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LOCALIZED OR DELOCALIZED PROTONS IN PHOTOPHOSPHORYLATION?

ON THE ACCESSIBILITY OF THE THYLAKOID LUMEN FOR IONS AND BUFFERS

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The deposition of protons inside thylakoids after flash excitation was measured photometrically with neutral red as pH indicator. In continuation of previous work (Junge, W., Ausländer, W., McGeer, A. and Runge, T. (1979) Biochim. Biophys. Acta 546, 121–141), we studied the influence of salts on neutral red binding and on the p*K* of the heterogeneous protonation-deprotonation of inside-bound neutral red as a function of salts. With freeze-thawed (cryoprotective dimethyl sulphoxide) or aged chloroplasts, we observed that the heterogeneous p*K* of inside-bound neutral red was salt dependent in a way which suggested that neutral red was bound close to the plane of negative fixed charges and that the adjacent inner aqueous phase could accommodate an extended ionic double layer. This, together with the known extremely rapid proton exchange between surface layer and adjacent bulk phase, led us to conclude that inside-deposited protons rapidly reached an aqueous inner bulk phase. This conclusion was corroborated by the observation that extremely hydrophilic buffers like phosphate quenched the transient internal acidification independent of whether proton deposition was due to water oxidation or to plastohydroquinone oxidation. Very different behaviour was observed for freshly prepared chloroplasts with broken outer envelope. Here, inside-bound neutral red was seemingly unaffected by salts and hydrophilic buffers failed to quench the internal acidification. The electrical conductivity and proton permeability of the thylakoid membrane, on the other hand, were as usual. We attributed the seeming inaccessibility of the internal phase to the failure to accommodate a sufficiently extended ionic cloud between the tightly appressed membranes. In such material we observed hindered lateral mobility of protons at the outer side of the thylakoid membrane. This was tentatively attributed to multiple binding-debinding at buffering groups. The consequences for the chemiosmotic theory are: There is one type of damaged chloroplast material, which is competent in photophosphorylation and where protons are deposited into an internal aqueous bulk phase in the orthodox sense. In more intact material, however, the internal space lacks the characteristic properties of an aqueous bulk phase and there is evidence for lateral diffusion limitation for protons. Here, the thermodynamics of photophosphorylation may be inadequately described by the proton-motive force between two aqueous phases which are each isopotential.

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Abbreviations: CF_0 integral membrane part of ATP synthase; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMSO, dimethyl sulphoxide; PS, photosystem; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Introduction

For more than a decade the pathway of energy flow in photophosphorylation and in oxidative phosphorylation has been under debate. The role of protons as obligatory intermediate between electron flow and ATP synthesis is now generally accepted. The experimental evidence for the generation of a proton-motive force across the thylakoid membrane [1–5] and for its use for photophosphorylation [6–9] has been amply reviewed. Concomitant with, or even preceding the hypothesis of Mitchell [10–13] of chemiosmotic energy conversion, came the hypothesis of Williams [14–17] that protons are pumped into small subcompartments instead of osmolar aqueous space. One salient difference between these concepts is the possibility that these subcompartments could sustain very large pH differences.

Most experiments, notably those on broken chloroplasts with swollen thylakoids, gave no evidence for proton release into subcompartments. Instead, it was observed that the electric component of the proton-motive force is delocalized over a membrane patch which contains more than 100 000 chlorophyll molecules [18] and that protons are released into an internal space which is osmotically variable and which accommodates small ions [19–22].

Observations by Prochaska and Dilley [23] and other [24,25] are disturbing this picture. Dilley's group found that chemical modifications of the water-oxidizing enzyme and of the 8 kDa subunit of the CF_0 part of the ATP synthase were affected by protons which were released during water oxidation but not by protons which were released during the oxidation of plastohydroquinone. This led them to postulate that protons from water oxidation were released into a sequestered domain which was not accessible to protons from the other site of proton release [24].

We have previously taken part in the discussion over localized versus delocalized protons in photophosphorylation by time resolving proton deposition in thylakoids after flashing light. We used the dye neutral red under selective buffering of the external phase as photometric indicator of transient pH changes inside thylakoids [22,26,27]. We found that proton deposition by water oxidation (100 μs–1 ms) was kinetically well distinguishable from deposition by plastohydroquinone oxidation (20 ms) [26,28]. The response of neutral red to flash excitation of chloroplasts decreased in magnitude upon addition of extremely hydrophilic buffers like phosphate and pyrophosphate, which were more efficient the larger was the internal osmolar volume [22,27]. This led us to conclude that even the most rapidly released protons from water oxidation equilibrated with the internal osmolar aqueous volume before they were consumed by the ATP synthase [22,27].

Hence, our conclusions were contradictory to those of Dilley and Homann and their co-workers [24,25]. As we could not see any obvious reason to refute the conclusions of the other authors we started to re-examine our side. This work is devoted to the following questions: (1) Is the indicator dye neutral red specific for protons in the aqueous inner volume, although it owes its sensitivity for pH changes inside to strong binding to thylakoid membranes? (2) are the discrepancies due to different chloroplast material?

We found huge differences in the behaviour of chloroplast preparations of different integrity. In freeze-thawed chloroplast (cryoprotective DMSO) neutral red indicated transients of the surface pH which rapidly equilibrated with the pH of an internal aqueous bulk phase. All our experiments clearly showed that protons were released into the inner aqueous volume. In freshly broken chloroplasts, but also in freeze-thawed ones with ethylene glycol as cryoprotective, on the other hand, it was difficult to make any assignment because the neutral red space was not intrinsically accessible to hydrophilic buffers nor was the surface potential modified by salts (if not under special conditions).

It is in more intact chloroplasts that we also recently observed peculiar effects of the water-oxidation protons. These, however, are presented elsewhere (Theg and Junge; and Qian, Hong and Junge, unpublished observations).

Materials and Methods

Chloroplasts and media. Spinach was purchased on the local market. The starting material for 'freeze-thawed chloroplasts (DMSO)' was prepared according to the procedure of Reeves and

Hall [29] which was modified as described elsewhere [28]. They were frozen and stored under liquid nitrogen until being thawed shortly before use. Since the observed behaviour was dependent on the freezing and storage conditions, we repeat information from Ref. 28: Chloroplasts were suspended in the following medium: 400 mM sorbitol, 10 mM NaCl, 4 mM $MgCl_2$, 4 g/l bovine serum albumin, 50 mM Hepes (pH 7.5) and 5% DMSO. They were frozen under liquid nitrogen and usually stored for more than 1 month before use. 'Fresh chloroplasts' were prepared as follows: Leaves were blended in an ice-cold medium with 0.4 M sucrose; 10 mM NaCl and 10 mM Tricine, pH 7.4. After passage through nylon tissue (mesh 25 μm) the juice was centrifuged at $1000 \times g$ for 1 min. The pellet was washed in the same medium to which was added 2 mM $MgCl_2$. This was followed by another centrifugation at $1000 \times g$ for 1 min. The pellet was resuspended and homogenized in the latter medium to which 1 mg/ml bovine serum albumin was added. For measurements of absorption changes chloroplasts were diluted at 10 μM Chl into media which are given in the figure legends. The length of the optical absorption cell was 2 cm. If not otherwise indicated 1.3 mg/ml bovine serum albumin was added routinely to buffer away pH transients in the external phase. The pH of the suspension was established by addition of KOH or HCl and it was stable for 5 min within 0.1 pH unit without need for further buffers. The rate of photophosphorylation was determined by ^{32}P according to a standard procedure [59] with 30 μM pyocyanine as cofactor for cyclic electron transport at a light intensity of 0.1 W/cm^2. Typical rates were: 1100 for fresh chloroplasts and 500–700 μM ATP/mg Chl per h for frozen ones.

Flash spectrophotometry. A chloroplast suspension in an optical cell was excited by repetitive flashes (15 μs full-width at half-maximum, 1 mJ/cm^2) at a period of 10–20 s. The measuring light was gated open only during the actual sampling interval (e.g., 500 ms) plus an allowance of about 10 ms to adapt the photomultiplier to the light. The d.c. level of the photomultiplier output was automatically set off and averaged as described previously [28]. Transients of the output voltage were amplified and passed through a high-frequency roll-off filter (band width setting reciprocal to the time-per-address of the averager). Repetitive transients were averaged on a TRACOR TN 1500. The $pH_{(in)}$-indicating absorption changes of neutral red resulted from subtraction of a signal obtained in the absence of this dye from another obtained in its presence, as described previously [22]. Controls for the absence of artefacts (e.g., a response of neutral red to redox transients) are given elsewhere [22].

Results and Discussion

Experiments with freeze-thawed chloroplasts

Neutral red binding to chloroplast membranes. We measured the amount of neutral red which was bound to thylakoid membranes by the following procedure: Chloroplasts were suspended at 10 μM Chl in 8 ml of the incubation medium which contained 20 μM neutral red. The salt content and the pH of the incubation medium were varied from sample to sample. The adjustment of the pH never required addition of more than 100 μM of HCl or KOH. After incubation for 5 min at room temperature the sample was centrifuged for 5 min at $4000 \times g$. The supernatant was discarded and the pellet was resuspended in 8 ml of 100 mM KCl at pH 8. This caused the greater part of bound neutral red to become detached from the membrane (see Fig. 1). Then followed another centrifugation step in which the supernatant was used. It was titrated to pH 5 in order to protonate neutral red and then the absorption was measured in a recording spectrophotometer (Aminco DW2a). The neutral red concentration was calculated on the basis of an extinction coefficient of $2.4 \cdot 10^4\ M^{-1} \cdot cm^{-1}$ (at 524 nm) as described previously [22]. The amount of neutral red which appeared in the supernatant approximated that which was bound to thylakoids during the incubation (error less than 5%). This procedure was carried out under dim light.

The results of such experiments are presented in Fig. 1. The percentage of neutral red which was bound relative to the total of 20 μM in the incubation medium was plotted as a function of the medium pH. The different sets of points corresponded to different salt concentrations in the incubation medium. Points are experimental and

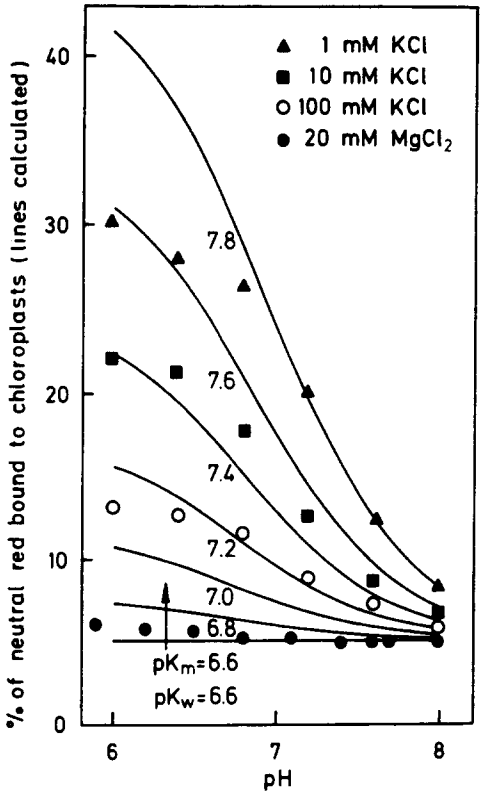

Fig. 1. Neutral red binding to freeze-thawed chloroplasts as a function of medium pH and of salts. Points, experimental; curves, calculated according to Eqn. A-5. pK values for neutral red in water (pK_w) and when bound to the membrane (pK_m) are given in the figure. Experimental procedure and conditions are described in the text.

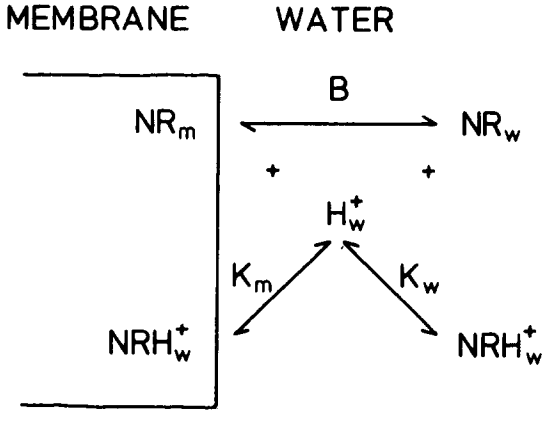

Fig. 2. Reaction scheme between protons in the aqueous bulk phase and neutral red in water and in the membrane.

curves are calculated based on the reaction scheme shown in Fig. 2. For the calculation we neglected any asymmetry between the two surfaces of the thylakoid membrane. We considered the homogeneous protonation-deprotonation reaction of water-dissolved neutral red and the heterogeneous reaction of membrane-bound neutral red with protons from the aqueous bulk phases. The distribution of neutral red between water and membrane and the protolytic reactions were assumed to be in equilibrium. Details are given in the Appendix.

What did we learn from an inspection of Fig. 1? In the presence of a divalent cation at relatively high concentration (20 mM $MgCl_2$) we found only very little influence of the pH on the distribution of neutral red between membrane and water. Under these conditions we expected only a very low surface potential at both interfaces of the membrane (see Ref. 31 for a comprehensive review). The lack of pH influence on the distribution implied that the heterogeneous dissociation constant of neutral red, if bound to the neutral(ized) membrane, equalled the homogeneous dissociation constant for neutral red in aqueous solution: K_m(20 mM $MgCl_2$) = K_w, which was 6.6 in our hands (see Ref. 22 for further references). This conclusion paralleled that by Gutman et al. [38] who found a similar coincidence between pK_w and the pK value for neutral red bound to micelles from neutral detergent. On the other hand, drastic redistribution of neutral red as a function of pH occurred at lower salt concentrations, i.e., in the presence of negative surface potential. The dependence of the heterogeneous dissociation constant on the surface potential, ϕ_s, is given in Eqn. 1:

$$pK_m(\phi_s) = pK_m(0) - (e/2.3kT)\phi_s \quad (1)$$

At a negative surface potential of 59 mV one expected that pK_m was shifted from 6.6 (its value at no surface potential) to 7.6. It was obvious from the reaction scheme in Fig. 2 that an increase in the negative surface potential or a decrease in the medium pH shifted the NR_m–NRH^+_m equilibrium towards the cationic form, which in turn caused the intake of neutral red (NR) from water to compensate for the loss of NR_m.

At low pH the experimental points deviated from the expectation. One possible reason for this

was increased protonation of negative fixed charged at lower pH. This was neglected when calculating the curves. The parameters underlying the fit were as follows: $pK_w = 6.6$, pK_m (neutral membrane) = 6.6, $BV_w/V_m = 19$ where B, K_m and K_w are as defined in Fig. 2 and V_m and V_w are the total volumes of membrane and water, respectively. Taking the specific volume of the thylakoid membrane as 5×22 nm^2 per Chl molecule [32] and considering the chlorophyll concentration in the incubation medium (10 μM) we calculated the ratio of water over membrane volume. For the neutral(ized) membrane the concentration ratio of membrane-bound neutral red over water-dissolved neutral red is then given by $1/B$. This amounts to 285 000, which corresponds to a surface density of neutral red of 7.5 pmol/cm^2. This load per unit area is of the same order of magnitude as observed for the binding of dipycrylamine to neutral bimolecular lipid membranes [33,34].

Which is the surface potential seen by membrane-bound neutral red? At 1 mM KCl and in the pH domain 7–8 we calculated a shift of pK_m by 1.2 units (Fig. 1). According to Eqn. 1 this is equivalent to -71 mV. Under the assumption that the charge on NRH_m^+ resided in the plane of the negative surface charges, the surface charge density of the thylakoid membrane, σ, could be calculated via the Gouy-Chapman equation for 1 : 1 electrolytes [35]:

$$136\sigma = \sqrt{c_{1,1}} \sinh(e\phi_s/2kT) \qquad (2)$$

We obtained 1 elementary charge per 220 nm^2 which is equivalent to -0.73 μC/cm^2. This was at the lower end of reported figures for the surface potential at the thylakoid membrane (compare with, e.g., -0.77 μC/cm^2 around PS I inside [36] and -2.5 outside [37]).

We concluded that neutral red was strongly bound to thylakoids. At least the protonated cation resided close to the membrane/water interface. Membrane-bound neutral red underwent a heterogeneous protonation-deprotonation reaction which was sensitive to the surface potential. And, therefore, it intrinsically indicated transients of the surface pH.

The influence of salts on the apparent pK of inside-bound neutral red. While it was shown in the foregoing section that the heterogeneous deprotonation constant of membrane-bound neutral red (total!) was sensitive to the surface potential, we considered whether the same hold true for the subset of bound neutral red molecules which were indicators of pH transients inside in flash spectrophotometric experiments. For this we measured the extent of the $pH_{(in)}$-indicating absorption changes (see Materials and Methods) as a function of the medium pH and under variation of the salt composition as parameter. The result is shown in Fig. 3. Points were obtained experimentally and curve were calculated according to Eqn. A-11 in Ref. 22. As previously established [22], the differential sensitivity of neutral red in response to the small pH decrease upon excitation of chloroplasts with a single-turnover flash was given by the first derivative of the titration curve of this dye with respect to pH. The pH at the maximum of such a bell-shaped curve equalled the heteroge-

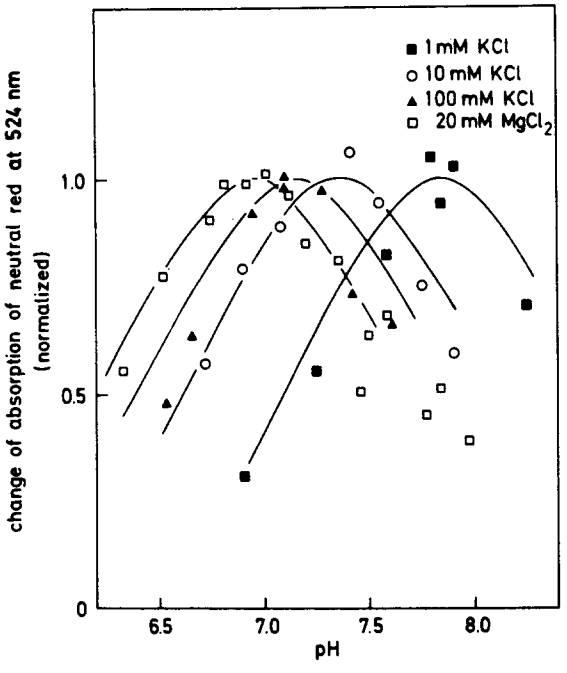

Fig. 3. Relative extent of the $pH_{(in)}$-indicating absorption changes of neutral as a function of medium pH and of salts for freeze-thawed chloroplasts. Points, experimental; curves, calculated according to Eqn. A-11 in Ref. 22 (normalized at maximum). 10 μM Chl, 13 μM neutral red, 1.3 mg/ml bovine serum albumin, 10 μM benzyl viologen. The extent of the $pH_{(in)}$-indicating absorption changes was taken at 500 ms after the flash.

neous pK of the membrane-bound dye, pK_m. From Fig. 3 we learned that pK_m was 6.95 at high concentrations of divalent cation and that it shifted to 7.85 at low salt (1 mM KCl). The trend followed by these figures agreed with what was obtained from the binding studies (see Fig. 1).

The above conclusion that neutral red indicated the surface pH was confirmed.. According to model studies by Gutman et al. [38] with neutral red bound to detergent micelles, we had to assume that the heterogeneous protonation of neutral red at the inner side of the thylakoid membrane was diffusion controlled and hence extremely rapid. This has made membrane-bound neutral red also an extremely rapid indicator of pH transients in the internal aqueous phase of thylakoids.

On the access of hydrophilic buffers to the aqueous spaces inside thylakoids wherefrom neutral red reports proton deposition. As cited in the Introduction [14–17], some authors had evidence that protons from water oxidation were deposited in another internal subcompartment than the one which accepted protons from plastohydroquinone oxidation. We investigated whether the obviously aqueous spaces into which protons were released could be distinguished, e.g., by their accessibility to buffers. We added phosphate to a chloroplast suspension in order to decrease the magnitude of the $pH_{(in)}$-indicating absorption changes of neutral red as describred previously [22]. The result is documented in Fig. 4. Upon addition of phosphate buffer, yielding a buffering capacity of the suspensions of 4.6 mM at the measuring pH, the extent of the absorption changes was halved, independent of whether protons were deposited by the action of both photosystems (above), PS II alone (middle) or PS I alone (below).

We found no difference in the action of this hydrophilic buffer on the release of protons by either photosystem. The most probable conclusion is that both photosystems deposited protons into the same inner aqueous space.

Experiments with freshly broken chloroplasts

The influence of salts on the apparent pK of inside-bound neutral red. Dilley and Homann and their coworkers [24,25], who observed special effects for protons released by water oxidation, used more intact chloroplasts than we did above and in previous studies. We considered whether the dis-

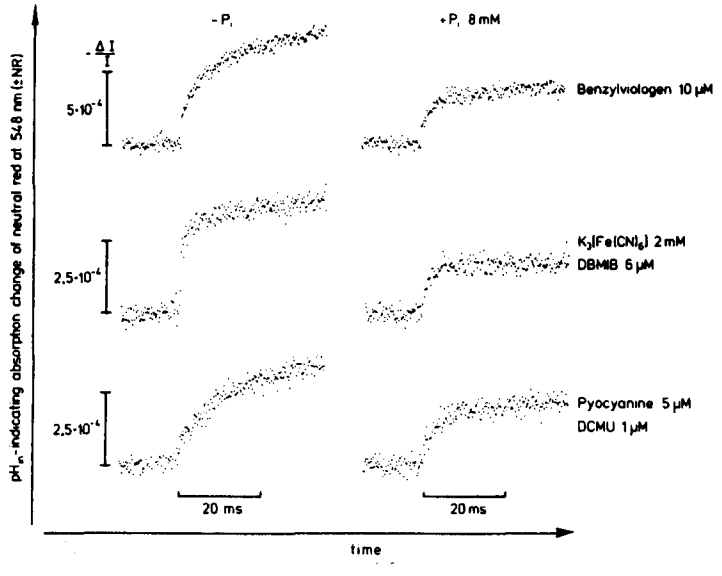

Fig. 4. Time course of the $pH_{(in)}$-indicating absorption changes of neutral red without (left) and with added phosphate buffer (right) in freeze-thawed chloroplasts. 10 µM Chl, 13 µM neutral red, 2.6 mg/ml bovine serum albumin, 10 µM benzyl viologen, pH 7. (Top) Benzyl viologen (10 µM) as electron acceptor, both photosystems release one proton. (Middle) DBMIB (6 µM) as acceptor and blocker, proton release only through water oxidation (in the presence of 2 mM $K_3Fe(CN)_6$). (Bottom) DCMU (1 µM) blocking PS II activity but PS I proton release reactivated by pyocyanine (5 µM).

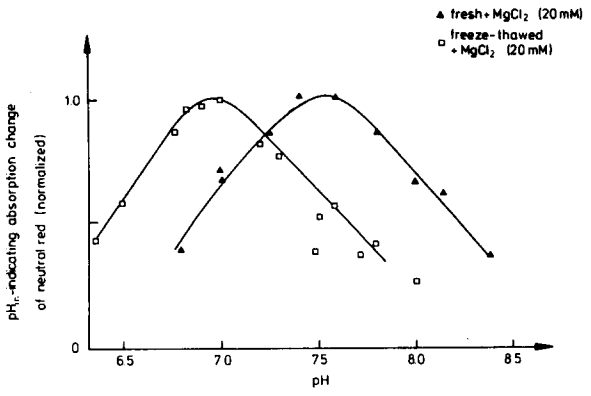

Fig. 5. Relative extent of the $pH_{(in)}$-indicating absorption changes of neutral red as a function of medium pH and of salts. Points, experimental; curves, calculated as in Fig. 3. (▲) Fresh class II chloroplasts. (□) Same chloroplast material but after freeze-thawing. 20 mM $MgCl_2$ and other conditions as in Fig. 3.

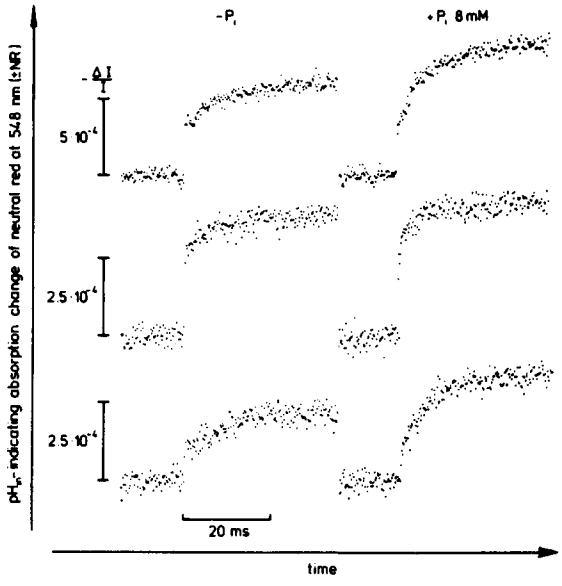

Fig. 6. Time course of the $pH_{(in)}$-indicating absorption changes of neutral red without (left) and with added phosphate buffer (right) in fresh class II chloroplasts. Other conditions as in Fig. 4. (Top) Benzyl viologen (10 μM) as electron acceptor, both photosystems release one proton (in the presence of 2 mM $K_3Fe(CN)_6$). (Middle) DBMIB (6 μM) as acceptor and blocker, proton release only through water oxidation. (Bottom) DCMU (1 μM) blocking PS II activity but PS I proton release reactivated by pyocyanine (5 μM).

crepancies resulted from different chloroplast material. Therefore, we repeated the experiments shown in Fig. 3 on class I chloroplasts which were broken to become class II chloroplasts just before the spectrophotometric experiments. The result, shown in Fig. 5, was a surprise. Even a high concentration of a divalent cation failed to decrease the apparent pK_m of neutral red, which remained at 7.6. Only after freeze-thawing of these chloroplasts or by aging at room temperature did the pK_m decrease to 6.95 units under the influence of 20 mM $MgCl_2$. Similar behaviour was observed for KCl.

This implied that the negative potential of the internal space where neutral red indicated pH transients could not be screened by Mg^{2+}.

On the access of hydrophilic buffers to the internal neutral red space. We also repeated the experiment shown in Fig. 4. The result is documented in fig. 6. The extent and the complex kinetics of the absorption changes of neutral red were similar to those observed in freeze-thawed chloroplasts. This also hold true for other small hydrophilic buffers (see Ref. 22 for a list). But again, to our surprise, the magnitude of the supposedly $pH_{(in)}$-indicating absorption changes of neutral red increased (only top and bottom) instead of decreasing as in freeze-thawed chloroplasts. To understand this behaviour we proceeded stepwise. At first we noted that the failure of phosphate to quench the supposedly $pH_{(in)}$-indicating absorption changes of neutral red was not due to the presence of intact chloroplast envelopes. The high rates of uncoupled reduction of $Fe(CN)_6^{3-}$ which were 200 nequiv./mg Chl per h and the unexpected stimulation of the neutral red response by phosphate argued against this, not to mention the hypo-osmolarity of the suspending medium and the appearance in a phase-contrast microscope. Secondly, we recalled from previous work [26] that neutral red intrinsically indicated pH transients in both aqueous phases. This is demonstrated in Fig. 7 (note: experiment on freeze-thawed chloroplasts). It was obvious that negatively directed signals, which were abolished upon addition of bovine serum albumin as buffer for pH changes outside, were superimposed on positively directed signals from inside, which were quenched only by a permeating buffer. If the buffering of the external phase were insuffi-

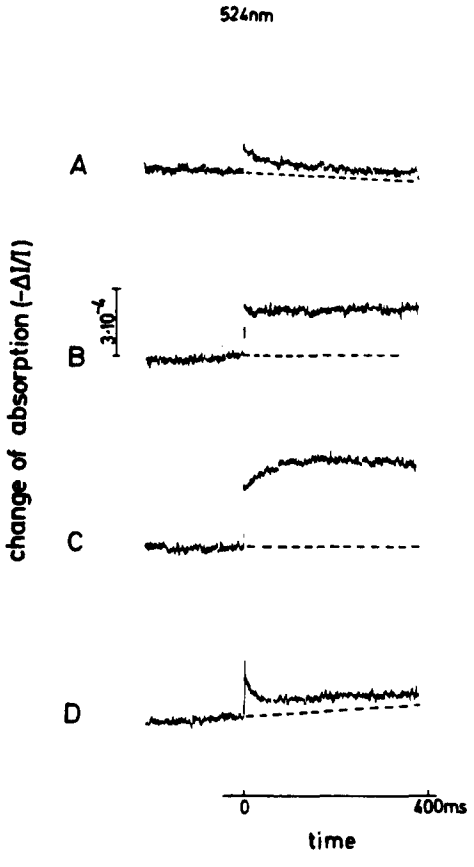

Fig. 7. Time course of the $pH_{(in)}$-indicating absorption changes of neutral red in a suspension of freeze-thawed chloroplasts. 10 μM Chl, 10 μM benzyl viologen, 3 mM $MgCl_2$, 30 mM KCl, pH 7. (A) No further additions. (B) Plus neutral red (13 μM). (C) Plus neutral red and plus bovine serum albumin (1.3 mg/ml). (D) Plus neutral red, plus bovine serum albumin and plus imidazole (3 mM). The difference between traces A and C represents the true $pH_{(in)}$-indicating absorption chanes of neutral red (the difference between C and D is less reliable, see below). The difference between traces B and C represents the $pH_{(out)}$-indicating absorption changes of neutral red. The difference between A and D is due to $pH_{(in)}$-indication by intrinsic chloroplast pigments (see also Fig. 2 in Ref. 22).

cient the supposedly $pH_{(in)}$-indicating absorption changes would seemingly come out too small. Hence, we interpreted the increase in the $pH_{(in)}$-indicating absorption changes of neutral red upon addition of phosphate in Fig. 6 by an additional buffering of the external phase, which cannot be fully buffered by bovine serum albumin. Here it is worth mentioning that an increase in the bovine serum albumin concentration up to 6 g/l did not counteract incomplete buffering. This interpretation also followed from inspection of Fig. 6 alone. It was apparent that there was almost no increase in the presence of DBMIB and $Fe(CN)_6^{3-}$. Under these conditions there was only proton release inside by water oxidation but no proton uptake from outside [26,39]. We expected and observed that insufficient buffering of the external phase should not matter in this particular case. It is worth noting that the insufficient buffering by bovine serum albumin occurred at pH 7.2 but not at pH 7.6. This might have been a result of tighter packing of thylakoid stacks under these conditions.

In class II chloroplasts we found that neutral red showed a similar extent and kinetics for proton deposition inside thylakoids, however, the internal space could not be buffered by phosphate. Moreover, protons from the outer bulk phase which was buffered by bovine serum albumin could not readily (i.e., within 20 ms) reach the sites of proton uptake at the outer side of the thylakoid membrane.

On the permeability of the thylakoid membrane to ions. It was puzzling that neither the surface potential inside nor the buffering capacity was influenced by ions in fresh thylakoids. There are two possible interpretations of this: Either the thylakoid membrane was very poorly permeable to ions (establishment of equilibrium took longer than 10 min), or the effect of ions was diminished for another reason (e.g., the extremely small thickness of an internal 'aqueous volume'). We studied the ion permeability by two indicators: (1) via the decay of the electrochromic absorption changes at 524 nm [18] which reflect the electric conductivity with contributions from all permeant ions, and (2) via the decay of the flash-induced pH difference inside. For the decay of the flash-induced electric potential difference we measured relaxation times ranging between 200 and 500 ms, depending on the preparation. Assuming a specific capacitance of 1 $\mu F/cm^2$ for the thylakoid membrane we calculated a specific electric conductance of $5 \cdot 10^{-6}\ \Omega^{-1} \cdot cm^{-2}$. The pH difference, on the other hand, had relaxed at 3–6 s as measured both with neutral red inside thylakoids and with cresol red or phenol red outside. It was evident that the

greater part of the electric conductivity was carried by some other ion than proton.

The lack of influence of small ions and hydrophilic buffers on the internal space was most likely attributable to an intrinsic property of this space rather than to limited permeability of the thylakoid membrane.

General Discussion

The dye neutral red has been the most convenient and the only kinetically competent indicator of proton deposition in thylakoids. However, it revealed its unique properties only in experiments aiming at the small pH changes which were induced upon flash excitation [22,26] but not for the larger pH changes under excitation with light gates [40]. Although it could, in principle, also act as a redox mediator, it did not do so with thylakoids in the presence of Hill reagents (see Fig. 2 in Ref. 22). Neutral red has been used by our group [26,44,45] and by others [41–43] mainly to study proton deposition due to photosynthetic water oxidation. Since there was no better way for such studies we continued to examine the basis for the functioning of this dye. To some colleagues it appeared as if there were a contradiction between the following two properties of neutral red: It bound strongly to thylakoid membranes but seemed to indicate pH transients in the internal aqueous phase, which we operationally defined as an osmolar space which was accessible to the phosphate anion [22]. It is known from studies with surface-adsorbed dyes [30] that these can undergo heterogeneous protonation-deprotonation reactions with protons from the aqueous phase. Under these conditions the heterogeneous pK_m depended on the surface potential and on the charging state of the dye. Such reactions were also established for neutral red adsorbed to detergent micelles and it was observed that the protonation was diffusion controlled, i.e., extremely rapid [38]. We previously applied neutral red to the disk membrane of retinal rods and we used the variation of its pK_m as a function of salts to gauge the interfacial potential at the disk membrane [46]. In this work we investigated whether the concept of a pH indicator adsorbed to a membrane close to the interface which sensed the bulk pH via the surface pH held true for neutral red in thylakoids. The results differed greatly depending on the intactness of thylakoids.

In freeze-thawed chloroplasts both the binding of the dye to the membrane as well as the pH and salt dependence of its transient response to flash excitation of chloroplasts were adequately described under the assumption that neutral red measured the surface pH. An estimate of the surface charge density seen by neutral red via the Gouy-Chapman theory yielded a figure within the range, but at the lower end of previously reported values ($-0.73\ \mu C/cm^2$; see Ref. 31 for a review). The applicability of the Gouy-Chapman theory to freeze-thawed thylakoids seemed warranted in view of their relatively large internal volume under our experimental conditions (30–60 l/mol Chl). Then it could be inferred from the model studies by Gutman et al. [38] that neutral red also was a kinetically competent indicator of pH transients in the internal bulk phase.

Since the pK of the membrane-adsorbed dye was sensitive to the surface potential, one might ask whether its transient response to flash excitation of chloroplasts was due to pH changes or rather to changes of the surface potential. We calculated that the pH changes were dominating even at low salt concentrations (1 mM 1 : 1 electrolyte) and that pK changes were negligible at usual salt concentrations (see Appendix).

In fresh chloroplasts the above controls for the location of neutral red at the membrane/water interface failed to operate. The '$pH_{(in)}$-indicating absorption changes of neutral red' were not quenched by added phosphate and the pK_m of the dye was seemingly unchanged by added salts. Even Mg^{2+} (20 mM) failed to operate, although there was good evidence that it was the major compensating ion for inwardly directed proton pumping in the intact chloroplasts [47–49]. This plus our observation of relatively rapid relaxation of the electric potential difference and the pH difference across the thylakoid membrane argued against the possibility that the membrane permeability hindered access of buffers and salts to the internal space. Instead, it seemed as if the very nature of the internal space was responsible for the exclusion of ions. One possible interpretation would be an extremely small spacing between the

two membranes of one thylakoid. For freshly broken chloroplasts Heldt et al. [50] reported specific inner volumes as low as 3 l/mol Chl. With the specific area per Chl molecule (2.2 nm^2) we calculated a thickness of less than 5 nm for the thylakoid lumen under these conditions. Considering that the applied technique tended to overestimate the internal volume, the gap could be even narrower. This would have the consequence that phosphate would indeed not buffer, even if it penetrated the membrane, since its buffering power decreased with decreasing internal aqueous volume [22]. The inability of ions to act on the surface potential might have been caused by the inability of a very narrow aqueous phase to accommodate an extended diffuse ionic double layer. It would be desirable if studies on electric phenomena in closely appressed model membranes were available (e.g., in extension of Ref. 51). Besides lacking clear concepts from model studies, we also were unable to quantify to what extent the freshly broken chloroplast retained a close appression of thylakoid membranes although they were suspended in hypoosmolar buffer (see Ref. 52 for similar difficulties). Specific experiments which are aimed at the electrostatics of thylakoids under strong osmotic pressure are under progress.

The consequences for the controversy over localized versus delocalized protons in photophosphorylation are as follows: There was one system, the admittedly damaged but for electron transport and ATP synthesis functional freeze-thawed chloroplast, which deposited protons in the internal aqueous bulk phase. The most rapid component of proton release which is linked to water oxidation had relaxation times of about 100 μs [28,44]. That protons went into an aqueous bulk phase was apparent from the buffering by phosphate and from the osmolar behaviour of this phase [22]. This was furthermore corroborated by the applicability of the Gouy-Chapman formalism to the effect of salts (this work). In this respect we regarded the freeze-thawed chloroplast as an example of an orthodox chemiosmotic system [10,11].

The orthodox behaviour was not to be established experimentally in class II chloroplasts. It is conceivable that an internal aqueous bulk phase did not exist in fresh thylakoids. Then the chemiosmotic hypothesis has to be modified for such a system. Although this would be a fine compromise after an extended controversy, it appeared to us as if the truth were approached from the chemiosmotic side, since photophosphorylation was functional in freeze-thawed chloroplasts where protons had to pass via the inner aqueous bulk phase (see above).

Subcompartmentation and lateral heterogeneity of the proton-motive force: From their experiments with hydrogen isotopes Haraux and De-Kouchkovsky [53,54] concluded that a drop of proton-motive force occurred from the source of protons to the sinks (ATP synthases) at the inner side of the thylakoid membrane. Van Dam et al. [55] also postulated lateral resistance for proton flow along the cristae membrane of mitochondria. In this work we observed largely delayed access for protons from the outer aqueous bulk phase (bovine serum albumin space) to the sites of proton consumption at the outer side of the thylakoid membrane. We tentatively attributed hindered lateral diffusion of protons to multiple binding-debinding by buffering groups for the following reason. If R is the binding ratio for protons in a given space (protons bound over protons free) the effective diffusion constant of protons is reduced by a factor $R + 1$ [56]. Based on our previous work on the buffering capacity of the thylakoid interior [22] we calculated a binding ratio of 10^4 for this space. This diminished the diffusion constant for protons from $9 \cdot 10^{-5}$ in pure water [57] to $9 \cdot 10^{-9}$ $cm^2 \cdot s^{-1}$ in this space. Hence, the proton diffused as slowly as a spherical protein with 4.7 nm diameter. During the typical cycle period of photosynthesis, 10 ms, the mean square displacement in approximately two-dimensional diffusion was only 190 nm, less than half the radius of a spinach thylakoid in a stack. From the results contained in this paper we do not infer any subcompartmentation but we also tend to deny that the internal and the external space of the thylakoid membrane are close to isopotential under continuous operation of the proton pumps and of the ATP synthase.

Appendix

Neutral red binding to membranes

We assumed the existence of only two extended phases, a lipophilic and an aqueous bulk phase.

The lipophilic phase modelled the membranes of chloroplasts. The interface was assumed to be loaded with negative fixed charges which created a negative surface potential which decayed into the aqueous phase. The indicator dye neutral red was assumed to exist only in two forms, the neutral unprotonated form (NR) and the cationic protonated one (NRH^+). Bartels [58] reported a more complicated protonation pattern, but we failed to confirm the involved titration behaviour that he had reported (see Fig. 1 in Ref. 22). Both forms of neutral red could exist in water (NR_w) or in the membrane (NR_m). We assumed that the most likely position of neutral red in membrane was with the protonated secondary amine function in the plane of the surface charges, hence close to the interface. Membrane-bound neutral red underwent protonation-deprotonation with protons from the interfacial layer. These, however, exchanged with protons from the aqueous bulk phase. The surface pH was a function of the bulk pH and of the surface potential. The equilibrium situation was given by the following four equations:

$$NR_w \times H_w^+ / NRH_w^+ = K_w \quad \text{(A-1)}$$

$$NR_m \times H_w^+ / NRH_w^+ = K_m \quad \text{(A-2)}$$

$$NR_w / NR_m = B \quad \text{(A-3)}$$

$$(NR_m + NRH_m^+) \times V_m + (NR_w + NRH_w^+) \times V_w = NR_t \times V_w \quad \text{(A-4)}$$

where NR, NRH^+ and H^+ are effective activities and V_m and V_w denote the volume of membranes and of water, respectively. The heterogeneous dissociation constant of membrane-bound neutral red, K_m, depended on the surface potential as given in Eqn 1.

We measured the ratio of neutral red bound to the membrane (both forms) over neutral red total (both forms). With Eqns. A1–A4 this was calculated:

$$BR = (1 + ((1 + H_m/K_w)/(1 + H_m/K_m)) \times B \times V_w/V_m)^{-1} \quad \text{(A-5)}$$

Only if $K_m = K_w$ does the binding ratio of neutral red became independent of the medium pH.

Transients of the surface potential versus pH changes in thylakoids

Proton deposition in thylakoids caused internal acidification, but it also decreased the magnitude of the negative surface potential inside. Since neutral red sensed the surface pH, we expected that it sensed the transient of the bulk pH inside as well as the transient of the surface potential. For the small changes that occurred under excitation of chloroplasts with a single-turnover flash, the differential absorption change of neutral red, dA, was expected to be proportional to the differential changes of bulk pH, dpH, and of the heterogeneous pK, dpK_m.

$$dA \propto dpK_m - dpH_w \quad \text{(A-6)}$$

We calculated the magnitudes of these variables by Eqns. 1 and 2 (Results and Discussion) with the following parameter assignments: The buffering capacity as defined in previous work [22], 1.2 mM/pH unit; the specific internal volume (see also Ref. 22), 50 l/mol Chl; the specific area per Chl molecule, 2.2 nm^2; the translocation of 2 protons per 600 Chl molecules and a surface density of 1 $\mu C/cm^2$. If the only electrolyte present was univalent the pK change inside was 0.018 units at 1 mM electrolyte, 0.012 at 10 mM and 0.003 at 100 mM. This is considerably smaller than the pH change which we determined as 0.05 units in the presence of neutral red [22].

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