INVOLVEMENT OF Ca^{2+} IN Cl^- BINDING TO THE OXYGEN EVOLVING COMPLEX OF PHOTOSYSTEM II.

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INTRODUCTION

Although numerous kinetic studies have sought to explain the mechanism of Cl^- activation of the oxygen-evolving complex (OEC) of Photosystem II (PS II) (1,2), a thorough understanding of the mechanism has been hindered by a lack of knowledge about Cl^- binding. The technique of ^{35}Cl-NMR was previously used to examine Cl^- binding in thylakoids from halophytes (3,4), but its application was restricted to plants requiring high Cl^- concentrations because of low instrumental sensitivity. We have succeeded in extending this NMR approach to measure Cl^- binding in spinach PS II particles by using a specially designed probe, which has enabled us to obtain ^{35}Cl-NMR spectra in the 0.1-10 mM range where oxygen evolution is activated. Results obtained by this method provide additional insight into the details of Cl^- binding in the OEC.

MATERIALS AND METHODS

PS II particles were prepared from market spinach by a modification of the method of Berthold et al. (5), using only a single Triton X-100 treatment which was followed by several washes. Depletion of Cl^- was achieved by incubating the particles in a Cl^--free buffer containing 50 mM Na_2SO_4, and/or by a brief high-pH treatment (20s at pH 8.2). Salt-washed particles (6) were prepared by incubation for 30 min in buffer containing 1.0 M NaCl on ice in the dark, followed by several washes in Cl^--free buffer. The particles were stored at 77K at a concentration of 2.5 mg Chl ml^{-1} in 400 mM sucrose, 20 mM MES at pH 6.0 until use.

The mean level of Cl^- depletion for the intact particles, as measured by the Hill activity $\pm Cl^-$ ($H_2O \rightarrow$ ferricyanide/2,6-dichloro-p-benzoquinone) was 20%. The mean Hill activity at pH 6.0 in the presence of 50 mM NaCl was 402 μmol O_2 (mg Chl)$^{-1}$ hr^{-1}. For the NaCl-washed particles, the mean activity (in μmol O_2 (mg Chl)$^{-1}$ hr^{-1}) was 28 in the presence of 50 mM NaCl, 102 in the presence of 50 mM NaCl/2.0 mM $CaSO_4$, and 198 in the presence of 25 mM $CaCl_2$.

The 8-ml NMR sample cell, 20 mm sideways-spinning probe, and 250 MHz NMR spectrometer are described in ref. (4,7). The net line-broadening ($\Delta\nu_t$) was calculated by measuring the full linewidth at half-maximum intensity for a spectrum of an NaCl-containing buffer solution; this value was then subtracted from the observed linewidth for each PS II particle suspension.

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RESULTS AND DISCUSSION

A simple two-state model for Cl^- binding to proteins (8) or thylakoid membranes (4,9) predicts that a plot of $\Delta\nu_t$ vs. $[Cl^-]$ will be a smooth, descending hyperbola. This is not the case for Cl^- binding to spinach thylakoids (data not shown) or PS II particles (Fig. 1, top).

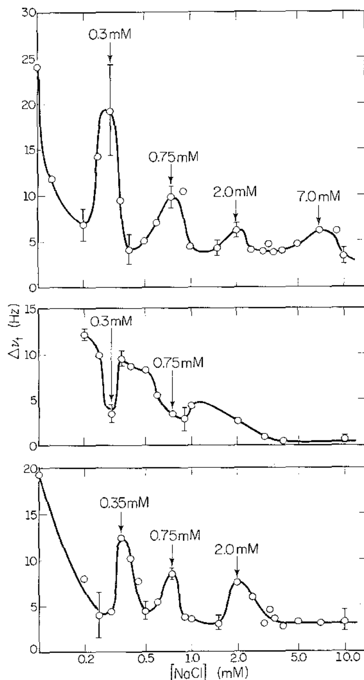

Figure 1. ^{35}Cl-NMR Binding Curves for Spinach PS II Particles. Top: Intact, Cl^--depleted particles. Middle: Particles washed with 1.0 M NaCl and then Cl^--depleted. Bottom: NaCl-washed particles with 2.0 mM $CaSO_4$ added to the NMR cell. Spectra were obtained at 24.51 MHz using a 33 μs 90° pulse and 360 ms recycle time. Signals were stored and averaged using a Nicolet 1180E computer. PS II particles were suspended in 400 mM sucrose, 20 mM MES at pH 6.0 and a Chl concentration of 0.5 mg Chl ml^{-1}. Error bars show the sample standard deviation for the mean value of $\Delta\nu_t$, with each point representing up to 7 different PS II preparations. The remainder of the error bars (which are approximately ±1.5 Hz) have been omitted for clarity. (After Coleman, Govindjee and Gutowsky, 1986; submitted for publication to Biochim. Biophys. Acta.)

In the latter case, the curve is interrupted by sharp increases in linewidth at 0.3 mM, 0.75-0.9 mM, and 2.0 mM, with an additional small broadening at 7.0 mM Cl^-. The appearance of these maxima probably reflects the presence of 3-4 distinct Cl^- binding sites within the OEC. We propose that they arise because of Cl^- induced changes in the affinity of the OEC for Cl^- (i.e. cooperative binding and/or alterations in the exchange of bound and free Cl^-).

In order to determine what proteins or cofactors might be involved in this phenomenon, we removed the extrinsic 18 and 24 kD polypeptides by washing with 1.0 M NaCl (6). As shown in Fig.1 (middle), NaCl-washing has two effects on the binding curve: 1) it lowers the overall curve, which probably reflects reduced affinity of the OEC for Cl^-, and 2) it creates sharp decreases in linewidth (minima) at 0.3 mM and 0.75-0.9 mM Cl^-. These binding effects are consistent with the decreased effectiveness of Cl^- as an activator of O_2-evolution in these particles.

When the assay mixture for the NaCl-washed particles was supplemented with 2.0 mM $CaSO_4$ in addition to 50 mM NaCl, the activity increased nearly four-fold. Likewise, addition of 2.0 mM $CaSO_4$ to the suspensions used in the Cl-NMR binding experiments also partially restored the linewidth maxima in the binding curve (Fig. 1, bottom).

The Ca^{2+}-dependent restoration of both O_2-evolution and Cl^- binding in NaCl-washed PS II particles strongly suggests that Ca^{2+} is required for Cl^- binding, either as a source of positive charge or as a stabilizer of protein conformation. Furthermore, since both the 18 and 24 kD polypeptides are absent, these results indicate that the 33 kD polypeptide or another intrinsic polypeptide is a functional location for Cl^- and Ca^{2+} binding.

Support for the 33 kD hypothesis can be found by examining the amino-acid sequence of the spinach 33kD polypeptide (10). In addition to potential Mn binding sites (10), this protein contains four regions that are rich in basic amino acids capable of binding Cl^-: 1) residues 1-20 (1 α-amino, 3 Lys, 1 Arg); 2) residues 41-80 (8 Lys, 1 Arg); 3) residues 101-161 (5 Lys, 3 Arg); and 4) residues 178-236 (7 Lys, 1 Arg).

There are also at least two regions which might function as Ca^{2+} binding sites (Fig. 2; see also ref. 11): residues 81-116 and 177-191. These potential Ca^{2+} binding sites overlap two of the Cl^- binding regions proposed above. The number of available Ca^{2+} ligands may not be optimal for tight Ca^{2+} binding, however, and this may explain why the 18 and 24 kD polypeptides appear to augment the ability of the depleted membrane to retain Ca^{2+} and Cl^- (see ref. 12 for a review). Furthermore, other evidence indicates that Ca^{2+} stimulates a low level of O_2-evolution at very high $[Cl^-]$ in the absence of the 33, 24, and 18 kD proteins (13). This suggests that intrinsic proteins such as D_2 may also be involved in Ca^{2+} and Cl^- binding.

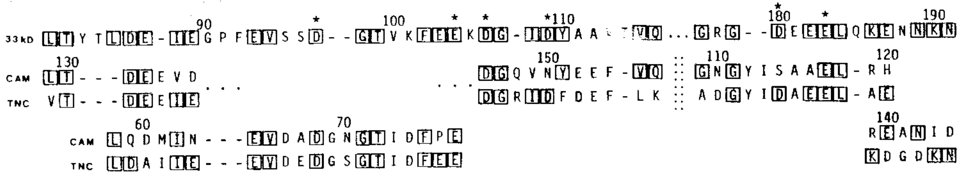

Figure 2. A Comparison of the Amino Acid Sequences for Two Regions of the 33 kD Polypeptide and Similar Sequences from Two Calcium Binding Proteins. The top line shows part of the sequence of the spinach 33 kD polypeptide ("33 kD"), as numbered in ref. 10 . The pairs of sequences arranged underneath it are those of mammalian calmodulin ("CAM") and troponin C from rabbit fast striated muscle ("TNC"). The numbering system for CAM and TNC is taken from ref. 11. Boxes over certain residues indicate homologies. Asterisks indicate potential Ca^{2+} ligands in the 33 kD protein, by analogy with similar binding sites in the two Ca^{2+}-binding proteins.

We have used information from our Cl-NMR binding studies, along with the published amino acid sequence, in order to show schematically how Cl^- and Ca^{2+} might interact with the 33 kD polypeptide (Fig. 3). As suggested by the close proximity in which we have placed the Cl^- and

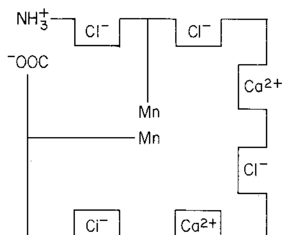

Figure 3. A Hypothetical Scheme for the Relative Positions of Cl^-, Mn, and Ca^{2+} Binding Sites on the 33 kD Polypeptide from Spinach PS II. Similar Scheme may apply if any of the binding sites are on other intrinsic polypeptides (D_1 or D_2).

Ca^{2+} binding sites, we speculate that the binding of Ca^{2+} is necessary to increase substantially the affinity of the protein for Cl^-. The binding of Cl^- to specific sites in turn activates the water-splitting reactions by accelerating the extraction of protons from water, as described earlier (14).

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