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THE EFFECT OF LOW CONCENTRATIONS OF UNCOUPLERS ON THE DETECTABILITY OF PROTON DEPOSITION IN THYLAKOIDS

EVIDENCE FOR SUBCOMPARTMENTATION AND PREEXISTING pH DIFFERENCES IN THE DARK

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Flash-induced pH changes inside thylakoids were measured with neutral red as an indicator in the presence and absence of low concentrations of uncouplers. We found that both carrier-type and pore-forming uncouplers caused the rapidly rising phase of the neutral red signal, previously attributed to proton deposition by water oxidation, to disappear. Gramicidin was particularly efficient in this respect, requiring only one molecule of uncoupler per 10^4 chlorophyll molecules to render the rapid proton deposition undectectable. This suggests that gramicidin did not act on each water-oxidizing enzyme individually, but rather at the level of the thylakoid membrane. In contrast to the effect on water-derived protons, the appearance of protons from plastoquinol was unaffected by gramicidin. Nor did gramicidin affect the rise of the neutral red signal due to proton deposition during two Photosystem I partial reactions with artificial donors. At the low gramicidin concentrations used, its effect on the neutral red signal could not be attributed to a general increase in proton permeability of the thylakoid membrane (acceleration of half decay from 9 to 0.8 s). The extent of alkalinization of the external medium during the first few hundred milliseconds following a light flash was unaltered by gramicidin, and we did not observe a kinetic correlation between the disappearance of the water proton and the decay of the transmembrane electric field. The last two findings suggest that the undetected protons had not crossed the thylakoid membrane, but instead were buffered away by some gramicidin-induced extra buffering power. pH titration of this extra buffering power revealed an apparent pK ranging between 7.2 and 7.7 and a stoichiometry of $2H^+$/site. The rapid phase of the neutral red signal regained 90% of its original amplitude after seven flashes were applied at 6.7 Hz repetition rate to a sample containing gramicidin. This suggests limits to the extra buffering power. One possible interpretation of our experiments is the following: Protons derived from water oxidation are initially deposited into extended and highly buffering special domains, and only escape into the common internal phase when the buffering capacity of the domains is saturated. As an alternative one may consider that the thylakoid lumen is partitioned into at least two domains, each dominated by different photosystems and with slow proton equilibration between them. Either view requires internal subcompartmentation. The consequences of such subcompartmentation for chloroplast bioenergetics are still obscure.

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Abbreviations: Chl, chlorophyll; CF_1, hydrophilic component of the chloroplast coupling factor; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; ETH 1001, *N*,*N*'-di[(1-ethoxycarbonyl)undecyl]-*N*,*N*'-4,5-tetramethyl-3,6-dioxaoctane amide; FCCP, carbonyl cyanide *p*-trifluorimethoxyphenylhydrazone; Pipes, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

The mechanism of energy transduction in photosynthetic and respiratory systems has long been the subject of intense investigation. Following Mitchell's introduction of the chemiosmotic coupling hypothesis [1–4], it has become generally accepted that the energy required for ATP synthesis is transiently stored as an electrochemical potential difference of protons established by electron transport between two aqueous bulk phases. The generation of such a proton-motive force in thylakoids by vectorial electron transport and its utilization have been reviewed [5–7]. There is still considerable discussion, however, concerning the location of the protons and the magnitude of the potential inside thylakoids.

In chloroplasts, the chemiosmotic hypothesis postulates that protons are liberated into the aqueous and osmolar thylakoid lumen by both protolytic reactions, photosynthetic water oxidation and the oxidation of plastoquinol. This view was supported by direct measurements of proton deposition in the thylakoid lumen using the pH-indicating dye neutral red [8,9]. An alternative to Mitchell's hypothesis was proposed by Williams [10–13], who suggested that protons are released within the membrane and travel intramembranously to the coupling factor. Kell [14] modified this viewpoint by postulating that protons follow restricted proton-conduction pathways that are in the Stern-Grahame layer adjacent to the inner surface of the membrane. The latter two hypotheses view the well established acidification of the aqueous lumen as a secondary consequence of proton release which is not essential for ATP synthesis.

Recent experiments in the laboratories of Dilley [15–18] and Homann [19–21] have been interpreted to indicate that a proton-motive force may exist in thylakoids even in the dark, and that there are further subcompartments in addition to the two aqueous phases of thylakoids. The putative proton gradient remained detectable for up to 15 min in the dark, and could be abolished by the addition of uncouplers, removal of the chloroplast coupling factor or by a short heat treatment. Experimentally, this metastable proton gradient was found to influence the formation of derivatives of thylakoid membrane proteins by acetic anhydride [15–17], the release of functional Cl^- associated with PS II [19] and the stability of the water-splitting enzyme toward manganese-extracting inactivation procedures [20].

An interesting feature of these experiments concerns the locus of the proton pool that makes up the acid component of the dark proton-motive force. Prochaska and Dilley [15,16] and Baker et al. [17] found that the pH-dependent reaction of acetic anhydride with both an 8 kDa subunit of the chloroplast coupling factor and a protein presumably involved in water oxidation was influenced more by protons released from PS II than by those derived from a cyclic PS I reaction. A similar observation was made for the light-dependent retention of Cl^- associated with PS II in chloroplasts diluted into Cl^--free media [21]. This suggested that the high-potential side of the dark proton-motive force was located neither in the aqueous thylakoid lumen nor in a common electrical double layer at the inner membrane surface.

The fact that protons derived from the two photosystems remained distinguishable at a site as distant as the coupling factor seemed incompatible with the chemiosmotic hypothesis. Therefore, Dilley and his collaborators [17,18] proposed a new model based on Williams' hypothesis of intramembranous proton processing. According to their model, protons released by water oxidation reach the coupling factor by travelling through special domains within the membrane. In the dark, the boundaries of such domains are thought to be nearly impermeable to protons unless the membrane is made leaky by mild heat treatment, addition of uncouplers or removal of the coupling factor. Although the model is hard to reconcile with the known lateral heterogeneity of the PS II and CF_1 distribution in the thylakoid membrane [22,23], it has proven to be useful in designing new experiments concerning the effects of uncouplers in dark-adapted chloroplasts.

As shown by Junge and co-workers [8,9,24], proton release during flash excitation of chloroplasts can be measured and kinetically resolved in the aqueous thylakoid lumen by monitoring the accompanying absorption changes of neutral red. This method has also been adopted in other laboratories [25–27]. The question of whether or

not protons are deposited into an aqueous osmolar volume was specifically studied by Junge et al. [9,28]. They found that the extent of the pH_{in}-indicating absorption changes of neutral red decreased in the presence of hydrophilic buffers like P_i, which were more effective the greater the internal osmolar volume of the thylakoids. This led them to answer their question in the orthodox chemiosmotic way. Recently, they further corroborated this view by showing that neutral red monitors the surface pH inside thylakoids [29], which is known from model studies of Gutman et al. [30] to equilibrate rapidly with the bulk pH. However, these conclusions were based on experiments performed with chloroplasts which had been stored frozen in the presence of dimethyl sulfoxide as a cryoprotective agent. In freshly prepared class II chloroplasts, or in those stored in the presenc of ethylene glycol, neither an effect of hydrophilic buffers nor the surface location of neutral red could be established by the same authors [29].

In this paper we address ourselves to the apparent discrepancies between experiments indicating localized protons and those in favor of delocalized ones.

Materials and Methods

Chloroplasts were prepared from market spinach or from 2–4-week-old pea seedlings by homogenizing the leaves and centrifuging the resulting slurry in a medium at pH 7.5 containing 200 mM sucrose, 25 mM Tricine, 5 mM $MgSO_4$ and 10 mM NaCl. The pellet thus obtained was resuspended in either the same medium, or one in which 25 mM Pipes, pH 7.0, replaced Tricine. Sometimes the pellet was washed and centrifuged once more before the resuspension step, and occasionally 1 mg/ml bovine serum albumin was included in the final medium. The chloroplasts were either placed on ice and used that day, or stored frozen in liquid N_2 in the presence of 30% ethylene glycol [31] for up to 60 days. The effects of uncouplers on the neutral red signal which are described below were not affected by the above-described slight variations in the chloroplast preparation method, and no qualitative differences were apparent between freshly prepared and freeze-thawed samples.

Measurements of absorption changes were performed in the apparatus described in Ref. 24. The 'pH_{in}-indicating absorption changes of neutral red' resulted from subtracting the absorption changes obtained in the absence of neutral red from those obtained in its presence. As previously demonstrated in Ref. 9, there was no redox response of neutral red. Saturating flashes were obtained with a xenon flashlamp ($\lambda > 615$ nm, 15 μs, 1 mJ/cm^2). For the experiments reported in Fig. 8, flashes were delivered with a fast-repetition xenon lamp (PRA 610, 430–475 nm, 15 μs, 0.4 mJ/cm^2). The intensity of the measuring beam was less than 5 μW/cm^2, which excited no more than 5% of the reaction centers per s [9]. Unless otherwise indicated, 5–20 signals were averaged and the flash repetition rate was less than or equal to 0.2 Hz.

The assay medium contained 10 mM NaCl, 5 mM $MgSO_4$ or $MgCl_2$, 2.6 mg/ml bovine serum albumin, and where indicated, 13 μM neutral red, 15 μM cresol red, 10 μM benzyl viologen, 10 μM DBMIB, or 2 mM $Fe(CN)_6^{3-}$. The pH of the medium was adjusted after the addition of chloroplasts (10 μM Chl) for each separate experiment. Uncouplers and ionophores were then added from ethanolic solutions, keeping the ethanol content of the final assay medium below 1%.

Results and Discussion

Gramicidin renders proton deposition by water oxidation transiently undetectable by neutral red

Absorption changes of neutral red in response to flash-driven electron transport from water to benzyl viologen appear in two kinetic phases [8,24]. The fast phase, complex in itself, has been attributed to water oxidation [8], while the slowly rising phase has been attributed to plastoquinol oxidation. When the electron-transport chain was interrupted before plastoquinone by DBMIB/$Fe(CN)_6^{3-}$, only the fast signal was apparent. Both situations were reproduced in the absence of uncouplers and are shown in the left-hand traces of Fig. 1. When gramicidin D was added in nanomolar concentrations the rapid component of the neutral red signal disappeared, regardless of the electron acceptor (middle traces). (The slow rise observed in the presence of DBMIB/Fe $(CN)_6^{3-}$ was not consistently reproducible.) The iono-

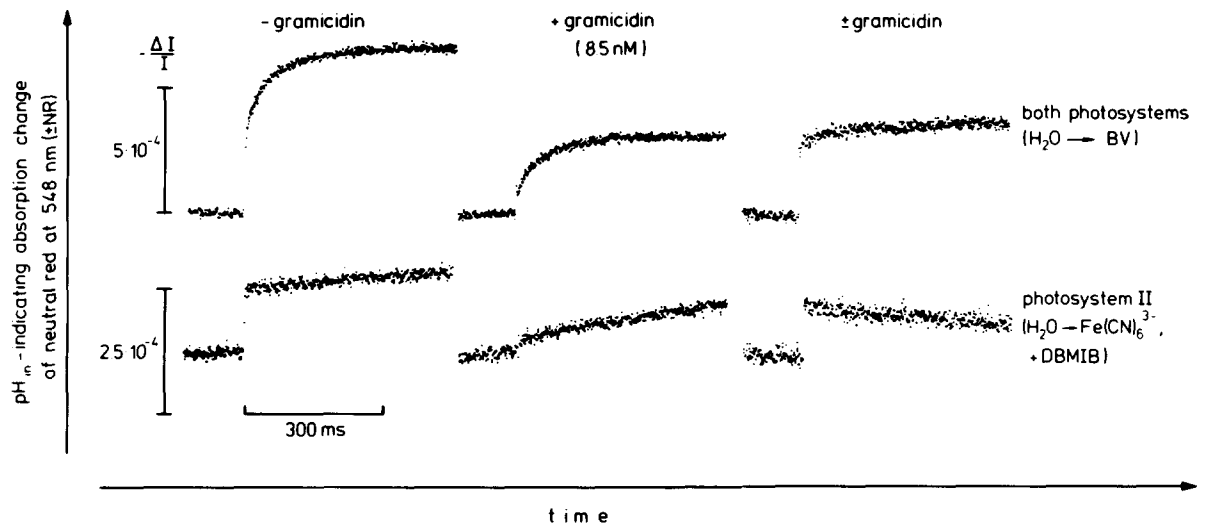

Fig. 1. Effect of gramicidin on flash-induced internal proton release from both photosystems (above) and from PS II alone (below). Left-hand and middle traces recorded between pH 7.79 and 7.81. Right-hand traces were obtained by subtracting the plus gramicidin curves from the appropriate controls. BV, benzyl viologen.

phores FCCP (100 nM), A23187 (1 μM) and nigericin (10 nM) plus KCl (10 mM) produced the same results as gramicidin (not shown).

Fig. 2 illustrates the concentration dependence of the gramicidin effect on the pH_{in}-indicating absorption changes of neutral red. The squares

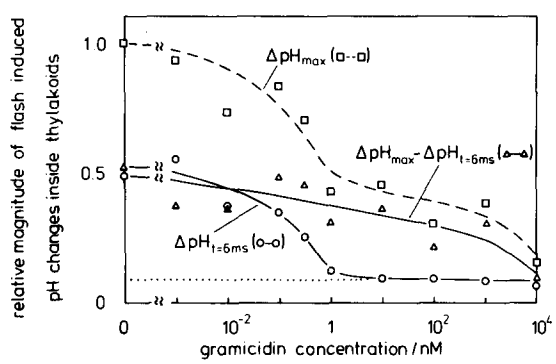

Fig. 2. Concentration dependence of the effect of gramicidin on internal proton release. (□) Amplitude of the neutral red signal 600 ms after the flash (both water and plastoquinol protons), (○) amplitude 6 ms after the flash (water protons), (△) difference between amplitudes 6 and 600 ms after the flash (platoquinol protons). The dotted line shows the expected contribution of the plastoquinol protons (release half-time, 50 ms) to the total signal 6 ms after the flash. All points obtained between pH 7.68 and 7.72 in the presence of 10 μM benzyl viologen.

show the full amplitude of the signal which developed within 600 ms after the flash was fired. The circles represent the amplitude 6 ms after the flash, which was primarily due to the water-derived proton [8,24]. The difference between the full amplitude and that at 6 ms (triangles) was attributed to protons released from plastoquinol. As can be seen, a 50% reduction of the neutral red signal from fast proton release occurred at approx. 0.5 nM uncoupler, i.e., one gramicidin molecule per $2 \cdot 10^4$ Chl or per 30–40 PS II reaction centers, and 1 nM completely eliminated the signal. This indicates that gramicidin did not act on the individual reaction centers, but on the thylakoid membrane. In contrast, the absorption change of neutral red attributable to the plastoquinol protons was significantly affected only at much higher uncoupler concentrations, when the signal's decay would be expected to compete with the slow rise.

The undetectability of the fast component of the internal acidification was apparently not due to the increased leak permeability for protons caused by gramicidin in the thylakoid membrane. We could measure this permeability via the decay of the neutral red signal due to plastoquinol oxidation, which proceeded with a half-time of 800 ms at 17 nM gramicidin (see Fig. 7).

Fig. 3 shows that the gramicidin effect on the

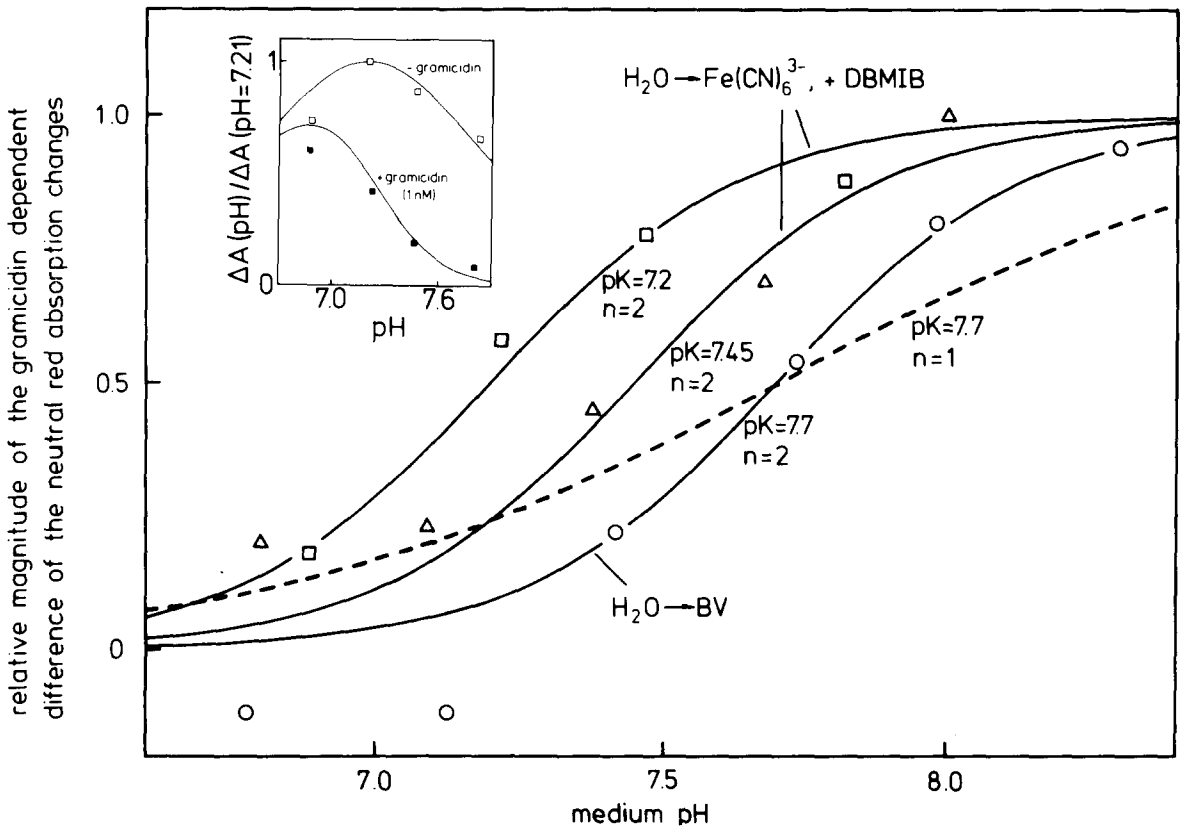

Fig. 3. The pH dependence of the effect of gramicidin on internal proton release in three different chloroplast preparations. (□ and △) $H_2O \rightarrow Fe(CN)_6^{3-}$, + DBMIB; 1 nM gramicidin when present. (○) $H_2O \rightarrow$ benzyl viologen (BV); 17 nM gramicidin when present. Solid curves were calculated according to Eqn. A3 using the indicated pK for a buffer which reacts with two protons at once ($n = 2$). The dashed line shows the predicted titration curve of the gramicidin effect with a pK of 7.7 for a buffer which binds only one proton. (inset) The pH dependence of the amplitude of the neutral red signal obtained with (■) and without (□) gramicidin. Same experiment as □ above. Lines were calculated from Eqns. A3–A5 using $pK_{NR} = 7.2$ and $pK_d = 7.2$.

water proton was pH dependent, being almost nonexistent at neutral pH and developing in alkaline media. The effect titrated with a single pK, which varied from 7.2 to 7.7 in three different experiments. For each, however, a titration curve calculated under the assumption that the buffering groups bound two protons at once (solid lines), rather than one (dashed line), gave the best fit to the data. For comparison, the inset shows the pH-dependent sensitivity curve of neutral red in thylakoids, which was as previously published [9] in the absence of gramicidin (open symbols), but different in its presence (closed symbols). It is significant that the apparent pK of the neutral red sensitivity curve and that of the gramicidin effect were not usually the same.

Specificity of the gramicidin effect for water-derived protons

Wagner and Junge [32] have reported a seemingly similar phenomenon in which the rapidly deposited proton escaped detection by neutral red after formation of the derivative of the chloroplast coupling factor with *N,N'*-(*o*-phenyl)dimaleimide. They found that the effect was not restricted to protons released by water oxidation, but that protons which were liberated during pyocyanine-mediated cyclic electron flow in the presence of DCMU also disappeared after *N,N*-(*o*-phenyl)dimaleimide treatment. This led them to postulate that *N,N*-(*o*-phenyl)dimaleimide treatment caused a transmembrane proton-conducting pathway to appear which closed after the passage of one pro-

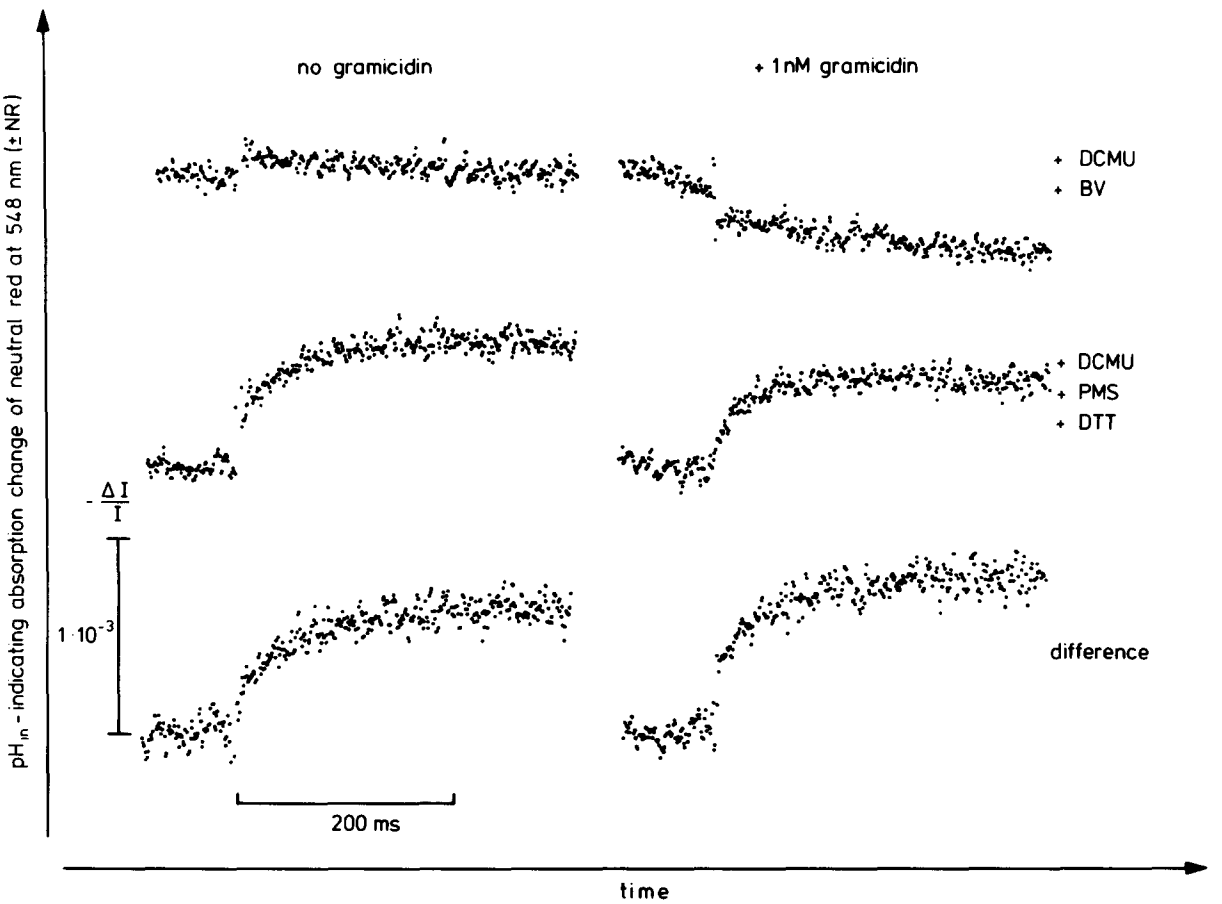

Fig. 4. Effect of gramicidin on protons release inside thylakoids as a result of phenazine methosulfate oxidation by PS I. 40 flashes averaged, sample changed after 20 flashes. Where indicated, assay mixtures contained 1 μM DCMU, 20 μM phenazine methosulfate (PMS) and 0.4 mM dithiothreitol (DTT). All traces recorded between pH 7.70 and 7.72. Lower traces were obtained by subtracting the upper from the middle traces.

ton. It was therefore necessary to determine whether gramicidin caused a gated proton channel to appear, similar to that apparently produced by *N,N*-(*o*-phenyl)dimaleimide, or whether its effect was exerted on the water-splitting proton specifically.

In contrast to the experments of Wagner and Junge, we found that gramicidin had no, or very little, effect on acidification of the thylakoid lumen by cyclic electron flow in the presence of DCMU and phenazine methosulfate/reductant (Fig. 4). A similar observation was made using the PS I partial reaction, DAD/reductant → benzyl viologen in the presence of DCMU (not shown). In this case, however, a gramicidin-dependent fast phase of proton release was noticeable at 0.2 Hz flash frequency. When the flash frequency was increased to 1 or 2 Hz, the fast phase disappeared and the remaining slow phase was insensitive to gramicidin. This led us to ascribe tentatively the fast phase to protons released by a PS II protolytic reaction which was observed by Hong et al. [33] to occur in the presence of DCMU, but further experiments are required to confirm this point.

As mentioned earlier, the ionophores FCCP, A23187 and nigericin also caused the fast phase of the neutral red signal to disappear. Even though the latter two uncouplers are supposedly electroneutral proton/cation exchange carriers, the possibility remained that increased membrane permea-

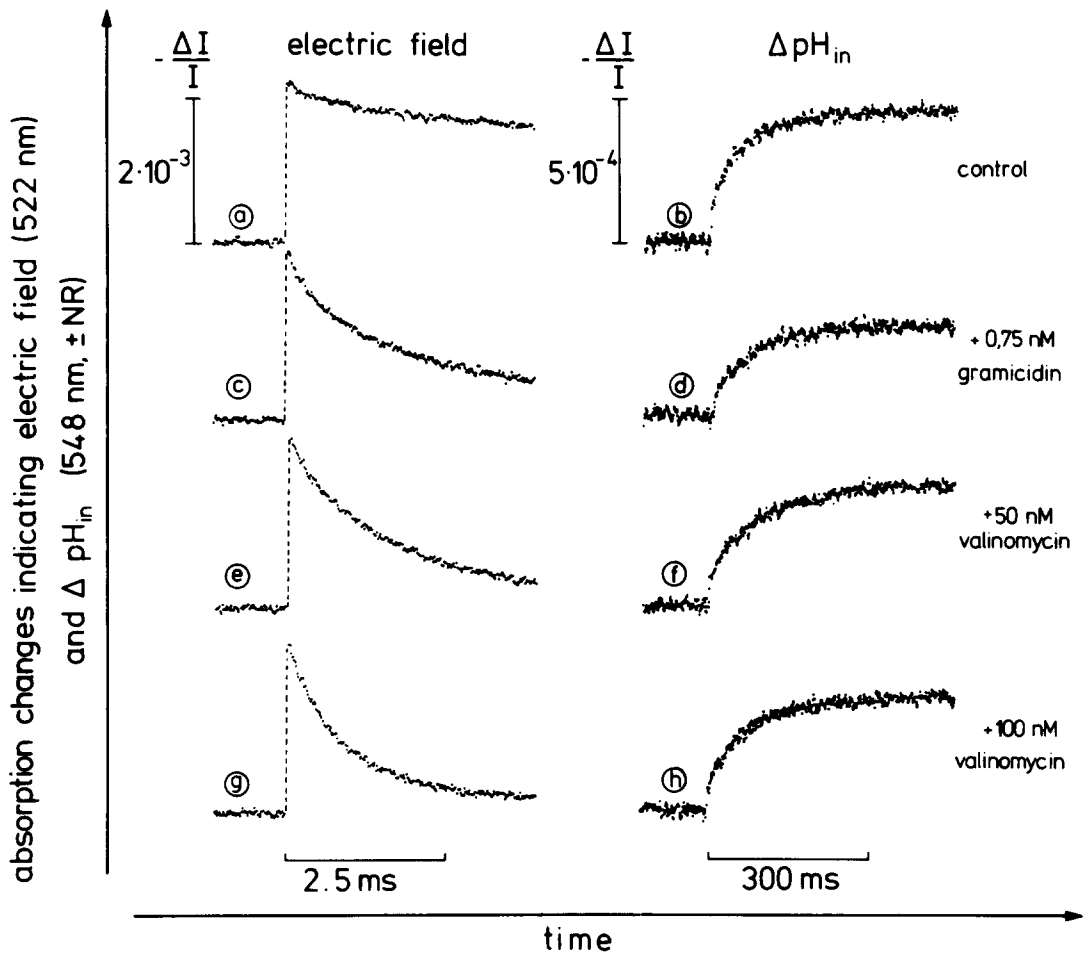

Fig. 5. Lack of correlation with increased ion permeability of the thylakoid membrane and the loss of the rapid phase of proton deposition. Internal proton release recorded between pH 7.78 and 7.88 in assay media containing 10 μM benzyl viologen, 10 mM KCl and the indicated additions; 1 ms/point, 0.3 kHz electrical bandwidth. Absorption changes at 522 nm recorded in an identical medium in which 25 mM Na^+-Tricine replaced the bovine serum albumin; 20 μs/point, 30 kHz electrical bandwidth.

bility to ions in general, rather than to protons specifically, was responsible for the disappearance of the water-derived proton. This was examined in the experiment shown in Fig. 5 using valinomycin. It is evident that at valinomycin concentrations which produced the same (50 nM) or even faster (100 nM) decay rates of the electrochromic absorption change at 522 nm as did 0.75 nM gramicidin, no effect on the rapid phase of the neutral red signal was seen. Similar results were obtained with the divalent cation-exchange ionophore ETH 1001 [34] (not shown). In one experiment, valinomycin at a higher concentation (0.2 μM), rendered the water-derived proton undetectable, probably as a result of its weak protonophoretic action [35].

The undetectable water-derived protons did not rapidly cross the thylakoid membrane

We asked whether the apparent disappearance of water protons was due to their very rapid passage across the membrane. Fig. 6 shows the pH_{in}-indicating absorption changes of neutral red (above) and the electrochromic absorption changes (below) at high time resolution (20 μs per address of the averaging computer and 30 kHz electrical

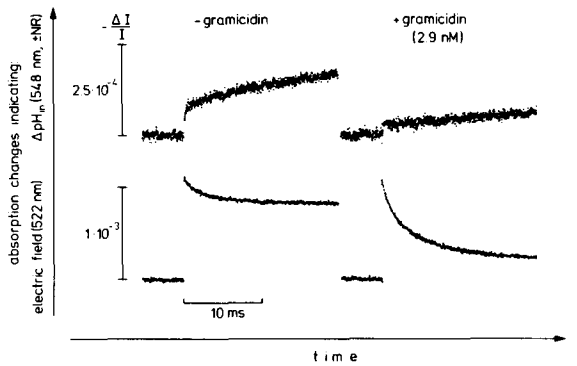

Fig. 6. High time resolution of internal proton release and electrochromic absorption changes at 522 nm in the presence and absence of gramicidin. All traces recorded at 20 μs/point, 30 kHz electrical bandwidth between pH 7.75 and 7.78 in the presence of 10 μM benzyl viologen.

bandwidth). Comparison of the upper and lower traces in the figure reveals that the rapidly released protons had become undetectable by neutral red before any appreciable decay of the electric field occurred, indicating that they had not crossed the thylakoid membrane, at least not electrically uncompensated.

To corroborate this point we measured flash-induced proton uptake from the external medium. The experiment was carried out at lower time resolution to find out whether the 'disappeared' protons reappeared at all in the outer bulk phase. The upper traces in Fig. 7, measured with cresol red [8], reveal that while the decay of the external

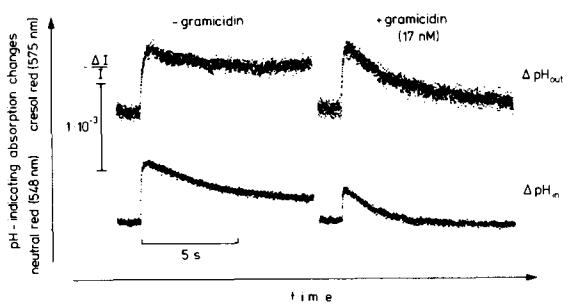

Fig. 7. Effect of gramicidin on internal and external flash-induced pH changes. All traces recorded between pH 7.73 and 7.80. The assay medium contained 10 μM benzyl viologen and 200 mM sucrose, and either 13 μM neutral red (pH_{in}) or 15 μM cresol red (pH_{out}). Bovine serum albumin was omitted for the measurements with cresol red.

alkalinization was accelerated by gramicidin, the initial extent of proton uptake was unchanged. Under the same conditions the neutral red signal was halved. Furthermore, the missing water protons apparently reappeared after a few seconds so that the net proton change in the solution was zero, just as in the absence of gramicidin. The latter two results are contrary to those obtained by Wagner and Junge [32] with *N,-N-(o*-phenyl)dimaleimide-treated chloroplasts, and indicate that the water protons had not immediately crossed the membrane. Instead, it appeared that low concentrations of gramicidin caused an increase in buffering capacity in some region in the thylakoid interior to which only water-derived protons had access.

The buffering region which transiently hides water-derived protons has a limited capacity

Fig. 8 shows that it was possible to recover the lost rapid phase of proton deposition when the samples were excited with a sequence of closely spaced flashes. The two traces depict the actual recordings (lines with noise), upon which are superimposed solid lines drawn from a simulation of the experiments according to the model described in the General Discussion and Appendix. The upper and lower traces were recorded in the presence and absence of gramicidin, respectively. It is immediately apparent that the fast phase of proton release was absent in the first few flashes when gramicidin was present (inset in the upper left-hand corner), but gradually reappeared in the later flashes. The reappearance of the rapid phase is shown more clearly in the lower right-hand inset, in which the relative magnitude of the signal arising from the water-derived proton (as determined from the calculated traces) is plotted as a function of flash number. This portion of the signal recovered from 40–90% of the control value during the first seven flashes (circles).

We found that the extent of restoration of the neutral red signal was controlled by the gramicidin concentration, pH, and to a lesser degree the flash frequency. The experiment in Fig. 8 was performed at pH 7.68 in the presence of 0.3 nM gramicidin; at 1 nM gramicidin concentration the signal did not recover its original amplitude even after 40 flashes.

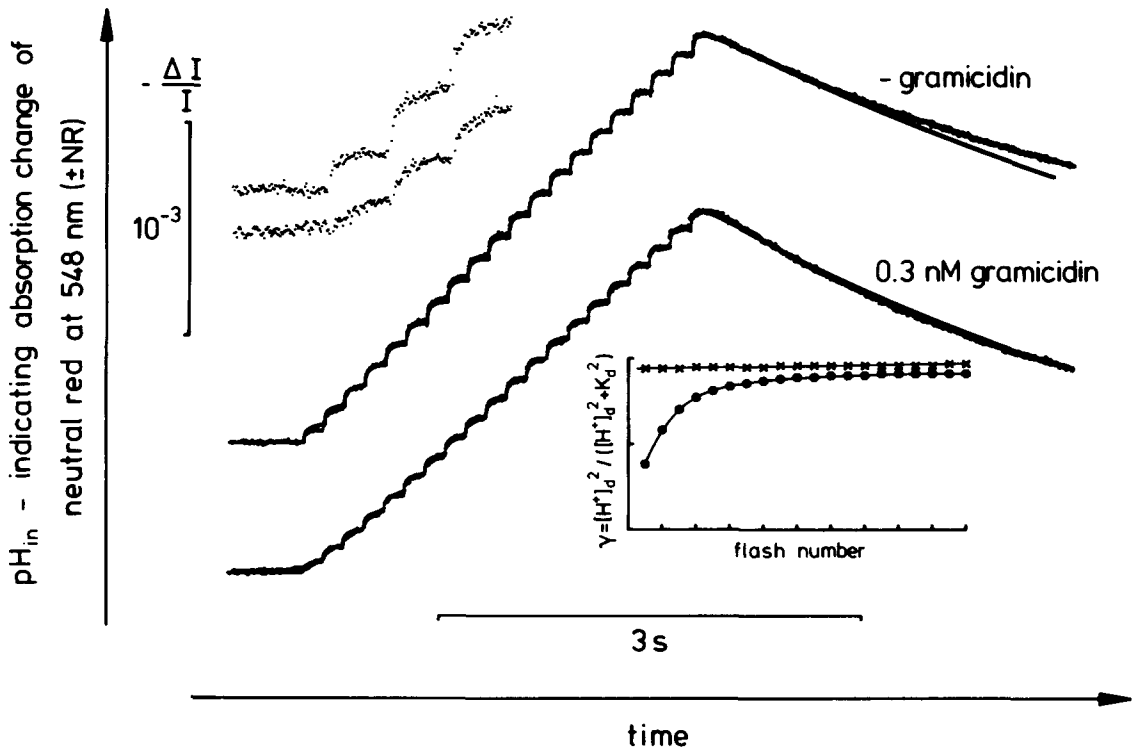

Fig. 8. Absorption changes of neutral red (NR) during $H_2O \rightarrow$ benzyl viologen electron transport driven by a sequence of closely spaced flashes. (a) No gramicidin, pH 7.68; (b) plus 0.3 nM gramicidin, pH 7.69. Chloroplasts flashed at a frequency of 6.67 Hz; 30 s separated the flash groups. Lines with noise are the actual data from the signal averager. The smooth lines were calculated as described in the Appendix, using the parameters listed in Table I (protons in the domain equilibrating to the outside). Inset in the upper left-hand corner: 3-fold expanded plots of the neutral red signals from the first three flashes. Inset in the lower right-hand corner: Calculated relative contribution of the water-derived proton to the fast phase of the neutral red signal. (×) −gramicidin, (●) +gramicidin.

General Discussion

In this paper we have investigated the effects of uncouplers on proton deposition into thylakoids using neutral red as a pH indicator under selective buffering of pH changes in the external medium. Under excitation of chloroplasts by repetitive flashes spaced 5 s or more apart, we made the following observations: (1) The rapid deposition of protons which results from water oxidation could no longer be detected in the presence of low concentrations of gramicidin, A23187, nigericin and FCCP, but was unaffected by the nonprotonophoric ionophores valinomycin (at less than 0.2 μM) or ETH 1001. (2) Under similar conditions, gramicidin caused no significant change in the slower rise of the neutral red signal due to internal acidification by either plastoquinol oxidation or protolytic PS I partial reactions. (3) Gramicidin did not affect the extent of external alkalinization as measured by the pH_{out}-indicating absorption changes of cresol red. (4) The undetectability of the water-derived proton in the lumen was not due to its rapid passage across the thylakoid membrane. (5) The lost rapid phase of the neutral red signal regained almost all of its original amplitude when flashes were delivered in closely spaced groups (20 flashes separated by 150 ms).

At first glance, our results are similar to those previously obtained by Wagner and Junge [32] for chloroplasts in which CF_1 formed a derivative with *N,N*-(*o*-phenyl)dimaleimide. There are, however, significant differences which are described in Results and Discussion. The major difference was

that gramicidin, at the low concentrations used in this study, did not cause rapid proton passage across the thylakoid membrane, while *N,N*-(*o*-phenyl)dimaleimide modification of CF_1 did.

Since the undetected water protons had not crossed the membrane, and since the release of PS I-related protons was not affected by gramicidin, we had to conclude that the water protons were buffered away by some extra buffering capacity which was activated by gramicidin. Gramicidin could not have caused the observed effects by direct action, as there was only one uncoupler molecule required per 10^4 Chl molecules, i.e., one gramicidin per 20 protons which disappeared. It is probable that gramicidin activated the extra buffering capacity by equilibrating the (possibly more acid) pH in a special domain with the pH of the internal and external bulk phases. We see two alternative possibilities to account for the special buffering domain: (1) If the thylakoid lumen is continuous and if protons from water and from plastoquinol rapidly distribute over its entire volume, then we have to postulate the existence of another domain located within the membrane into which protons from water oxidation are initially released. This is closely related to an earlier hypothesis by Dilley and co-workers [17,18]. (2) If the thylakoid lumen is not continuous, we have to assume that it consists of at least two subcompartments, one receiving protons from water and the other protons from plastoquinol. A diffusion barrier for protons must separate the subcompartments from each other. We also have to assume that there are two corresponding pools of neutral red, each of which responds to protons from different photosystems.

Based on our detailed previous work [8,9,29], we can accept the second alternatively only under severe restrictions. As in those studies, we have observed that the flash-induced absorption changes of neutral red occurred in two kinetic phases, each contributing approximately equally to the total amplitude. These two phases were previously correlated with the two sites of proton release in the electron-transport chain [8,24]. This implies that the ratio of the surface densities of neutral red times the number of proton pumps divided by the number of buffering groups is the same for both subcompartments. Moreover, the relaxation rate of the pH difference across the respective membrane patches would have to be very similar in the two subcompartments. Although we could not exclude such an explanation, we found it the least likely of the two possibilities described above, and therefore attempted to interpret our data according to a model of intramembranous buffering domains. To this end we made the following assumptions: (1) The thylakoid membrane contains special domains into which protons derived from water oxidation are initially deposited, and from which neutral red is excluded. (2) In the dark, the special domains are isolated from the aqueous bulk phases by nearly proton-impermeable boundaries, such that $\tilde{\mu}_{H^+}$ (domain) does not necessarily equal $\tilde{\mu}_{H^+}$ (bulk) unless uncouplers are present. (3) Protonatable groups reside within the domains, imparting to them a high buffering capacity. (4) Only those protons which are not trapped by buffering groups within the domains after a flash are rapidly released into the thylakoid lumen (overflow hypothesis).

According to this model, uncouplers would act to equilibrate the intrinsically nonequilibrium electrochemical potentials of protons in the domains and the bulk phases. It can be inferred from the pH dependence of the gramicidin effect shown in Fig. 3 that the original pH of the special domains was close to 7.0 in our samples, which is the same values as that estimated by Theg et al. [20] from their investigation of the uncoupler sensitivity of the water-splitting enzyme toward inactivation. Presumably this value is a function of the pH at which the chloroplast preparations were stored (cf. Ref. 20).

We could also use the above model to predict, in a semiquantitative fashion, the recovery of the neutral red signal in a series of closely spaced flashes (see Appendix). A close correlation between the experimental points and calculated lines in Fig. 8 could be obtained by assuming that the initial pH in the domains was 7.0, and that the buffering groups residing therein bound two protons at a time (see Fig. 3). From the number of flashes required before 90% recovery was achieved, we could calculate a gramicidin-induced deficit of protons in the domains of 12 nmol/μmol Chl, a number compatible (but at the lower end) with those arrived at by Dilley and his collaborators

[15,17,18] (15–30 nmol H^+/μmol Chl), and by Theg et al. [20] (10–100 nmol H^+/μmol Chl).

Although the above model of intramembranous buffering domains is able to explain our data, we find it difficult to accept the following structural and kinetic properties it imposes: (1) The extra compartment must link at least 20 PS II reaction centers (if gramicidin acted as a dimer as in model membranes, even 40 centers would have to be linked). There is no independent structural evidence for such a large subcompartment. (2) In the absence of gramicidin, proton transfer across the boundaries of these special domains and the thylakoid membrane is characterized by three very different time constants: more than 15 min for the equilibration with the bulk phases [20], 100 μs to 2 ms for the 'overflow' of protons into the lumen (Ref. 24, and this work) and about 5–10 s for the outflux of protons from the lumen into the external medium phase (Ref. 8, and this work). The discrepancy between the first and the latter two time domains requires the assumption of a 'permeability switch' (by some conformational change in the overflow mode). One may also envisage the possibility that there is no very tight permeability barrier, but rather a slowly operating proton pump which competes well with the 5–10 s exchange time constant between lumen and external bulk, thereby keeping the pH of the extra domain more acid. There is evidence for ATPase-related [36,37] and respiratory [38] proton pumps in chloroplasts of living algae, but methods similar to those used in the above-cited studies provided no evidence for such activities in isolated chloroplasts [39].

Our data lead us to believe that the thylakoid membrane contains photosystem-specific subcompartments into which protons are deposited by electron transport. We are also led to accept the existence of special domains which, in the dark, contain protons which are not in equilibrium with those in the bulk phases. These conclusions were also drawn by other authors [15–21] using very different (biochemical) experimental approaches. However, the physical nature of the special domains remained obscure. Saturation of these domains after acceptance of a few protons makes them scarely disturb the orthodox picture of steady-state phosphorylations.

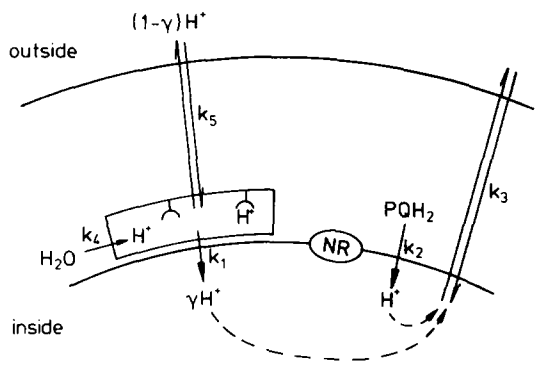

Fig. 9. Model of proton pathways used to simulate the data of Fig. 8. Details are given in the text.

Appendix

The solid lines in Fig. 8 were calculated using equations derived from the scheme in Fig. 9. Water-derived protons are assumed to be deposited into an intramembranous domain, where a fraction, γ, are buffered and $1-\gamma$ are released into the thylakoid lumen. The external phase is considered to be well buffered by bovine serum albumin.

The following symbols are used: N, flash number; $[H^+]_l$ and $[H^+]_d$, the free proton concentration in the lumen and domain, respectively; pH_e, pH_l, and pH_d, the pH values of the external medium, lumen and domain, respectively. The superscript o refers to initial conditions: $\Delta pH_{w\to l}$ and $\Delta pH_{w\to d}$, the maximum flash-induced pH jump produced by the water-derived proton in the lumen and domain, respectively; $\Delta pH_{PQ\to l}$, the flash-induced pH jump produced in the lumen by the proton released from plastoquinol, $\Delta pH_{PQ\to l} = \Delta pH_{w\to l}$; K_{NR}, acid dissociation constant of neutral red in the lumen; K_d acid dissociation constant of buffering groups in the domain; γ, the fraction of water-derived protons buffered in the domain: ΔA, absorption change of neutral red.

The pertinent parameters to calculate are the instantaneous pH values of the lumen and domain. From consideration of the proton pathways given in Fig. 9 they are:

$$pH_i(t) = pH_i^\circ(N)[e^{-k_3 t}] + \frac{k_1}{k_3 - k_1}\gamma(\Delta pH_{w\to i}) \times \left[e_1^{-k_1 t} - e^{-k_3 t}\right] + \frac{k_2}{k_3 - k_2}(\Delta pH_{PQ\to i}) \times [e^{-k_2 t} - e^{-k_3 t}] + pH_e[1 - e^{-k_3 t}] \quad (A1)$$

$$pH_d(t) = pH_d^\circ(N)[e^{-k_5 t}] + \frac{k_4}{k_5 - k_4}(1-\gamma)(\Delta pH_{w\to d}) \times [e^{-k_4 t} - e^{-k_5 t}] + pH_e[1 - e^{-k_5 t}] \quad (A2)$$

To calculate these pH changes in response to a number of flashes, N, pH_i° and pH_d° are considered to be functions of N, taking the last value of the instantaneous pH before the Nth flash is fired.

The fraction of water-splitting protons that are bound in the domain is determined by the pK of the gramicidin effect, and was calculated at the beginning of each successive flash. As mentioned before, the buffering groups in the domain appear to bind two protons at once (see Fig. 3), such that:

$$\gamma = [H^+]_d^2/\left([H^+]_d^2 + K_d^2\right) \quad (A3)$$

Finally, the response of neutral red to pH changes in the lumen is pH dependent (cf. Fig. 3, inset and Ref. 9). Therefore, a correction was applied to account for this dependence, namely:

$$\Delta A(t) = \alpha(pH_e - pH_i(t)) \quad (A4)$$

where:

$$\alpha = 4K_{NR}[H^+]_i(t)/([H^+]_i(t) + K_{NR})^2 \quad (A5)$$

In practice, ΔA was initially calculated without considering the effect of the domain, i.e., with γ set equal to one. The rate constants k_1 and k_2 were determined separately, and k_3 was chosen to give the same rate of decay of the neutral red signal

TABLE I

PARAMETERS USED TO SIMULATE THE TRACES SHOWN IN FIG. 8 OBTAINED IN THE PRESENCE AND ABSENCE OF GRAMICIDIN.

Parameter	Minus gramicidin			Plus gramicidin, with domain equilibrating to	
	Without domain	With domain equilibrating to		Outside	Lumen
		Outside	Lumen		
Plotter gain (relative)	0.98	1	1	1	1
Lumen parameters					
$pH_e^\circ(N=1)$ *	7.68	7.68	7.68	7.69	7.69
$\Delta pH_{w\to i}$ **	−0.015	−0.015	−0.015	−0.015	−0.015
$\Delta pH_{PQ\to i}$ **	−0.015	−0.015	−0.015	−0.015	−0.015
k_1 (s^{-1}) *	150	150	150	150	150
k_2 (s^{-1}) *	15	15	15	15	15
k_3 (s^{-1})	0.15	0.15	0.15	0.185	0.185
pK_{NR} **	7.16	7.16	7.16	7.16	7.16
$pH_i^\circ(N=20)$	7.24	7.25	7.25	7.31	7.31
Domain parameters					
$pH_d^\circ(N=1)$	–	7.0	7.0	7.69	7.69
$\Delta pH_{w\to i}$		−0.3	−0.3	−0.3	−0.3
k_4 (s^{-1})	–	150	150	150	150
k_5 (s^{-1})	–	0.05	0.05	0.25	0.40
pK_d *	–	7.58	7.58	7.58	7.58
$pH_d^\circ(N=20)$	–	6.85	6.83	7.08	7.05

* Values that could be determined experimentally. ** Values that were varied to fit the trace obtained in the absence of gramicidin, but left unchanged for the plus gramicidin trace.

after the final flash as was recorded experimentally in Fig. 8. * Then pK_{NR}, $\Delta pH_{PQ\rightarrow l}$ (and $\Delta pH_{w\rightarrow l}$) and the gain of the plotter were varied until a good fit with the experimental recording was obtained. Finally, the effect of varying λ was determined by arbitrarily setting pH_d^o to 7.0 and k_5 to 0.05 s^{-1}, and then readjusting the plotter gain. The value of pK_d was calculated from the ratio of the amplitudes of the first flashes in the presence and absence of gramicidin with the aid of Eqn. A3.

The data obtained in the presence of gramicidin were simulated by varying $\Delta pH_{w\rightarrow d}$ and k_5. The plotter gain was left unchanged, k_3 was increased by 20% and k_4 was arbitrarily given the same values as k_1.

The parameters used for these calculations are listed in Table I. Those which could be determined experimentally are indicated by one asterisk, while others that were varied to fit the trace obtained in the absence of gramicidin, but then unchanged to simulate the plus gramicidin trace are marked by two asterisks, Table I also shows that it mattered little if protons buffered in the domain were considered to equilibrate with those in the lumen rather than in the external medium; k_5 was the only parameter requiring adjustment.

The use of pH in the calculations, rather than $[H^+]$, is only accurate when the buffering capacity is constant over the pH range investigated. This condition has been shown to hold for pH changes in the thylakoid lumen [9], but surely it is not met in the domains. Therefore, we did not attempt to interpret the kinetic parameters determined by the simulation. The calculations do show, however, that the basic postulate of the model, namely that water-derived protons first enter a buffering domain before reaching the lumen, is not unreasonable.

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* The decay of the neutral red signal after the twentieth flash appeared to be biphasic. We did not consider the slow phase in our calculations, as it probably contributed very little to the signal's kinetics in the 150 ms that separated the flashes.

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