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THE CHLORIDE REQUIREMENT FOR PHOTOSYNTHETIC OXYGEN EVOLUTION

ANALYSIS OF THE EFFECTS OF CHLORIDE AND OTHER ANIONS ON AMINE INHIBITION OF THE OXYGEN-EVOLVING COMPLEX

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In the presence of Cl^-, the severity of ammonia-induced inhibition of photosynthetic oxygen evolution is attenuated in spinach thylakoid membranes (Sandusky, P.O. and Yocum, C.F. (1983) FEBS Lett. 162, 339–343). A further examination of this phenomenon using steady-state kinetic analysis suggests that there are two sites of ammonia attack, only one of which is protected by the presence of Cl^-. In the case of Tris-induced inhibition of oxygen evolution only the Cl^- protected site is evident. In both cases the mechanism of Cl^- protection involves the binding of Cl^- in competition with the inhibitory amine. Anions (Br^- and NO_3^-) known to reactive oxygen evolution in Cl^--depleted membranes also protect against Tris-induced inhibition, and reactivation of Cl^--depleted membranes by Cl^- is competitively inhibited by ammonia. Inactivation of the oxygen-evolving complex by NH_2OH is impeded by Cl^-, whereas Cl^- does not affect the inhibition induced by so-called ADRY reagents. We propose that Cl^- functions in the oxygen-evolving complex as a ligand bridging manganese atoms to mediate electron transfer. This model accounts both for the well known Cl^- requirement of oxygen evolution, and for the inhibitory effects of amines on this reaction.

Introduction

Amine-induced inhibitions of the photosynthetic oxygen-evolving complex may be divided into two distinct categories. One category (exposure to NH_2OH in the dark or to 0.8 M Tris in the light) is characterized by a strong irreversible inactivation of oxygen evolution accompanied by the release of functional manganese from the thylakoid membrane [1–6]. The second category (exposure to NH_3, NH_2CH_3 or low concentrations of Tris) produces a freely reversible inhibition of oxygen evolution [7,8]. An examination of reversible amine inhibition has shown that Z^+, the oxidized form of the primary donor to P^+-680 is induced with a slow decay and a high microwave power saturation requirement [8,9], indicating that the pool of mangense involved in oxygen evolution has not been perturbed. In addition evidence has been presented that amine binding occurs via a Lewis-acid/Lewis-base mechanism, indicating that

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone; DMSO, dimethyl sulfoxide (Me_2SO); ADRY, acceleration of the deactivation reactions of the water-splitting enzyme system Y; PS, photosystem; Chl, chlorophyll; DCBQ, 2,6-dichloro-*p*-benzoquinone; ANT_{2a}, 2-(3-chloro-4-trifluoromethyl)anilino-3,5-dinitrothiophene; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; Chl, chlorophyll.

manganese is in fact the site of amine attack [8]. These results confirm those of Izawa et al. [7], who first showed that the site of reversible amine inhibition of PS-II activity was located on the oxidizing side of the photoact.

Velthuys and Zankel [10–12] explored the effects of amines on the yield of delayed luminescence from PS-II, and Velthuys concluded from his studies that both the S_2 and S_3 states of the oxygen-evolving reaction are susceptible to attack by amine inhibitors. The hypothesis proposed by Velthuys for the efficacy of ammonia binding to the oxygen-evolving complex is as follows: NH_3 is isoelectronic with water and is, as such, a substrate analog, and, in addition, unspecified higher oxidation states of manganese present in the S_2 and S_3 states favor the binding of amines to manganese owing to the associated ligand field stabilization energies. Frasch and Cheniae [4] have extended Velthuys' results by showing that Tris inactivation required the S_2 state, and they also showed that ammonia inhibited Tris inactivation.

Extensive work by several groups has confirmed the initial observation by Wearburg that Cl^- is required for photosynthetic oxygen evolution [13–19]. Izawa et al. [7] have shown that Cl^- depletion of thylakoid membranes produces a situation analogous to NH_3 inhibition, namely that in both cases these reversible inhibitions induce a lesion on the oxidizing side of PS II. Further research by Kelly and Izawa [15] has shown that a limited group of anions (Br^-, NO_3^-, HCO_3^-, I^- and HCO_2^-) can substitute with varying effectiveness for Cl^- in restoring oxygen evolving activity. Other anions (F^-, OH^-, $CH_3CO_2^-$, PO_4^{2-} and SO_4^{2-}) are ineffective (see also Ref. 18). While there is no consensus at this time as to the mechanism of the chloride effect on the oxygen-evolving complex, several hypotheses have been advanced. Johnson et al. [17] have proposed that Cl^- neutralizes charge and thereby stabilizes a hypothetical supermolecular conformation required for oxygen evolution. Alternatively, it has been suggested that Cl^- stabilizes higher oxidation states of manganese [20].

It is interesting to note that the presence of one of a group of Lewis bases (Cl^-, Br^-, I^-, NO_3^-, HCO_3^-, HCO_2^-) is essential for oxygen evolution, whereas a second group of Lewis bases (the amines) is inhibitory. This suggested to us that anions like Cl^- and amines might have a common site of action in the oxygen-evolving complex. We [21] have recently shown that Cl^- depletion and amine inhibition of the oxygen-evolving complex produce similar effects on the induction of Z^+ and that Cl^- acts competitively to protect the oxygen-evolving complex against NH_3 inhibition. In this communication we present a further characterization of the effects of Cl^- on amine inhibition of the oxygen-evolving complex and extend our analysis of the role of Cl^- in photosynthetic oxygen evolution.

Materials and Methods

Chloroplast thylakoid membranes were isolated from market spinach as previously described [22]. Chloride depletion of these preparations was carried out by the procedure in Ref. 21. Oxygen-evolving PS-II membranes were prepared by the method of Berthold et al. [23] with the modification described in Ref. 24. Stock solutions of $(Tris)_2SO_2$ and $(NH_4)_2SO_4$ were adjusted to pH 7.5 with NaOH, whereas stock solutions of Tris free base were adjusted to pH 7.5 with acetic acid. All reagents were prepared so as to minimize Cl^- contamination. Stock solutions of ANT_{2a} and CCCP were prepared in DMSO.

Measurements of oxygen-evolution activity were carried out in a thermostated (25 °C) Clark-type electrode [25]. In assays determining the effects of Cl^- or other anions on the inhibition of the oxygen-evolving complex by amines or ADRY reagents, thylakoid membranes or PS-II preparations (30 μg Chl) were incubated directly in the electrode cuvette under dim light. The incubation medium contained 50 mM Hepes and the various salts indicated in the figures. After 1 min of incubation, DCBQ and $Fe(CN)_6^{-3}$ (final concentrations, 250 μM and 2.5 mM, respectively) were added, and illumination for assay of activity was begun. In experiments investigating the effect of NH_3 on Cl^--induced reactivation of Cl^--depleted thylakoid membranes, the depleted membranes (1.5 mg Chl/ml) were incubated with varying concentrations of Cl^- and NH_3 under continuous illumination (white light, $2 \cdot 10^3$ J · m^2 · s^{-1}) from an Oriel model 6325 light source. After 2 min, 40

μl of this material was transferred to the oxygen-electrode cuvette where activity was assayed in the presence of 50 mM Hepes (pH = 7.5), 250 μM DCBQ and 2.5 mM $Fe(CN)_6^{3-}$. (This transfer effected a 1 : 40 dilution of Cl^- and NH_3). Experiments determining the effect of Cl^- on NH_2OH inactivation were carried out by exposing either thylakoid membranes or Cl^--depleted thylakoid membranes (300 μg Chl per ml) to varying concentrations of NH_2OH in the dark at 25 °C. The incubation medium contained 100 mM Hepes (pH, 7.5) and 40 mM sucrose. After 5 min, 100 μl of this incubation mixture was transferred to the electrode cuvette for assay under conditions described for the Cl^- reconstitution experiments. (This transfer effected a 1 : 16 dilution of NH_2OH.)

All chemicals used in these studies were of the purest grades commercially available. The ADRY reagents, ANT_{2a} and picrate, were a generous gift from Dr. D.F. Ghanotakis.

Results

Cl^--induced stabilization of the oxygen-evolving complex against inhibitory amines

Since ammonia inhibition of the oxygen-evolving complex can be attenuated by the presence of Cl^-, we reasoned that the reversible inhibition induced by low concentrations of Tris [4,8] should be similarly affected. As shown in Fig. 1, Cl^- completely protects the oxygen-evolving complex against Tris inhibition. We also observed a Cl^--insensitive inhibition of oxygen-evolution activity which can be induced by either $(Tris)_2SO_4$ or $(Na)_2SO_4$ (data not shown). This sulfate-dependent effect produces a maximal inhibition of 20% at the sulfate concentrations used in our assays, so the data shown in Fig. 1 were collected at a constant (200 mM) sulfate concentration to correct for this inhibitory effect. Note the similarity between the data of Fig. 1 and the results of Mual-lem et al. [26], obtained under considerably different conditions. Fig. 2 shows the effect of varied Cl^- concentration on the inhibition of oxygen-evolving activity induced by 400 mM Tris. We observe that complete protection against Tris attack is obtained with concentrations of Cl^- between 10 and 20 mM. The residual 20% inhibition of activity remaining at high Cl^- concentrations is

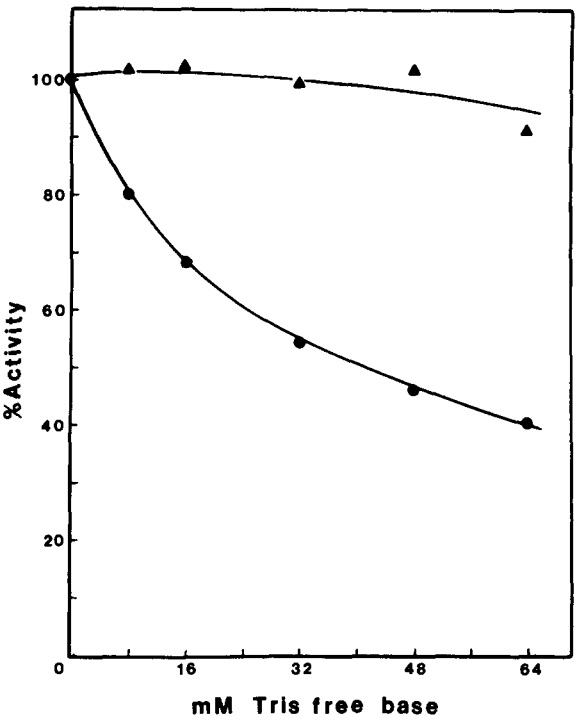

Fig. 1. Effects of chloride ((●), 0.4 mM Cl^-; (▲), 100 mM Cl^-) on $(Tris)_2SO_4$ inhibition of photosynthetic oxygen-evolution activity. Assays were performed as described in Materials and Methods at a constant sulfate concentration (200 mM). The control (100%) activity was 270 μmol O_2/h per mg Chl.

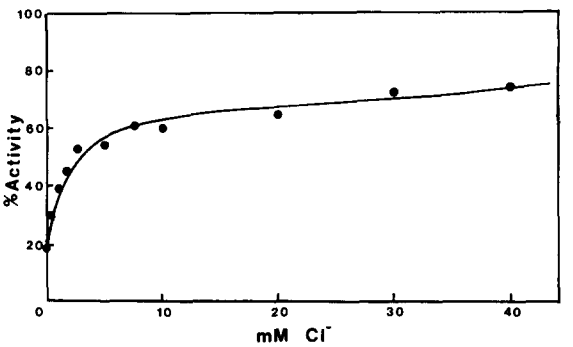

Fig. 2. Effect of varied chloride concentration on the inhibition of oxygen-evolution activity by $(Tris)_2SO_4$. Failure to obtain 100% protection is due to the inhibition of activity by sulfate, not Tris. Activity was calculated as the rate of oxygen evolution in the presence of 200 mM $(Tris)_2SO_4$ (and the concentration of Cl^- shown), divided by the rates obtained (with the same concentrations of Cl^-) in the absence of $(Tris)_2SO_4$, and multiplied by 100 to yield percentage. Control (100%) activity at 40 mM chloride (-sulfate) was 250 μmol O_2/h per mg Chl.

the surfate-induced inhibition described above. Our results with Tris inhibition should be contrasted with the results obtained when NH_3 is used as the inhibitory species [21]. We have observed that Cl^- affords only partial protection of activity against NH_3 inhibition in either the presence or absence of sulfate (data not shown).

In order to establish the range of protective effects of Cl^-, we have also examined NH_2OH-induced inactivation of the oxygen-evolving complex and the effects of ADRY reagents on oxygen evolution. The data in Fig. 3 show that Cl^- does not affect the inhibition induced by an ADRY reagent, even at high-light intensity used here. (See Ref. 27 for a discussion of light intensity on the ADRY effect.) In contrast to this result we have observed that Cl^- impedes the NH_2OH-induced inactivation of the oxygen-evolving complex in agreement with the earlier results presented by Kelly and Izawa [15] (data not shown).

The data of Table I summarize the results of experiments designed to compare the effectiveness of various anions in protecting the oxygen-evolving complex against Tris inhibition. For these experiments we have utilized PS-II membranes rather than whole thylakoid membranes in order to compensate for any possible differences in permeability of the anionic species. As the data in Table I show, Br^- and NO_3^- are effective surrogates for Cl^-, whereas formate and acetate are substantially less effective. Note also that NO_3^-, acetate and formate are inhibitory by themselves in this assay system.

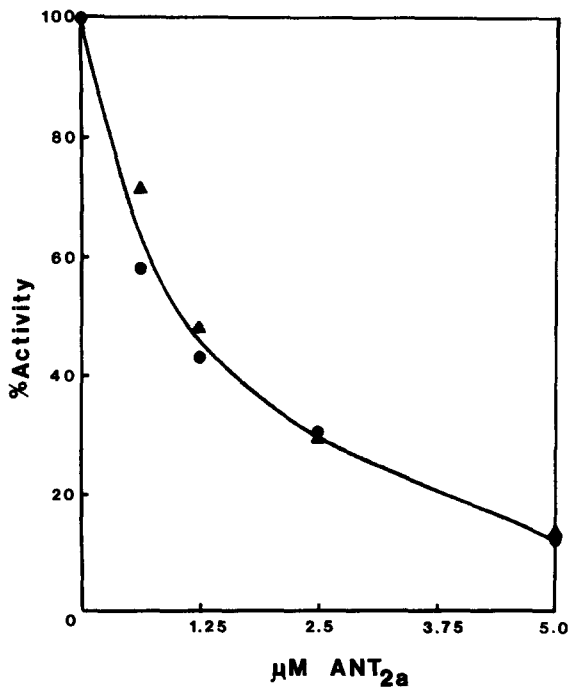

Fig. 3. Failure of chloride ((●), 0.4 mM Cl^-; (▲), 100 mM Cl^-) to protect against ADRY inhibition of oxygen-evolution activity. Similar results were observed with picrate and CCCP (data not shown). The control rate (100%) was 445 μmol O_2/h per mg Chl.

TABLE I

EFFECTIVENESS OF VARIOUS ANIONS T0 PROTECT AGAINST INHIBITION BY TRIS

Assays were carried out as described in Materials and Methods using PS-II membranes.

Added anion (50 mM)	Activity (μmol O_2/h per mg Chl)		Percentage inhibition
	− Tris	+ 50 mM Tris acetate	
None	216	26	88
Cl^-	220	162	26
Br^-	214	132	38
NO_3^-	90	54	40
Formate	58	0	100
Acetate	51	0	100

Steady-state kinetic analyses of the effects of Cl^- on Tris or NH_3 inhibition of the oxygen-evolving complex

In our previous report [21] we showed that NH_3 and Cl^- acted in the oxygen-evolving complex in a competitive fashion. Subsequently we have extended and refined our initial kinetic experiments. Correcting for SO_4^{2-}-induced inhibition and reducing the range of Cl^--concentrations, we have examined both $(Tris)_2SO_4$- and $(NH_4)_2SO_4$-induced inhibitions. In order to distinguish pure competitive inhibition from mixed-type inhibition we have followed the procedure outlined by Cornish-Bowden [28], who recommends the use of a $1/v$ vs. (I) plot (Dixon plot) together with a $(s)/v$ vs. (I) plot (Cornish-Bowden plot) for this purpose. These two plots allow the determination of the dissociation constants for both the enzyme-substrate-inhibitor complex and the enzyme-in-

hibitor complex:

$$E \cdot S \cdot I \rightleftharpoons E \cdot S + I$$

$$K_i' = \frac{[E \cdot S][I]}{[E \cdot S \cdot I]} = \text{intersection of Cornish-Bowden plot}$$

$$E \cdot I \rightleftharpoons E + I$$

$$K_i = \frac{[E][I]}{[EI]} = \text{intersection of Dixon plot}$$

Thus a K_i' much larger than the K_i is indicative of pure competitive binding of substrate and inhibitor.

Fig. 4 shows both Dixon and Cornish-Bowden plots for the effect of Cl^- on Tris inhibition of the oxygen-evolving complex at constant SO_4^{2-} concentration (200 mM). Clearly Cl^- and Tris binding are purely competitive ($K_i = 12$ mM Tris free base, $K_i' \geqslant 300$ mM). When the NH_3-Cl^- interaction was examined at a constant SO_4^{2-} concentration (40 mM), substantially different results were obtained. As shown in Fig. 5, the Dixon plot gives a value of 220 μM for K_i. However the Cornish-Bowden plot indicates that there is NH_3 binding which is not competitive with Cl^- ($K_i' = 580$ μM). We interpret this to indicate that there are two sites of inhibitory ammonia binding in the oxygen-evolving complex, only one of which is the Cl^- binding site (see Ref. 29 for a discussion of this type of inhibition). The inhibitions indicated by the data in Fig. 5 are represented by the equations below, where the oxygen-evolving complex is abbreviated as OEC:

$$\begin{array}{lll} \text{Inactive} & \text{OEC}\cdot NH_3 & \\ & \text{I} \quad \downarrow\uparrow & \\ & NH_3 & \\ & + & \\ & \text{OEC} & +Cl^- \rightleftharpoons \text{OEC}\cdot Cl^- \text{ Active} \\ & + & + \\ & NH_3 & NH_3 \\ & \text{II} \quad \downarrow\uparrow & \text{II} \quad \downarrow\uparrow \text{ Inactive} \\ & \text{OEC}\cdot NH_3 + Cl^- & \rightleftharpoons \text{OEC}\cdot Cl^- \cdot NH_3 \end{array}$$

I: binding of NH_3 at Cl^- binding site, competitive with Cl^-, $K_i = 220$ μM
II: inhibitory binding of NH_3, not competitive with Cl^-, $K_i' = 580$ μM

The K_i values obtained for Tris and NH_3 in our Cl^- competition experiments are compared in Table II with data from other investigations. The higher values obtained in the previous studies presumably arise from the presence of Cl^- (as the counteranion) added with the inhibitory amine.

The existence of two inhibitory binding sites for

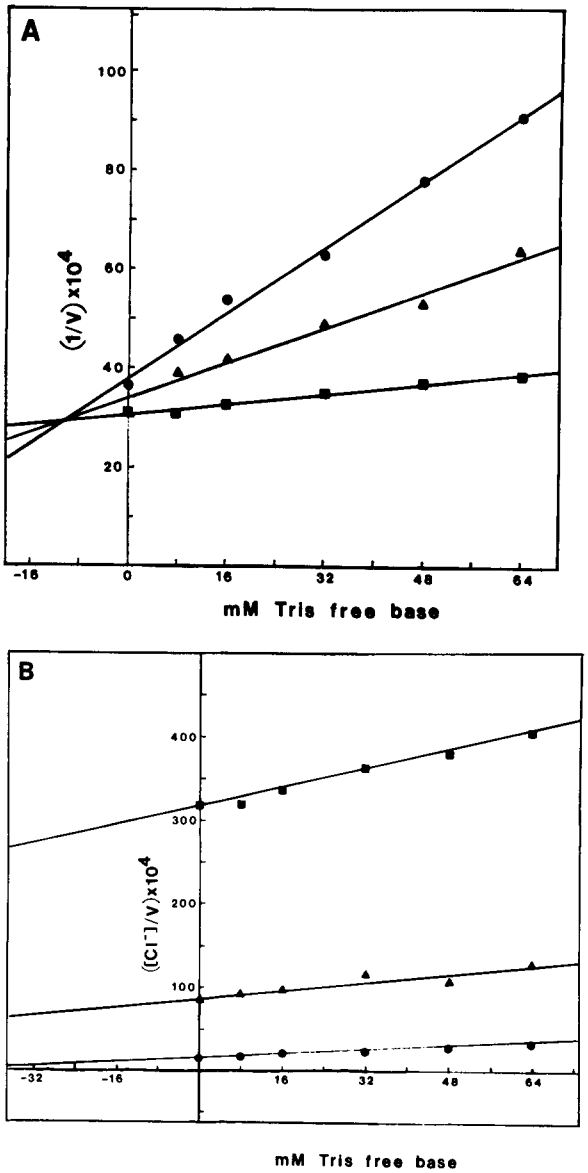

Fig. 4. (A) Dixon plot of Tris inhibition of oxygen-evolution activity at various chloride concentrations. (B) Cornish-Bowden plot of Tris inhibition at various chloride concentrations. ((●), 0.4 mM Cl^-; (▲), 2.4 mM Cl^-; (■), 10.4 mM Cl^{-1}). Assays were carried out as described in Materials and Methods at a constant sulfate concentration (200 mM).

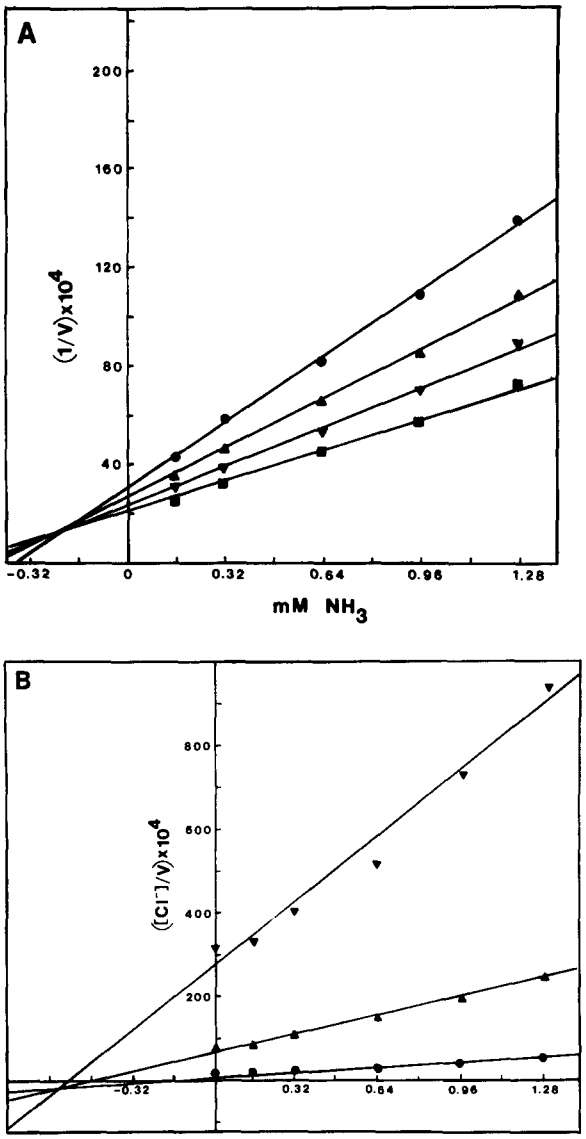

Fig. 5. (A) Dixon plot of NH_3 inhibition of oxygen-evolution activity at various chloride concentrations. (B) Cornish-Bowden plot of NH_3 inhibition at various chloride concentrations. The sulfate concentration was held constant at 40 mM in all experiments shown in (A) and (B). (●), 0.4 mM; (▲), 2.4 mM; (▼), 10.4 mM and (■), 50.4 mM Cl^-.

NH_3 in the oxygen-evolving complex was further tested by examining the effect of NH_3 on Cl^--induced reactivation of oxygen evolution in Cl^--depleted thylakoid membranes. In this experiment the amine concentration after reactivation with Cl^- is lowered 40-fold by dilution into the assay

TABLE II

COMPARISON OF K_i VALUES FOR NH_3 AND TRIS INHIBITION OF THE OXYGEN-EVOLVING COMPLEX

Inhibitory reagent	K_i	Reference
$(NH_4)_2SO_4$	220 μM	this work
NH_4Cl	400 μM	10
$(Tris)_2SO_4$	12 mM	this work
Tris-Cl	50 mM	8

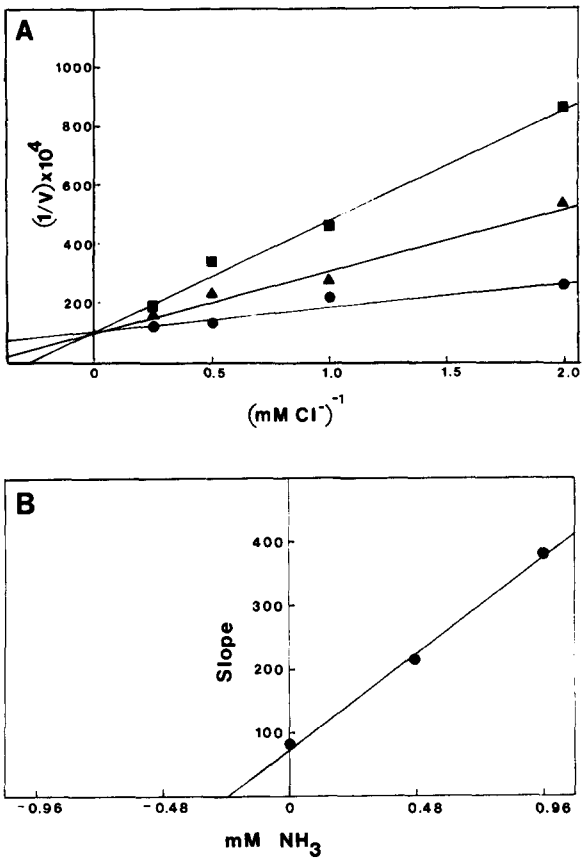

Fig. 6. (A) Lineweaver-Burk plot of NH_3-induced inhibition of chloride-induced reactivation of chloride-depleted chloroplast thylakoid membranes. Assays were carried out as described in Materials and Methods; The NH_3 and chloride concentrations shown in the figure refer to the concentrations of these species in the incubation mixture prior to transfer to the assay cuvette ((●) No NH_3; (▲), 0.48 mM NH_3; (■), 0.96 mM NH_3). (B) Replot of data from (A), showing a $K_i(NH_3)$ of 0.24 mM for inhibition of chloride reactivation of oxygen-evolution activity.

buffer, so the only effect seen here should be NH_3 interference with the Cl^--reactivation process. We are unable to detect any residual interference by NH_3 in the activity assay. If such interference exists, it has disappeared within the response time (9 s) of our oxygen electrode. The results are shown in Fig. 6. Fig. 6A (a Lineweaver-Burk plot) shows a competitive interaction, while the slope replot (Fig. 6B) gives a K_i value of 240 μM, close to the K_i value (220 μM) obtained from the NH_3/Cl^- competition experiments performed on Cl^--sufficient membranes.

Discussion

Protection by Cl^- and other anions against amine inhibition of the oxygen-evolving complex

Lewis bases (such as amines and halides) will form stable bonds with metals such as manganese (a Lewis acid), and it is therefore not surprising to find that Cl^- and certain other anions will protect the oxygen-evolving complex against inhibition by Tris and NH_3. In fact, the strength of an amine inhibitor's binding to the Cl^- site is related to the basicity of the amine; we have shown that NH_3 with a pK_a of 9.25 binds much more tenaciously to the Cl^- site (K_i, 220 μM) than does Tris (pK_a, 8.1, K_i, 12 mM). The presence of Cl^- also stabilizes the oxygen-evolving complex against inactivation by NH_2OH (data not shown), a treatment known to release manganese [1,2,6]. Among the various anions we have tested, Cl^-, Br^- and NO_3^- are the most effective in protecting against Tris attack (Table I). These results are in reasonable agreement with those of Kelly and Izawa [15], who showed that these anions could restore oxygen-evolving activity to Cl^--depleted thylakoid membranes, with the order of effectiveness $Cl^- > Br^- > NO_3^-$. As we show in Fig. 3, the ADRY effect in steady-state light [27] is insensitive to the presence of Cl^-, and we therefore conclude that ADRY reagents (ANT_{2a}, CCCP, Picrate) act at a site in the oxygen-evolving complex which is different from the Cl^- binding site.

Our steady-state kinetic experiments (Figs. 4 and 5), conducted at constant concentrations of SO_4^{2-}, refine our earlier data [21] and show clearly that the free base species of NH_3 and Tris compete with Cl^- to occupy the Cl^- cofactor site in the oxygen-evolving complex. This competition may follow an Sn1 mechanism as proposed by Velthuys [10]. In such a mechanism one ligand must vacate a site before the substituting ligand binds. We would identify the leaving ligand as Cl^-, rather than water, the ligand implied by Velthuys. In fact, our data on the NH_3/Cl^- antagonism have revealed a second, Cl^- insensitive, inhibitory NH_3 binding site ($K_i' = 580$ μM). We suspect, but cannot prove at this time, that this second, Cl^--insensitive site is the water-oxidizing site in the oxygen-evolving complex. Tris ($K_i' \geqslant 300$ mM), probably on account of its bulk, cannot gain access to this binding site.

The role of Cl^- in photosynthetic oxygen evolution

Our conclusions regarding the mechanistic implications of the competition between Cl^- and amines for manganese binding sites in the oxygen-evolving complex were addressed in an earlier communication [21]. Other studies [30,31] have shown that an extensive group of anions (including those shown to be effective in restoring oxygen-evolution activity to Cl^--depleted thylakoid membranes) can mediate electron transfer between transition-metal complexes in solution, and for Cl^- in the oxygen-evolving complex, we would propose the diagram shown in Fig. 7 to represent the chloride-manganese interaction. The binding activities of sites I and II are summarized in the legend of Fig. 7. This model (Fig. 7) represents the 4-manganese metal cluster [32,33,34] found in the oxygen-evolving complex in a fashion similar to that of other tetrametal redox assemblies (cytochrome oxidase, laccase, ascorbate oxidase) in

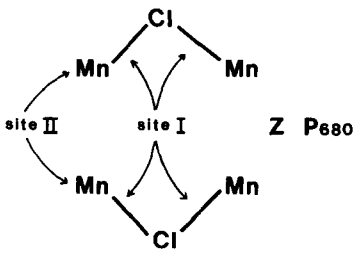

Fig. 7. Model for the interaction of chloride with manganese in the oxygen-evolving complex. Ligands bound at site I would include both anions (Cl^-, Br^-, NO_3^-) and the inhibitory amines (Tris, NH_3). Binding at site II is restricted to NH_3 and perhaps H_2O.

which water and oxygen are substrates and/or products [35,36]. For manganese in the oxygen-evolving complex, we would suggest that the site II metals bind H_2O and that the Cl^- bridge across site I permits transfer of electrons from the site of H_2O oxidation through the second pair of manganese atoms to photoinduced Z^+. Although this model is undoubtedly an oversimplification of the actual mechanism, and is not intended to represent the actual Cl^- stoichiometry in the oxygen-evolving complex, it does explain several experimental observations, as we noted earlier [21].

Two further aspects of the model of Fig. 7 deserve mention here. (1) Ionic radii of bridging anion ligands are critical for activity. The Pauling radii of Cl^- and Br^- are 1.81 and 1.95 Å, respectively. Other anions which are either marginally effective or inhibitory have much smaller ionic radii (1.4 Å for F^-; 1.38 Å for ^-OH). This point has been addressed in detail by Critchley et al. [18] and subsequently by Critchley [19], who examined the relationship between Cl^- and ^-OH with regard to oxygen-evolution activity. The preference of the oxygen-evolving complex for Cl^- (among various effective anions) may be due to the existence of a specific transport system for this anion in the thylakoid membrane [38]. (2) If halides act as bridging ligands to manganese, then spectroscopic probes (EPR, NMR) of the oxygen-evolving complex might provide evidence of an interaction between the Cl^- nucleus ($I = 3/2$) and manganese. A hyperfine structure has been reported [39] to be associated with the low-temperature multiline EPR signal attributed to the S_2 state. Critchley et al. [18] reported a pH-dependent broadening of the ^{35}Cl NMR signal from mangrove thylakoid membranes; the origin of this broadening might be due to a $^{35}Cl^-$-Mn(II) interaction.

We feel that the hypothesized bridging ligand function for Cl^- in the oxygen-evolving complex represents the most straightforward explanation for a number of results now available in the literature, and we have tried to present our ideas in forms that can be tested by further experimentation. It is important to point out that there may be alternative explanations for Cl^- function in the oxygen-evolving complex. Chloride and various amines can also affect the redox potentials of metals [40], and it is possible that NH_3 displacement of Cl^- produces an inhibition of oxygen evolution by altering the redox potential of manganese in the oxygen-evolving complex. It is also possible that Cl^- is not required for a direct role in electron transport but that it is required for charge neutralization or to stabilize the oxygen-evolving complex [17]. Further experimentation is now in progress to refine and test the bridging ligand hypothesis.

Acknowledgements

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