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Studies on well-coupled Photosystem I-enriched subchloroplast vesicles. Neutral red as a probe for external surface charge rather than internal protonation

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The occurrence of short-range proton displacements in Photosystem I (PS I) -enriched subchloroplast vesicles was studied under single-turnover conditions, using the pH indicators Cresol red and Neutral red. Bulk proton translocation was not detectable with Cresol red. Neutral red (externally added) was adsorbed to the vesicles in a salt-reversible way. It showed a flash-induced absorbance transient, recorded between 545 and 550 nm, consisting of a fast decrease (halftime < 5 ms) followed by an increase (halftime 20–40 ms). On the basis of the effects of uncoupler, ionophores, buffers and pH, it was concluded that the fast decrease represents Neutral red reduction and the following increase mainly reflects Neutral red protonation (and includes its reoxidation). The results further demonstrate that Neutral red is adsorbed to external membrane sites that have different accessibility towards various buffers. Bovine serum albumin, in a concentration at which it hardly contributed to external bulk buffering, stimulated the Neutral red response in parallel with the electric potential, indicating carotenoid response. This was presumably due to a general stabilizing effect. Our results strongly suggest that in these Photosystem I-enriched vesicles Neutral red senses flash-induced changes of external membrane surface charge, rather than intravesicular acidification, due to proton displacements across the membrane. This behavior is in many respects similar to that of the aminoacridine probes.

Introduction

Subchloroplast vesicles, derived from the exposed thylakoid membranes by mild digitonin treatment, offer a most useful object for mechanistic studies of the primary events in electron transfer-associated energy transduction. Peters et al. [1–6] in our group have optimized the isolation of these Photosystem I-enriched vesicles [1–3] and analyzed their composition [3], their photophosphorylation activities [4], electric potential generation [5] and cytochrome redox reactions [6].

These subchloroplast vesicles have several advantages over intact or broken chloroplasts for such investigations: (1) they are simple, unstacked vesicles, with a relatively long lifetime; (2) they contain only one type of photosystem, PS I, avoiding complications of multiple redox pathways; (3) they contain all components, required for cyclic electron flow and ATP synthesis, except ferredoxin and (4) they show a relatively large redox-asso-

Abbreviations: Chl, chlorophyll; PS I, II, Photosystem I, II; P-700, primary electron donor of Photosystem I; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide.

ciated electric potential ('slow component') as induced by single-turnover flashes.

In the presence of ferredoxin and NADPH, these vesicles are capable of active ATP synthesis [4]. Interestingly, bulk proton uptake, measured with a pH electrode under continuous illumination, is extremely small in such a system, compared to chloroplasts or to PS I vesicles in the presence of the proton-carrying redox mediator phenazinemethosulphate (Refs. 1 and 2, and Krab, K., Hotting, E.J. and De. Wolf, F.A., unpublished data). While these electrode measurements give only information on external bulk pH and are relatively slow, it would be interesting to study the possible occurrence of proton displacements close to the membrane and in response to single-turnover flashes under optimal conditions for the 'slow' electric potential formation and ATP synthesis [4–6].

For such a kinetic analysis of proton displacements on the millisecond time-scale we have to put up with pH-indicator dyes that usually show unwanted side-effects not related to pH changes (e.g., binding or redox changes). For the present work we have chosen the positively charged Neutral red and the negatively charged Cresol red for measurements close to the membrane surface and in the bulk phase, respectively. These probes have been used, with satisfaction, in other membrane systems [7–24]. Commonly, Neutral red is thought to permeate the membrane and would therefore be useful to monitor intravesicular pH changes, at least after appropriate buffering of extravesicular changes [8,9–14,21,25]. Cresol red, on the other hand, can be expected to remain in the external bulk and should give similar net responses as the H^+-sensitive glass electrode. However, older work of Azzone's group [17–20] with submitochondrial vesicles convincingly demonstrated an energy-dependent increase of Neutral red binding to the external membrane surface by electrostatic interaction with nucleophilic sites. This caused protonation and other changes of the dye molecules that were not related to pH changes of their environment. Van Walraven et al. [22,23], using both pH indicators with ATPase proteoliposomes, could clearly discriminate between extraliposomal and intraliposomal pH changes (induced by ATP-hydrolysis) with the externally added or internally trapped pH indicators, respectively. They also observed marked kinetic differences with both pH indicators; Neutral red sensing the pH changes at the two membrane-medium interfaces, and Cresol red sensing the pH changes in both bulk phases.

In the present paper, we evaluate the behavior of these pH indicators with flash-illuminated PS I vesicles in an attempt to gain more insight into the involvement of proton displacements in the course of the primary energy-transducing steps and the apparent difference between these vesicles and chloroplasts with respect to bulk proton movements.

Materials and Methods

PS I vesicles were isolated from market spinach by digitonin fractionation (0.2% digitonin (w/v) at a digitonin/chlorophyll ratio (w/w) of 1) mainly as described previously [3]. However, in that paper [3], the summary and legend to Fig. 1 inconsistently stated that the chlorophyll concentration was 0.2 mM. This should be 2 mM, and hence the digitonin/chlorophyll ratio used was indeed 1, rather than 10 (this error was kindly brought to our attention by Dr. B. Andersson, Sweden). We have now slightly modified the procedure in that during the digitonin incubation and subsequent steps, the medium contained 1 mm Tes-KOH buffer, pH 7.0–7.2 for Neutral red and pH 7.5–8.0 for Cresol red, instead of 20 mM Tes-KOH buffer (pH 7.8); the storage medium contained, in addition, 250 mM sorbitol. The vesicles were stored under liquid nitrogen.

In the reaction mixture, the oxygen concentration was controlled by a Clark-type oxygen electrode and was maintained at about 100 μM by periodic stirring. Neutral red or Cresol red were added just before experimentation. All experiments were carried out at 20°C in a thermoelectrically controlled multipurpose cuvette (vol. 1.8 ml) [26]. The light path of the measuring beams in the cuvette was 1 cm. The actinic light, which was passed through a filter, cutting off below 695 nm (Schott, Mainz, F.R.G.), was provided from the bottom of the cuvette via a fiber-optic light guide. Actinic flashes (tail depressed, 5 μs at half maximal amplitude) were generated by a General Electric FT-230 xenon flash tube; the output was about

200 J per flash. Flash-induced absorbance transients were recorded with a laboratory-built dual-wavelength kinetic flash spectrophotometer, fitted with Philips XP 2020 photomultiplier tubes. These were protected against the actinic light by two broadband blue filters (Corning), cutting off above 600 nm. Transients, following 16 flashes, fired at 0.1 Hz, were averaged. On-line processing was carried out by a microcomputer-minicomputer system as described before [27].

The pK_a of Neutral red, in the presence of PS I vesicles, was determined from spectrophotometric data (550–570 nm), as recorded with an Aminco DW-2a spectrophotometer, also equipped with the above-mentioned multipurpose cuvette.

Binding of dyes was determined by adding vesicles (20 μg Chl/ml) to a solution of 20 μM Cresol red, 20 μM Neutral red or 125 μM Neutral red, incubating for 5 min and precipitating the vesicles by centrifugation (1 h at 129000 $\times$ g). The dye absorbances before addition and after precipitation of the vesicles were compared and the differences were corrected for nonspecific binding, using the following extinction coefficients: 5.8 $mM^{-1}\,cm^{-1}$ for Cresol red (pH 7.0) at 572 nm; 14.2 $mM^{-1}\,cm^{-1}$ for Neutral red (pH 7.0) at 524 nm; 21.9 $mM^{-1}\,cm^{-1}$ for Neutral red (pH 6.0) at 524 nm; 25.0 $mM^{-1}\,cm^{-1}$ for Neutral red (pH 5.0) at 524 nm.

Paper electrophoresis of Cresol red and Neutral red was carried out in 0.1 M sodium acetate buffer (pH 5.0, 6.0 and 7.0) and in 0.1 M borate buffer (pH 8.0) on Whatman 3 paper, using a Pharmacia EPS 500/400 power supply, run at 200 V (pH 5.0–7.0) or 400 V (pH 8.0) and at 40 mA.

Spinach ferredoxin, bovine serum albumin, Neutral red (hydrochloride, used without further purification) and Cresol red (sodium salt) were obtained from Sigma (St. Louis, MO, U.S.A.); NADPH, nigericin and valinomycin from Boehringer (Mannheim, F.R.G.). S-13 was kindly donated by Dr. P.C. Hamm (Monsanto Co., St. Louis, MO, U.S.A.). Digitonin, from Merck, and was recrystallized twice from ethanol. All other chemicals were purchased from Merck (Darmstadt, F.R.G.).

Results

Comparison of Cresol red and Neutral red responses in PS I vesicles

When PS I vesicles, supplemented with ferredoxin and NADPH, were illuminated with continuous light (steady-state conditions), only very small pH changes could be observed with a pH electrode (Krab, K., Hotting, E.J. and de Wolf, F.A. unpublished data). The pH indicator Cresol red failed to show clear absorbance changes (detection limit: $2 \cdot 10^{-1}$ protons/P-700) that could be discerned from the nonspecific light-induced changes. Clear changes were only observed with Cresol red when ferredoxin was replaced by phenazine methosulfate (not shown), in line with earlier measurements with a pH electrode [1,2]. No flash-induced Cresol red response could be observed (detection limit: $2 \cdot 10^{-2}$ protons/P-700).

Under single-turnover conditions, Neutral red does show a multiphasic absorbance change in response to a flash. This response is masked, to a large extent, by the electric potential-indicating carotenoid absorbance changes in the same wavelength region. Fig. 1 shows the absorbance transients at 520 nm (mainly carotenoids) and 550 nm (mainly Neutral red, if present) in the absence and

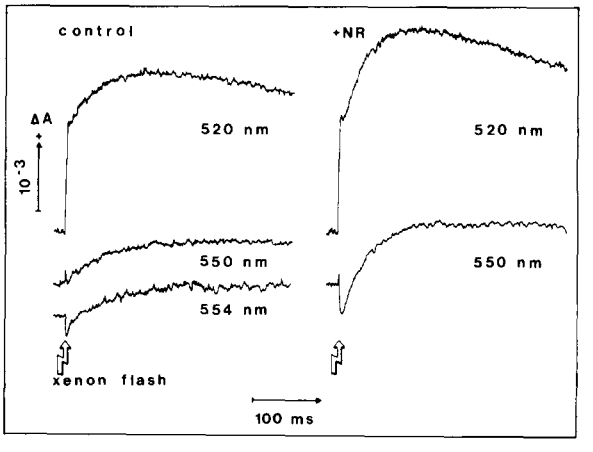

Fig. 1. Flash-induced absorbance transients in the absence or presence of 10 μM Neutral red (NR). Average of 16 transients, following successive flashes, fired at 0.1 Hz. The reaction mixture contained 250 mM sorbitol/20 mM NaCl/20 mM KCl/5 mM $MgCl_2$/2.5 mM KH_2PO_4/1 mM Tes-KOH buffer (pH 7.2)/500 μM NADPH/5 μM ferredoxin/vesicles at 50 μg Chl per ml.

presence of Neutral red. At 554 nm the cytochrome *c*-554 redox changes are visible. The spectral characteristics of the absorbance changes, determined at 10 and 80 ms after the flash, are shown in Fig. 2. The measurements could not be extended below 500 nm, due to the noise level in that wavelength region (detection limit below 500 nm: $\Delta A \geqslant 4 \cdot 10^{-4}$). Between 500 and 550 nm (depending on the batch of vesicles) the effect of Neutral red (i.e., the absorbance changes in the presence of the dye, corrected for those in its absence) mainly reflects an attenuation of the membrane potential indicating carotenoid absorbance changes (Fig. 2A). This attenuation largely masked the absorbance changes of the dye itself. Therefore, we have tried to diminish the masking effect. Noticing that the largest changes of the carotenoid absorbance occur shortly after the flash, the Neutral red absorbance changes (at 10 and 80 ms after the flash) can also be expressed as the difference of the absorbance levels at the end of the registrations (400 ms). The resulting data are shown in Fig. 2B. At wavelengths of more than 550 nm, where no carotenoid changes are observed, these data are identical to those in Fig. 2A. Comparing Fig. 2B and 2C, it can be seen that the spectrum of the flash-induced Neutral red absorbance increase at 80 ms after the flash (Fig. 2B) resembles the absolute acid minus base spectrum of Neutral red in solution (Fig. 2C). Thus, it seems to reflect Neutral red protonation. The disappearance of the absorbance of Neutral red at an ambient pH between 6 and 7 (see Fig. 2C) might cause a spectrum as shown in Fig. 2B, lower trace (flash-induced decrease at 10 ms after the flash). The ambient (bulk) pH was 7.2, but in the presence of vesicles the protonation level of Neutral red may be elevated due to surface charge effects. The shown disappearance of Neutral red absorbance (10 ms) may reflect reduction of the dye, since reduced Neutral red is colourless (see below). Lastly, it should be noticed that it cannot be decided from these data whether flash-induced Neutral red aggregation (stacking) occurs.

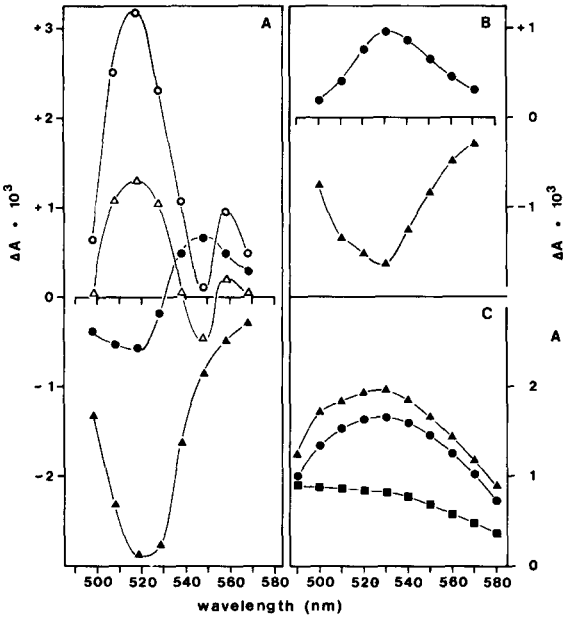

Fig. 2. Spectra of flash-induced absorbance changes obtained with PS I vesicles (A, B), and absorbance spectra of Neutral red (C). (A) Changes determined at 10 ms (△, ▲) and 80 ms (○, ●) after the flash, with respect to the absorbance level before the flash, in the absence of dye (△, ○) and in the presence of 20 μM Neutral red (▲, ●) after correction for nonspecific changes occurring in the absence of the dye. (B) Neutral red-dependent changes as in A; however, with respect to the absorbance level at 400 ms after the flash: $A_{10ms} - A_{400ms}$ (▲) or $A_{80\ ms} - A_{400\ ms}$ (●). (C) Absorbance spectra of 100 μM Neutral red in the absence of vesicles at pH 6 (▲), pH 7 (■) and pH 4–pH 7 difference spectrum (●). Further conditions as in Fig. 1.

Although the acid minus base absorbance differences of Neutral red are maximal at 530 nm (Fig. 2 B, C) we did not choose this wavelength, to avoid interference with the carotenoid absorbance changes. The best wavelength for the registration of Neutral red responses was somewhere between 545 and 550 nm, depending on the batch of vesicles, where the intrinsic absorbance changes of the vesicles were minimal. Therefore, the measuring wavelengths may differ slightly in the presented experiments. It is appropriate to report on some necessary control experiments that are required for the correct interpretation of further flash-photometric analyses.

pK_a and binding of Cresol red and Neutral red to PS I vesicles

Cresol red (which has a pK_a of 8.2) is, negatively charged in the pH region 5 to 9 (checked with paper electrophoresis). At 20 μM, no detectable amounts bind to the PS I vesicles, not even when the $MgCl_2$ concentration is increased to 20 mM [28] (cf. Ref. 23) presumably because it is

repulsed by the negatively charged membrane. Thus, Cresol red is a clear bulk-phase probe.

On the other hand, Neutral red, which is positively charged at pH values lower than 7 (checked as for Cresol red), is readily adsorbed to PS I vesicles. At 20 μM Neutral red and in the presence of 5 or 20 mM $MgCl_2$, this binding amounts to 225 and 115 nmol/mg Chl, respectively. Its binding at 125 μM, as a function of Mg^{2+} or Ca^{2+} concentration and at different pH is shown in Fig. 3. From these data, we can conclude that the protonated (positively charged) form of Neutral red is preferentially bound, in accordance with its behavior in chloroplasts [12] and submitochondrial particles [17–20].

Literature reveals a variation of observed pK_a values for Neutral red, between pH 4 and 9. Single pK_a values were reported to be 6.6 [11], 6.7 [20] or 6.8 [25] and a pair of pK_a values was spectrophotometrically observed at 5.89 (in fact, not corresponding to a protonation-deprotonation equilibrium) and 7.38 [29]. We have therefore redetermined the pK_a value of Neutral red at low concentration (20 μM) from spectrophotometric data. This resulted in a value of 6.60 $\pm$ 0.09 (S.D.) ($N = 4$). The variation of the pK_a values, as revealed by the literature, may be caused by the following factors that disturb accurate titrations: (1) variable and irreversible absorbance changes often occurred, following each pH adjustment; (2) spontaneous aggregation of Neutral red occurred (see also Refs. 17–20, 30) above pH 7.2 and was already substantial at 75 μM; this aggregation is accompanied by proton ejection, (3) also in potentiometric redox titrations, slow irreversible changes have been observed [31] yielding a fluorescent product. Apparently, several forms of Neutral red exist which are not in equilibrium during the titrations and which may be irreversibly changing from one form to another during the titrations. Considering these problems, we must treat the results of these pK_a analyses with caution. For our studies, the pK_a of about 6.6 seems to be the most relevant one.

In harmony with earlier observations [12,20,32] we observed (Fig. 4) that in the presence of PS I vesicles, this pK_a of Neutral red is shifted upward (to 6.9 at 2.5 μg Chl/ml and to 7.2 at 50 μg Chl/ml). In another experiment we observed an upward pK_a shift of 0.1 unit, due to PS I vesicles

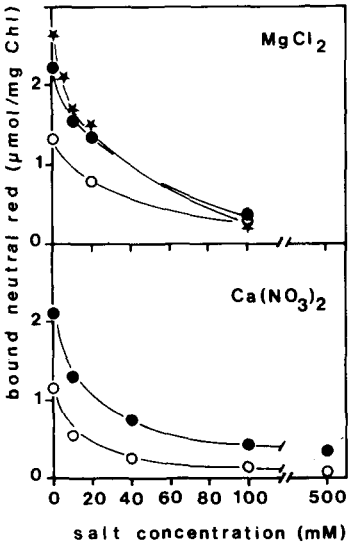

Fig. 3. Amount of Neutral red bound to PS I vesicles in the dark, as a function of bivalent cation concentration and at different pH values. The medium contained either $MgCl_2$ or $Ca(NO_3)_2$/250 mM sorbitol/1 mM Hepes-KOH buffer, pH 5 (*), pH 6 (●), pH 7 (○)/125 μM Neutral red.

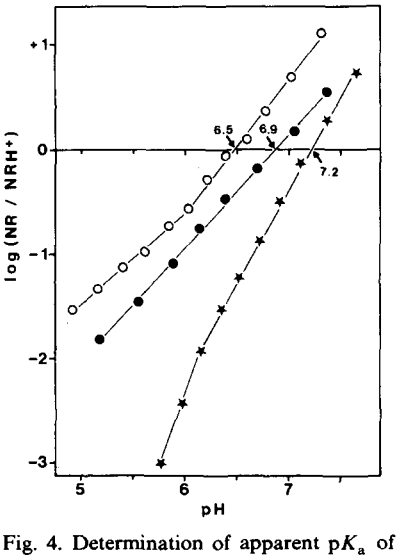

Fig. 4. Determination of apparent pK_a of Neutral red in the absence (○) and presence of PS I vesicles in the dark. The plots were obtained from spectrometric data. The medium contained 250 mm sorbitol/5 mM MgCl/250 μM NADPH/5 μM ferredoxin/20 μM Neutral red/vesicles at 2.5 (●) or 50 (*) μg per ml.

(13 μg Chl/ml) in the dark, and a further upward shift of 0.2 unit, induced by continuous illumination, suggesting an energization-dependent binding of the protonated form of the dye.

Flash-induced Neutral red responses in PS I vesicles

As already seen in Fig. 1, the Neutral red absorbance at 550 nm first rapidly decreases (halftime < 5 ms), then increases again (overall halftime about 25 ms). The relative extents and rates of these two events depended on the conditions applied (Figs. 6, 8, 9 and 10) and varied with the batch of vesicles.

In Fig. 5, the relative amplitudes of the fast absorbance decrease (measured at 10 ms) and of the slower increase (at 80 ms) are plotted as a function of Neutral red (A) and of $MgCl_2$ (B) concentration. Above 20 μM, Neutral red decreases its own response but the fast process is less sensitive than the slow process: above 70 μM Neutral red only the fast process remains. At these concentrations the dye acts as an uncoupler, this is also reflected in a progressive inhibition of the slow-rising component of the carotenoid absorbance change at 520 nm. Increasing $MgCl_2$ concentration diminishes both parts of the Neutral red response to roughly the same extent, in accordance with the repulsive effect of Mg^{2+} on Neutral red binding (Fig. 3). This result shows that no flash-induced pH changes are experienced in the external bulk solution. (The detection limits in these experiments varied from $1 \cdot 10^{-2}$ to $2 \cdot 10^{-1}$ protons/P-700, due to noise). Moreover, the effect of Mg^{2+} suggests that Neutral red did not permeate the vesicle membrane but interacted electrostatically with the external membrane surface [18–20] (contrasting view in Refs. 10–14).

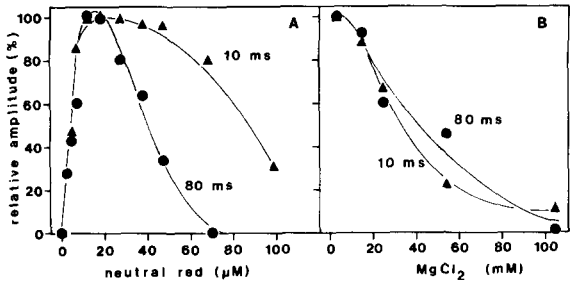

Fig. 5. Relative amplitude of flash-induced Neutral red absorbance changes (measured from initial absorbance level) at 548 nm as a function of Neutral red (A) or $MgCl_2$ (B) concentration. Values, obtained at 10 ms and 80 ms after the flash, were corrected for nonspecific changes occurring in the absence of Neutral red. Conditions as in Fig. 1; however, 1 mM Hepes was used for part A instead of Tes.

The effect of the buffer Hepes (50 mM) is shown in Fig. 6. Only the slower Neutral red absorbance increase is sensitive to buffers (also to Tes, Tricine and phosphate), showing that this component reflects Neutral red protonation. The effect of buffering with Tricine or phosphate is shown in Fig. 7. The zwitterionic Tricine inhibits at much lower buffer capacity (50% at 0.35 mM) than the negatively charged phosphate (50% at 3.8 mM), which is not unexpected considering their different structures. Tricine was shown to act as a permeant buffer in ATPase proteoliposomes [23] and will presumably penetrate into the membrane interface domain more easily than phosphate. In contrast, Hepes was shown to be impermeant in those proteoliposomes [23]. This also suggests that the Neutral red response is generated at the exposed membrane surface in our case. This was further substantiated by the fact that all the buffers tested inhibited instantaneously, showing that they reached the energization-monitoring dye without detectable hindrance due to a diffusion barrier in these vesicles that are well coupled (Refs. 1, 2, 4,

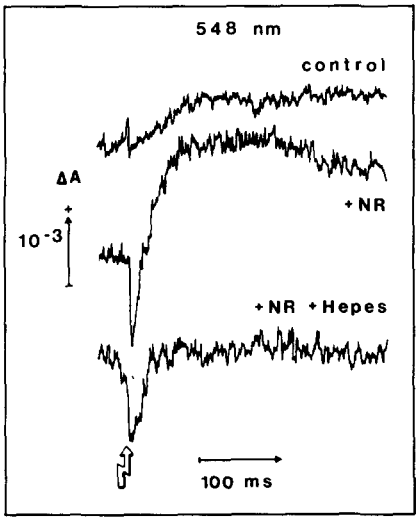

Fig. 6. Flash-induced absorbance transients in the absence and presence of 40 μM Neutral red (NR) and 50 mM Hepes-KOH buffer (pH 7.2) (buffer capacity 25 mM). Further conditions as in Fig. 1.

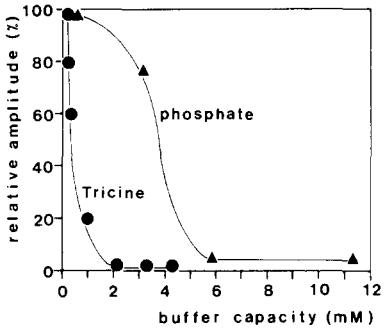

Fig. 7. Relative amplitude of flash-induced Neutral red absorbance changes (measured from initial absorbance level) at 548 nm as a function of the buffer capacity (expressed as amount of acid or base added per 1 per unit pH change [44]). Buffering of the medium was effected either by Tricine (pH 7.2), or phosphate (pH 7.0). The values, obtained 80 ms after the flash, were corrected for nonspecific effects. Neutral red concentration was 20 μM. Further conditions as in Fig. 1.

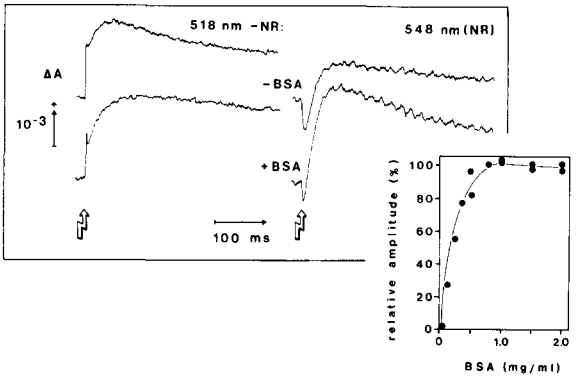

Fig. 8. Flash-induced carotenoid (518 nm) and Neutral red (548 nm) absorbance transients, both in the same batch of vesicles, in the absence and presence of bovine serum albumin (BSA). Carotenoid transients: pH 8.0, further conditions as in Fig. 1; Neutral red transients: conditions as in Fig. 5A. Inset: relative enhancement of flash-induced Neutral red absorbance changes at 548 nm as a function of BSA concentration. Values, corrected for nonspecific effects, were obtained 80 ms after the flash.

and 5, and De Wolf, F.A., Galmiche, J.M. and Girault, G., unpublished data).

Bovine serum albumin has been used earlier [10–14] as impermeant 'broadband buffer' in chloroplasts, in order to eliminate residual external pH indicator responses. These authors observed a bovine serum albumin-induced stimulation of Neutral red protonation [10] but others observed an inhibition [25] in chloroplasts. For comparison, we have also tested the effect of bovine serum albumin on the Neutral red response with our PS I vesicles. As Fig. 8 shows, it stimulates the Neutral red absorbance increase with maximal effect at about 0.75 mg/ml (inset) which corresponds to a buffer capacity of only 0.33 mM. In parallel, the carotenoid response (slow component) is stimulated. Therefore, the stimulation of the Neutral red response by bovine serum albumin is unlikely to be due to any buffering effect of this protein but rather to its beneficial effect on membrane potential formation (Fig. 8) and on membrane processes in general [33–36] (e.g., scavenging of free fatty acids [34–36]). Thus, both Neutral red and carotenoid responses which are sensitive to the energy state of the membrane may be stabilized in the presence of bovine serum albumin.

The fast absorbance decrease is insensitive to buffers but also to uncoupler (0.1 μM S-13) and ionophores (0.1–1.0 μM valinomycin or 0.05–1.0 μM nigericin). Its amplitude was not enhanced around the pK_a of about 6.6 (Fig. 9). Therefore, this fast event cannot reflect protolytic effects. It most likely reflects Neutral red reduction, a process earlier observed by Lynn [9] in continuously illuminated chloroplasts (reduced Neutral red is colourless [31,37]; we tested it, using sodium dithionite). The redox midpoint potential of Neutral red is about −370 mV at pH 7.0 [37], making it a suitable electron acceptor for PS I [38]. We found that its amplitude increased with decreasing pH, probably reflecting the increasing extent to which it could be reduced by PS I as its E'_m increased from −420 mV at pH 8 to −180 mV at pH 4 [37]. Following the fast flash-induced decrease, the Neutral red absorbance rapidly returned to its original level in the presence of buffers (Fig. 6) or uncoupler (Fig. 10), or at low pH (Fig. 9), when no proteolytic events are observed. Possibly this reflects rapid Neutral red reoxidation. If no reoxidation would follow the Neutral red reduction, a considerable decrease of extent of the flash-induced Neutral red response after a large number of flashes would be expected by the progressive Neutral red absorbance decrease. Such a decrease was not observed even after 100 flashes, while the total amount of Neutral red present per P-700 (mol/mol) was 100 to 140.

Fig. 9 also shows that the slower Neutral red absorbance increase is maximal (with respect to the baseline) between pH 6 and 7 (thus, around its pK_a), supporting the notion that it monitors Neutral red protonation. Assuming that the initial part of the absorbance increase is due partly to (uncoupler and buffer-insensitive) Neutral red reoxidation, the overall halftimes do not reflect the true rate of Neutral red protonation. After correction for the signals occurring in the presence of Hepes (Fig. 6), phosphate, or S-13 (Fig. 10), a halftime of 10–40 ms was obtained for this protonation rate. The protonation-associated part of the Neutral red response was also very sensitive to the uncoupler S-13 (0.1 μM) and was stimulated by 0.1 μM valinomycin (Fig. 9). Nigericin (0.1 to 1.0 μM) had no effect, as was also tested in several experiments: within the limits set by the noise, the registrations superpose (see for example the dashed line in Fig. 10). Under similar conditions in these PS I vesicles, it was observed earlier that valinomycin inhibits the slow component of the carotenoid response while nigericin only showed some stimulation at suboptimal electron flow. This

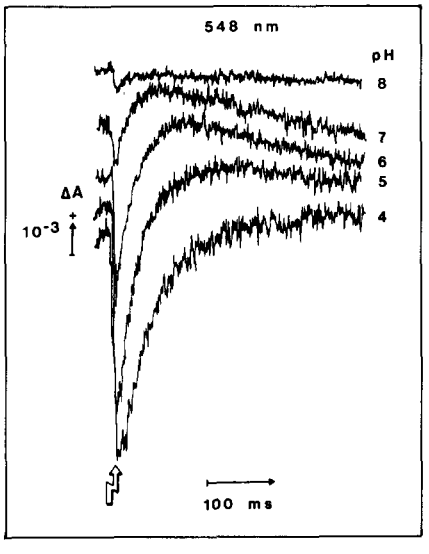

Fig. 9. Flash-induced Neutral red absorbance transients corrected for nonspecific effects, at different pH values. Neutral red concentration was 20 μM. Further conditions as in Fig. 1, except for the buffers: 1 mM succinate (pH 4.0 and 5.0), 1 mm maleate (pH 6.0) and 1 mM Tes-KOH (pH 7.0 and 8.0). The buffer capacity ranged from 0.3 mM (pH 5.0) to 0.7 mM (pH 4.0).

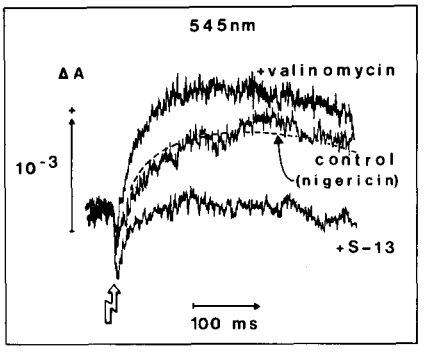

Fig. 10. Flash-induced Neutral red absorbance transients corrected for nonspecific effects in the presence or absence of 0.1 μM Valinomycine (val), 0.1 μM S-13 or 0.1 μM nigericin (dashed line). Neutral red concentration was 20 μM. Further conditions as in Fig. 1.

lack of effect of nigericin under optimal conditions was explained by assuming that the flash-induced electric potential reached a maximum level [5]. A close similarity was observed between the corrected halftimes of Neutral red protonation and those of the slow carotenoid absorbance rise (between 10 and 80 ms after the flash): their ratio was 1.09 ± 0.11 (S.D. of three experiments; the correlation coefficient was 0.96, corresponding to a correlation probability greater than 95%).

Finally, we have investigated whether the flash-induced Neutral red response is dependent on the optimal cyclic electron flow as earlier devised by Peters et al. [4]. The requirement of ferredoxin (4 μM) and NADPH (500 μM) as a redox poiser was the same for the Neutral red response as for maximal electric potential formation (results not shown).

Discussion

The presented experiments were aimed at the resolution of possibly occurring flash-induced proton displacements in PS I vesicles localized at the membrane level. Previous findings [1,2] and some of our present results clearly show that in these small vesicles (50 nm diameter) trans-membrane proton translocation is not detectable under single-turnover conditions; only a very small proton uptake is observed under steady-state conditions in the same system (Krab, K., Hotting, E.J.

and De Wolf, F.A., unpublished data). Yet, the membrane integrity of these vesicles is of such quality that the electric potential generation (slow component) is more prominent than in chloroplasts [5] and that appreciable ATP synthesis can occur (Ref. 4, and De Wolf, F.A., Galmiche, J.M. and Girault, G., unpublished data).

From the effects of buffers, ionophores (uncoupler) and bulk pH, we concluded that the observed flash-induced Neutral red absorbance changes reflect protonation of the dye. This conclusion is restricted to the absorbance increase (corrected for the initial absorbance increase, presumably reoxidation). The fast decrease is attributed to Neutral red reduction. We cannot exclude that Neutral red senses a local acidification in its restricted environment. However, it is unlikely that the observed Neutral red protonation was caused directly by translocated protons: (1) the instantaneous effect of (hydrophilic) buffers and bivalent cations (no detectable hindrance due to a diffusion barrier) shows that Neutral red was exclusively present outside these vesicles; (2) proton translocation, if any occurs, is directed inward. It could be argued that external Neutral red protonation is due to proton extrusion by a population of inside-out vesicles. This argument can be refuted because, firstly, the used fractionation technique is very unfavourable for the formation of inside-out vesicles in sufficient amounts to produce the observed effects and, secondly, any inside-out vesicles cannot function with added ferredoxin and NADPH and have also lost their plastocyanin. Both the Neutral red absorbance increase and the carotenoid response require the presence of ferredoxin and NADPH.

Thus, since Neutral red protonation did not seem to be caused by the appearance of translocated protons in its environment, an alternative explanation should be looked for. Protonation of cationic pH indicators like Neutral red may be caused by an increase of the net negative charge of the vesicle membrane surface. Upon such an increase, protonated Neutral red will be attracted to the membrane surface by coulombic interaction and hence the indicator will undergo an apparent upward pK_a shift and take up protons. This view is consistent with earlier notions [17–20,32,39]. The observed upward pK_a shift of Neutral red in the presence of the vesicles in the dark (Fig. 4) and the further light-induced pK_a shift support such a mechanism. In chloroplasts, a light- or ATP-dependent increase of the net negative surface charge was earlier observed by free-flow electrophoresis [39,40] (not observed in Ref. 41). Like the Neutral red response in PS I vesicles, this increase of surface potential was stimulated by low concentrations of valinomycin (Kraayenhof, R., De. Wolf, F.A., Van Walraven, H.S. and Krab, K., unpublished data). The close similarity between the halftimes of Neutral red protonation and carotenoid (or oxonol VI, Refs. 5 and 6) responses would suggest that the primary cause of the slow Neutral red absorbance increase (protonation) is somehow related to electrical potential generation. This relation may of course be indirect due, for instance, to surface charge effects related to membrane potential formation.

Our interpretation of the behavior of Neutral red in the presence of PS I vesicles differs from that given by others [9–11,25] for the case of chloroplasts. These authors all neglect the influence of surface charge and attribute the Neutral red signal to protolytic events occurring in the chloroplast lumen. Arguments for this were that the Neutral red response did not precisely follow the external pH [9] or that it was not sensitive to hydrophilic buffers in the outside medium [25]. Junge et al. [10,11] found that the flash-induced Neutral red absorbance increase (i.e., Neutral red protonation) was sensitive to a large number of buffers, including hydrophilic ones, but not to bovine serum albumin, which stimulated the net increase (contrasting results in Ref. 25). They concluded from this that the fraction of Neutral red sensing the acidification had entered the thylakoid lumen or internal interface domain and that this compartment was accessible to all buffers, except bovine serum albumin.

On the basis of our results with PS I vesicles, chloroplasts and ATPase proteoliposomes, we challenge this conclusion. Firstly, the bovine serum albumin effect upon the Neutral red response is possibly due to a general stabilization of membranes rather than to buffering. Secondly, the (possible) presence of a barrier for bovine serum albumin, which is permeable to buffers, does not imply that this barrier is the thylakoid membrane

proper. Biomembranes should not be considered as smooth septa, but rather as inhomogeneous lipid-protein assemblies with ruffled surfaces, creating cleft- or pocket-like domains. Such domains, on the exposed membrane surface may be accessible to a variety of molecules, depending on their size, form, electric charge, etc. It would be quite feasible that externally adsorbed Neutral red has different accessibility for different cation and buffer species (Fig. 7) while the large bovine serum albumin molecules are fully excluded from its sites. The Neutral red responses found earlier [10,12], said to demonstrate PS II-induced fast proton release inside thylakoid interior domains, may as well reflect electric charge rearrangements at the thylakoid exterior domains in the way described here for subchloroplast vesicles and by Azzone's group for submitochondrial vesicles [17–20]. In this respect Neutral red seems to behave like the structurally similar aminoacridine probes [42]. Lastly, the variation of the amplitude of the flash-induced signals as a function of flash number, as observed by Junge et al. in chloroplasts [11], is not necessarily indicative of a pH-indicating behavior of those signals: (1) starting at intermediate pH (7), the amplitude will also decrease with the flash number if (protonated) Neutral red is accumulated, due to electrostatic interaction at the membrane surface (Neutral red depletion in medium and/or electrostatic screening of surface charges); (2) starting at relatively high pH (8), the amplitude may increase if the surface charge is repeatedly increased after several flashes, thereby attracting protons and, consequently, Neutral red. In PS I vesicles we did not observe such a variation of amplitude, presumably as the flash-induced changes are completely relaxed before each new flash.

In PS I vesicles, we did not observe flash-induced proton translocation monitored with Cresol red neither did the observed Neutral red response reflect the appearance of translocated protons. Therefore, our results support the view that vectorial proton translocation is not a prerequisite for energy conservation under single-turnover conditions [43].

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