Ca^{2+} Function in Photosynthetic Oxygen Evolution Studied by Alkali Metal Cations Substitution

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ABSTRACT Effects of adding monovalent alkali metal cations to Ca^{2+}-depleted photosystem (PS)II membranes on the biochemical and spectroscopic properties of the oxygen-evolving complex were studied. The Ca^{2+}-dependent oxygen evolution was competitively inhibited by K^+, Rb^+, and Cs^+, the ionic radii of which are larger than the radius of Ca^{2+} but not inhibited significantly by Li^+ and Na^+, the ionic radii of which are smaller than that of Ca^{2+}. Ca^{2+}-depleted membranes without metal cation supplementation showed normal S_2 multiline electron paramagnetic resonance (EPR) signal and an $S_2Q_A^-$ thermoluminescence (TL) band with a normal peak temperature after illumination under conditions for single turnover of PSII. Membranes supplemented with Li^+ or Na^+ showed properties similar to those of the Ca^{2+}-depleted membranes, except for a small difference in the TL peak temperatures. The peak temperature of the TL band of membranes supplemented with K^+, Rb^+, or Cs^+ was elevated to ~38°C which coincided with that of $Y_D^+Q_A^-$ TL band, and no S_2 EPR signals were detected. The K^+-induced high-temperature TL band and the $S_2Q_A^-$ TL band were interconvertible by the addition of K^+ or Ca^{2+} in the dark. Both the Ca^{2+}-depleted and the K^+-substituted membranes showed the narrow EPR signal corresponding to the $S_2Y_Z^+$ state at $g = 2$ by illuminating the membranes under multiple turnover conditions. These results indicate that the ionic radii of the cations occupying Ca^{2+}-binding site crucially affect the properties of the manganese cluster.

INTRODUCTION

Photosynthetic oxygen evolves in the oxygen-evolving complex (OEC) of photosystem (PS)II. The process is catalyzed by a tetranuclear manganese cluster, the oxidation state of which changes with the period of four during the photochemical oxidation of substrate water molecules to yield oxygen. The change in the oxidation state of the manganese cluster corresponds to that of the kinetically determined intermediate oxidation states of OEC termed S_i ($i = 0-4$). Upon illumination, each S state advances stepwise by absorbing successive photons to the highest oxidation state, S_4, which is an unstable intermediate state and, in turn, decays to the S_0 state in the dark with the release of molecular oxygen. Because the S_1 state is thermally stable, a molecular oxygen is evolved after three flashes when dark-adapted PSII is illuminated with a series of single-turnover flashes (Debus, 1992; Britt, 1996; Witt, 1996; Renger, 1997).

Ca^{2+} is an indispensable metal cofactor required for oxygen evolution that is inhibited by the depletion of one Ca^{2+}/PSII and is restored by the reconstitution with Ca^{2+} (Yocum, 1991; Debus, 1992). Extended x-ray absorption fine structure spectroscopy (EXAFS) (Yachandra et al., 1993; Latimer et al., 1995; Cinco et al., 1998) and Fourier transform infrared (FTIR) (Noguchi et al., 1995) studies indicate that Ca^{2+} is closely associated with the manganese cluster, presumably through a carboxylate bridge to form a multimetal center (Hatch et al., 1995; Riggs-Gelasco et al., 1996). Recently, crystal structure of oxygen-evolving PSII was reported at 3.8 Å resolution, but no electron density could be assigned to the Ca^{2+} ions (Zouni et al., 2001). In the absence of Ca^{2+}, the electronic structure of the manganese cluster is changed (Ono et al., 1991; Ono et al., 1993; Latimer et al., 1998) and the oxidation process of the cluster beyond the S_2 or S_3 state is interrupted, concomitant with the oxidation of Y_Z and/or an auxiliary redox component(s) of PSII (Boussac et al., 1989, 1990b; Sivaraja et al., 1989; Ono and Inoue, 1990; Hallahan et al., 1992; Ono et al., 1993; Gilchrist et al., 1995; Tang et al., 1996; Mino et al., 1998). Although mutagenesis studies suggest that Asp59 and 61 in the AB-loop of the D1 protein might bind Ca^{2+} (Chu et al. 1995), the identity of the ligand for Ca^{2+} binding in PSII remains unresolved. Ca^{2+} is not exchangeable for aqueous ions but becomes extractable when the 24-kDa protein is absent in the OEC of higher plants (Ghanotakis et al., 1984b; Ono and Inoue, 1988). Therefore, novel procedures for Ca^{2+} depletion in PSII samples include a step for the liberation of this protein (Yocum, 1991); the released protein is isolated from the membranes by NaCl treatment (Ghanotakis et al., 1984a; Miyao and Murata, 1984) but rebound again to the membranes by low-pH treatment (Ono and Inoue, 1988). The redox properties of the Ca^{2+}-depleted OEC come to be influenced by the binding of the 24 kDa protein (Ono et al., 1992). The oxidation potential of the manganese cluster in the Ca^{2+}-depleted S_2 state is abnormally low, and the threshold temperature for formation of the S_2 state is markedly upshifted in the presence of the 24-kDa protein. In addition to the effects of the 24-kDa protein, some organic chelating agents seem to interact with

Received for publication 1 November 2000 and in final form 14 June 2001.

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0006-3495/01/10/1831/10 $2.00

the Ca^{2+}-depleted manganese cluster to modify its electron paramagnetic resonance (EPR) properties (Boussac et al., 1990a).

In general, the Ca^{2+} binding sites in various enzymes can be occupied by other metal cations. The function of Ca^{2+} can be evaluated by studying the influence of the cation substitution on the structure and function of the enzymes, although the function of the enzyme may not necessarily be preserved after cation substitution. The first experimental indication for the binding of another cation to the Ca^{2+} site in OEC has been provided by analyzing the effects of divalent cations on photoligation of the manganese cluster (Ono and Inoue, 1983), where Mn^{2+} binds to the putative Ca^{2+} site to inhibit the appearance of O_2-evolving activity (Ono and Inoue, 1983; Chen et al., 1995). Development of reliable procedures for specific depletion of the functional Ca^{2+} in OEC allows us to study the effects on OEC of Ca^{2+} depletion and replacing Ca^{2+} with other cations.

Among cations studied so far, Sr^{2+} is the only divalent cation that can functionally substitute for Ca^{2+} to some extent (Ghanotakis et al., 1984; Boussac and Rutherford, 1988a, 1988b; Ono and Inoue, 1988, 1989b). The Sr^{2+}-substituted S_2 state, however, causes an enhanced $g = 4$ EPR signal and a multiline EPR signal with modified hyperfine splitting (Boussac and Rutherford, 1988b; Boussac et al., 1989; Ono and Inoue, 1989b; Sivaraja et al., 1989), indicating that the electronic structure of the manganese cluster is somehow modified by Sr^{2+} occupation of the Ca^{2+} site. The Sr^{2+} substitution provides a potent experimental tool to study the structure and function of the Mn-Ca catalytic core using EPR (Boussac and Rutherford, 1988b; Boussac et al., 1989; Ono and Inoue, 1989b; Sivaraja et al., 1989), x-ray absorption spectroscopy (Yachandra et al., 1993; Latimer et al., 1995; Riggs-Gelasco et al., 1996; Cinco et al., 1998), and FTIR (Chu et al., 2001). Other divalent cations may also bind to the Ca^{2+} site although it cannot support O_2 evolution. Formation of the S_2 state in the cation-substituted OEC is suggested by the thermoluminescence (TL) study (Ono and Inoue, 1989b). Trivalent lanthanide cations may occupy the Ca^{2+} site, but the binding seems to be largely irreversible (Ghanotakis et al., 1985; Bakou et al., 1992; Ono, 2000). Lanthanide-substituted OECs show no S_2 EPR signals (Ghanotakis et al., 1985; Bakou et al., 1992; Hatch et al., 1995), although lanthanide (La^{3+} or Dy^{3+}) substitution induces no significant change in the features of the EXAFS spectra of the manganese cluster (Hatch et al., 1995; Riggs-Gelasco et al., 1996).

Na^+, K^+, and Cs^+ have been suggested to associate with the Ca^{2+} site on the basis of the inhibition of Ca^{2+}-dependent restoration of O_2 evolution in a competitive-like manner by these monovalent cations (Waggoner et al., 1989; Yocum, 1991). The reversibility of the inhibition by monovalent cations may render them useful tools for probing the function of Ca^{2+} in the water oxidation. However, no attempt has been made to characterize the binding of the monovalent cations and the properties of OEC, including the structure and the function of the manganese cluster of the monovalent cation substituted PSII. Notably, it has been reported that highly concentrated Na^+ reactivates O_2 evolution in PSII core complexes of cyanobacteria, where O_2 evolution activity is very low in the absence of Ca^{2+} and restored by Ca^{2+} as in the same type of core preparations from higher plants (Pauly et al., 1992; Witt, 1996).

This study examines the effects of replacing Ca^{2+} with alkali metal cations on the properties of the manganese cluster to elucidate the Ca^{2+} function in the Mn-Ca catalytic center in OEC. To avoid further complexity caused by the 24-kDa extrinsic protein and chelating agents when evaluating the effects of the binding of monovalent cations, we depleted Ca^{2+} using a high salt concentration under weak light, followed by extensive washing with a buffer medium free of metal cations and chelators. The results indicated that the properties of the manganese cluster and the redox event in OEC are significantly modified in OEC substituted with alkali metal cations of which effects are closely correlated with their ionic radii.

MATERIALS AND METHODS

Sample preparations

BBY type O_2 evolving PSII membranes prepared from spinach as described (Ono et al., 1992) were washed twice with a medium containing 400 mM sucrose, 20 mM NaCl, and 20 mM 2-[*N*-morpholino] ethanesulfonic acid (MES)/NaOH (pH 6.5) (buffer A), then suspended in the same medium. To deplete Ca^{2+}, the membranes were suspended in a medium containing 2 M NaCl, 200 mM sucrose, and 20 mM MES/NaOH (pH 6.5) at 0.5 mg of chlorophyll (Chl)/ml. The suspension was gently stirred for 20–30 min at 0°C under fluorescent light in a flat tray, then Na-EDTA was added at 1 mM, and the mixture was further incubated for 5 min in the dark. The following manipulations proceeded in the dark or under dim green light unless otherwise noted. The membranes were washed three times with a medium containing 400 mM sucrose (SigmaUltra), 20 mM 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol(bis-tris)/HCl (pH 6.5) (buffer B), and finally suspended in the same buffer. The membranes (Ca^{2+}-depleted membranes) were incubated in the dark for >3 h for relaxation to the S_1 state. To deplete the manganese cluster, the membranes were suspended in a medium containing 2 M NaCl, 200 mM sucrose, and 20 mM MES/NaOH (pH 6.5) at 1 mg of Chl/ml and incubated for 20 min at 0°C in the dark. The membranes were then treated with 1 mM NH_2OH for the depletion of the manganese cluster as described (Ono and Mino, 1999). The resulted Mn-depleted membranes were finally suspended in buffer B. For EPR measurements, the dark-adapted membrane samples in buffer B at approximately 0.5 mg of Chl/ml were supplemented with alkali metal cations (chloride salt) at the indicated concentrations. 50 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU; 10 mM dimethyl sulfoxide solution as stock) was included in the sample suspension when indicated. The membranes were precipitated by centrifugation and resuspended in the supernatant to an appropriate Chl concentration, transferred to Spracil quartz EPR tubes of 4-mm inner diameter, then stored in liquid N_2. Phenyl-*p*-benzoquinone was added to the sample at 1 mM when indicated.

Activity measurements

TL signals were measured using apparatus specifically designed in-house. Samples (0.2 mg of Chl/ml) in buffer B supplemented with various

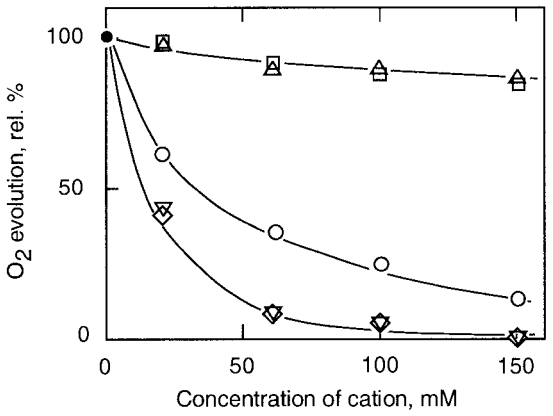

FIGURE 1 Inhibition of Ca^{2+}-dependent O_2 evolution by addition alkali metal cations. O_2 evolution activity in Ca^{2+}-depleted PSII membranes was measure in the presence of 1 mM Ca^{2+} and various concentration of Li^+ (△), Na^+ (□), K^+(◇), Rb^+ (▽), and Cs^+ (○), respectively. O_2 evolution activity of Ca^{2+}-depleted membranes was 280 and 30 μmol O_2 (mg of Chl/ml)$^{-1}$ in the presence and absence of 1 mM Ca^{2+}. Data are presented after subtracting the residual activity found in the absence of Ca^{2+}.

concentrations of alkali metal cation and 20 μM DCMU were illuminated at 5°C under continuous light (>600 nm) for 5 s, then quickly frozen in liquid N_2 in the dark unless otherwise noted. Alternatively, the Mn-depleted membranes were illuminated at 77 K for 1 min. Low-temperature X-band EPR spectra were measured with a Jeol JES-FE1XG EPR spectrometer or a Bruker E580 EPR spectrometer equipped with an Oxford-900 continuous flow cryostat. The samples were illuminated from both sides for 20 s at 0°C with 650 W projectors, quickly frozen in ethanol/dry ice bath in the dark, and transferred into liquid N_2 unless otherwise noted. The O_2 evolving activity was measured using a Clark-type oxygen electrode in buffer B supplemented with various concentrations of monovalent cations and 0.2 mM phenyl-*p*-benzoquinone at 25°C under saturating light conditions. The activity of the untreated control PSII membranes in buffer A and B were essentially the same. Alkali metal cations were added as chloride salts with a grade of 99.9% purity except for RbCl (99.8% purity).

RESULTS

O_2-evolving activity in the Ca^{2+}-depleted membranes sample was inhibited but considerably restored by Ca^{2+}. Fig. 1 shows the effects of alkali metal cations on the O_2-evolving activity restored by 1 mM Ca^{2+}. The Ca^{2+}-dependent O_2 evolution was largely inhibited by K^+, Rb^+, and Cs^+ in the following order: $K^+ = Rb^+ > Cs^+$. The inhibitory effect of these cations is reversible because Ca^{2+} dependent O_2 evolution was restored when excess alkali metal cations were removed by washing the sample membranes (data not shown). In contrast, Li^+ and Na^+ slightly inhibited the O_2 evolution, which was suppressed only by 10%, even in the presence of 100 mM concentrations of these two cations. This indicates that the inhibition by K^+, Rb^+, and Cs^+ is not attributable to nonspecific effects of highly concentrated salts. It is of note in this context that no alkali metal cations (added as chloride salts) restored O_2 evolution (data not shown) in contrast to the reported findings for core preparations of cyanobacteria (Pauly et al., 1992; Witt, 1996).

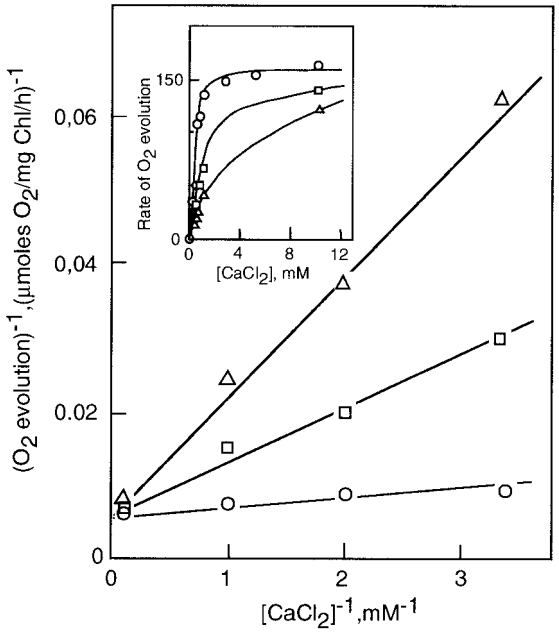

FIGURE 2 Competitive-type inhibition of Ca^{2+}-dependent O_2 evolution by K^+. Dependence of O_2 evolution on Ca^{2+} concentration was measured in the presence of 0 mM (○), 10 mM (□), and 30 mM (△) K^+. The reciprocal of O_2 evolution activity was plotted against the reciprocal of Ca^{2+} concentration. O_2 evolution activity is plotted against Ca^{2+} concentration in the inset.

This suggests that the proposed salt-dependent conformational change in cyanobacterial OEC is not responsible for the Ca^{2+}-dependent restoration of O_2 evolution in higher plant OEC.

Fig. 2 shows the effects of the Ca^{2+} concentration on the restored O_2 evolution in the presence K^+ at various fixed concentrations. The restoration of O_2 evolution was saturated at ~3 mM Ca^{2+} and activity remained constant up to 20 mM Ca^{2+} in the absence of K^+ as shown in the inset figure. The apparent Km of Ca^{2+} for the restoration of O_2 evolution was 0.2 mM in this membrane preparation (data not shown). A double reciprocal plot of the Ca^{2+}-dependent O_2-evolution rate as a function of the Ca^{2+} concentration at varying K^+ concentrations yielded linear lines with a crossing point on the *y* axis, indicating that K^+ competitively inhibits the Ca^{2+}-dependent O_2 evolution. The competitive-type inhibition was shown with either Rb^+ or Cs^+. Apparent K_i values were estimated to be 3 mM, 3 mM, and 8 mM for K^+, Rb^+, and Cs^+, respectively, based on the value of the crossing point in the Dixon-type plot. Data presented in Fig. 1 indicate that the K_i values for Li^+ and Na^+ are much larger than the value obtained for Rb^+, but we could not obtain reliable values for Li^+ and Na^+, most likely because of the side effects of salts on the activity at very high concentrations. The results indicate that K^+, Rb^+, and Cs^+ associate with the binding site for Ca^{2+} in competition with Ca^{2+} to inhibit O_2 evolution. Li^+ and Na^+ might associate

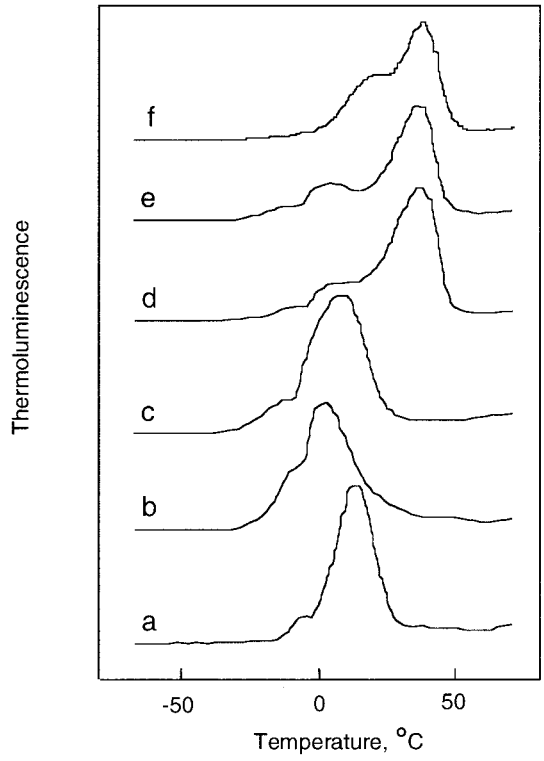

FIGURE 3 Effects of alkali metal cations on thermoluminescence glow curves in Ca^{2+}-depleted PSII membranes. None (*a*); addition of Li^+ (*b*); Na^+ (*c*); K^+ (*d*); Rb^+ (*e*); and Cs^+ (*f*). Sample membranes (0.3 mg of Chl/ml) were supplemented with 300 mM respective metal cations, incubated at 0°C for 5 min and illuminated at 5°C for 5 s. Reaction mixture included 50 μM DCMU.

to the Ca^{2+} site, but the putative affinities for these ions must be very low. The inhibition of Ca^{2+}-dependent O_2 evolution by alkali metal cations has been previously reported (Waggoner et al., 1989; Yocum, 1991), in which K^+, Cs^+, and Na^+ similarly inhibited the O_2 evolution. K^+ and Cs^+ act as competitive-type inhibitor with higher K_i value in K^+ than in Cs^+, and Na^+ acts as mixed-type inhibitor of which K_i value (8 mM) is close to that of K^+. The ion species-dependent inhibition in the present study is in conflict with the previously reported inhibitory effects, which are independent of ion species. Therefore, it is likely that our present observations are the phenomena that have not previously reported, although the inconsistency may be partly ascribed to the experimental conditions; the sample membranes were depleted of both Ca^{2+} and Cl^- (Waggoner et al., 1989) or were depleted of only Ca^{2+} in this study.

Fig. 3 shows the TL glow curves of the Ca^{2+}-depleted membranes supplemented with various alkali cations at 300 mM. The sample membranes were illuminated in the presence of DCMU, which ensures a single turnover of PSII. Ca^{2+}-depleted membranes without metal cation supplementation generated a TL band at ~12°C. The peak temperature was comparable with that of the $S_2Q_A^-$ band (Rutherford et al., 1982), which was induced at 10°C in untreated control membranes. The TL peak temperature was upshifted to 38°C by supplementation with K^+, Rb^+, or Cs^+, although a shoulder remained in the membranes supplemented with Cs^+. The small shoulder at ~0°C, found in K^+ and Rb^+ supplemented membranes, is an artifact attributable to the heating rate being altered by melting ice. Modifications of the TL band suggest that the binding of K^+, Rb^+, and Cs^+ to the Ca^{2+} site alters the redox properties of OEC. Supplementation with Li^+ or Na^+ shifted the TL peak to 3 and 10°C, respectively. Because the affinity of these two cations for the Ca^{2+} site seems to be low, as shown in Fig. 1, whether these changes are because of the binding of Li^+ or Na^+ to the Ca^{2+} site, or whether they are caused by another effect of these cations remains unclear. Taking into account the small but appreciable difference in TL peak temperature between Li^+ and Na^+ supplemented membranes, these two cations may affect OEC differently.

Fig. 4*A* shows the effects of the K^+ concentration on TL glow curves. Upon adding K^+, a high-temperature peak developed concomitantly with the decrease in intensity of the 12°C peak with increasing K^+ concentration. The 12°C peak, then, became negligibly small upon supplementation with 200 mM K^+ (trace *d*). A small shoulder at ~0°C in the 300 mM K^+ supplemented membranes (trace *e*) is attributable to a change in heating rate by melting ice. Fig. 4 *B* shows that the intensity of the high-temperature peak was inversely proportional to that of the 12°C peak, suggesting that the high-temperature peak reflects the OEC with a Ca^{2+} site occupied by K^+.

The Ca^{2+}-depleted PSII membranes showed the TL band with elevated peak temperature in the presence of K^+, Rb^+, or Cs^+. A quite similar TL band with elevated peak temperature has been induced by illuminating the membranes depleted of Ca^{2+} by low pH (Ono and Inoue, 1989a; Ono et al., 1991, 1992). The upshifted TL band is ascribed to the charge recombination between Q_A^- and Y_D^+ (Demeter et al., 1993; Johnson et al., 1994); illumination induces the modified S_2 state whose oxidation potential becomes more negative than that of Y_D^+. As shown in Fig. 5, illumination at 77 K induced the typical $Q_A^-Y_D^+$ band at ~40 C in the Mn-depleted membranes in the presence of 300 mM K^+. Close similarity between the high-temperature band in K^+-substituted OEC and the $Q_A^-Y_D^+$ band suggests that the $Q_A^-Y_D^+$ recombination is responsible for the high-temperature TL band found in K^+-, Rb^+-, and Cs^+-substituted OECs. In fact, preliminary result showed that the Y_D^+ EPR signal decayed with approximately the same course as that of the high-temperature TL band.

Fig. 6 shows the EPR spectra of the Ca^{2+}-depleted membranes (light minus dark) supplemented with various alkali metal cations at a concentration of 300 mM. The sample membranes were illuminated at 0°C with continuous light in the presence of DCMU, conditions that are comparable with those for TL measurement. The Ca^{2+}-depleted membranes

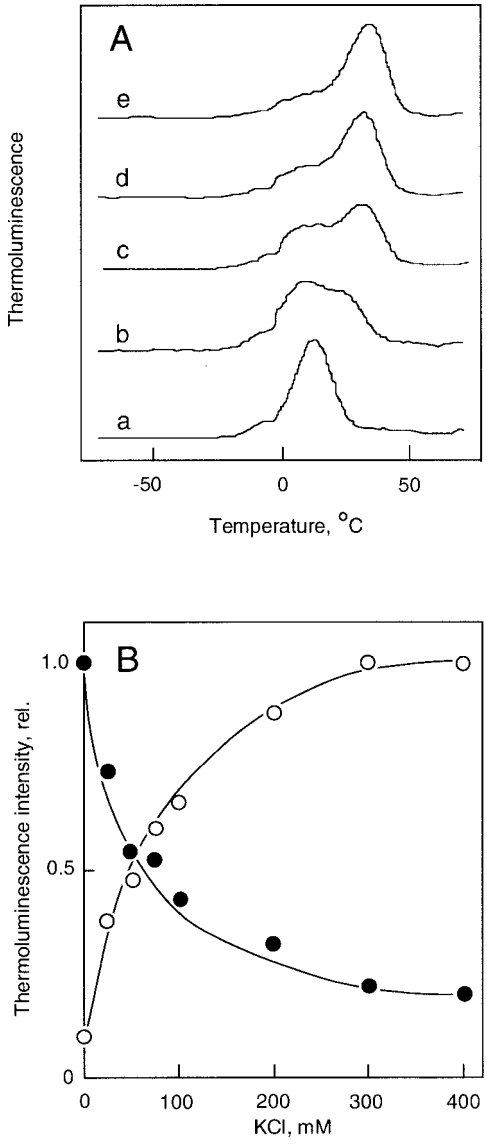

FIGURE 4 Effects of K^+ concentration on TL glow curves in Ca^{2+}-depleted PSII membranes. (*A*) TL glow curves induced by illuminating the sample membranes (0.3 mg of Chl/ml) at 5°C for 5 s in the presence of 0 mM (*a*); 50 mM (*b*); 100 mM (*c*); 200 mM (*d*); and 400 mM (*e*) K^+. 50 μM DCMU was included in the reaction mixture. (*B*) K^+-dependent change in intensity of Ca^{2+}-depleted (●) and K^+-substituted (○) TL glow components. Intensity of Ca^{2+}-depleted and K^+-substituted glow components was estimated from amplitudes of glow curves shown in *A* at 10 and 38°C, where overlap of both components was negligibly small. TL intensity was normalized by intensity of the component at 0 mM K^+ and 10°C and that at 400 mM K^+ and 38°C, respectively.

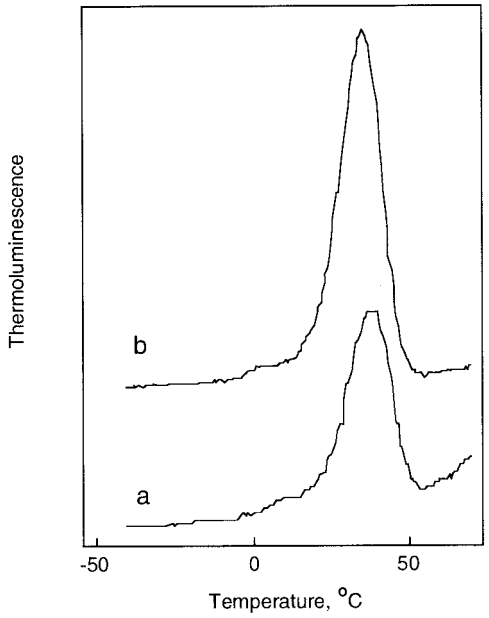

FIGURE 5 Thermoluminescence glow curves of manganese-depleted PSII membranes (*a*) and Ca^{2+}-depleted PSII membranes (*b*). Sample membranes (0.3 mg Chl/ml) were supplemented with 300 mM K^+, incubated at 0°C for 5 min, then illuminated at 77K for 1 min (*a*) or at 0°C for 5 s (*b*). Reaction mixture included 50 μM DCMU.

without metal cation supplementation generated a multiline S_2 signal with spectral features that were indistinguishable from those induced in the untreated control and Ca^{2+}-supplemented membranes, although a g = 4 S_2 signal was not induced in both Ca^{2+}-depleted and Ca^{2+}-supplemented membranes. The S_2 multiline signals of Li^+- and Na^+-supplemented membranes were also normal. In contrast, neither a multiline nor a g = 4 S_2 signal was induced in K^+-, Rb^+-, and Cs^+-supplemented membranes. The multiline signals in the Ca^{2+}-depleted and cation-supplemented membranes disappeared almost completely after dark incubation for 20 min at 20°C as expected by the normal-like $S_2Q_A^-$ TL-bands as shown in Fig. 3. It has been reported that the S_2 state formed in the Ca^{2+}-depleted membranes prepared by a procedure similar to that of the present study is rather stable (Ono and Inoue, 1990). The S_2 state, thus formed, shows a modified multiline signal with narrower hyperfine splitting and an $S_2Q_A^-$ TL-band peaking at ~22°C, which is 10°C higher than that of the corresponding band in this study. These differences in the S_2 properties between the two studies may be attributable to the presence of a chelator, which was always included in the sample suspension in the previous study (Ono and Inoue, 1990) but strictly omitted in the present study. A chelator may influence the S_2 properties by associating with the manganese cluster (Boussac et al., 1990a; Zimmermann et al., 1993). A relatively intense signal was induced at lower magnetic fields (800–1000 gauss region) although the signal intensity altered from sample to sample. Because this signal was distinctly observed in Ca^{2+}-supplemented membranes, Ca^{2+} depletion and cation supplementation are not directly responsible for induction of the signal, although the origin of this signal is not clear. $Fe^{2+}Q_A^-$ signals were similarly induced in all sample membranes, indicating that an electron was delivered from the oxidizing side of PSII to Q_A in agreement with the generation of pronounced TL bands in

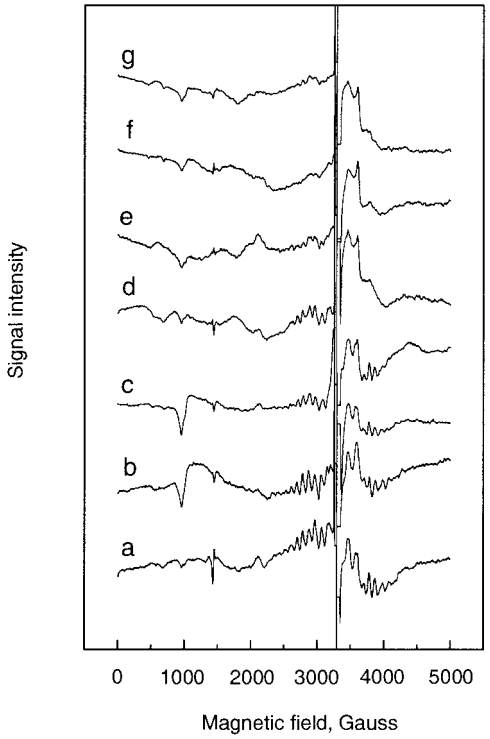

FIGURE 6 Effects of alkali metal cations on S_2 EPR spectra (light minus dark) in Ca^{2+}-depleted PSII membranes. None (*a*); addition of Ca^{2+} (*b*); Li^+ (*c*); Na^+ (*d*); K^+ (*e*); Rb^+ (*f*); and Cs^+ (*g*). Sample membranes (3.6–3.8 mg Chl/ml) were supplemented with 30 mM Ca^{2+} or 300 mM alkali metal cations, then illuminated for 20 s at 0°C. Reaction mixture contained DCMU. See text for other details. Instrumental settings: temperature, 6 K; microwave power, 2 mW; microwave frequency, 9.09 GHz; modulation frequency and amplitude, 100 kHz and 20 G, respectively.

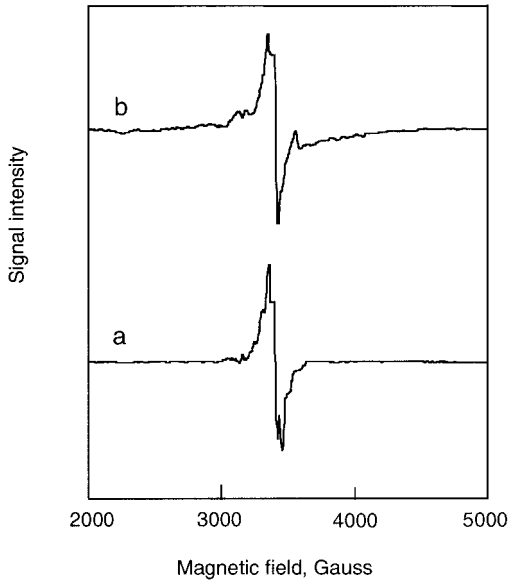

FIGURE 7 g = 2 narrow slit-type EPR signals in Ca^{2+}-depleted PSII membranes. Sample membranes (3.6–3.8 mg Chl/ml) were supplemented with 300 mM K^+ (*a*) and no cation (*b*), then illuminated for 60 s at 0°C. Reaction mixture contained 1 mM phenyl-*p*-benzoquinone. Spectrum *b* is presented after magnification by a factor of 1.5 in intensity. See text for other details. Instrumental settings: temperature, 8 K; microwave power, 1 mW; microwave frequency, 9.50 GHz; modulation frequency and amplitude, 100 kHz and 16 G, respectively.

the membranes supplemented with cations (see Fig. 3). Other spectral changes were not reproducibly obtained.

Fig. 7 shows the g = 2 split-type EPR signals of the Ca^{2+}-depleted membranes. The sample membranes were illuminated at 0°C with continuous light in the presence of phenyl-*p*-benzoquinone. Illumination induced a narrow split-type signal at g = 2 with an approximate line width of 150 G in both the Ca^{2+}-depleted membranes supplemented with no cation (trace *a*) and 300 mM K^+ (trace *b*). The signal intensity was relatively higher in the presence of 300 mM K^+ than in the absence of K^+ with slight difference in line shape. These may be caused by the difference in properties of the S_2 sate manganese cluster, but further studies will be required to elucidate which and how differences in manganese cluster influence the observed g = 2 signal. It is of note that any of the g = 2 signal is not induced in the presence of DCMU even after prolonged illumination at 0°C.

Fig. 8 shows the effects of dark additions of K^+ and Ca^{2+} to the membranes pre-illuminated on TL glow curves. In these experiments, K^+ was added after illuminating the Ca^{2+}-depleted membranes without supplementation of cations, or Ca^{2+} was added after illuminating the depleted membranes supplemented with K^+. The K^+ addition converted a TL band for an $S_2Q_A^-$ charge pair (curve *a*) to a high-temperature TL band (curve *d*), of which the peak temperature coincided with that of the TL band induced by illuminating membranes supplemented with K^+ (curve *e*). Similarly, the Ca^{2+} addition converted the K^+-supplemented high-temperature band to the normal $S_2Q_A^-$-band (curve *c*). It is of note that the Ca^{2+} addition reduced the K^+ concentration to 10 mM which did not affect the O_2 evolution supported by 10 mM Ca^{2+}. The results demonstrated that the high-temperature and $S_2Q_A^-$-bands are interconvertible in the dark in the presence of K^+ and Ca^{2+}.

Alkali metal cation-dependent changes in the properties of OEC are summarized in Table 1, which also includes the ionic radius of each monovalent cation. The effects of alkali metal cations are correlated closely with the ionic radius of the cations. The cations Li^+ and Na^+, that have ionic radii smaller than that of Ca^{2+}, had low affinity for the Ca^{2+} site and little effect on the O_2 evolution restored by Ca^{2+}, and the membranes supplemented with these cations generated a TL band with peak temperatures similar to that of the normal $S_2Q_A^-$ band and a normal S_2 multiline signal. In contrast, K^+, Rb^+, and Cs^+, of which the ionic radii are larger than the one of Ca^{2+}, significantly inhibit Ca^{2+}-dependent O_2 evolution. Further, the membranes supplemented with these cations generated the abnormal TL bands

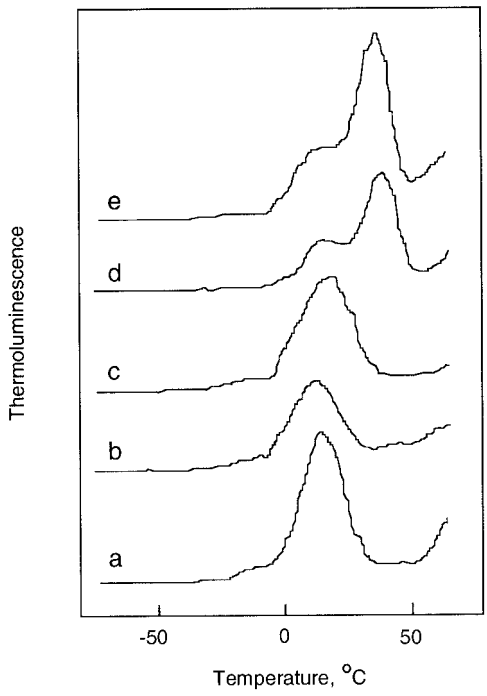

FIGURE 8 Effects of K^+ post-addition and Ca^{2+} post-addition on TL glow curves in Ca^{2+}-depleted PSII membranes. Sample membranes were illuminated with no cations (*a*); with 10 mM Ca^{2+} and 10 mM K^+ (*b*); with 300 mM K^+, followed by the addition of 10 mM Ca^{2+} in the dark (*c*); with no cations, followed by the addition of 300 mM K^+ in the dark (*d*); with 300 mM K^+ (*e*). Sample membranes in buffer B at 2 mg of Chl/ml supplemented with cations were illuminated at 6°C for 5 s, then 29 vols of buffer B was added immediately after illumination followed by dark incubation for 10 s, and freezing in liquid N_2. Buffer B contained sufficient amounts of cations to obtain desired cation concentrations after dilution. For *a*, *b*, and *e*, illuminated membranes were diluted 30-fold with buffer B containing the same cation constituents as that of the illuminated suspensions. 100 μM DCMU was included in the illuminated sample suspensions.

with an elevated peak temperature but no appreciable S_2 EPR signals. The ionic radius of Na^+ is most similar to that of Ca^{2+} among the alkali metal cations, and is only slightly smaller that of Ca^{2+}.

DISCUSSION

Alkali metal cations have similar chemical properties to those of their alkaline earth metal congener because these cations all have the same electronic configuration in the same period. This renders alkali metal cations useful for probing the function of Ca^{2+} in OEC. The present study demonstrated that alkali metal cations associate with the Ca^{2+} site in their specific manner, although their affinities for the Ca^{2+} site are relatively low, presumably because of the one unit charge decrease. The binding of the cations modifies the redox and magnetic properties of the manganese cluster, where the ionic radii of the alkali metal cations are crucial for their modification effects as well as association with the Ca^{2+} site. Cations with an ionic radius larger than Ca^{2+}, such as K^+, Rb^+, and Cs^+, interact with the Ca^{2+} site as indicated by the upshifted TL band and the disappearance of the S_2 EPR signals in OEC substituted with cations. In contrast, Li^+ and Na^+, with an ionic radius smaller than Ca^{2+}, affect little the EPR and TL properties of the S_2-state manganese cluster except for a slight but appreciable downshift of the peak temperature of the TL bands. This suggests that Li^+ or Na^+ binding to the Ca^{2+} site induces little perturbation in the S_2-state manganese cluster. However, we can not exclude the possibility that these two cations do not directly associate with the Ca^{2+} site because Li^+ and Na^+ have much lower affinities for the Ca^{2+} site than K^+, Rb^+, and Cs^+. These results are reminiscent of the finding that Sr^{2+}, an alkaline earth metal cation with a larger ionic radius than Ca^{2+}, can support O_2 evolution whereas that with a smaller ionic radius, such as Mg^{2+}, can not. It has also been reported that Ki values for the inhibition of Ca^{2+}-dependent O_2 evolution by lanthanide ions correlate with their ionic radii (Ono, 2000). Further, a similar ionic radius-dependent effect has been found in the apparent overall binding constant of alkali metal cations for the inhibition of OEC photoactivation (Ananyev et al., 1999), although the observed constant may reflect cation binding to the site for Mn^{2+} present in apo-OEC before photoactivation.

TABLE 1 Effects of alkali metal cations on properties of OEC in Ca^{2+}-depleted PS II membranes

Added cation	Ionic radius (Å)	Competition with Ca^{2+}	K_m or K_i (mM)	TL peak (°C)	Multiline S_2 EPR signal	O_2 evolution
None				12	Yes (normal)	No
Ca^{2+}	0.99		0.2	8	Yes (normal)	Yes
Li^+	0.60	N.D.	>100*	2	Yes (normal)	No
Na^+	0.95	N.D.	>100*	10	Yes (normal)	No
K^+	1.33	Yes	3	38	No	No
Rb^+	1.48	Yes	3	38	No	No
Cs^+	1.69	Yes	8	38	No	No

*Estimate.
N.D., not determined.

At first glance, the absence of S_2 EPR signals may imply that the manganese cluster is not oxidized by illuminating K^+-, Rb^+-, or Cs^+-substituted membranes. However, the interconversion between the $S_2Q_A^-$ TL band and the high-temperature TL band in the dark demonstrated that the manganese clusters in those membranes are oxidized to the S_2 state which does not generate an S_2 EPR signal. This view is further confirmed by the finding that the $g = 2$ split-type signal was induced in the K^+-substituted membranes as shown in Fig. 7. This split-type signal is induced upon illuminating the Ca^{2+}-depleted, Cl^--depleted, and acetate-treated membranes (Boussac et al., 1989, 1990b; Sivaraja et al., 1989; MacLachlan et al., 1993, 1994; Szalai and Brudvig, 1996a), in which the normal oxidation process of the manganese cluster beyond the S_2 state is interrupted. The signal is thought to originate from the interaction between Y_Z^+ and the S_2 state manganese (Boussac et al., 1990b; Hallahan et al., 1992; Gilchrist et al., 1995; Tang et al., 1996; Szalai and Brudvig, 1996b; Force et al., 1997; Peloquin et al., 1998). Contribution of another signal to the $g = 2$ region has been also proposed, where a dipole-dipole interaction between Y_Z^+ and another organic radical is responsible for the signal formation (Astashkin et al., 1997; Mino et al., 2000). However, we emphasize that the formation of the S_2 state manganese cluster is also prerequired for the alternative $g = 2$ signal. Preliminary field-swept pulsed EPR measurements did not find a $g = 2$ S_2 signal in K^+-substituted membranes (data not shown), indicating that a broadened hyperfine structure is not the direct cause of the absence of the multiline. Presumably, the absence of the S_2 signals can be ascribed to faster relaxation of the $g = 2$ S_2 signal. Alternatively, the spin state of the cluster is changed from the $S = 1/2$ ground state to a higher spin state because of the structural modification of the cluster. It is notable in this context that an interaction between Y_Z^+ and the oxidized manganese cluster with $S = 1$ has been proposed as the origin of the $g = 2$ signal (Gilchrist et al., 1995). Subtle change in the spin-exchange interactions between weakly coupled manganese ions will account for the putative spin-state change (Hasegawa et al., 1999).

As shown in Fig. 5, close similarity between the high-temperature band in K^+-substituted OEC and the $Q_A^-Y_D^+$ band suggests that the high-temperature TL band induced in K^+-, Rb^+-, and Cs^+-substituted membranes is ascribed to $Q_A^-Y_D^+$ recombination. It has been reported that the high-temperature band preferentially accompanies the PSII with the $g = 1.82$ form of $Q_A^-\cdot Fe^{2+}$ which is not a native form and induced by the change in the nonheme iron environment (Demeter et al., 1993). The appearance of the band might be caused by a cation-inducing modification on the acceptor side of PSII, which converts $Q_A^-\cdot Fe^{2+}$ from the $g = 1.9$ to $g = 1.82$ form. However, this does not seem to be the case, because little $g = 1.82$ signal was induced in K^+-, Rb^+-, and Cs^+-substituted OECs as shown in Fig. 5. Therefore, $g = 1.9$ form of $Q_A^-\cdot Fe^{2+}$ must be responsible for the high-temperature band in the cation-substituted OECs. It is of note in this context that the redox potential of these two forms of $Q_A^-\cdot Fe^{2+}$ is almost the same (Hubbard et al., 1989). The result also indicates that K^+ substitution does not much influence the redox potential difference between the Q_A/Q_A^- and Y_D/Y_D^+ couples. Therefore, we may conclude that a larger stabilization energy for the Q_A^-/S_2 state, caused by the change in oxidation potential of the S_2 state to a value more negative than that of Y_D^+, is responsible for the high-temperature TL bands.

The marked decrease in oxidation potential of the S_2 state manganese cluster in K^+-, Rb^+-, and Cs^+-substituted OEC as indicated by the upshifted TL band is very similar to that observed in the Ca^{2+}-depleted sample membranes prepared by low-pH treatment (Ono and Inoue, 1989a; Ono et al., 1992), in which binding of the 24 kDa protein to Ca^{2+}-depleted OEC causes abnormalities of the manganese cluster, including the alteration of the magnetic properties (Ono and Inoue, 1989a; Ono et al., 1992). Interestingly, the K-edge of the Mn XANES spectrum of low-pH treated membranes downshifts, indicating the modified ligation structural of the manganese cluster such as a broken ligation bond to the cluster (Ono et al., 1991, 1993; Latimer et al., 1995; Cinco et al., 1998), whereas no such spectral change has been identified in Ca^{2+}-depleted samples devoid of the 24-kDa protein (Hatch et al., 1995; Riggs-Gelasco et al., 1996). EXAFS (Yachandra et al., 1993) and FTIR (Noguchi et al., 1995) studies indicated that Ca^{2+} is connected with the manganese cluster via a carboxylate bridge, and that this structure is responsible for the conformational change that appears upon S_2 state formation by breaking the coordination to Ca^{2+}, whereas Ca^{2+} depletion at low-pH liberates the carboxylate ligand from the manganese cluster (Noguchi et al., 1995). Taking into account the striking resemblance of the TL properties of K^+-, Rb^+-, and Cs^+-substituted membranes to those of the low-pH–treated membranes, it may be presumed that the manganese cluster in all these membranes are similarly modified. Presumably, binding of K^+, Rb^+, or Cs^+ induces constraint in the vicinity to the Ca^{2+} site as that by the binding of the 24-kDa protein because of their ionic radii larger than Ca^{2+}, and effects in modifications on the manganese cluster. However, Ca^{2+}-depleted membranes without metal cation supplementation show the normal multiline signal and TL bands, indicating that the structure and the redox properties of the manganese cluster in the S_2 state are not much modified compared with those of the untreated control membranes, in contrast to those in K^+-, Rb^+-, and Ca^+-substituted and low-pH–treated OECs.

As shown in Fig. 1, 100 mM K^+ was sufficient for the almost complete inhibition of O_2 evolution supported by 1 mM Ca^{2+}, whereas a higher K^+ concentration was required to develop the high-temperature TL band even in the absence of Ca^{2+} (Fig. 4). The difference in the effective K^+ concentrations between the two measurements was similar

for Rb^+ and Cs^+. This apparent inconsistency, however, can be attributed to the assay conditions. First, only the S_1 and S_2 states are realized during the TL assay, whereas the S-state cycle turns numerous times for the O_2 assay. An aqueous solution may access the Ca^{2+} site more easily at higher S states (Boussac and Rutherford, 1988c), thereby continuous illumination facilitates the release of Ca^{2+} from the site (Boussac et al., 1985; Miyao and Murata, 1986). Second, the TL and O_2 assays were performed at 5°C and 25°C, respectively. The exchange rate will generally accelerate with increasing temperature. Therefore, a cation may more easily access the Ca^{2+} site under the assay conditions for O_2 than for TL. Over 60% of the O_2 evolution activity was preserved, even in the presence of 200 mM K^+ in the untreated control membranes which retain the 24 kDa extrinsic protein (unpublished data). If the assumed K^+ concentration in chloroplasts is relatively high, one physiological role of the 24-kDa protein may be to prevent replacement of the functional Ca^{2+} by K^+ during the S-state cycling.

This research was supported by a grant for the Frontier Research System at RIKEN given by Ministry of Education, Culture, Sports, Science, and Technology of Japan. A.R. is the recipient of the STA fellowship.

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