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Fluoride inhibition of oxygen evolution; new evidence from ^{35}Cl-NMR measurements

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^{35}Cl-NMR was used to investigate the nature of F^--induced inhibition of oxygen-evolution in thylakoids from the mangrove, *Avicennia marina*. These studies showed a correlation between F^- inhibition of oxygen evolving activity and increased bulk Cl^- relaxation, possibly associated with the formation of a new class of high-affinity Cl^--binding sites, or a change in the nature of the existing binding sites, induced by F^-. The presence of added Cl^- did not alter the F^--induced inhibition of oxygen evolution. Increased Cl^- relaxation and F^--induced inhibition of oxygen evolution occurred at lower concentrations of F^- at pH 7.8 than at pH 6.3. In mangrove thylakoids. F^--induced inhibition of oxygen evolution does not appear to be due to competition with Cl^- for Cl^- binding sites, but instead involves some other interaction close to the oxygen-evolving complex.

Introduction

Chloride has been shown to be an essential cofactor for photosynthetic oxygen evolution, although its mode of action is still uncertain [1]. Replacement of Cl^- by other halide ions has been attempted [2–4] in an effort to investigate the Cl^- effect. Of these ions, only Br^- has been found to significantly replace Cl^- in function with F^- being inhibitory. This inhibition by F^- occurs in both the presence [4] and absence [2] of Cl^-, and has been localised to the oxygen-evolving centre by donor studies [3]. It has been suggested [4] that F^- inhibits oxygen evolution through displacement of Cl^- from the oxygen-evolving centre, and that, because of its small size, it is unable to replace Cl^- function.

^{35}Cl-NMR has been used recently to study interactions between Cl^- and thylakoid membranes [4–6]. Baianu et al. [6] indicated that their measurements were restricted by sensitivity limitations to high NaCl and chlorophyll concentrations, both of which created problems in measurement. These studies have indicated that less Cl^- is bound to the thylakoid membranes in the presence of F^-.

We have used ^{35}Cl-NMR spectroscopy with lower Cl^- and chlorophyll concentrations than previously used to investigate F^--induced inhibition of oxygen evolution by thylakoids isolated from the mangrove, *Avicennia marina*. The high Cl^- requirement (100–500 mM) of these membrane preparations make them ideal for use in ^{35}Cl-NMR experiments in our NMR set up, where the Cl^- lineshape is difficult to observe if the Cl^-

Abbreviations: Chl, chlorophyll; Mes, 4-morpholineethanesulphonic acid.

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concentration is too low. The effects of pH and light intensity on this inhibition have also been studied.

Materials and Methods

Avicennia marina propagules and young seedlings were collected from a mangrove swamp at Cullendulla Creek, Batemans Bay, New South Wales, Australia, and grown as described previously [7].

Thylakoids were isolated from freshly picked leaves as described in [8], except the wash and final resuspension buffer consisted of 50 mM Hepes-KOH (pH 7.3)/5 mM EDTA.

^{35}Cl-NMR spectra were recorded at 25°C at 19.6 MHz on a Bruker CXP- 200 wide bore instrument using 10 mm tubes. 3-ml samples were used with 150–170 μg Chl · ml^{-1}, 20% ^2H_2O and 4 mM EDTA. Buffers, pH and NaCl concentrations are indicated in figure legends. Samples were allowed to equilibrate in the magnet for 10 min at 25°C before experiments commenced. The number of scans varied from 1000 to 10000, depending on the NaCl concentration, and 3 Hz exponential filtering was applied. Samples were spun in the magnet at 30 rev · s^{-1}.

Oxygen evolution was measured in a Clark-type oxygen electrode at 25°C illuminated with red light (1000 μE · m^{-2} · s^{-1} quantum-flux density). Buffers, pH, NaF and NaCl concentrations are indicated in the figure legends. 0.5 mM ferricyanide plus 0.13 mM phenyl-*p*-benzoquinone were used as electron acceptors. 50 μg Chl was incubated in 3 ml of assay medium for 10 min prior to assay, unless otherwise indicated. Light intensity was varied by Schott neutral density filters. Chlorophyll concentrations were determined as in Ref. 9.

^{35}Cl-NMR data for Figs. 1, 2 and 4 are presented as the specific linewidth increment on a chlorophyll basis, v, defined as ΔHz · mg Chl^{-1} · ml, where ΔHz is the linewidth increase over the thylakoid background value. This latter value varied from 13.5 to 25.7 Hz · mg Chl^{-1} · ml depending on pH and Cl^- concentration, but, for any one treatment, the variation was less than 0.2 Hz · mg Chl^{-1} · ml. The data in Fig. 3 are presented as $v \cdot [Cl^-]$, which is proportional to the total Cl^- binding to a particular class of sites when, as here, the site concentration is much less than the total Cl^- concentration. Oxygen evolution data for Figs. 1, 2 and 4 are presented as percent inhibition. Again there are differences between preparations in initial activity and also Cl^- concentration greatly alters the activity of these thylakoids, as does pH (see Ref. 7). Presentation of data in this form allows comparison between different treatments as well as with ^{35}Cl-NMR data.

Results

Chloride binding

Incubation in the dark with NaF induced an increase in the bulk Cl^--relaxation rate due to the thylakoid membranes as measured by the increase in ^{35}Cl-NMR linewidth (Fig. 1). As NaF was increased in the presence of 70 mM NaCl, the linewidth increased in a hyperbolic fashion, reaching a plateau between 50 and 100 mM NaF. The F^--induced increase in linewidth had an apparent affinity for NaF of about 5 mM. NaF also inhibited oxygen-evolution activity at similar concentrations.

Previous reports [4–6] have indicated a narrowing of ^{35}Cl-NMR linewidth on F^- incubation of thylakoids which has been interpreted to be a

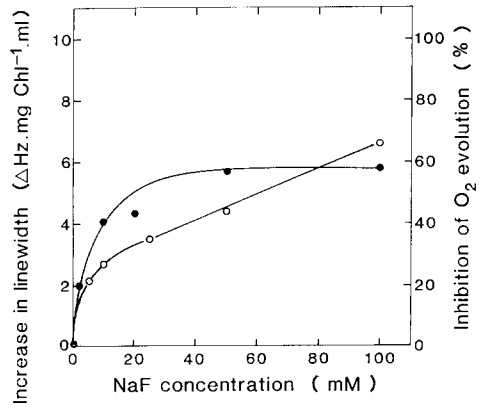

Fig. 1. Inhibition of oxygen evolution and increase in linewidth caused by NaF. Assay buffers were 25 mM Hepes-aminomethylpropanediol (pH 7.3)/70 mM NaCl for oxygen-evolution mesurements and 50 mM Hepes-KOH (pH 7.3)/4 mM EDTA/70 mM NaCl for ^{35}Cl-NMR measurements. Increase in linewidth (●); inhibition of oxygen evolution (○).

result of a decrease in Cl^- bound to the membranes. We have been able to obtain a similar result provided the samples were not treated with EDTA. As this EDTA concentration does not alter rates of O_2 evolution in these preparations in the presence or absence of F^- (Preston, C., unpublished data), we can only conclude that this difference is due to a Cl^- population not exchanging sufficiently rapidly on the NMR time-scale with the bulk Cl^- in non-EDTA-treated samples. This non-exchangeable Cl^- may be trapped in the thylakoid lumen space with slow transport across the membrane (see Ref. 10), and may become continuous with the bulk Cl^- following EDTA treatment. EDTA washing has been used to deplete thylakoids of Cl^- [3,11], presumably acting by permeabilising the membrane. Upon treating the same preparation with EDTA, we were able to obtain the data indicated in Fig. 1. EDTA itself increased the Cl^- relaxation of thylakoids to a small extent as is common with several other amines (Preston, C. and Pace, R., unpublished data). The F^--induced increase in Cl^- relaxation was not a result of the reversal of any effects of EDTA.

The increase in linewidth due to NaF seemed to be dependent on the Cl^- concentration (Fig. 2A). The largest increase in linewidth occurred in the presence of 50 mM NaCl and the smallest in the presence of 250 mM NaCl. With both 50 and 100 mM NaCl, the linewidth increased with NaF concentrations above 100 mM; however, with 250 mM NaCl the opposite occurred, although here the high total salt concentration may have induced non-specific effects. The inhibition of O_2 evolution due to NaF was independent of the NaCl concentration (Fig. 2B), indicating that F^- inhibition is non-competitive with Cl^-.

In Fig. 3, the detailed activation behaviour of the F^--induced relaxation is shown. At low NaCl concentrations, the curve is concave up, but F^--induced increase in linewidth becomes saturated at 50 mM NaCl. The data from Fig. 2 are included in Fig. 3B and show the F^--induced increase in linewidth is saturated from 50 to 250 mM NaCl. The sigmoidal behaviour of the F^--induced increase in linewidth is in contrast to the Cl^- dependence of the binding of Cl^- to thylakoids in the absence of F^- which shows low affinity behaviour. This suggests that F^- induces the formation of a new class of Cl^- binding site which may be co-operative in nature. The Cl^- concentra-

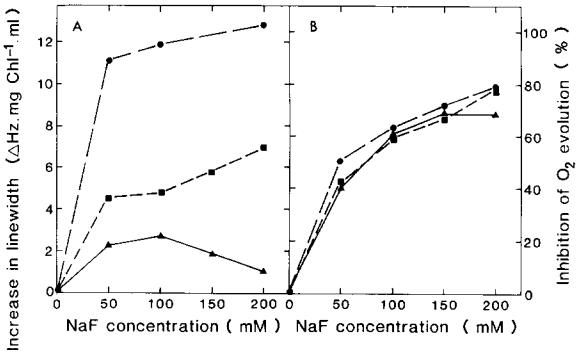

Fig. 2. Effect of NaCl concentration on the increase in linewidth (A), and the inhibition of oxygen evolution (B) caused by NaF. Assay buffers were 50 mM Hepes-KOH (pH 7.3)/4 mM EDTA for ^{35}Cl-NMR measurements and 25 mM Hepes-aminomethylpropanediol (pH 7.3) for oxygen evolution measurements at NaCl concentrations of 50 mM (●— —●); 100 mM (■- - - - -■); and 250 mM (▲———▲).

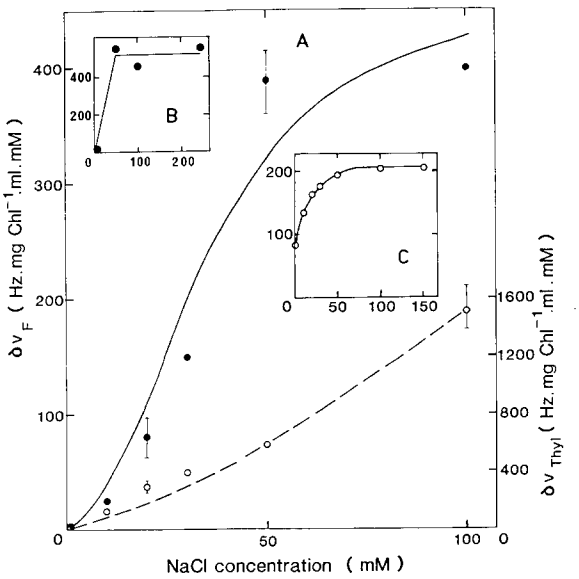

Fig. 3. F^--induced Cl^- binding as a function of Cl^- concentration. δv_{Thyl} is binding to thylakoids alone (○), δv_F is binding caused by 50 mM NaF (●). The curve through the δv_F points is given by $\delta v_F = \delta_{max}[Cl]^n/1+([Cl]/K_{1/2})^n$, where $\delta_{max} = 480$ Hz·mg Chl^{-1}·ml·mM, $K_{1/2} = 35$ mM and $n = 2$. (B) The data from Fig. 2 plotted as δv_F vs. NaCl concentration. (C) The rate of oxygen evolution of the thylakoids in μmol O_2 evolved·mg Chl^{-1}·h^{-1} as a functon of NaCl concentration in mM. Assay buffers were as for Fig. 2.

tion over which this increase in binding occurs is similar to that over which oxygen evolution is activated by Cl^- (Fig. 3C).

pH altered the response of F^--induced Cl^- binding as a function of NaF concentration (Fig. 4A). At pH 7.3, lower concentrations of NaF (up to 100 mM) increased apparent Cl^- binding to a plateau value, while higher concentrations of NaF produced a steep rise in linewidth. This pattern was repeated at pH 6.3, except that the plateau was shifted to higher NaF concentrations. At pH 7.8, the plateau region was reduced in size and shifted to lower NaF concentrations. This indicates that the apparent affinity of the F^--binding site is dependent on pH, being higher at high pH. The increase in linewidth at high (300 mM) NaF was high for all pH values, suggesting a general poisoning by F^- at such high concentrations.

The inhibition of oxygen evolution activity by NaF was also influenced by pH (Fig. 4B). The greatest inactivation occurred at pH 6.3 and the least at pH 7.8. There were also differences in inhibition of oxygen evolution at low NaF with different pH (Fig. 4B inset). Initially, the greatest inhibition occurred at pH 7.8 and least at pH 6.3. While there are clear differences in detail between the responses to NaF of O_2 evolution and ^{35}Cl-NMR linewidth at high NaF concentrations, at low (less than 50 mM) NaF concentrations, the qualitative similarities of the two sets of data at all pH values tested are quite striking (Fig. 4A and B, insets). This suggests that, at low NaF concentrations at least, the interaction responsible for the increase in linewidth is also reponsible for the inhibition of O_2 evolution.

Incubation of thylakoids with 100 mM NaF in the absence of Cl^- greatly inhibited oxygen evolution at all pH values tested. Addition of NaCl just prior to assay could increase activity at pH 6.3 or

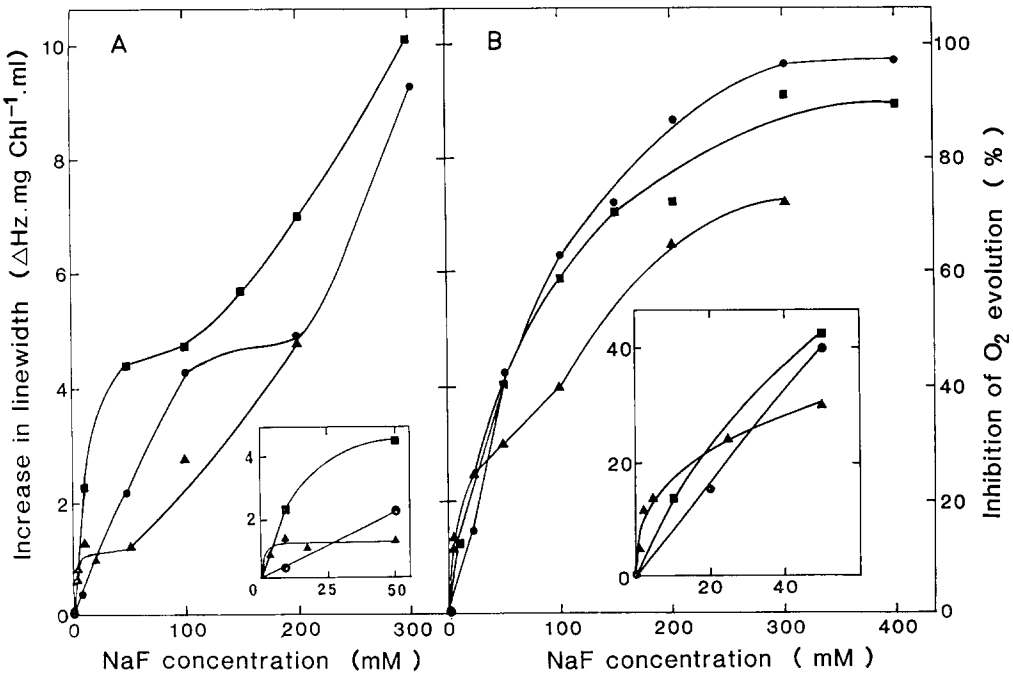

Fig. 4. Effect of pH on the increase in linewidth (A), and the inhibition of oxygen evolution (B) caused by NaF. Assay buffers were 50 mM Mes-KOH (pH 6.3)/4 mM EDTA/100 mM NaCl, 50 mM Hepes-KOH (pH 7.3)/4 mM EDTA/100 mM NaCl or 50 mM Hepes-KOH (pH 7.8)/4 mM EDTA/100 mM NaCl for ^{35}Cl-NMR measurements and 25 mM Mes-aminomethylpropanediol (pH 6.3)/100 mM NaCl, 25 mM Hepes-aminomethylpropanediol (pH 7.3)/100 mM NaCl or 25 mM Hepes-aminomethylpropanediol (pH 7.8)/100 mM NaCl. for oxygen evolution measurements. pH 6.3 (●); pH 7.3 (■); and pH 7.8 (▲). The insets show expansions of the effects of NaF on ^{35}Cl-NMR linewidth and on oxygen evolution at low concentrations of NaF. (Axes on the insets are the same as for the main figures.)

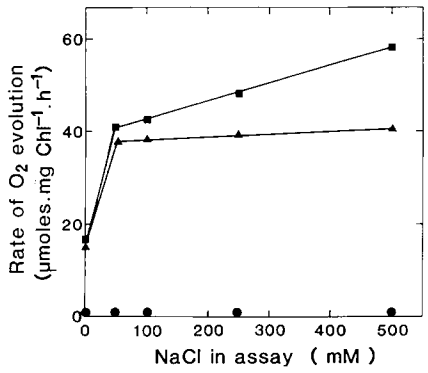

Fig. 5. Inhibition oxygen evolution by NaF incubation in the absence of NaCl at different pH. Thylakoids were incubated in 25 mM Mes-aminomethylpropanediol (pH 6.3), 25 mM Hepes-aminomethylpropanediol (pH 7.3) or 25 mM Hepes-aminomethylpropanediol (pH 7.8) with 100 mM NaF. NaCl was added just prior to assay. pH 6.3 (■); pH 7.3 (▲); and pH 7.8 (●).

pH 7.3, but not at pH 7.8 (Fig. 5). At pH 7.3, there was little increase in activity between 50 and 500 mM NaCl, but at pH 6.3, activity continued to increase in this region.

Light intensity

F^- only inhibited oxygen evolution at high light intensities above 180 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Fig. 6). At lower light intensities, there was no difference in the activity of thylakoids treated with 100 mM NaF for 10 min and that of untreated thylakoids. Above this intensity, the untreated thylakoids had

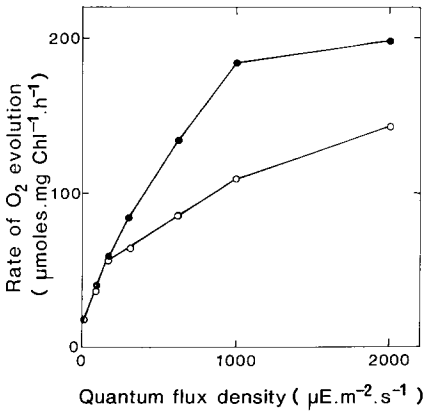

Fig. 6. The effect of NaF on oxygen evolution at different light intensities. Thylakoids were incubated in 25 mM Hepes-aminomethylpropanediol (pH 7.3)/150 mM NaCl in the absence (●) or presence (○) of 100 mM NaF.

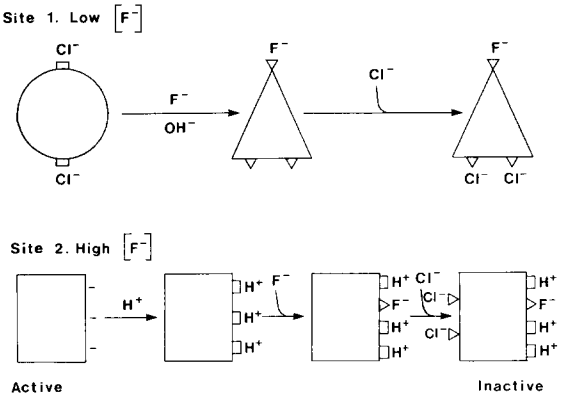

Fig. 7. A model detailing the interactions of F^-, pH and Cl^- on the thylakoid membrane. Two sites of F^- inhibition are shown. Site 1 is a site with high affinity for F^-. This site in the presence of F^- undergoes a conformational change which induces Cl^- to bind, creating a Cl^- binding but inhibited form. The F^- binding to this site is more effective at high pH and could be aided by OH^-. Site 2 has much lower affinity for F^- and may require protonation of negative charges to aid F^- binding. Once F^- has bound, Cl^- is induced to bind, creating an inactive form.

increasingly more activity, although activity of NaF-treated thylakoids did increase at higher light intensities.

Discussion

F^- inhibits oxygen evolution by mangrove thylakoids in a complex manner that is inconsistent with a competitive interaction with Cl^- for Cl^- binding sites. Moreover, the F^--induced enhancement of ^{35}Cl-NMR relaxation gives no indication of any competitive effects. At least two interpretations of this relaxation enhancement are possible. Firstly, since only the bulk Cl^- signal is seen in these experiments, tightly bound Cl^- which is exchanging at a rate of less than 10^4 s^{-1} will not be detected, assuming the binding-site concentration is comparable to that of the oxygen-evolving complex in our preparations (10^{-6} M). Thus, F^- poisoning of the oxygen-evolving site might mobilise tightly bound Cl^- from this site. This Cl^- pool would be different to the highly labile pool usually associated with oxygen evolution [1]. Alternatively, F^- binding to thylakoids in the dark (S_1 and S_0) state might induce a conformational change in one or more of the proteins of

the oxygen evolving complex, creating an inhibited but Cl^--binding form. That the effect indeed occurs at or near the oxygen-evolving site is suggested by the similar F^- dependence of the ^{35}Cl-NMR relaxation and oxygen-evolution inhibiton effects at low NaF concentrations (Fig. 1). In either case, it is clear, however, that F^- binding creates a class of labile Cl^--binding sites which is distinct from the F^- site. This is at variance with previous studies [4–6] where F^- inhibition of oxygen evolution was interpreted, in terms of Cl^--relaxation changes, to occur through a reduction in Cl^- binding. We have used much lower salt and chlorophyll concentrations, and the addition of EDTA rendered the membranes sufficiently Cl^--permeable to allow rapid exchange of internal Cl^- with the bulk. This is necessary to detect internal binding sites, and Baianu et al. [6] have conceded that conditions of sufficiently rapid exchange may not have always applied in the their study.

Our results appear also to be inconsistent with a recent study [12] on spinach PSII particles, which suggests that F^- inhibition of O_2 evolution is competitive with Cl^-. However, the authors acknowledge that this conclusion is merely a possible interpretation of their data, which apply only to a single F^- concentration. To establsih competition unambiguously, a range of F^- and Cl^- concentrations on the same material must be examined, as was done here. Another study on spinach PSII particles [13] also suggests competition between F^- and Cl^-. The discrepancies between our results and those in Ref. 13 are surprising. An intriguing possible explanation is that the O_2-evolving system of mangrove thylakoids is sufficiently different to that of spinach for the F^- inhibition to occur through a different mechanism. Alternatively, the incubation time used may have influenced the F^- inhibition. A competitive interaction may be apparent after the short, less than 1 min, incubation used in [13] which is not present after the full 10-min incubation required for maximum inhibition [4] which was used here. The sigmoidal titration behaviour of the F^--induced relaxation enhancement shown in Fig. 3 indicates that the stoichiometry of binding (n) to the labile Cl^- sites is more than 2, and assuming $n = 2$, the $K_1/_2$ per ion (see caption, Fig. 3) is 35 mM. This is comparable to the $K_{1/2}$ for Cl^- stimulation of oxygen evolution under the same conditions (20 mM, Fig. 3C). It is thus likely that the sites are closely related and that there are multiple Cl^--activating sites per oxygen evolving complex (see Ref. 14).

pH is well known to affect the interaction between oxygen evolution and Cl^- [1–4,7,8,15,16]. Interpretation of the effects of pH on F^- inhibition of O_2 evolution are complicated by the marked influence pH has on the Cl^- requirement for O_2 evolution (see Ref. 1 for review). Here, we have obtained two opposing effects of pH on F^- inhibition which are dependent on the F^- concentration. At low NaF concentrations, inhibition was more severe at high pH; however, at higher NaF concentrations, inhibition was more severe at low pH. The NMR data appears to indicate two effects of F^- depending on NaF concentration. These are a high affinity saturable component and another component with much lower affinity. A possible description of the pH effects is illustrated in Fig. 7, and could involve these two components having different pH optima, with the saturable component having higher affinity for F^- at high pH (see Fig. 4A). At low NaF concentrations, OH^- may be required to assist F^- binding specifically to a component of the oxygen-evolving centre. F^- binding then induces a change in the site into an inhibited but Cl^--binding form. At high NaF concentrations, F^- may be reacting more generally with components of the thylakoid membrane and may require protonation of negative charges to interact more effectively. The effects of pH seen in the absence of Cl^- during incubation with NaF (Fig. 5) appear at first inconsistent with the data in Fig. 4; however, it should be noted that at pH 7.8 these preparations are functionally Cl^- depleted and have no activity [7,15]. In this situation, the F^- incubation may drive all the O_2-evolving centres into a conformation that is unable subsequently to become activated.

F^- is only inhibitory at high light intensities (Fig. 6), which suggests that the effect of F^- may be to slow down turnover of the oxygen-evolving centre. At low light intensities, electron transport proceeds at a slow light-limited rate [17], and the NaF-inhibited water-splitting complex can apparently supply sufficient electrons. At high light

intensities, the NaF-inhibited system cannot supply electrons rapidly enough, which reduces the rate of oxygen evolution.

The mechanism of F^- inhibition of oxygen evolution appears to involve Cl^- binding or Cl^- mobilisation from a class of high-affinity sites. As seen by NMR spectroscopy, this seems to proceed via a two-step process; the first with high affinity for F^- and the second with much lower affinity for F^-, and which may be related to a general poisoning of thylakoid activity. We do not, however, rule out some other interactions of Cl^- and F^- binding to thylakoids that are non-competitive in nature.

We suggest that F^- binds to a component of the oxygen-evolving system, possibly the manganese complex which undergoes the S-state transitions [18], and that this induces Cl^- to bind to the thylakoids. We have evidence that a Cl^--binding site is formed during the progression of the S-states [19] and it may be this site on S_2 and its associated pool of Cl^- which is affected by F^-. Casey and Sauer [20] have shown that F^- prevents the oxygen evolving complex from advancing to S_2, at cryogenic temperatures, which is consistent with our proposal of F^- inhibition occurring at S_2. As yet, the inhibition of oxygen evolution by high concentrations (above 200 mM) of NaF has not been fully investigated, but a specific effect of F^- at these concentrations seems unlikely.

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References

1 Critchley, C. (1985) Biochim. Biophys. Acta 811, 33–46
2 Hind, G., Nakatani, H.Y. and Izawa, S. (1969) Biochim. Biophys. Acta 172, 277–289
3 Kelley, P.M. and Izawa, S. (1978) Biochim. Biophys. Acta 502, 198–210
4 Critchley, C., Baianu, I.C., Govindjee and Gutowsky, H.S. (1982) Biochim. Biophys. Acta 682, 436–445
5 Govindjee, Baianu, I.C., Critchley, C. and Gutowsky, H.S. (1983) in The Oxygen Evolving System of Photosynthesis (Inoue, Y., Crofts, A.R., Govindjee, Murata, N., Renger, G. and Satoh, K., eds.), pp. 303–315, Academic Press, Tokyo
6 Baianu, I.C., Critchley, C., Govindjee and Gutowsky, H.S. (1984) Proc. Natl. Acad. Sci. USA 81, 3713–3717
7 Critchley, C. (1983) Biochim. Biophys. Acta 724, 1–5
8 Critchley, C., Andersson, B., Ryrie, I.J. and Anderson, J.M. (1984) Biochim. Biophys. Acta 767, 532–539
9 Arnon, D.I. (1949) Plant Physiol. 24, 1–15
10 Ball, M.C., Mehlhorn, R.J., Terry, N. and Packer, L. (1985) Plant Physiol. 78, 1–3
11 Izawa, S., Heath, R.L. and Hind, G. (1969) Biochim. Biophys. Acta 180, 388–398
12 Damoder, R., Klimov, V.V. and Dismukes, G.C. (1986) Biochim. Biophys. Acta 848, 378–391
13 Sandusky, P.O. and Yocum, C.F. (1986) Biochim. Biophys. Acta 849, 85–93
14 Homann, P.H. (1985) Biochim. Biophys. Acta 809, 311–319
15 Critchley, C. (1982) Nature 298, 483–485
16 Theg, S.M. and Homann, P.H. (1982) Biochim. Biophys. Acta 679, 221–234
17 Bjorkman, O. (1981) in Encyclopedia of Plant Physiology: New Series, Vol. 12A (Lange, O.L., Nobel, P.S., Osmond, C.B. and Ziegler, H., eds.), pp. 57–107, Springer-Verlag, Berlin
18 Govindjee and Wydrzynski, T. (1981) in Photosynthesis II. Electron Trnasport and Photophosphorylation (Akoyunoglou, G., ed.), pp. 293–305, Balaban International Services, Philadelphia, PA
19 Preston, C. and Pace, R.J. (1985) Biochim. Biophys. Acta 810, 388–391
20 Casey, J.L. and Sauer, K. (1984) Biochim. Biophys. Acta 767, 21–28