Biochimica et Biophysica Acta, 682 (1982) 436–445
Elsevier Biomedical Press

BBA 41190

THE ROLE OF CHLORIDE IN O_2 EVOLUTION BY THYLAKOIDS FROM SALT-TOLERANT HIGHER PLANTS

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(Received July 1st, 1982)

Key words: Photosynthesis; Oxygen evolution; Cl effect; ^{35}Cl-NMR; Halophyte

(1) Thylakoids isolated from leaves of two salt-tolerant higher plant species were found to require high (greater than 250 mM) concentrations of Cl^- for maximal rates of photosynthetic O_2 evolution and maximum variable chlorophyll *a* fluorescence yield. These activities were also tolerant to extremely high (2–3 M) salt concentrations. Their pH dependence was markedly different in the absence and presence of sufficient salt levels. (2) When Cl^- was provided as $CaCl_2$, as opposed to $MgCl_2$, KCl or NaCl, higher rates of O_2 evolution were obtained, suggesting that Ca^{2+} has an important role in Photosystem II reactions. (3) The site of Cl^- action was located on the electron donor side of Photosystem II. (4) O_2 evolution in the presence of optimal Cl^- concentrations showed a pH dependence closely matched by that of ^{35}Cl-NMR line broadening, which is indicative of Cl^- binding. This pH-dependent ^{35}Cl-NMR line-width broadening was not altered significantly by treatment of the thylakoids with EDTA; it was, however, abolished by heat treatment. (5) Only anions with similar ionic radii (Br^-, NO_3^-) were effective in replacing Cl^-. Small anions such as F^- and OH^- were inhibitory; larger ions had no effect. The inhibition by F^- is due, at least in part, to displacement of Cl^-. The selectivity is attributed to a combination of steric and ionic field effects. (6) It is proposed that Cl^- facilitates Photosystem II electron transport by reversible ionic binding to the O_2-evolving complex itself or to the thylakoid membrane in close proximity to it.

Introduction

Surprisingly little attention has been paid to the study of photosynthetic properties of salt-tolerant (halophytic) higher plants considering the comparatively extensive literature dealing with salt and/or cation effects on photosynthesis in non-salt-tolerant (glycophytic) systems [1]. Two recent reports showed that thylakoids isolated from salt-tolerant higher plant species have different and unusual properties compared to those from non-salt-tolerant species [2,3]. Thylakoids isolated from a salt-secreting mangrove, *Avicennia marina*, were found to have a remarkable pH-dependent chloride requirement for maximal photosynthetic O_2-evolution activity [3].

Similar results were obtained by Wignarajah and Baker [2] using thylakoids from leaves of a salt-marsh species, *Aster tripolium*. Although a specific requirement for Cl^- was not reported, it can be inferred from the data that at least part of

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Abbreviations: Chl, chlorophyll; DBDMQ, methylenedioxybenzoquinone; DMQ, 2,5-dimethylquinone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine.

0005-2728/82/0000–0000/$02.75

the effects were due to Cl^-. Cl^- was the anion in all of the salts tested and assays were carried out at one pH value only [2]. In both species the salt requirement was approximately two orders of magnitude greater than that reported previously for photosynthetic O_2 evolution in spinach thylakoids [2,4,5].

In the present paper we report effects of Cl^- on thylakoids from leaves of *Avicennia germinans* and *A. tripolium*. The tolerance of the mangrove thylakoids to Cl^- allowed the use of sufficiently high Cl^- concentrations for the ^{35}Cl line shapes to be readily observed by high-field Fourier transform NMR. Therefore, the observations include not only the pH dependence of O_2 evolution and Chl *a* fluorescence yield but also their relationship to the ^{35}Cl line width. Comparison of our results with reports on other anions and thylakoids from other species provides evidence about the specificity of the Cl^- requirement. Anions larger than Cl^- are ineffective for steric reasons while those that are smaller inhibit the action of Cl^-. The mechanism of the Cl^- effect appears to be reversible ionic binding by the O_2-evolving complex, or by the thylakoid membrane in its vicinity, in a manner that facilitates electron transfer through the S-states (also see Ref. 6).

Materials and Methods

Branches from *Avi. germinans* plants, growing in their natural swamp habitat in Miami, FL, were picked and sent to the laboratory. These branches, kept in plastic bags with sufficient moisture at room temperature, did not lose significant photosynthetic activity for about 2–3 weeks. *A. tripolium* was grown in pots in the greenhouse under natural light conditions. Thylakoids were isolated from leaves of *Avi. germinans* or *A. tripolium* according to procedures previously described [2,3].

Chlorophyll was determined by the method of Arnon [7]. O_2 evolution was measured in a Hansatech O_2 electrode at 25°C and in saturating heat-filtered white light (400 $\mu E \cdot m^{-2} \cdot s^{-1}$). The basic reaction medium (1 ml total volume) contained 50 mM Tricine-NaOH (pH 7.8, or as stated in figure legends), 100 mM sucrose, 1 mM $MgCl_2$ and 1 mM ferricyanide. Chlorophyll concentration was 30 $\mu g/ml$.

Chl *a* fluorescence induction was measured at room temperature in an apparatus described by Munday and Govindjee [8]. The excitation light (110 $\mu E \cdot m^{-2} \cdot s^{-1}$) was a broad-band blue beam (Corning filters CS 4-76 and 3-73) and fluorescence was detected through a Corning CS 2-64 filter with a monochromator set at 688 nm (bandwidth approx. 10 nm). The fluorescence transients were recorded on as Esterline-Angus chart recorder or photographed from an oscilloscope screen. Oxygen yield per flash was measured as a function of the number of 3-μs light flashes (spaced 1 s apart), with a Joliot-type electrode [9] on a laboratory-assembled instrument as described before [10]. ^{35}Cl-NMR spectra were recorded as described elsewhere [11]. Fluorescence transients, Hill reaction rates and ^{35}Cl-NMR line widths were measured in parallel on the same preparations.

The Cl^- concentrations given in the text and figures are those of the salt added to the reaction medium used to suspend aliquots from the stock chloroplasts. A volume ratio of 50 : 1 (1 ml medium to 20 μl of stock suspension) was ordinarily employed (see Ref. 3) and the $[Cl^-]$ in the stock chloroplasts was less than approx. 50 mM. Therefore, the actual $[Cl^-]$ differs from that added to the medium by no more than 1 mM or 2%.

Results

Photochemical reactions

Hill reaction and Chl a fluorescence induction. Fig. 1 shows the dependence upon Cl^- concentration of electron-transport activities (as measured by the Hill reaction – Fig. 1A, and Chl *a* fluorescence transients – Fig. 1B) in thylakoids from the two salt-tolerant higher plants, *Avi. germinans* and *A. tripolium*. In thylakoids isolated in the presence of 50 mM NaCl from leaves of both species, optimal rates of photosynthetic O_2 evolution, measured with ferricyanide as the Hill acceptor, were obtained with 500 and 250 mM NaCl, respectively (Fig. 1A). In contrast, spinach thylakoids not only lacked this requirement of high $[Cl^-]$ but were adversely affected by salt concentrations in excess of about 150 mM NaCl (Fig. 1A). Chl *a* fluorescence induction in thylakoids from *Avi. germinans* showed a salt requirement (Fig. 1B, inset) very similar to that of the Hill reaction. In this case, the

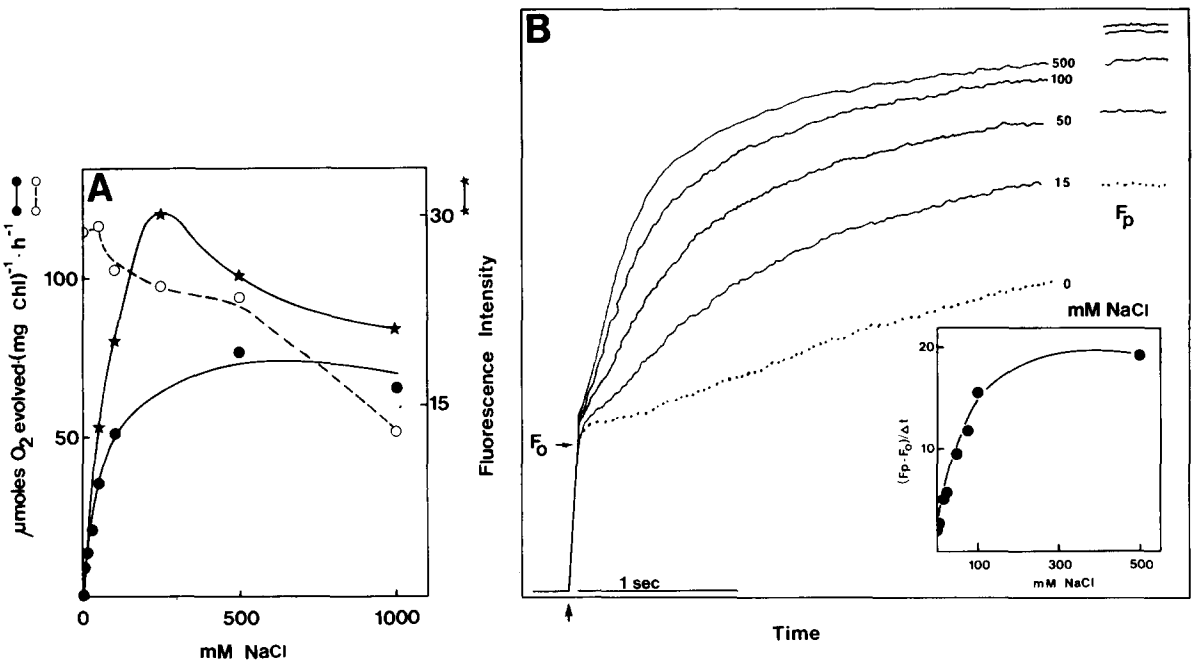

Fig. 1. Dependence of photochemical activity on NaCl concentration in the assay medium. (A) O_2 evolution with ferricyanide as electron acceptor in thylakoids (pH 7.8) from *Avi. germinans* (●——●), *A. tripolium* (★——★) and spinach (○——○); (B) the inset gives the rate of the variable fluorescence rise, O → P, in thylakoids (pH 7.6) from *Avi. germinans*. The figure shows tracings of the actual Chl *a* fluorescence induction curves from which the values in the insert were calculated; F_p, peak fluorescence intensity (or P level); F_0, fluorescence intensity of the '0' level.

variable part of the chlorophyll fluorescence induction curve ($F_p - F_o$) increases with added NaCl, the concentration dependence (Fig. 1B and inset) paralleling that of the O_2 evolution (Fig. 1A).

Tolerance to high salt levels. Thylakoids from *Avi. germinans* not only required high Cl^- concentrations for O_2 evolution but also were unusually tolerant toward high levels of salt in the assay medium. Their O_2 evolution and Chl *a* fluorescence induction were not inhibited by NaCl concentrations as high as 3 M (data not shown). Despite these exceptional properties, thylakoids from *Avi. germinans* had the normal periodicity of 4 in flash-induced O_2 yield (O_2 yield/flash as a function of flash number) in the presence of sufficient NaCl (i.e., 250 mM), and showed the typical changes in the flash pattern when treated with low concentrations of NH_2OH [12] (data not shown). No electron acceptor was added to these samples, so the flash patterns show a faster damping of the oscillations in O_2 yield than normally observed [13].

The calcium effect. The Cl^- concentration dependence of the ferricyanide Hill reaction in *Avi. germinans* thylakoids was markedly different when Ca^{2+} was the accompanying cation (open circles in Fig. 2), than when Mg^{2+}, Na^+ or K^+ (other symbols in Fig. 2) were used. Over the entire range of [Cl^-] tested, Ca^{2+} produced considerably higher rates than Mg^{2+}, Na^+ or K^+, thereby effectively reducing the Cl^- required for a given O_2 evolution. Quinone-type electron acceptors (DMQ and DBDMQ) were also tested to determine whether the effect depends upon the nature of the electron acceptor in the Hill reaction. In the presence of 500 mM NaCl (pH 7.8), the rates of electron flow, in μmol O_2 evolved/mg Chl per h, with 0.5 mM DMQ or DBDMQ were 61 or 108, and were quite comparable with 1 mM $K_3Fe(CN)_6$ (73) and 0.5 mM DMQ + 1 mM $K_3Fe(CN)_6$ (101). Thus, there is no apparent correlation between the effectiveness of the PS II electron acceptors and their hydrophilic, or hydrophobic, character. This supports the interpretation that the calcium effect is

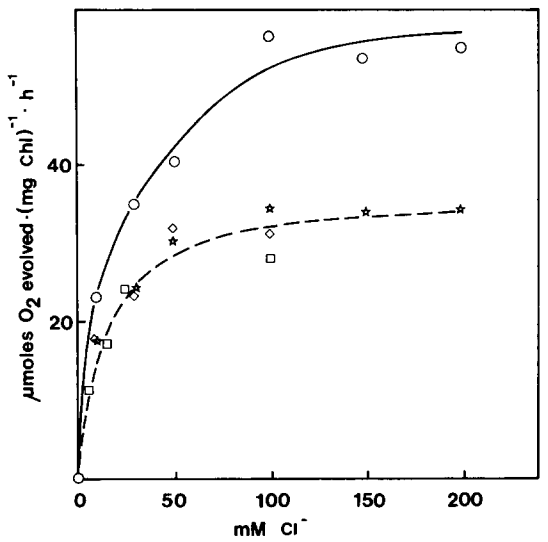

Fig. 2. Rate of O_2 evolution of *Avi. germinans* thylakoids at pH 7.0 with ferricyanide as electron acceptor as a function of Cl^- concentration for several cations: Ca^{2+} (○——○), Mg^{2+} (□——□), Na^+ (☆——☆), and K^+ (◇——◇).

intrinsic and may have physiological significance, as suggested recently for other systems [14,15].

pH dependence. The effect of Cl^- on O_2 evolution during the ferricyanide Hill reaction in thylakoids from *Avi. germinans* and *A. tripolium* is pH dependent in a manner similar to that reported for *Avi. marina* thylakoids prepared at pH 7.6 [3]. The dependence of O_2 evolution upon pH in the range 5.9 to 8.2 is shown in Fig. 3A for thylakoids from *Avi. germinans*, prepared and kept at pH 7.6 prior to the assay. These samples were assayed at different pH in the absence (●) and in the presence of high NaCl concentrations (☆, 0.5 M; and ○, 1 M). In the absence of added salt the O_2-evolution rates for pH values from 7.4 to 8.4 were very unstable and thus only the initial rates are plotted. In the presence of high salt concentrations all measured rates were stable and higher by approx. 30 μmol O_2/mg Chl per h.

The rate of Chl *a* fluorescence rise $(F_p - F_0)/\Delta t$ (equivalent to the initial slope) in *Avi. germinans* isolated at pH 7.0, with added salt, increases with increasing pH up to 7.6 (Fig. 3B) while that without decreases. The pH dependence of O_2 evolution in *A. tripolium* thylakoids, prepared and kept at pH 7.0, and assayed with or without salt is presented in Fig. 3C.

The photochemical efficiency [16], $1 - (F_o/F_p)$, of PS II showed the same trends with regard to Cl^- concentration and pH (data not shown) as those given in Fig. 3.

Site of Cl^- effect. Loss of chlorophyll fluorescence induction (see reviews in Refs. 17 and 18) is often due to inhibition of electron donation from water to the PS II reaction center. However, such a loss is also observed when electron flow out

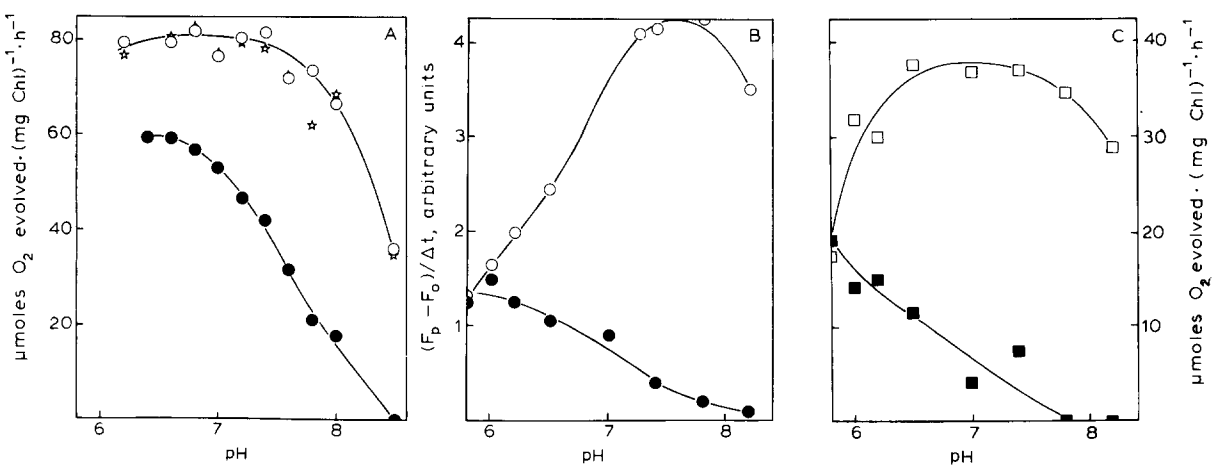

Fig. 3. The pH dependence of electron-transport activity in the absence (closed symbols) or presence (open symbols) of added 0.5 M (☆——☆) or 1.0 M (○——○ and □——□) NaCl: (A) O_2 evolution in thylakoids from *Avi. germinans* isolated at pH 7.6; (B) the initial rate of O → P fluorescence rise in thylakoids from *Avi. germinans* isolated at pH 7.0; and (C) O_2 evolution in thylakoids from *A. tripolium* isolated at pH 7.0. Buffers (50 mM): Mes (pH 5.8–6.7), Hepes (pH 6.8–7.8), Tricine (pH 7.6–8.4), at the pH values indicated. Δt: time difference between the two points used for fluorescence measurements in B.

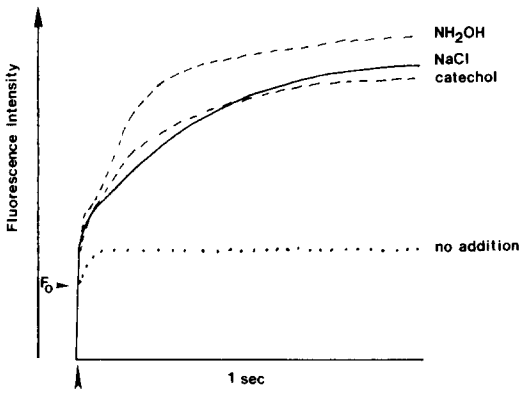

Fig. 4. Chl *a* fluorescence transients of *Avi. germinans* thylakoids in the absence of added Cl^- ('no addition'); in the presence of 250 mM Cl^- (NaCl); and in the presence, without added NaCl, of the electron donors to PS II, NH_2OH (50 mM) and catechol (500 μM, plus 1 mM ascorbate).

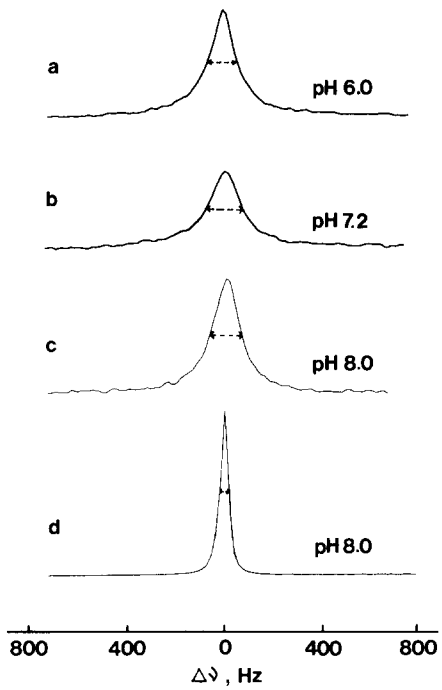

Fig. 5. ^{35}Cl-NMR spectra of suspensions containing 5 mg/ml Chl (*Avi. germinans* thylakoids) and 600 mM NaCl at three pH values (a–c) and of a 600 mM NaCl solution in H_2O (d). Buffer compositions as in Fig. 3. (For details of NMR measurements see Ref. 11.)

of Q^- is enhanced by the addition of artificial electron acceptors. A choice between these two options can be based on the use of artificial electron donors to PS II [19]. When an electron donor, e.g., catechol [20], diphenylcarbazide [21] or hydroxylamine [22] was added to thylakoid samples in the absence of added Cl^-, the variable fluorescence was reestablished to the same extent that it is by adding Cl^- (Fig. 4; data for diphenylcarbazide not shown). This indicates that PS II electron transport is impaired in the vicinity of the water-oxidation site by the absence of sufficient Cl^-.

^{35}Cl-NMR

In the present study, we found that the pH dependence of PS II electron transport parallels that of the ^{35}Cl-NMR line width, the first observations of which are reported elsewhere [11]. Typical ^{35}Cl-NMR spectra of thylakoids from *Avi. germinans* in the presence of 600 mM NaCl are shown in Fig. 5. That for pH 7.2 is broader than for 6.0 and 8.0 while all three are several-fold broader than the line for aqueous NaCl. The line widths of the ^{35}Cl-NMR peaks, defined as the full width of the line at half the maximum intensity, $\Delta\nu$ (in Hz), were determined as described in Ref. 11. The results are plotted as a function of pH in Fig. 6 for two species of halophyte thylakoids prepared at pH 7.6 and/or 7.0. As shown in Fig. 6, there is a close correlation for the various preparations between the pH dependence of Hill reaction rates (open circles) and that of $\Delta\nu$ (open squares).

When a single peak is present in the ^{35}Cl-NMR spectrum, as in Fig. 5, its broadening beyond that of aqueous Cl^- is indicative of Cl^- binding [11]. Such broadening was largest in the pH range where maximal O_2 evolution occurs (Fig. 6). The insert in Fig. 6B shows the pH dependence of the ^{35}Cl-NMR line width measured 1 h after the first measurement (Fig. 6B), indicating that there was no significant change over that period of time. Instrumental broadening of the line shapes was about 20 Hz in Fig. 6A, with 12-mm sample tubes, and 10 Hz in Fig. 6B and C, with 5-mm tubes.

Removal of very loosely bound Mn^{2+} by EDTA washing [13] did not significantly alter the pH dependence (Fig. 7A), indicating that any contributions by this paramagnetic species to the ^{35}Cl line broadening are small. (Points in Fig. 7A are corrected for small differences in instrumental

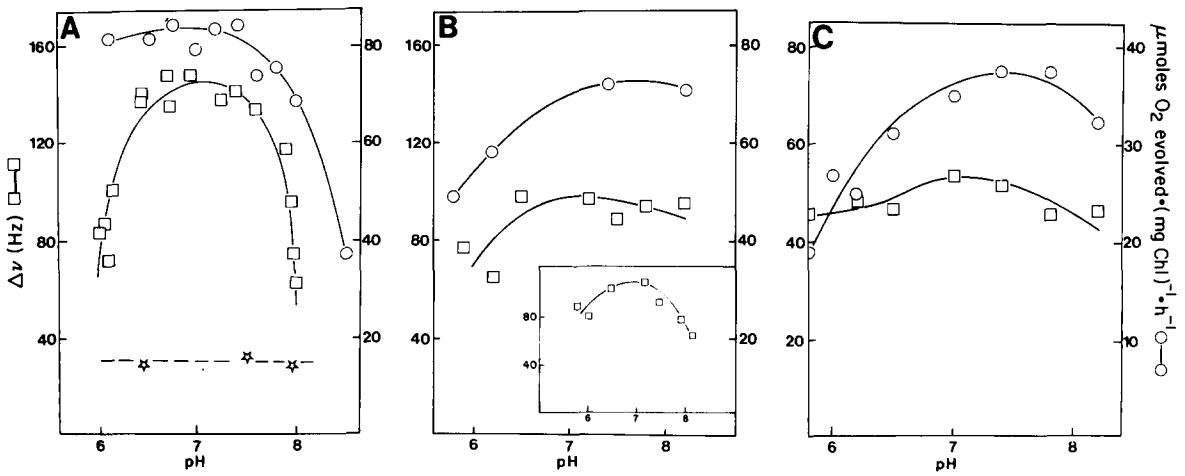

Fig. 6. The pH dependence of O_2 evolution with ferricyanide as electron acceptor (open circles) and ^{35}Cl-NMR line widths (open squares) in thylakoids: (A) from *Avi. germinans*, isolated at pH 7.6, with 6 mg Chl/ml and 1 M NaCl; (B) from *Avi. germinans*, isolated at pH 7.0, with 4 mg Chl/ml and 1 M NaCl; and (C) from *A. tripolium*, isolated at pH 7.0, with 3 mg Chl/ml and 0.5 M NaCl. The points (☆) at the bottom of A are for 1 M NaCl solution, without thylakoids. The inset in B is the pH dependence of the ^{35}Cl line width observed in the same samples 1 h after the first measurements. Assays were carried out with media as in Fig. 3.

broadening.) Heat treatment of the thylakoids at 48°C for 3.5 min abolished the O_2 evolution completely (data not shown) and also the pH dependence of the ^{35}Cl-NMR line width (Fig. 7B). In this case, the line width for the heat-treated thylakoids (47 Hz), while 5–10 Hz less than that of the untreated thylakoids, is still approx. 30 Hz greater than that (17 Hz) for aqueous Cl^-. This

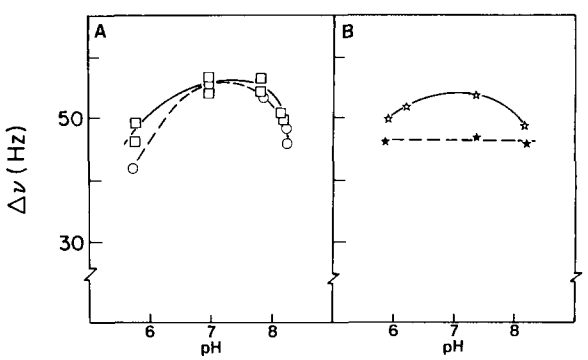

Fig. 7. pH dependence of ^{35}Cl-NMR linewidth in thylakoids from *Avi. germinans*: (A) isolated in the presence (O------O) or absence (□———□) of 2 mM EDTA in the grinding and washing media (Chl concentration, 3 mg/ml; NaCl, 0.5 M); (B) before (☆———☆) or after (★------★) heating to 48°C for 3.5 min, both in the presence of 2 mM EDTA (Chl concentration, 2.9 mg/ml; NaCl, 1 M).

shows that although the heat treatment does affect the binding sites, the Cl^- binding to the treated thylakoids is still extensive.

The ^{35}Cl-NMR spectra obtained for Figs. 6 and 7 were single lines of the shape shown in Fig. 5. However, spectra obtained [11] for *A. tripolium* and spinach thylakoids at lower Cl^- (250 mM, pH 7.2) had not only a strong line of width comparable to those in Fig. 5 but also a weaker, symmetrically placed broader line. For *A. tripolium* (3.1 mg Chl/ml) the line widths were 45 and 180 Hz with relative integrated intensities of 3 : 1; and for spinach (3.6 mg Chl/ml), 60 and 150 Hz and intensities of 8 : 1. These results show that there are at least two sets of sites for the Cl^- in both spinach and *A. tripolium*. Moreover, each must have its own pool of aqueous Cl^- with which it exchanges, while the rate of exchange between the two aqueous pools is smaller than that between each set of sites and its pool. The Cl^- which gives the broader line seems to be associated with the regions of the stacked thylakoids [11].

Anion specificity

The mechanism of the Cl^- action should be related in some way to its specificity. For *Avi. marina* thylakoids the specificity was very pro-

nounced, such that Br^- and I^-, for example, could not substitute for Cl^- [3]. In the present study, with thylakoids from *Avi. germinans*, Br^- and NO_3^- were found to stimulate Chl *a* fluorescence transients and O_2 evolution to a substantial degree, as shown in Fig. 8. On the other hand both F^- and SO_4^{2-} have no significant stimulating effect on either the O_2 evolution or the Chl *a* fluorescence. For Br^- the fluorescence yield is low compared with the O_2 evolution, possibly because Br^- is a quencher of fluorescence (Thorne, S.W., personal communication to C.C.).

One might expect the specificity of the anion to depend upon factors such as its size, shape, hydration and charge. In Fig. 9A we have plotted the O_2 evolution, in relative units, reported for spinach thylakoids in Ref. 5 vs. the anion volume; the latter was calculated for spherical ions from the Pauling radius. A similar plot is given in Fig. 9B for our mangrove data. The figure shows that the Cl^- volume of 25 Å^3 is optimum for O_2 evolution by spinach as well as mangrove thylakoids. The photochemical activity falls off progressively for smaller as well as for larger anions, with a somewhat more rapid fall-off for mangroves than for spinach chloroplasts with ions larger than Br^-.

The decreasing effectiveness of the larger ions is consistent with steric effects, either at the binding site itself or in the access of the ion to the site through a channel of restricted size. But the ineffectiveness of the smaller ions must be caused by

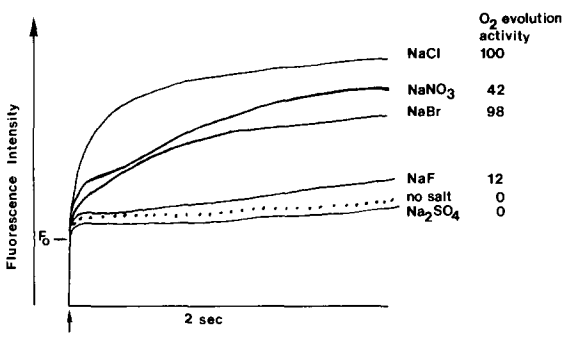

Fig. 8. Chl *a* fluorescence transients at pH 7.8 in suspensions of *Avi. germinans* thylakoids in the absence of salt (dotted line) or upon the addition of various sodium salts (continuous lines). Monovalent salts, 500 mM; divalent salt, 250 mM. The corresponding rates of O_2 evolution (μmol/mg Chl per h) are shown on the right for each anion (500 mM).

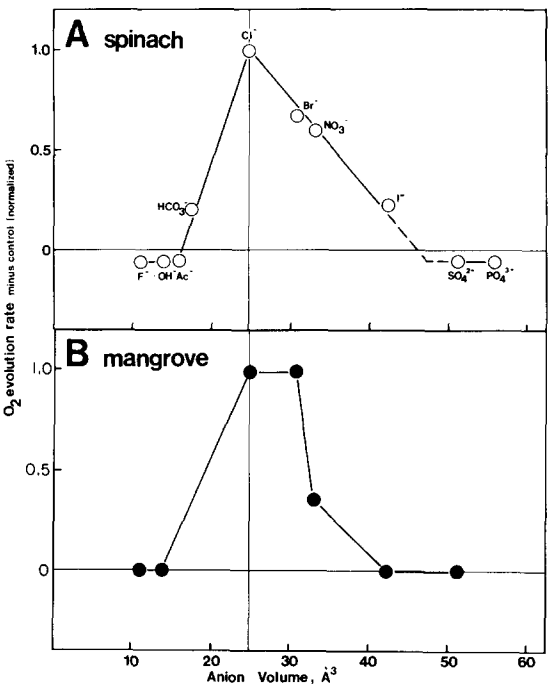

Fig. 9. Hill reaction rates (O_2 evolution with ferricyanide as electron acceptor) vs. the anion volume calculated for a spherical ion from the Pauling radius: (A) of spinach thylakoids at pH 7.2, O_2 data from Ref. 5; and (B) of mangrove thylakoids at pH 7.8 from this work (Fig. 8) plus I^- from Ref. 3. The points in B are for the ions directly above in A.

something else. F^- not only is unable to substitute for Cl^- but also has a strong inhibitory effect on the photochemical activity in the presence of Cl^-. As shown in Fig. 10, the inhibition increased both with concentration of F^- and with time. For *Avi. germinans* thylakoids with 250 mM NaCl, it took about 3–5 min for the inhibition to attain half its maximum effect (Fig. 10A). Nearly complete inhibition was obtained with comparable concentrations (250 mM) of F^- and Cl^- after a few minutes (Fig. 10B). By contrast, in Cl-depleted spinach thylakoids at pH 7.0, 10 mM F^- was found sufficient to inhibit completely the Hill reaction after about 3 min dark incubation [5].

We have obtained some information about the mechanism for the inhibition by F^- from its effect upon the ^{35}Cl NMR lineshape. 250 mM F^- (NaF) was added to the *A. tripolium* and spinach thylakoid samples described at the end of the preceding section. F^- reduced O_2 evolution of *A.*

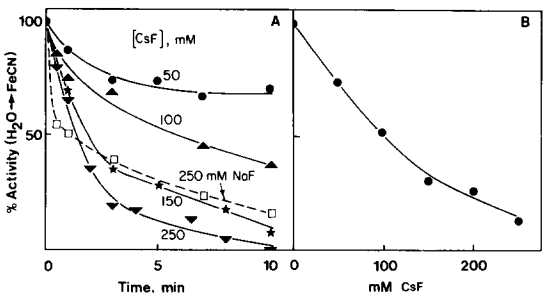

Fig. 10. Effect of F^- on O_2 evolution activity in thylakoids from *Avi. germinans* at pH 7.5 in 250 mM NaCl: (A) as a function of time after adding various concentrations of F^-; and (B) as a function of [CsF], after a 3 min incubation time.

tripolium by nearly 50% and of spinach by 90%. In both cases, the ^{35}Cl central line became narrower (by approx. 15 and 30 Hz, respectively) while the broad line decreased in intensity. These results show that F^- displaces part of the bound Cl^-. However, at least for spinach the inhibition of O_2 evolution is significantly greater than the fraction of Cl^- displaced, indicating that the inhibition may involve other factors as well.

Discussion

O_2 evolution in thylakoids isolated from *Avi. germinans* and *A. tripolium* has qualitatively similar requirements for high Cl^- concentrations as did those from *Avi. marina* [3]. These findings lend support to the suggestion that in some halophytes, Cl^- might accumulate in the thylakoids. Earlier observations of Cl^- accumulation in chloroplasts from salt-tolerant species have been reported [23,24].

The site of Cl^- action in mangrove thylakoids was localized at, or very close to, the donor side of PS II because electron transport through the reaction center was fully operative in the presence of artificial electron donors. The site of the Cl^- effect in Cl^--depleted spinach thylakoids is also the donor side of PS II [5]. Thus, the Cl^--depleted spinach thylakoids show similarities to those from salt-tolerant plants, but there are also striking differences. Similarities include the localization and pH dependence of the effect, while differences include the magnitude, the concentration dependence, the greater specificity, and the kinetics. Kelley and Izawa [5] reported that reactivation of spinach thylakoids by readdition of Cl^- required an incubation time of 2 min, whereas in the mangrove thylakoids no more than 15 s is required (data not shown). This suggests that diffusion of Cl^- is faster in mangrove than in spinach thylakoids.

It was proposed earlier [3] that Cl^- might act by binding to specific sites on the O_2-evolving complex itself, or the thylakoid membrane in close proximity to it. The broadening of the ^{35}Cl-NMR line width is interpreted to be due to Cl^- binding [11] and this was indeed confirmed under several experimental conditions employed here. The pH

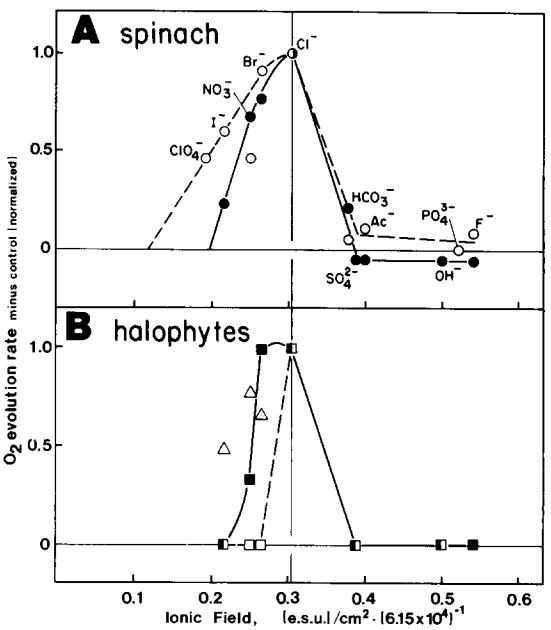

Fig. 11. Hill reaction rates (O_2 evolution with ferricyanide as electron acceptor) vs. the ionic field of the anion calculated at its Pauling radius, r_A, from the charge center: (A) for spinach thylakoids (●———●) isolated at pH 7.4, assayed at 7.2, 10 mM anion, O_2 data from Ref. 5; (○------○) prepared at pH 8.0, assayed at pH 8.3, 5 mM anion, O_2 data from Ref. 4; and (B) for halophyte thylakoids (□-----□) *Avi. marina* isolated at pH 7.6, assayed at pH 7.8, 500 mM anion, O_2 data from Ref. 3; (△———△) *A. tripolium* prepared at pH 7.6 or 7.8 (Br^-), assayed at pH 7.8, 250 mM anion; (■———■) *Avi. germinans* prepared at pH 7.0 or 7.6 and assayed at pH 7.0 or 7.8, respectively, 500 mM anion. The points in B are for the ions directly above in A. 'Minus control' on the ordinate indicates that the Hill reaction rates without added anion were subtracted from those with the anions.

range of maximal broadening of the ^{35}Cl-NMR line width is the same as for the highest Hill reaction rates. It appears, therefore, that the Cl^- is bound ionically in a reversible [11], pH-dependent fashion to the thylakoid membrane and/or the O_2-evolving complex, and that its binding facilitates electron transport through the S states (also see Ref. 6).

The specificity of the requirement for Cl^- is quite sharp. Steric factors can account for the ineffectiveness of the larger ions, but the inhibitory effects of the smaller ions require a different explanation. A likely possibility is suggested by Fig. 11 in which O_2-evolution data (only partially from Fig. 9) are plotted vs. the ionic field of the anion for spinach thylakoids (Fig. 11A) and halophytes (Fig. 11B). The ionic field E_A is defined as:

$$E_A = (Q_A / e_S r_A^2)$$

where Q_A is the anion charge and r_A is the Pauling radius for simple anions (pp. 61–73 in Ref. 25). For the complex anions such as SO_4^{2-} and NO_3^-, r_A was derived from either thermochemical data (pp. 282–290 in Ref. 25) or polarization calculations [26]. e_S is the differential dielectric 'constant' of water [25] which as an approximation [29], we took to be 78. Only the smaller anions are hydrated to an appreciable degree [27], and the change in e_S is too small (less than approx. 10%) to affect our conclusions (see pp. 288 and 597 in Ref. 25).

One might expect the binding of an anion to a membrane site with a positive charge q_M to be directly proportional to the ionic field of the anion. The Coulomb attractive force would be $q_M E_A$. However, the plots in Fig. 11 of O_2 evolution vs. ionic field of the anion are quite similar to those vs. anion volume in Fig. 9; they have a maximum for Cl^- and fall off rapidly for anions with larger as well as smaller E_A values. It is noteworthy that for spinach (Fig. 11A) the curve at alkaline pH (8.3) is broader than that at neutral pH (7.2). Moreover, the curves for the halophytes are narrower than those for spinach, the highest specificity being in *Avi. marina* where Br^- has no effect.

A comparison of Figs. 9 and 11 reveals some interesting similarities and differences in the response of thylakoids to particular anions. In Fig. 11, Br^-, NO_3^-, I^- and ClO_4^- have ionic fields progressively less than the 0.3 e.s.u. optimum value of Cl^-. Their effectiveness decreases monotonically both with decreasing ionic field and with increasing volume (Fig. 9). On the other hand, the anions with ionic fields greater than the optimum (Fig. 11) include large ions of multiple charge (SO_4^{2-} and PO_4^{3-}) as well as the small ions of single charge (F^-, OH^-, Ac^- and HCO_3^-). This shows graphically that while some anions are inactive because of steric factors preventing their binding to the membrane, other anions block the O_2 evolution by binding too tightly to the membrane, displacing the Cl^- and preventing electron transfer.

Further evidence in the matter comes from experiments with SO_4^{2-}. Although not tested for its inhibitory action in halophyte thylakoids, with spinach membranes at 100 mM Cl^- and neutral pH, SO_4^{2-} is neither stimulatory nor inhibitory (data not shown). This behavior is quite distinct from the inhibition by F^- of O_2 evolution in halophyte and spinach [5] thylakoids (Fig. 10). The simplest interpretation is that the size of SO_4^{2-} prevents it from affecting the membrane sites involved in the electron transfer.

Inhibition of the Hill reaction at alkaline pH values has been reported previously [28,29]. The pH dependences of the O_2 evolution and Cl^- binding given in Fig. 6 for mangrove thylakoids are a measure of the effects of OH^-, at least for the higher pH values. The data in Fig. 6A show that both O_2 evolution and Cl^- binding are optimal at neutral pH values (approx. 7.2). Increasing the pH to above approx. 8 causes a sharp reduction in both O_2 evolution and Cl^- binding. This is probably an inhibitory displacement of bound Cl^- by OH^-. However, the mechanism must differ from that of F^-, since only 0.003 mM OH^- is required for 50% inhibition of the O_2 evolution compared to approx. 100 mM F^-, even though OH^- has a somewhat smaller ionic field than F^-. Furthermore, in some cases (Figs. 3 and 6) pH changes decrease substantially the Cl^- binding but have little effect upon the O_2 evolution.

These differences suggest that changes in pH not only displace the Cl^- but also modify the charge distribution in the thylakoids and/or the

binding sites. Moreover, in the case of spinach, evidence is found for conformational changes as well. In Fig. 11A, the plot of O_2 evolution vs. ionic field of the anion is significantly broader at pH 8.3 than at 7.2. Also, both curves for spinach are broader than those for halophytes. This implies that the binding sites are more selective in halophytes than in spinach. Nonetheless, halophyte thylakoids seem to have a 'looser' overall structure inasmuch as the reactivation time for Cl^--depleted thylakoids is shorter for halophytes than spinach. Both differences may be part of the adaptation to high salt concentrations.

We conclude that a model combining ionic field and steric factors offers promise for interpreting the available data on anion interactions with thylakoid membranes. The physiological reasons for the extreme Cl^- requirement for O_2 evolution in thylakoids from halophytes are yet to be understood. The tolerance of these membranes towards molar salt concentrations is possibly even more startling. Whether these properties are a reflection of a true physiological requirement in response to adaptation to high salinity has yet to be established. Direct observation of Cl^- in thylakoids in situ [23] is difficult at present. However, ^{35}Cl-NMR and ^{36}Cl radioactive labeling of thylakoids [30] provide unique opportunities for studying the role of anions in O_2 evolution.

Acknowledgments

We thank in particular Mrs. Mary E. Collins of the Fairchild Tropical Garden, Miami, Florida, for a constant supply of *Avi. germinans* branches and Dr. John Cheeseman for plants of *A. tripolium*. We are thankful to William Coleman for his help; he was supported by a grant from the Campus Research Board to G. (1981–82). This work was supported by NSF grant PCM 79-11148 to H.S.G. and by grant PCM 78-24532 to G. Also, the work has benefitted from use of facilities made available through the University of Illinois-NSF Regional NMR Instrumentation Facility (grant CHE 79-16100).

References

1 Flowers, T.J., Troke, P.F. and Yeo, A.R. (1977) Annu. Rev. Plant. Physiol. 28, 89–121.
2 Wignarajah, K. and Baker, N.R. (1981) Physiol. Plant. 51, 387–393
3 Critchley, C. (1982) Nature 298, 483–485
4 Hind, G., Nakatani, H.Y. and Izawa, S. (1969) Biochim. Biophys. Acta 172, 277–289
5 Kelley, P.M. and Izawa, S. (1978) Biochim. Biophys. Acta 502, 198–210
6 Muallem, A., Farineau, J., Laine-Böszormenyi, M. and Izawa, S. (1981) in Photosynthesis II. Photosynthetic Electron Transport and Photophosphorylation (Akoyunoglou, G., ed.), pp. 435–443, Balaban International Science Services, Philadelphia
7 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
8 Munday, J.C., Jr. and Govindjee (1969) Biophys. J. 9, 1–21
9 Joliot, P. (1967) Brookhaven Symp. Biol. 19, 418–433
10 Stemler, A., Babcock, G.T. and Govindjee (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 4679–4683
11 Baianu, I.C., Critchley, C., Gutowsky, H.S. and Govindjee (1982) Proc. Natl. Acad. Sci. U.S.A., in the press
12 Bouges, B. (1971) Biochim. Biophys. Acta 234, 103–112
13 Radmer, R. and Cheniae, G. (1977) in Primary Processes of Photosynthesis (Barber, J., ed.), pp. 303–348, Elsevier, Amsterdam
14 Troxel, K.S., Barr, R., and Crane, F.L. (1980) Proc. Indiana Acad. Sci., 89, 343–349
15 Barr, R., Troxel, K.S. and Crane, F.L. (1982) Biochem. Biophys. Res. Commun. 104, 1182–1188
16 Malkin, S., Armond, P.A., Mooney, H.A. and Fork, D.C. (1981) Plant Physiol. 67, 570–579
17 Papageorgiou, G. (1975) in Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 319–371, Academic Press, New York.
18 Lavorel, J. and Etienne, A.L. (1977) in Primary Processes of Photosynthesis (Barber, J., ed.), pp. 203–268, Elsevier, Amsterdam
19 Izawa, S. (1980) Methods Enzymol. 69, 413–434
20 Ort, D.R. and Izawa, S. (1974) Plant Physiol. 53, 370–376
21 Vernon, L.P. and Shaw, E.R. (1969) Plant Physiol. 44, 1645–1649
22 Mohanty, P., Mar, T. and Govindjee (1971) Biochim. Biophys. Acta 253, 213–221
23 Ziegler, H. and Lüttger, U. (1967) Planta 74, 1–17
24 Larkum, A.W.D. (1968) Nature 218, 447–449
25 Conway, B.E. (1981) Ionic Hydration in Chemistry and Biophysics, Elsevier, Amsterdam
26 Masterton, W.L., Bolocofsky, D. and Lee, T.P. (1971) J. Phys. Chem. 75, 2809–2815
27 Baianu, I.C., Boden, N., Lightowlers, D. and Mortimer, M. (1978) Chem. Phys. Lett. 54, 169–175
28 Reimer, S. and Trebst, A. (1975) Biochem. Physiol. Pflanz. 168, 225–232
29 Maison-Peteri, B., Vernotte, C. and Briantais, J.-M. (1981) Biochim. Biophys. Acta 637, 202–208
30 Theg, S.M. and Homann, P.H. (1982) Biochim. Biophys. Acta 679, 221–224