spinach thylakoids was found to depend on the degree of Cl^- depletion in the sample. In partially Cl^--depleted thylakoids, mild heating (38 °C, 3 min) was found to eliminate inflections in plots of both Hill activity versus [Cl^-] (at low light intensity) and excess ^{35}Cl-NMR linewidth versus [Cl^-] (in the dark). In PS II membranes, the same treatment reduced the differences between the linewidth maxima and minima, particularly in the region of 0.3 mM and 7.0 mM Cl^-, as compared to unheated membranes. These results indicate that mild heating affects the Cl^--binding domains within the oxygen-evolving complex, OEC. EPR measurements of the temperature dependence of Mn release from heated thylakoids show that Mn release begins to correlate with the loss of Hill activity only at higher temperatures, where the OEC is already substantially inactivated. We conclude from these studies that the Cl^--binding domains of the OEC constitute a principal site of damage by heat treatment.

Introduction

Mild heat treatment of thylakoids and Photosystem II (PS II) membranes has been used for many years as both a convenient means to inactivate the water-splitting reactions and to study the structure of the oxygen evolving complex (OEC) (Katoh and San Pietro 1967, Yamashita and Butler 1968, Lozier et al. 1971, Nash et al. 1985). Most studies have focused on the stability of a pool of bound manganese (Mn) that is believed to be directly involved in the light-driven oxidation of water, since this Mn is released by

heating (Cheniae and Martin 1966, Lozier et al. 1971, Kimimura and Katoh 1972, Wydrzynski and Sauer 1980, Cramer et al. 1981, Wydrzynski 1982). Detachment of catalytic Mn from its binding site has been proposed as the chief reason for the irreversible loss of enzyme activity in thylakoids (Kimimura and Katoh 1972).

The available evidence for a direct correlation between Mn release and inhibition of the Hill reaction in thylakoids is somewhat contradictory. Kimimura and Katoh (1972), using *Euglena* chloroplasts, and Cramer et al. (1981), using spinach chloroplasts, both reported that Mn release occurs in the same temperature range as the loss of Hill activity. Cheniae and Martin (1966), however, found that in chloroplast particles from *Scenedesmus* the rate of inactivation of the Hill reaction at 50 °C was much faster than the rate of Mn loss.

Recent indications that bound Cl^- and Ca^{2+} ions are essential for OEC function (for a review, see Homann 1987) make it necessary to determine whether they also contribute to the stability of the complex. In the case of Cl^- a new perspective on this problem was provided by the discovery that Cl^- depletion increases the sensitivity of O_2 evolution to thermal inactivation. Coleman et al. (1984) and Nash et al. (1985) found that the increased sensitivity is reversible, such that addition of NaCl to Cl^--depleted membranes prior to heating reduces the level of inactivation. This protective effect of Cl^- indicates that it helps to stabilize the structure of the OEC. Since stabilization is likely to result from Cl^- binding to the native complex (see discussion in Coleman et al. 1987a), we decided to examine the effect of heating on both Cl^--binding (by ^{35}Cl-NMR) and Cl^- activation of O_2 evolution in EDTA-washed preparations, and to relate these effects to the loss of catalytic Mn (measured by EPR).

Materials and methods

Sample preparation. Procedures for preparing and assaying Cl^--depleted thylakoids and PS II membranes from market spinach are described elsewhere (Coleman et al. 1987a). Thylakoids, partially depleted of Cl^-, were prepared by the same procedure, except that the washed thylakoids were simply centrifuged and washed again in Cl^--free buffer, and were not subjected to high-pH/Gramicidin treatment. The latter procedure produced a level of Cl^- depletion (as measured by the Hill activity $\pm Cl^-$) of about 35% in the thylakoids. For the Hill assays, the level of saturating light intensity (approximately 700 mW cm^{-2} of red light) was determined by measuring the Hill activity as a function of light intensity, using a series of calibrated neutral density filters.

Heating methods. For heating large numbers of small aliquots (experiments in Figs. 1, 7 and 8), glass tissue grinders (Kontes Glass) were used to spread the sample into a very thin, uniform layer between the pestle and the inner wall of the holding tube. For larger aliquots, the thylakoids or PS II membranes were spread in a thin layer at the bottom of a large glass beaker. Because of the slightly greater thickness of the layer in the latter set-up, the level of inhibition produced by the heat treatment was slightly less. Heating vessels were allowed to equilibrate with the bath temperature before membrane suspensions were added. This was done to insure uniformity of treatment at equilibrium conditions. The vessels containing the membrane suspensions were heated in a water bath whose temperature was controlled by a water bath-circulator (Lauda-Brinkmann RC-3) with a stability of ± 0.1 °C. The temperature was continually monitored by a Solomat T135 digital thermometer. Aliquots to be heated were first removed from the ice and allowed to warm to room temperature for 5 min before being placed in the water bath for 3 min. All treatments were done in darkness. Heated aliquots were immediately returned to the ice.

Manganese release. Mn release from thylakoids was determined by difference in the total Mn content, measured by EPR, of samples before and after heat treatment. A small aliquot of thylakoids (780 μl at 3.5 mg Chl ml^{-1}) in buffer consisting of 0.2 mM ethylene diaminetetraacetic acid (EDTA) and 50 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) (pH 7.2) was mixed with 20 μl of 2 M NaCl/buffer (50 mM Cl^- final concentration) or buffer without Cl^-, and allowed to incubate for 5 min at room temperature. A small amount of EDTA was included in the buffer in order to prevent any Mn that might be released from the OEC from rebinding to non-specific sites on the membrane (Khanna et al. 1983). After heating for 3 min, the sample was quenched on ice for 10 min. A small aliquot was removed for the Hill reaction assay, and the remainder was centrifuged for 6 min at 9000 × g to remove all unbound Mn. The pellet was rinsed and resuspended in buffer, and the chlorophyll (Chl) concentration was measured again for each sample. Immediately prior to EPR measurement, each sample was treated with HCl at a final concentration of 0.1 N in order to release all of the bound Mn. Additional HCl did not increase the signal amplitude. Also, a check was made prior to HCl treatment, on the completeness of removal of non-specifically bound Mn. Washing the heated thylakoids (66 °C, $+Cl^-$) with 1 mM $MgCl_2$ (to displace any loosely-bound Mn) did not affect the Mn content of the membranes.

EPR Measurements. The hexaquo-Mn^{2+} concentration was measured at room temperature by the EPR method (Yocum et al. 1981, Khanna et al. 1983, Miller and Cox 1984). A set of Mn^{2+} standards in buffer (0.5 to 4.0 μg

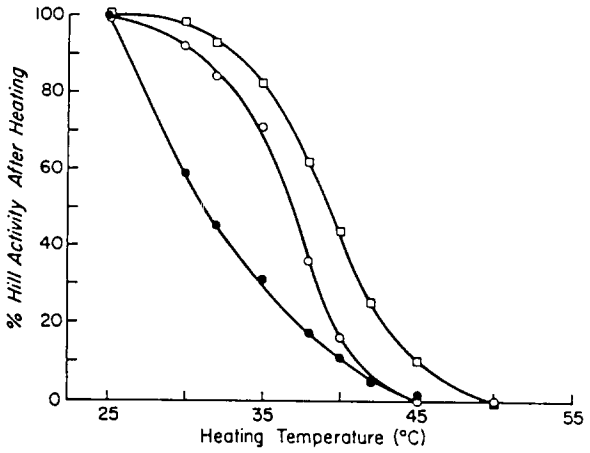

Fig. 1. Effect of Cl^- depletion on the thermal inactivation of the Hill reaction in thylakoids. Partially Cl^- depleted samples were heated for 3 min in buffer containing 50 mM Cl^- (□) or no added Cl^- (○). Thoroughly Cl^--depleted samples (●) were heated without Cl^-. Activity was measured by 2,6-dichlorophenolindophenol (DCIP) reduction in buffer containing 70 mM Cl^-. The mean activity after treatment at 25 °C was 561 μmol DCIP reduced (mg Chl)$^{-1}$ hr^{-1}. The light intensity was 700 mW cm^{-2}.

Mn ml^{-1}) was prepared by serial dilution of a concentrated atomic absorption standard solution (Spex, Inc., Metuchen, NJ). These solutions were used to generate a standard curve to calibrate the peak-to-trough height of the 2nd low-field line of the Mn EPR signal. EPR spectra were obtained at X-band frequency using a quartz aqueous TM flat cell. The spectrometer was a Varian E-line instrument equipped with a Varian TM cavity. Spectrometer settings were as follows: scan range: ±500 G, field set: 3300 G, modulation frequency: 100 kHz, modulation amplitude: 10 G, time constant: 0.064 s, scan time: 4 min, power: 80 mW, frequency: 9.413 GHz.

Unheated thylakoids were found by the above method to contain an average of 5.5 Mn per 400 Chl. This is within the range of 4–6 Mn per 400 Chl commonly reported for EDTA-washed thylakoids (Mansfield and Barber 1982, Yocum et al. 1981).

NMR measurements. ^{35}Cl-NMR line-broadening measurements were performed in darkness as described by Coleman et al. (1987a).

Results

Figure 1 shows that progressive removal of Cl^- from thylakoid membranes has a dramatic effect on the temperature sensitivity of the OEC between 25 °

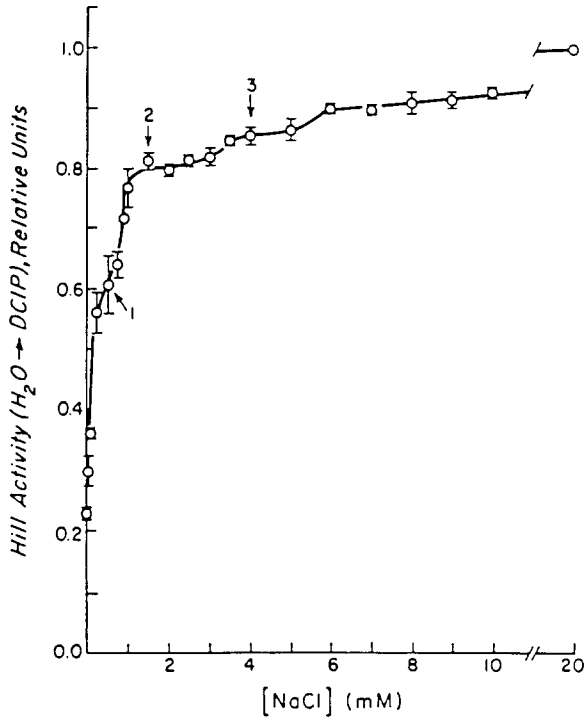

Fig. 2. Cl^- activation of the Hill reaction in unheated thylakoids at 5.5% of saturating light intensity (38 mW cm $^{-2}$). Arrows indicate the locations of the intermediary plateaus. The maximum activity (at 20 mM Cl^-) was 252 μmol DCIP reduced (mg Chl) $^{-1}$ hr $^{-1}$. Error bars show the sample standard error for separate measurements on two different preparations.

and 50 °C. In Cl^--sufficient membranes (50 mM Cl^-), the inactivation follows a fairly sharp sigmoid curve with a transition midpoint (T_m) of 39.4 °C. In partially Cl^--depleted membranes, the curve is still sigmoidal, but the T_m is shifted downward by 2.5 °, to 36.9 °C. In thoroughly Cl^--depleted thylakoids, the T_m drops to 31.4 °C, and the sigmoidicity is much less apparent.

Mild heating also affects the steady-state kinetics of Cl^- activation of O_2 evolution. Figure 2 shows the Cl^- concentration dependence for the Hill reaction in unheated, Cl^--depleted thylakoids. Under these conditions, the response of the oxygen-evolving enzyme to changes in [Cl^-] is highly cooperative, since its apparent affinity for Cl^- changes as the [Cl^-] increases. The type of cooperativity shown by the enzyme in Fig. 2 is commonly referred to as 'complex kinetic cooperativity' because of the presence of intermediate plateaus in the curve. In enzyme systems where preparation artifacts and steady-state kinetic effects can be ruled out, the appearance of

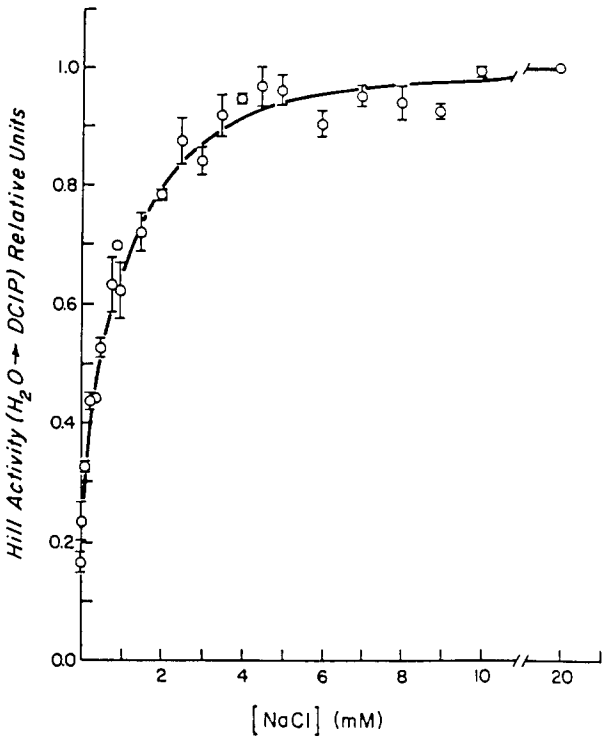

Fig. 3. Cl^- activation of the Hill reaction in heated thylakoids at 5.5% of saturating light intensity (38 mW cm^{-2}). The thylakoid suspension (1.5 mg Chl ml^{-1}) was heated for 3 min at 38 °C without Cl^-. The maximum activity (at 20 mM Cl^-) was 121 μmol DCIP reduced (mg Chl)$^{-1}$ hr^{-1}. Error bars show the sample standard error for separate measurements on 2–3 different preparations.

intermediate plateaus in plots of rate vs. ligand concentration has been suggested to arise from several sources, including: 1) interacting binding sites on different subunits, whose intrinsic ligand dissociation constants alternately increase and decrease as the sites are sequentially filled; 2) multiple non-identical sites with different degrees of cooperativity in their response to the ligand; or 3) multiple forms of the enzyme which undergo slow interconversion after ligand binding (Kurganov 1982). Figure 3 shows that heating at 38 °C for 3 min, besides decreasing the activity by 51%, removes all of the plateaus (numbered 1–3 in Fig. 2) that were observed in the Cl^- activation curve in the native system (see also Coleman et al. 1987a). The elimination of the apparent cooperativity results in a plot of the relative Hill activity vs. log [Cl^-] (Fig. 4) that is a smooth sigmoid. Intermediate plateaus would stand out clearly as deviations from sigmoidicity (bumps) in this kind of plot (Coleman et al. 1987a). The heated enzyme behaves as

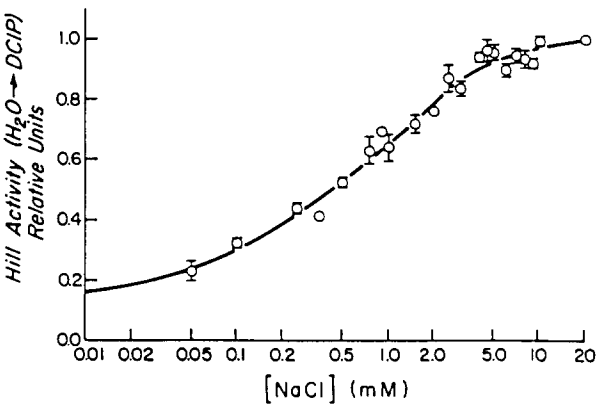

Fig. 4. Relative Hill activity vs. log [Cl^-] for heated thylakoids at 5.5% of saturating light intensity (38 mW cm^{-2}). See Fig. 3 for other details.

though it has a single K'_A (apparent activator dissociation constant) for Cl^- of about 0.5 mM at this light intensity.

A useful way to examine Cl^- binding to proteins more directly is to measure the ^{35}Cl-NMR line-broadening (excess linewidth) as a function of [Cl^-]. This technique has been used to study Cl^- binding in thylakoids from halophytes (Critchley et al. 1982, Baianu et al. 1984) and in thylakoids and PS II membranes from spinach (Coleman et al. 1987a, b). For relatively simple systems, in which bound Cl^- exchanges freely with Cl^- in solution, a plot of the excess ^{35}Cl-NMR linewidth vs. [Cl^-] is a smoothly descending hyperbola (Chiancone et al. 1972, Baianu et al. 1984). However, in spinach thylakoids and PS II membranes (which were studied at much lower [Cl^-]), this curve is interrupted by sharp increases in linewidth (linewidth maxima) at particular concentrations of added Cl^- (Coleman et al. 1987a). In spinach thylakoids, for example, the linewidth maxima appear at 0.3, 0.75, 3.25 and 7.0 mM Cl^-.

We have proposed that linewidth maxima occur because the addition of Cl^- to Cl^--depleted membranes exposes previously non-exchanging Cl^--binding sites within the OEC (Coleman et al. 1987a, b). When these sites become accessible, Cl^- ions can bind to them and exchange with Cl^- in the bulk solvent, thereby contributing additional broadening to the $^{35}Cl-NMR$ linewidth. It is possible that these sequestered Cl^--binding sites are organized into four domains that are linked by salt-bridge contacts (Coleman and Govindjee 1987).

In order to determine whether the correlation between increased sensitivity to heating and Cl^- depletion directly involves the Cl^--binding sites, we measured the excess ^{35}Cl-NMR linewidth as a function of Cl^- for thorough-

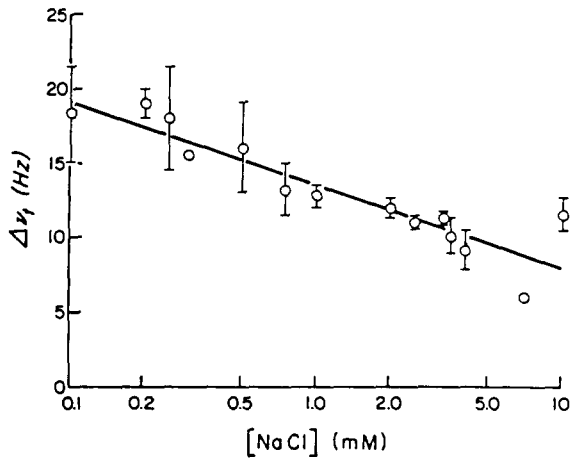

Fig. 5. ^{35}Cl-NMR binding curve for heated thylakoid. Samples at 1.0 mg Chl ml^{-1} were heated at 38 °C for 3 min in the absence of Cl^-. For a discussion of the significance of $\Delta\nu_t$ and details of the ^{35}Cl-NMR measurements, see Coleman et al. (1987a). Error bars show the sample standard error for 2–7 measurements of $\Delta\nu_t$ from 7 different preparations.

ly Cl^--depleted thylakoids that had been heated at 38 °C. Figure 5 shows that heat treatment eliminates the linewidth maxima that we had observed in the native system (see Coleman et al. 1987a). As a result, the heated thylakoids behave more like bovine serum albumin in terms of their Cl^--binding behavior (Coleman et al. 1987a), although the linewidths for the thylakoids are much broader. PS II membranes (Fig. 6) were found to be less

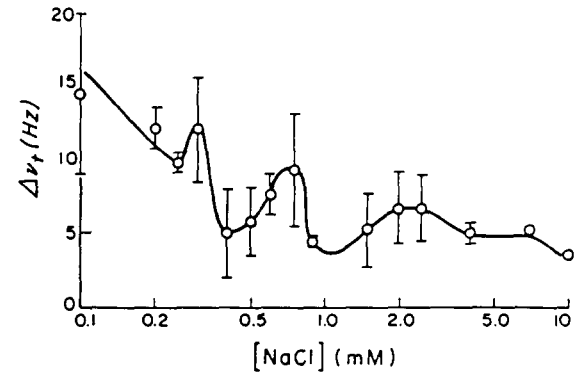

Fig. 6. ^{35}Cl-NMR binding curve for heated PS II membranes. Samples at 0.5 mg Chl ml^{-1} were heated at 38 °C for 3 min in the absence of Cl^-. For details of $^{35}Cl^-$-NMR assays, see 'Materials and methods' in Coleman et al. (1987a). Error bars show the sample standard error for 2–4 measurements of $\Delta\nu_t$ from 8 different preparations. The mean activity of the preprations was 313 μmol O_2 (mg Chl)$^{-1}$ hr^{-1}.

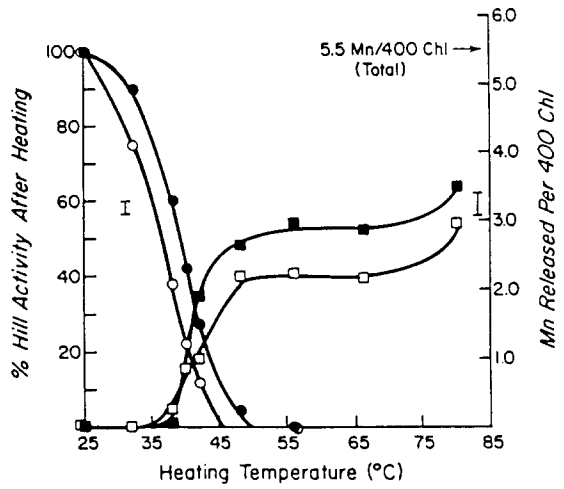

Fig. 7. Effect of partial Cl^- depletion on the loss of Hill activity and the release of functional Mn in heated thylakoids. Hill activity was measured in 50 mM Cl^- after heating for 3 min in the presence (●) or absence (○) of 50 mM Cl^-. The mean Hill activity after treatment at 25 °C was 369 μmol DCIP reduced (mg Chl)$^{-1}$ hr^{-1}. Mn content of the identical thylakoid samples was measured by the EPR method after heating in the presence (■) or absence (□) of 50 mM Cl^-. The light intensity was approximately 700 mW cm^{-2}. See 'Materials and methods' for other experimental details.

sensitive to 38 °C heat treatment than were the thylakoids, a difference which is consistent with the high concentration of sucrose in the PS II buffer. Sucrose is known to stabilize proteins against thermal denaturation by altering the structure and properties of the solvent environment (Lee and Timasheff 1981). Although the PS II membranes were less sensitive to mild heating, their ^{35}Cl-NMR binding curve still shows considerable flattening of the maxima and minima. Heating reduces the differences between the linewidth maxima and minima to such an extent that a smooth curve could also be drawn to fit the points. However, the linewidths in the vicinity of the maxima at 0.3 mM and 7.0 mM Cl^- appear to be altered more significantly than the others.

Since previously published reports have suggested that Cl^- might function as a ligand to the catalytic Mn (Sandusky and Yocum 1983, Critchley and Sargeson 1984), we simultaneously examined the effect of partial Cl^- depletion on the heat release of Mn and the inactivation of the Hill reaction. Figures 7 and 8 show that although there is a significant effect of Cl^- on the loss of activity between 25 ° and 35 °C, there is no detectable Mn release in this temperature range. In Cl^- -sufficient samples, Mn release is not correlated with loss of Hill activity until 37% of the activity is already gone. In

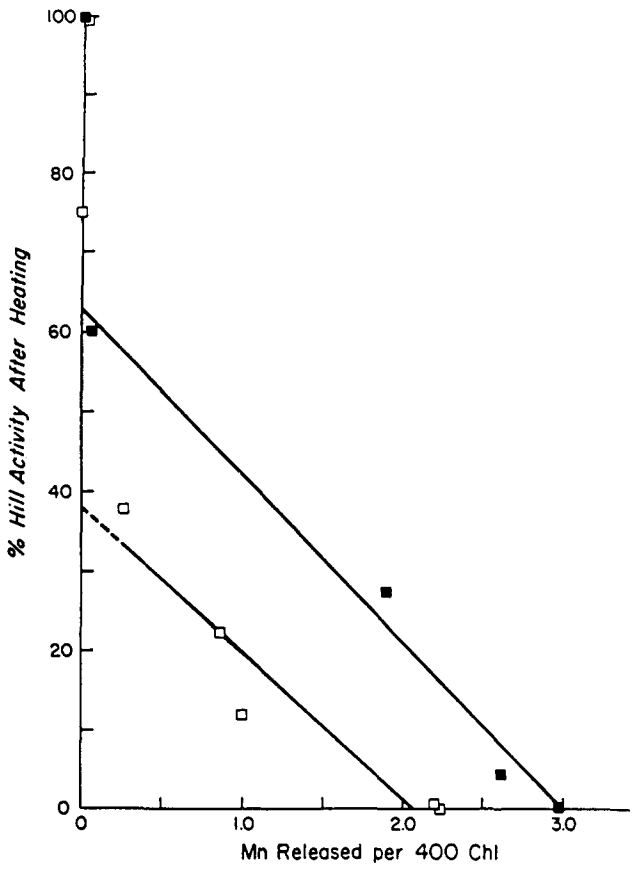

Fig. 8. Effect of partial Cl^- depletion on the correlation between the loss of Hill activity and the release of functional Mn in heated thylakoids. Data are taken from Fig. 7. (■) heated in 50 mM Cl^-; (□) heated in the absence of Cl^-.

partially Cl^--depleted samples, the two processes are not correlated until 62% of the Hill activity is lost.

In Cl^--sufficient samples, Hill activity is completely eliminated when 3.0 Mn per PS II have been released. In Cl^--depleted samples, Hill activity is eliminatd when only about 2 Mn have been released. The amount of Mn released at any given temperature is generally higher when Cl^- is present, contrary to what might be expected if Cl^- were acting as a stabilizing ligand to Mn. Even after treatment at 65 °C, only about half of the total Mn is removed from the membrane, in agreement with Nash et al. (1985), although the amount released begins to increase again at 80 °C.

Discussion

The results of these heating experiments indicate that Cl^- binding plays an important role in stabilizing the structure of the OEC. As shown by the data in Figs. 3–6, the Cl^- binding sites themselves appear to be directly affected by heat treatment. The experiments in Fig. 1 suggest that the inactivation process is more highly cooperative when Cl^- is present, creating a fairly sharp transition from the active to the inactive state (see Hinz 1986). Studies on a great number of proteins have demonstrated that ligand binding often stabilizes the protein structure against thermal denaturation (see, e.g. Webster and Gross 1965, Zyk et al. 1969, Lee et al. 1973, Fukada et al. 1983). Fukada et al. have found, for example, that the enthalpy of unfolding measured for the L-arabinose binding protein from *E. coli* increases in the presence of arabinose, and that this increase is due to the additional enthalpy of dissociation of the ligand. This effect may have a parallel in the OEC, since Hind et al. (1969) have noted that raising the temperature enhances the apparent dissociation of Cl^- from the thylakoid membrane. If Cl^- must first dissociate from the OEC before unfolding can occur, then this might explain why Cl^- raises the T_m for inactivation of the Hill reaction.

A previous study has indicated that the level of protection of the Hill activity in Cl^--depleted thylakoids is anion-specific, and that Cl^- is the most effective anion (Coleman et al. 1984). The effectiveness of other anions (Br^-, NO_3^-, and SO_4^{2-}) was found to correlate with their ability to stimulate O_2 evolution in unheated thylakoids.

In Cl^--sufficient thylakoids, there is an additional protective effect of monovalent and divalent cations when they are added to the heating medium (Weis 1982). Krishnan and Mohanty (1984) have also reported that treatment with 150 mM $MgCl_2$ after heating partially reactivates O_2 evolution in thylakoids.

The loss of kinetic cooperativity following mild heat treatment, if it is analogous to the classical "desensitization" of allosteric enzymes (Gerhart and Pardee 1962, 1963; Stadtman 1966, 1970; Kurganov 1982 (pp. 24–28)), suggests that Cl^- binding may involve both an active site and allosteric sites. There is a difficulty in interpreting such experiments, however, if the cooperativity involves subunit-subunit interactions, since the loss of coopertivity following a particular treatment may simply result from dissociation of the enzyme subunits (Gerhart and Pardee 1963), rather than from specific modification of the allosteric binding site(s). In PS II membranes, there is evidence that treatment at moderately high temperatures ($>40\,°C$) releases a fraction of the 24 and 33 kD extrinsic polypeptides from the OEC (Nash et al. 1985).

Our observation that the ^{35}Cl-NMR linewidth maxima can be reduced (Fig. 6) or eliminated (Fig. 5) by heating in the absence of Cl^- indicates that this treatment alters the structure of the Cl^--binding domains (a direct effect) and/or their sensitivity to Cl^- (an indirect effect). Monitoring of the protein conformation itself would be useful in clarifying this point. Based on our earlier ^{35}Cl-NMR study of the OEC, we proposed that the four sequestered Cl^--binding domains are located on the 33 kD extrinsic polypeptide (Coleman et al. 1987b). The modification of the 1st and 4th linewidth maxima relative to the 2nd 3rd suggests that the four putative domains might be arranged into two intramolecular dimers (cf. the model of Coleman and Govindjee 1987), which is a common protein folding motif, particularly in Ca^{2+}-binding proteins (Privalov 1982).

Our EPR measurements of the heat release of Mn from EDTA-washed thylakoids indicate that dissociation of Mn from the OEC is correlated with a decline in Hill activity at heating temperatures above 37 °C. At lower temperatures, the Cl^--binding sites are primarily affected. This result (see Fig. 8) is in qualitative agreement with the finding of Nash et al. (1985; compare with the inset to their Fig. 4), who noted that the decline in O_2 evolution with heating temperature for PS II membranes was steeper than the decline in Mn content, although they could not fully account for the disparity. The loss of Cl^--binding capacity that we have observed by NMR may help to explain this finding.

Our observation of an incomplete correlation between the loss of Hill activity and the loss of catalytic Mn in thylakoids is in disagreement with several earlier studies, which employed a different method for preparing thylakoids. Previous indications of a direct correlation between the two phenomena (Kimimura and Katoh 1972, Cramer et al. 1981) were probably affected by the presence of very loosely bound, non-catalytic Mn (Wydrzynski 1982), which was substantially removed by EDTA treatment of our thyakoid preparations. It is also possible that EDTA treatment enhances the permeability of the membrane to Mn^{2+}. Using PS II membranes (from thylakoids isolated without EDTA treatment), Nash et al. (1985) reported a correlation between loss of O_2 evolution and loss of Mn at high temperatures, as we also have observed, but they apparently did not examine the effect at lower temperatures.

EPR measurement of Mn release also raises the question of whether some of the liberated Mn was trapped by the thylakoid membrane. However, this idea needs to be reconsidered in the light of recent evidence. Previous reports have indicated that Mn remains in the lumen after disruptive treatments

because of the impermeability of the membrane itself (Blankenship and Sauer 1974). In the case of heat treatment, however, Miller and Cox (1984) have reported that endogenous Mn released by heating at 45 °C passes rapidly across the membrane. Exogenously added Mn^{2+} also equilibrates rapidly across the thylakoid membrane (Miller and Cox 1982) and is effective (in micromolar concentrations) as an electron donor to Z^+ (Velthuys 1983), which is situated on the inner side. Other studies have shown that exogenous Mn^{2+} added in the dark to EDTA-washed, Cl^--depleted thylakoids interferes with Cl^- activation of O_2 evolution (Izawa et al. 1983). Chloride release from EDTA-washed thylakoids at alkaline pH also appears to be accelerated in darkness by exogenous Mg^{2+} (Homann et al. 1983), and it is difficult to explain how Mg^{2+} could cross the membrane if Mn^{2+} cannot.

The most reasonable explanation for the observed sequestering of endogenous Mn after it is released from its binding site is that in some cases (perhaps more commonly after Tris treatment than after heat treatment) it becomes bound to non-specific sites on the OEC proteins. This could explain, for example, why the extracted 33kD extrinsic polypeptide has sometimes been found to contain Mn (Abramowicz and Dismukes 1984). Because of this possibility, we followed the method of Khanna et al. (1983), and deliberately added 0.2 mM EDTA to the suspension buffer, so that enough chelator would be present in the heating medium to drive Mn^{2+} into solution. Our observation that washing the heated membranes with a small amount of Mg^{2+} did not affect their Mn content indicates that we were successful in thoroughly removing any non-specifically bound, heat-released Mn. For these reasons, we believe that our quantitation of Mn release from EDTA-washed thylakoids is no less accurate than measurements using PS II preparations, particularly since there is less likelihood of disrupting the PS II polypeptides during preparation.

Taking these considerations into account, the data in Figs. 7 and 8 suggest that Cl^- may not be dirctly involved in ligating or stabilizing the catalytic Mn, since the stability of the Hill reaction shows a definite dependence on Cl^- in the temperature range(25 °–35 °C) at which no Mn release is observed. This finding is consistent with the results of EXAFS studies by Yachandra et al. (1986), in which it was shown that Cl^- is not coordinated to the EPR-observable Mn in the S_1 state. We observed, moreover, that Mn release at higher temperatures is slightly greater when Cl^- is present. Our finding that the release of Mn as a function of heating temperature is biphasic is consistent with other reports of the heterogeneous nature of Mn binding to the OEC (for a review, see Dismukes 1986).

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