Proton efflux from thylakoids induced in darkness and its effect on photosystem II

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1. INTRODUCTION

Alkaline conditions have a profound influence on the properties of the oxygen-evolving photosystem II. It is known for some time that such conditions may cause an inactivation of the water-oxidizing enzyme complex [1,2], especially when it is in its S_2-state [3]. This inactivation is probably caused by a release of bound manganese [3]. Alkaline media also facilitate the removal of functional Cl^- from photosystem II [4,5], and lead to a deprotonation of certain amino groups whose acetylation inactivates the oxygen-evolving mechanism [6,7].

It is not clear whether a direct relation exists between all these events since the pH necessary to bring them about varies considerably. For example, Cl^- release occurs already at pH 7.8 [5], while the inactivating attack on the S_2 state requires a medium pH of 9 [3]. There is one common feature, however. In each case, the action of high pH is greatly enhanced by the addition of uncouplers [1,5,7]. This interesting effect suggests that the H^+-concentration of the medium is not readily felt by the target site(s) of photosystem II unless H^+-equilibrium across the thylakoid membrane is facilitated.

The apparent protection of the water-oxidizing enzyme from the pH of the bulk solution has been used as one of the arguments for its location on the inner surface of the thylakoid membrane [1,3], or inside a special intramembranous domain [5–7].

In the present paper, we describe two additional inactivating treatments of photosystem II which are facilitated by uncouplers but do not require as high a pH as most of those mentioned above. We also describe experiments which provide information about the quantity and nature of the intrathylakoidal protons which the uncouplers dissipate. These data may provide the basis for a unifying concept concerning the effect of alkaline pH on photosystem II.

2. MATERIALS AND METHODS

Thylakoids from leaves of *Pisum sativum* var. Progress 9 were isolated in a medium containing 200 mM sucrose, 25 mM Na–Tricine (pH 7.6), 5 mM $MgSO_4$ and 10 mM NaCl. Heat inactivation was carried out in buffered media containing 40 mM sucrose, 10 mM KCl, and 5 mM $MgSO_4$. The remaining Hill activity was measured in the presence of nigericin as previously described [5].

If thylakoids were to be incubated with $MgSO_4$, the pellet was divided and suspended at pH 7.0 or 8.0 with Na–Hepes or Na–Tricine as the respective buffers (approx. 0.6 mg Chl/ml). The $MgSO_4$ incubation itself was initiated by adding 0.2 ml 1.25 M $MgSO_4$ to 0.8 ml thylakoid suspension and the mixture kept in very dim ambient light at room temperature. 50 μl samples were periodically withdrawn for assay of gramicidin-uncoupled electron transport activity with DCIP as acceptor [5].

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Abbreviations: Chl, total chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DCIP, 2,6-dichlorophenol-indophenol

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For the H$^+$-release experiments, the pellet was washed twice and resuspended at pH 6.5 in 400 mM sorbitol, 6 mM buffer (Na–Pipes, Na–Hepes, Na–Tricine or Na–imidazole), 2 mM $MgSO_4$ and 10 mM KCl. Storage (at 0°C) and treatments occurred in darkness or very dim ambient light. Various checks did not reveal significant differences between the results obtained under either condition. The release of H$^+$ was determined with a Beckman Model 1019 pH meter. 0.2 ml chloroplast suspension (200–300 μg Chl) were injected into 4.5 ml of a medium containing 1 mM $MgSO_4$, 10 mM KCl, variable amounts of NaOH and, where indicated, 400 mM sorbitol. After usually 1.5 min, 5 μg gramicidin D (in 5 μl ethanol) were injected, and the recorded pH change converted to $\Delta[H^+]$ by comparison with the response to injections of known amounts of HCl.

Table 1

Nigericin effect on heat inactivation of electron transport

pH	Time at high temp. (s)	Rates equiv./mg h		% Remaining activity		% +nig. / % −nig.
		+nig.	−nig.	+nig.	−nig.	
8.0	0	852	973	–	–	–
8.0	90	104	437	12	45	0.27
7.0	0	976	1112	–	–	–
7.0	90	934	1039	96	94	1.03
7.0	180	672	650	69	58	1.19

Thylakoids (34 mg Chl) were injected into cuvette containing 1 ml incubation medium buffered with 25 mM of either Na–Tricine (pH 8) or Na–Hepes (pH 7) (pH measured at room temperature). After the specified heating time at 43°C, 1.5 ml identical medium at room temperature was added for assay 1 min later in presence of 1 μg/ml nigericin. +nig. and −nig. refer to presence or absence of 2.5 μg/ml nigericin during heating. Each entry is an average of two determinations.

3. RESULTS

3.1. *Uncoupler sensitivity of photosystem II*

One test for uncoupler effects on photosystem II of isolated thylakoids was a heat treatment which is known to release most of the functional Mn from thylakoids [8]. In fig.1 it can be seen that the loss of Hill activity at 42°C in uncoupler-containing media proceeded faster as the pH of the suspension medium was raised. In the absence of uncouplers, the results were qualitatively similar except that the rate of inhibition at pH > 7 was always somewhat slower. The data of table 1 confirm this by showing that nigericin caused a more rapid inactivation of thylakoids heated in alkaline media, but had relatively little effect at neutral pH. Interestingly, it was occasionally observed that nigericin protected the water-splitting enzyme against heat denaturation at pH 6.0. Several uncouplers were tested as substitutes for nigericin with qualitatively the same effect.

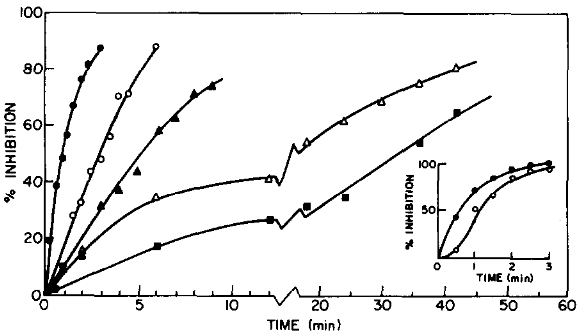

Fig.1. Effect of pH on the rate of heat inactivation of electron transport. pH during incubation at 42°C: ■, 5.8; △, 6.2; ▲, 7.0; ○, 7.3; ●, 7.8. Incubation medium buffered with 25 mM phosphate, and containing 1.25 μg nigericin/ml. At times indicated, a sample was withdrawn and injected into a medium at room temperature for assay of DCIP Hill activity. Curves were obtained with three different batches of chloroplasts, and [Chl] (μg/ml) during the heating period and assay, respectively, were: pH 7.3–8.9, 147 and 3; pH 6.2 and 7.0, 91 and 3.6; pH 5.8, 84 and 4.2. Inset: effect of 1 μg/ml nigericin (●) on heat inactivation (38°C) at pH 8.5. Thylakoids (128 μg Chl/ml) heated as described above in medium buffered with 25 mM Na–Tricine.

As is evident from the inset of fig.1, nigericin accelerated the heat inactivation at pH 8.5 by eliminating a lag of approximately 30 s in the inhibition process. Presumably, this lag was a reflection of the time required for heat-induced H$^+$-equilibration which is known from other studies [6]. To avoid this complication, we also investigated whether uncouplers influence the inactivation of photosystem II by high Mg^{2+} concentrations

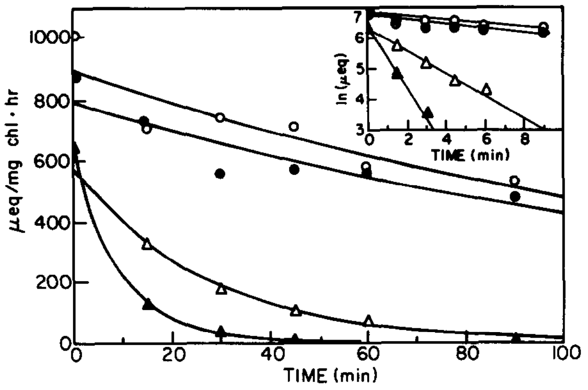

Fig.2. Uncoupler effect on the rate of inhibition of electron transport by 250 mM $MgSO_4$ at neutral and alkaline pH. Thylakoids incubated at pH 7.0 (●, ○) or 8.0 (▲, △) with (filled symbols) or without 25 μM gramicidin D (see Materials and Methods). Inset: Logarithmic plot of Hill activity *vs* time. All lines were computer-drawn according to a first order rate equation with rate constants determined from half-times of inactivation taken from least square analyses of the points in the inset (k (h^{-1}): ○, 0.39; ●, 0.38; △, 2.31; ▲, 6.03).

which, like the inactivation by heat, is associated with a release of functional Mn [9]. Figure 2 shows that the loss of Hill activity in the presence of 250 mM $MgSO_4$ followed first-order kinetics and responded to pH and uncoupler (gramicidin D) in a manner qualitatively similar to that observed during heat inactivation. These data corroborate, therefore, the results of the previously described experiments and establish that uncouplers accelerate inactivating treatments affecting the water-splitting apparatus even at pH 8.

3.2. *Uncoupler-induced proton release from thylakoids in the dark*

The apparent slow equilibration of the pH between the external medium and the intrathylakoidal space around photosystem II suggested that protons remained sequestered inside the thylakoids for a considerable time after transfer to slightly alkaline media. Indeed, Dilley and his coworkers [6,7] have reported that 20–40 neq. H^+/mg Chl were released by gramicidin from a thylakoid suspension at pH 8.5 which had not been preilluminated. We have confirmed this observation using uncouplers like gramicidin, nigericin, FCCP and quinacrine derivatives. Surprisingly, H^+ efflux from thylakoids under our experimental conditions was also caused by agents like diphenyliodonium chloride, *N*-methylphenazinium methosulfate (PMS), tetraphenylborate and DCMU, all at 50–100 μM.

The amount of H^+ which was set free by an uncoupler declined with the time elapsed after thylakoid injection into the assay medium, but occasionally was still considerable after 10 min. Figure 3 shows that gramicidin induced H^+ efflux was a function of pH and osmolarity of the assay solutions. The absence of sorbitol as osmoticum had two effects: First, more protons were released, and second, only into such media were significant amounts of H^+ set free when assay and storage pH were identical. The latter aspect was verified

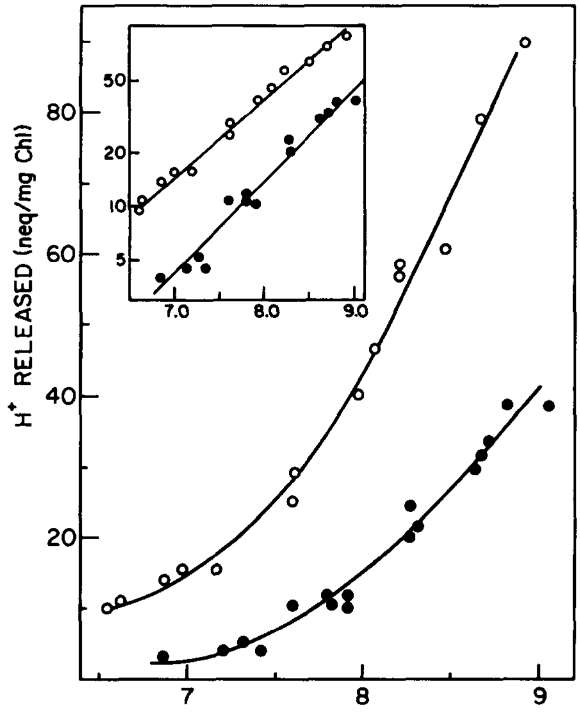

Fig.3. Release of H^+ from thylakoids by gramicidin in the dark. 0.2 ml thylakoids (315 μg Chl) stored at pH 6.6 were injected into an assay medium containing (●) or not containing (○) 400 mM sorbitol plus NaOH to attain the assay pH given on the abcissa, followed by gramicidin addition as described in Materials and Methods. Inset: same data plotted semilogarithmically.

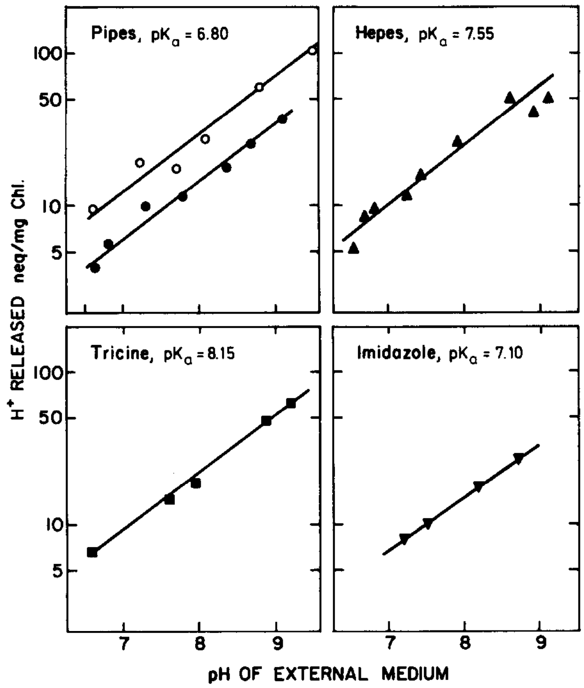

Fig.4. Gramicidin-induced proton release from thylakoids stored in the presence of different buffers. Thylakoids were stored at pH 6.4 to 6.7 in the presence of 6 mM of the indicated buffers, and treated as described in legend to fig.3. All assay media containing 400 mM sorbitol except for (○). The experiment with imidazole was performed with chloroplasts from a different leaf harvest.

for storage pH values between 6.5 and 7.8 (not shown). Interestingly, the number of H^+ released was an exponential function of the medium pH, regardless of the external buffer, all of which had p*K* values within the examined pH range (fig.3, inset; fig.4). This finding ruled out that the protons were liberated from any added buffer molecules which had become trapped in the thylakoid lumen. In agreement with such conclusion we found that a 5-fold increase of the Na–Hepes in the storage suspension led only to a 30% greater efflux of H^+.

4. DISCUSSION

Our data show that a large amount of thylakoid-associated protons equilibrates only slowly with the external bulk medium unless agents like uncouplers are added. The very large number of protons which may be set free from thylakoids (> 1 $H^+/10$ Chl), regardless of the presence of permeant buffers, suggests that most originate from a bound pool. Any attempt to localize this pool may disregard the diffuse electrical layer, or general cation binding to the membrane surfaces, since neither 100 mM KCl nor 50 mM $MgCl_2$, were found to cause a release of the protons detectable after uncoupler addition.

The observed exponential relation between the amount of liberated H^+ and external pH would, if valid for any pH-range, imply an inexhaustible H^+-resevoir. This is similar to situations in which the buffering capacity, $\partial[H]_{total}/\partial pH$, or the H^+-binding ratio, $\partial[H]_{total}/\partial[H^+]_{free}$, are constants independent of pH. Haraux and de Kouchkovsky [10] encountered the latter phenomenon in the thylakoid lumen when the extent of light-driven H^+ pumping was controlled so that the internal pH varied by as much as three units. These authors, probing a more acid pH range than us, attributed their observations to a continuous unmasking of additional H^+-binding groups as the negative charges of surface-exposed anions became neutralized. For our experiments we may assume that more buffering groups, possibly $-NH_3$ [6,7], became available for deprotonation as the pH, and hence the H^+-binding capacity of the medium was increased. When thylakoids were swollen in hypotonic media, a larger number of ammonium groups apparently could become exposed and deprotonated. Consequently, H^+-release into hypotonic media was especially large and significant even when their pH was the same as that during thylakoid storage.

At present it is difficult to understand what prevents the large reservoir of protons to readily equilibrate with the external bulk phase. The accelerating influence of compounds other than protonophoretic uncouplers speaks against a simple permeability problem. It is more likely that all these agents facilitate conformational changes which are necessary to expose hidden protonated groups of membrane proteins. As discussed by others [11,12], electrical surface properties of membranous systems are sensitive to the modifying effect of uncouplers on the protonic resistance of the membrane, to the closeness of individual membranes as a consequence of stack formation or osmotic swelling, and to interactions with organic amphiphiles or chaotropes.

Dilley and his co-workers [6,7] have postulated that the water oxidizing enzyme is located in a H^+-sequestering, intrathylakoidal domain. Our experiments suggest that at least a portion of the newly exposed protonated groups were originally part of such a domain. A certain degree of protonation of these groups appears to be important for the function and stability of the oxygen evolving mechanism. In our previous article [5] we overlooked the many similarities between the earlier data [1,2] on the uncoupler-sensitivity of the water-splitting enzyme at high pH, and our experimental procedure to create a Cl^--deficient photosystem II without first removing the coupling factor from the thylakoids. Indeed, as is suggested also by recent results of Kelley and Izawa [13] and Izawa (pers. commun.), the functional Cl^- may be a primary target of alkaline conditions. In the early experiments mentioned above, the Cl^- of photosystem II may have become detached at the rather alkaline pH around 9 even though Cl^- was abundant in the medium. When the water-oxidizing enzyme was in its S_2-state, or illuminated at the elevated pH, oxygen evolving activity was irreversibly lost [1,3]. We showed later that, in a Cl^--free medium containing an uncoupler, Cl^--deficiency could be created already at pH 7.8, most rapidly in the S_2-state [5,14]. Again, such Cl^--deficient state is made irreversible by light [4].

Most, if not all, the irreversible inactivations of photosynthetic water oxidation dealt with in this article involve the loss of functional manganese [3,8,9]. The concept emerging from the old and new data discussed here, therefore, should be a valuable asset in our quest to understand organization and function of the entire photosynthetic water-oxidizing system.

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