

# Monovalent cations ( $\text{Na}^+$ , $\text{K}^+$ , $\text{Cs}^+$ ) inhibit calcium activation of photosynthetic oxygen evolution

Charlene M. Waggoner, Vincent Pecoraro and Charles F. Yocum

*Departments of Biology and Chemistry, The University of Michigan, Ann Arbor, MI 48109-1048, USA*

Received 19 December 1988

In the absence of the 17 and 23 kDa water-soluble polypeptides, the oxygen-evolving complex of photosystem II requires addition of  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  to maximize rates of oxygen evolution activity. We have found that monovalent cations ( $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Cs}^+$ ) inhibit the calcium-binding site responsible for activation of the oxygen-evolving complex.  $\text{K}^+$  inhibits in a purely competitive manner, while  $\text{Na}^+$ , with an ionic radius much closer to that of calcium, displays mixed-type inhibition kinetics. The  $K_i$  values for  $\text{Na}^+$  and  $\text{K}^+$  have been estimated to be in the range of 8–10 mM. These values are well below the concentrations of these ions commonly used in buffer titrations and  $\text{Cl}^-$  additions to standard oxygen evolution assay media and as such could alter the interpretation of data regarding calcium activation of oxygen evolution activity. An organic cation, tetramethylammonium, does not inhibit calcium activation and can be substituted for the inorganic cation as a  $\text{Cl}^-$  counterion in oxygen evolution assays.

Oxygen evolution; Photosystem II; Binding site inhibition; Monovalent cation effect;  $\text{Ca}^{2+}$  activation

## 1. INTRODUCTION

Removal of the 23 kDa and 17 kDa extrinsic polypeptides from PS II by exposure to conditions of elevated ionic strength inhibits oxygen evolution activity [1]. Investigations in several laboratories have shown that addition of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  after polypeptide depletion reconstitutes substantial rates of oxygen evolution activity [2–5];  $\text{Ca}^{2+}$  has been established as an essential cofactor for the oxygen-evolving reaction [2]. Of the two polypeptides removed by salt washing, it is the 23 kDa protein which appears to be required for retention of calcium at its site of action in the oxygen-evolving complex [6]. Several investigators [2,7,8] have employed salt-washed PS II preparations as model systems for examining the roles played by  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  in the process of oxygen evolution. They (see [2,7,8]) have also examined the  $\text{Ca}^{2+}$  require-

ment for oxygen evolution in salt-washed PS II membranes. While a consensus exists that the presence of the ion is necessary for activity, there is a lack of agreement about the optimal concentration for  $\text{Ca}^{2+}$ , its  $K_d$ , and its stoichiometry with regard to the other components (e.g. manganese) at the site of action in PS II.

In the course of experiments intended to explore further the roles of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  in the oxygen-evolving reaction, we have found that monovalent cations such as  $\text{Na}^+$  and  $\text{K}^+$ , commonly used as  $\text{Cl}^-$  counterions in kinetic experiments and in the preparation of buffer solutions, act as inhibitors of the  $\text{Ca}^{2+}$ -dependent activation of oxygen evolution activity in salt-washed PS II membranes. The interference exhibited by these monovalent cations is competitive, and affects the estimated  $K_d$  for  $\text{Ca}^{2+}$  at its site of action in the oxygen-evolving reaction. We suggest that this interference may explain at least part of the confusion surrounding the nature of the interaction of  $\text{Ca}^{2+}$  with PS II. These problems can be avoided by the use of an organic salt of chloride which, as we describe here, does not affect the kinetics of the binding of  $\text{Ca}^{2+}$  to PS II which activates oxygen evolution.

*Correspondence address:* C.M. Waggoner, Departments of Biology and Chemistry, The University of Michigan, Ann Arbor, MI 48109-1048, USA

*Abbreviations:* PS, photosystem; TMACL, tetramethylammonium chloride; Mes, 2-(*N*-morpholino)ethanesulfonic acid

## 2. MATERIALS AND METHODS

PS II membranes were isolated according to Berthold et al. [9], and depleted of the 17 and 23 kDa extrinsic proteins by exposure to 2 M NaCl and 1 mM EGTA at pH 6.0 on ice in room light for 1 h at a chlorophyll concentration of 1.5 mg/ml. The salt-washed samples were washed in a  $15 \times$  volume of 20 mM Mes-Tris, pelleted, and stored at 3 mg chlorophyll/ml in 20 mM Mes-Tris. Oxygen evolution activity was determined using a Clark-type oxygen electrode with 2,6-dichloro-*p*-benzoquinone as the acceptor. The standard assay medium contained 20 mM Mes buffer whose pH had been adjusted to 6.0 with the free base form of Tris to avoid inclusion of monovalent metal cations. Under these conditions, the concentration of the Tris free base species was 100  $\mu$ M, which is insufficient to interfere with oxygen evolution activity [10]. This was verified by assays employing salt-washed PS II membranes with either Tris or  $\text{Na}^+$  (from NaOH) present.  $\text{Ca}^{2+}$  was added to assays in the form of calcium hydroxide buffered with Mes to pH 6, or as the chloride salt.  $\text{Na}^+$ ,  $\text{K}^+$ , or  $\text{Cs}^+$  were added as the hydroxide salts, neutralized with Mes as described for  $\text{Ca}^{2+}$  above.  $\text{Cl}^-$  additions were made using the metal cation described in the figure legends or, alternatively, as the tetramethylammonium salt. In all cases, the final ionic strength of the assay solutions was the same.

## 3. RESULTS

Control PS II membranes, prior to removal of extrinsic 17 and 23 kDa polypeptides, exhibited oxygen evolution activities of 750–800  $\mu$ mol  $\text{O}_2$ /mg chlorophyll per h. Salt washing as described in section 2 produced an apparent loss of essentially all of the 17 and 23 kDa water-soluble polypeptides, as assessed by polyacrylamide gel electrophoresis followed by staining with Coomassie blue. In the absence of added  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  in the assay media, the salt-washed PS II preparations retain 5–10% of control PS II oxygen evolution activity. This activity is also hydroquinone-insensitive, indicating that 5–10% of the 23 kDa water-soluble polypeptide is present [6] even though undetected by Coomassie blue staining on gels. The addition of 30 mM  $\text{Cl}^-$  and 15 mM  $\text{Ca}^{2+}$  produces a reconstitution of 55–60% of the control PS II activity.

Double-reciprocal plots ( $1/\text{activity}$  vs  $1/[\text{CaCl}_2]$ ) from experiments with the salt-washed PS II membranes in the presence of varied concentrations of NaCl and KCl reveal an inhibitory behavior by these cations. The data of fig.1 show that  $\text{K}^+$  is a competitive inhibitor [11] of  $\text{Ca}^{2+}$  activation of oxygen evolution activity. The  $K_i$  for  $\text{K}^+$ , as obtained from a Dixon plot (not shown) is estimated to be

10 mM.  $\text{Na}^+$  is also inhibitory, displaying mixed-type inhibition kinetics (fig.2) which indicate that at least two types of inhibition or binding sites are

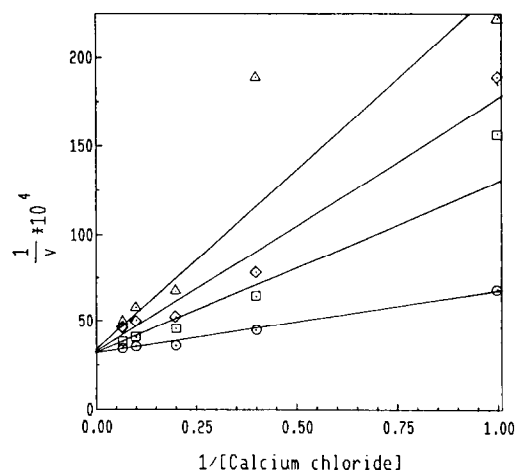


Fig.1. Inhibition of  $\text{Ca}^{2+}$ -activated oxygen evolution by  $\text{K}^+$ . The assay media contained a mixture of Mes-Tris and Mes-KOH (pH 6.0). All sets of data have had baseline rates of oxygen evolution in the absence of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  subtracted; this rate is produced by intact centers containing both the 17 and 23 kDa polypeptides. (Circles) 30 mM Mes-Tris; (squares) 10 mM Mes-KOH, 20 mM Mes-Tris; (diamonds) 20 mM Mes-KOH, 10 mM Mes-Tris; (triangles) 30 mM Mes-KOH.

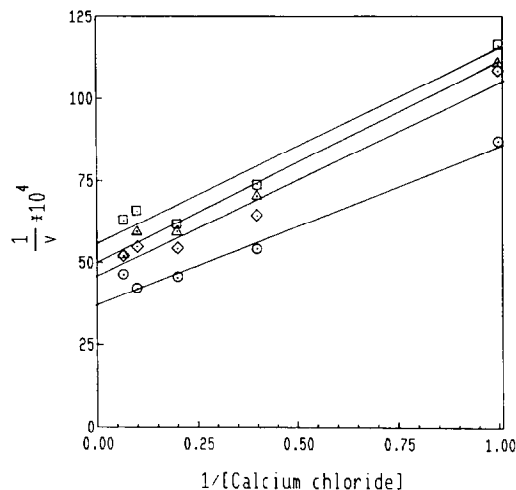


Fig.2. Inhibition of  $\text{Ca}^{2+}$ -activated oxygen evolution by  $\text{Na}^+$ . Data calibration was performed as in the legend to fig.1. Assay media contained a mixture of Mes-Tris and Mes-NaOH to the designated concentrations. (Circles) 30 mM Mes-Tris; (diamonds) 10 mM Mes-NaOH, 20 mM Mes-Tris; (triangles) 20 mM Mes-NaOH, 10 mM Mes-Tris; (squares) 30 mM Mes-NaOH.

involved. The estimated  $K_i$  for  $\text{Na}^+$  is in the range of 8 mM. Finally,  $\text{Cs}^+$  is also a competitive inhibitor of oxygen-evolving activity, exhibiting the strongest inhibition of the monovalent cations tested (not shown).

The data presented above reveal a previously unsuspected complication in studies where the amounts of  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  must be varied independently in experiments with salt-washed PS II preparations, or where zwitterionic buffers neutralized with NaOH or KOH are components of assay mixtures. We have found that the water-soluble organic salt tetramethylammonium chloride, which does not alter the pH of assay solutions, can serve as a noninhibitory  $\text{Cl}^-$  donor in our reconstitution experiments. The tetramethylammonium ion itself does not interfere with the  $\text{Cl}^-$ -dependent reconstitution of oxygen evolution activity in salt-washed or control PS II membranes (fig.3), in marked contrast to the situation observed with  $\text{Na}^+$  or  $\text{K}^+$ . A comparison of tetramethylammonium chloride and NaCl as  $\text{Cl}^-$  donors in assays of activity (fig.4) reveals that the tetramethylammonium ion does not inhibit oxygen evolution activity even at a concentration of 60 mM, which is much greater than the values

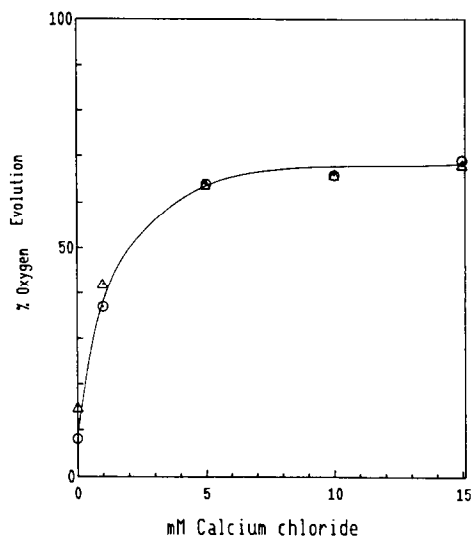


Fig.3. Effect of tetramethylammonium ion on oxygen evolution. Rate of oxygen evolution is given as a percent of the rate of oxygen evolution of untreated intact PS II membranes ( $735 \mu\text{mol O}_2/\text{mg chlorophyll per h}$ ). (Circles) 30 mM Mes-Tris; (triangles) 30 mM Mes-TMAOH.

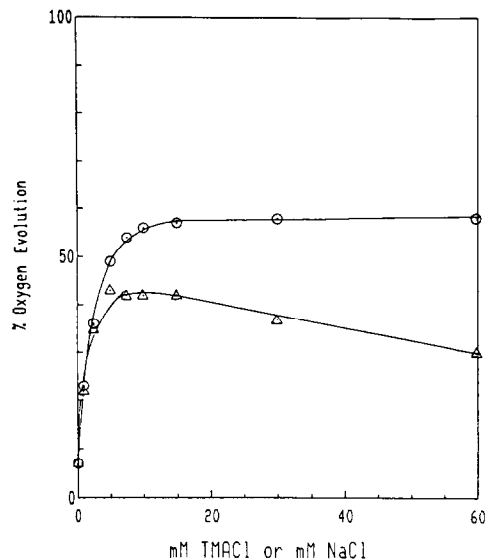


Fig.4. Effect of tetramethylammonium and  $\text{Na}^+$  as  $\text{Cl}^-$  counterions. All assays were carried out in media containing 1 mM  $\text{Ca(OH)}_2$ -Mes and 20 mM Mes-Tris at pH 6.0. Rates of oxygen evolution are given as a percent of the rate of oxygen evolution of untreated intact PS II membranes ( $768 \mu\text{mol O}_2/\text{mg chlorophyll per h}$ ). (Circles) Tetramethylammonium chloride; (triangles) NaCl additions.

usually found in oxygen evolution assay media. However,  $\text{Na}^+$  inhibits oxygen evolution at concentrations well below the level of NaOH required to titrate 50 mM Mes buffer to pH 6.0, and it becomes increasingly more inhibitory as the concentration increases. A double-reciprocal plot of the data in fig.4 (not shown) demonstrates that NaCl produces kinetics indicative of non-competitive inhibition of the  $\text{Cl}^-$ -binding site. This inhibition is attenuated by increasing the concentration of  $\text{Ca}^{2+}$  in the assay media (not shown), which indicates that  $\text{Na}^+$  is not competing for the  $\text{Cl}^-$ -binding site. This finding eliminates the  $\text{Cl}^-$ -binding site in the oxygen-evolving reaction as the site at which  $\text{Na}^+$  inhibits, and reinforces our conclusion, based on the other data presented here, that it is the  $\text{Ca}^{2+}$ -binding site in the oxygen-evolving reaction which is the site at which monovalent metal cations interfere with oxygen evolution activity.

#### 4. CONCLUSIONS

The reactivation of salt-washed PS II mem-

branes by  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  has been well documented. However, all reports to date utilized assay media containing an inhibitory ion such as  $\text{Na}^+$ . Not only is  $\text{Na}^+$  added in the form of  $\text{NaCl}$  used to balance  $\text{Cl}^-$  concentrations, but it is also commonly used in Mes-NaOH buffer systems. The  $K_i$  for monovalent cation inhibition of the  $\text{Ca}^{2+}$  activation process we report here is estimated to be in the range of 8–10 mM, a concentration which is well below the cation level that would be present in Mes buffer at a concentration of 50 mM at pH 6. Since, in the absence of cationic inhibitors, approx. 2 mM  $\text{Ca}^{2+}$  saturates the oxygen-evolving reaction, high concentrations of inorganic cations such as  $\text{Na}^+$  or  $\text{K}^+$  will alter the kinetics of  $\text{Ca}^{2+}$ -activated oxygen evolution. In the light of these observations, all estimates of  $\text{Ca}^{2+}$ -binding constants, the functional role of the cation and its interactions with the other components of the oxygen-evolving complex made in the presence of  $\text{Na}^+$  need to be reexamined. Our preliminary experiments suggest that exclusion of  $\text{Na}^+$  in  $\text{Ca}^{2+}$ -binding measurements yields an estimated  $\text{Ca}^{2+}$   $K_d$  of  $\leq 20 \mu\text{M}$  which is considerably lower than that observed with  $\text{NaCl}$  and Mes-NaOH present under standard assay conditions.

Although cation-binding sites have high specificities for select ions, it is not unusual for  $\text{Ca}^{2+}$ -binding proteins to be affected by other cationic species including mono- and trivalent cations [12], and our findings and those published earlier [13] show that both monovalent and trivalent metals can bind to the site normally occupied by  $\text{Ca}^{2+}$  in the oxygen-evolving complex. In contrast to the lanthanides, which produce an irreversible inhibition characterized by loss of manganese, the monovalent cations we have examined produce a freely reversible inhibition. Like

calmodulin and other  $\text{Ca}^{2+}$ -binding proteins that have been characterized [12], the  $\text{Ca}^{2+}$ -binding site(s) in PS II appear(s) to accommodate a range of alternative metal ions, the notable differences being the higher apparent  $K_d$  value obtained for  $\text{Ca}^{2+}$  when it acts as a promoter of oxygen evolution activity in PS II.

*Acknowledgements:* This research is supported by the Competitive Research Grants Office of the United States Department of Agriculture (G-86-CRCR-1-2025 and 88-37130-3546).

## REFERENCES

- [1] Akerlund, H.-E., Jansson, C. and Andersson, B. (1982) *Biochim. Biophys. Acta* 681, 1–10.
- [2] Ghanotakis, D., Babcock, G. and Yocum, C. (1984) *FEBS Lett.* 167, 127–130.
- [3] Ghanotakis, D., Topper, J., Babcock, G. and Yocum, C. (1984) *FEBS Lett.* 170, 169–173.
- [4] Ikeuchi, M. and Inoue, Y. (1986) *Arch. Biochem. Biophys.* 247, 97–107.
- [5] Boussac, A., Maisson-Peteri, B., Etienne, A.-L. and Verrotte, C. (1985) *Biochim. Biophys. Acta* 808, 231–234.
- [6] Waggoner, C. and Yocum, C. (1987) in: *Progress in Photosynthesis Research* (Biggens, J. ed.) vol. I, pp. 685–688, Nijhoff, Dordrecht.
- [7] Preston, C. and Critchley, C. (1985) *FEBS Lett.* 184, 318–322.
- [8] Cammarata, K. and Cheniae, G. (1987) *Plant Physiol.* 84, 587–595.
- [9] Berthold, D., Babcock, G. and Yocum, C. (1981) *FEBS Lett.* 134, 231–234.
- [10] Sandusky, P. and Yocum, C. (1986) *Biochim. Biophys. Acta* 849, 85–93.
- [11] Segel, I. (1975) *Enzyme Kinetics*, Wiley, New York.
- [12] Levine, B. and Williams, R. (1982) in: *Calcium and Cell Function* (Cheung, W. ed.) vol. II, pp. 2–38, Academic Press, London.
- [13] Ghanotakis, D., Babcock, G. and Yocum, C. (1985) *Biochim. Biophys. Acta* 809, 173–180.