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# Stoichiometry of proton release during photosynthetic water oxidation: a reinterpretation of the responses of Neutral red leads to a non-integer pattern

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**We studied the stoichiometry of proton release into the thylakoid lumen as caused by a series of one-electron turnovers of the photosynthetic water oxidase. Earlier results obtained with Neutral red as an indicator for pH transients in the thylakoid lumen led to a 1:0:1:2 pattern of proton release for the $S_0 \rightarrow S_1$:$S_1 \rightarrow S_2$:$S_2 \rightarrow S_3$:$S_3 \rightarrow (S_4) \rightarrow S_0$ transitions of the water oxidizing enzyme. Recently, a new interpretation of the Neutral red signals has been presented, that led to a non-integer stoichiometry of proton release (Lavergne, J. and Rappaport, F. (1990) in Current Research in Photosynthesis (Baltscheffsky, M., ed.), Vol. I, pp. 873–876, Kluwer, Dordrecht). This new interpretation was based on the proposal that in stacked thylakoids the absorption changes of Neutral red reflect not only pH transients in the lumen, but also pH transients in the external partitions between the appressed membranes in grana stacks. The latter space was not accessible to the buffer bovine serum albumin which was used to quench pH transients in the bulk. This interpretation was scrutinized with both, stacked and totally unstacked membranes. In unstacked membranes, bovine serum albumin quenched pH transients at the outer side of the membrane very rapidly. Then, the pattern of proton release as detected with Neutral red revealed the proposed non-integer stoichiometry, in which a fraction of a proton (about 0.5) is released upon the $S_1 \rightarrow S_2$ transition and less than two protons (about 1.5) during the oxygen-evolving $S_3 \rightarrow (S_4) \rightarrow S_0$ step.**

## Introduction

Photosynthetic water oxidation in higher plants is catalyzed by a manganese cluster which is part of the Photosystem II complex (for recent reviews see, for example, Refs. 1, 2). Two molecules of water are oxidized in a four-step reaction yielding four protons and one molecule of dioxygen. Following the model of Kok et al. [3] the oxygen-evolving system cycles through four (five) oxidation states $S_0$, $S_1$, $S_2$, $S_3(S_4)$ with $O_2$ being released during the $S_3 \rightarrow (S_4) \rightarrow S_0$ transition. The stoichiometry of proton release during the four successive redox transitions has been under discussion for a long time. Finally, a pattern of 1 : 0 : 1 : 2, starting from the $S_0$ state, has been favoured by different authors [4–8] and seemed to be established with the time-resolved measurements (by the pH-indicating dye, Neutral red) of proton release into the thylakoid lumen [9]. The relaxation times in the latter study were in general agreement with those of electron transfer between the oxygen-evolving system and the more primary donors of Photosystem II [10,11]

Neutral red (NR) is an amphiphilic dye that is adsorbed to both sides of the thylakoid membrane. In the presence of bovine serum albumin (BSA) as non-permeating buffer, NR was used to probe proton release into the thylakoid lumen [12]. After addition of DNP-INT, an inhibitor of the plastoquinol oxidation [13], the absorption changes of NR should reflect exclusively proton release due to water oxidation [9]. When dark-adapted thylakoids, with the water oxidase mainly in the $S_1$-state, are excited with a series of short flashes, NR exhibited a kinetically multiphasic acidification after

Abbreviations: BSA, bovine serum albumin; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DNP-INT, dinitrophenyl ether of iodonitrothymol; DMQ, 2,6-dimethyl-*p*-benzoquinone; NR, PR, Neutral red, Phenol red; PS II, PS I, Photosystem II, I.

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each flash (see Fig. 1, top). The fast phase ( < 5 ms) was clearly attributable to protons which were liberated during water oxidation (see Fig. 1, bottom) and the oscillating pattern appeared consistent with a 1 : 0 : 1 : 2 stoichiometry. However, a portion of the signals (half-rise time about 100 ms) could not be attributed to other known events [9].

Polle and Junge [14] showed that the alkalinization in the partition region, that is caused by the reduction of bound quinone ($Q_B$), relaxes with a half-decay time of about 100 ms into the bulk. This led Lavergne and Rappaport [15] to reinterpret the observed absorption changes of Neutral red as a superposition of responses arising both from the lumenal acidification and from the transient alklinization in the partition region. As illustrated in Fig. 2, the pH-indicating dye NR reports from three compartments: the lumen, the partition region and bulk water. The protonic equilibrium between

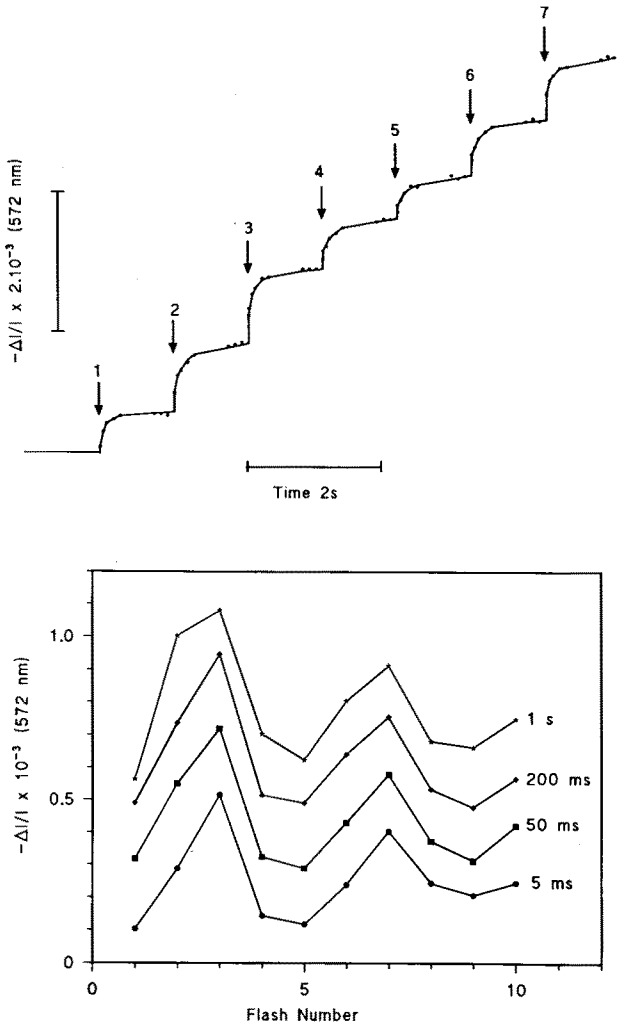


Fig. 1. Absorption changes of Neutral red upon a train of single-turnover flashes (1.1 s apart). Top: Original data obtained with dark-adapted stacked thylakoid membranes in a BSA-buffered medium (2.6 mg/ml) at pH 7.0. Bottom: the flash-induced changes at 5 ms, 50 ms, 200 ms and 1 s, replotted as a function of flash number.

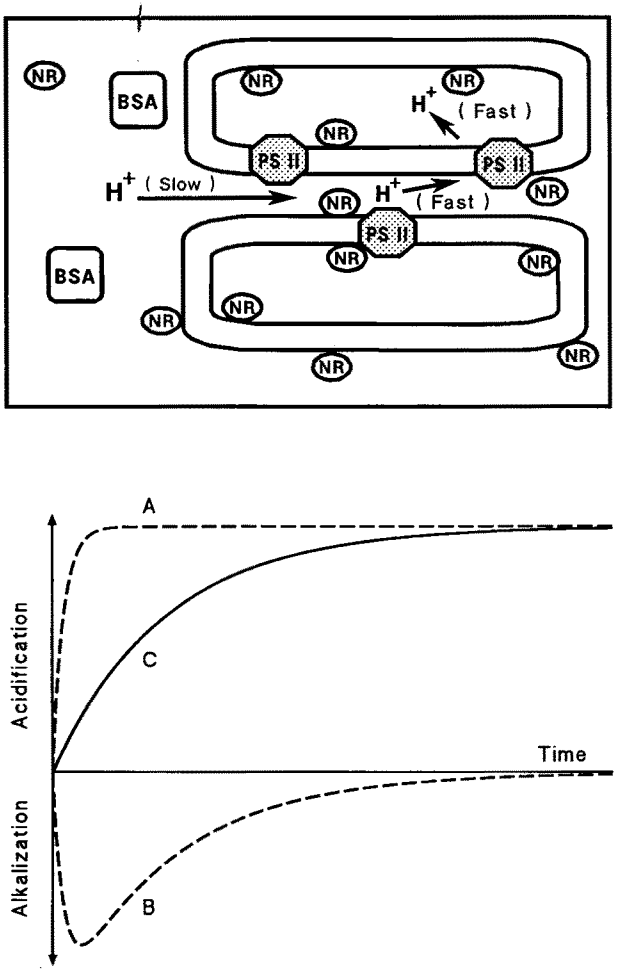


Fig. 2. Scheme illustrating the interpretation of the Neutral red (NR) response proposed in Ref. 15. Top: the picture shows a grana stack with NR adsorbed on both sides of the membrane. It is assumed that BSA remains confined to the bulk aqueous phase and does not penetrate the appressed external regions (partitions). The adsorbed dye responses on a fast time-scale to both proton release in the lumen and proton uptake in the partitions. The latter transient relaxes together with the slower equilibration between partitions and the BSA-buffered bulk. Bottom: scheme of the kinetics of NR response in the lumen (A), in the partitions (B) and their sum (C). The relative amplitudes of (A) and (B) were chosen so as to mimic the typical result observed on the first flash at pH 7, i.e., no (or little) fast phase on the observed response (C). As explained later in the text, this coincidence does not hold at other pH values.

these compartments is not adjusted immediately: the relaxation time between lumen and bulk water is in the order of about 10 s [16] and the one between partitions and bulk water in the order of 100 ms, as mentioned [14,17]. The superposition of the different Neutral red responses is illustrated in Fig. 2 (bottom). From the lumen, in the presence of an inhibitor of plastoquinol oxidation (DNP-INT), one expects an acidification response in the ms range due to proton release from water oxidation. The acidification lasts for about 10 s. From the partition one expects to see a fast alkalinization due to the reduction of $Q_B$ at the PS II acceptor side (rise

time about 2 ms [14]), relaxing within 100 ms by lateral diffusion of $H^+$ and $OH^-$ along partitions to and from the bulk buffer medium. In the presence of BSA (> 1.3 mg/ml) pH transients in the bulk are practically buffered away. The NR response would then be contributed both by pH changes in the lumen and in the partitions, and the true extent of the lumenal change would appear when the partition transient had relaxed. This view has an important consequence for the determination of the stoichiometry of proton release upon the successive transitions of the oxygen-evolving system. If correct, one should take into account the total change (fast + slow phases) rather than solely the fast phase. As may be seen in Fig. 1 (bottom), the pattern obtained for the total changes showed less contrasted oscillations than previously thought. They could not be accommodated by the 1 : 0 : 1 : 2 model. This implied a non-integer stoichiometry in which a fraction of a proton was released upon the $S_1 \rightarrow S_2$ step, and less than two protons were released during the oxygen-evolving $S_3 \rightarrow S_0$ step. As rough approximation, a 1 : 0.5 : 1 : 1.5 stoichiometry has been proposed [15].

We scrutinized the above interpretation of the NR signal in three ways: (1) With stacked thylakoids the extent and the kinetics of the slow phase were correlated with the slow alkalinization of the bulk as seen by the external dye Phenol red (PR). (2) Again in stacked thylakoids the protonation of $Q_B$ and thus the alkalinization of the partitions was suppressed to eliminate any response of NR other than from the lumen. And (3) the buffering action of BSA was speeded up by complete destacking of the membranes. Under destacked conditions, the slow phase of the NR response was suppressed while the total extent was conserved. All results strongly supported the proposed interpretation and the non-integer stoichiometry.

## Material and Methods

Stacked thylakoid membranes and EDTA-treated unstacked membranes were prepared from 12–14-day-old pea seedlings (*Pisum sativum*) as previously described [14]. The resulting pellets were diluted with 0.4 M sucrose, 10 mM NaCl and 2 mM Tricine/NaOH (pH 7.8) (for stacked membranes 5 mM $MgCl_2$ was added) to yield final chlorophyll concentrations between 1.7 and 2 mM. These stock solutions were stored at −80°C for up to 2 months. For flash photometric measurements thylakoids equivalent to 10 μM Chl were suspended in 10 mM NaCl (or additionally 5 mM $MgCl_2$ for stacked membranes). At pH 7.0 to 7.2 we measured pH transients in the lumen by absorption changes of Neutral red at 548 nm in the presence of 2.6 mg/ml BSA [12] or pH transients in the medium by Phenol red at 559 nm [14]. pH-specific absorption changes were obtained by subtracting a signal in the absence from a signal detected in the presence of the respective indicator dye.

For all experiments membranes were dark-adapted for 15 min at room temperature. Except for the experiment in Fig. 8, hexacyanoferrate III (2 mM) served as electron acceptor and 10 μM DNP-INT and 5 μM DMQ were present. Further additions are indicated in the figure legends.

Flash photometric experiments were carried out using either of two different techniques: (a) with gated (but then continuous) measuring light plus short exciting xenon flashes as in Ref. 9 (Figs. 3–5) and (b) with the Joliot-type spectrophotometer [18,19] in which the absorption was sampled at discrete times by short monochromatic flashes (Figs. 1, 7 and 8). With the latter apparatus, NR absorption changes were obtained at 572 nm and in presence of 1 μM valinomycin.

## Results and Discussion

The problem of superposition of NR absorption changes resulting from pH transients in partitions with those from the lumen is most pronounced in stacked thylakoid membranes. Therefore, we started with this material. Fig. 3 shows the absorption changes of NR (in the presence of BSA, upper trace) and PR (in the absence of BSA, middle trace) induced by a train of four flashes in dark-adapted stacked membranes. PR is a hydrophilic (sulphonic acid) dye which is selective for pH transients in the bulk phase (see Ref. 14). Both measurements were carried out in presence of 10 μM DNP-INT in order to abolish the oxidation of plastoquinol at the cytochrome $b_6/f$ complex [13] together with the concomitant proton release into the lumen. The NR signal showed the typical biphasic behaviour, with a slow phase (about 100 ms) most pronounced on the first flash. This slow component decreased with successive flashes. With PR we obtained traces indicating slow proton uptake, again larger upon the first flash and smaller in the following ones. This pattern seemed similar to that of the slow component of NR. For comparison, we normalized both signals to the same total extent for the first flash. The difference, NR minus PR, which is shown in the lower trace of Fig. 3, showed only rapid transients, while slow components were cancelled almost perfectly. This implied that both, the time course and the relative extent (as function of flash number) of the PR signal were in good agreement with the slow phase of the NR signal. The somewhat faster kinetics of the NR transients was most probably due to the action of NR as mobile buffer in the partitions, as described more detailed for phosphate (compare Fig. 7) later.

A second attack on the problem was to prevent proton uptake at the acceptor side of PS II. Then, pH transients in the partitions and transient absorption

changes of NR in this compartment, as well, should also be abolished. We used DCMU to inhibit proton uptake at the $Q_B$ site. Because DCMU prevents electron transfer from $Q_A$ on, it allows a single charge separation step upon flash number one. Fig. 4 shows the absorption changes of PR (upper trace) and NR (lower trace) in the presence of DCMU. Again, dark-adapted stacked thylakoid membranes were excited by a train of four flashes. There was very little proton uptake (as seen by PR) upon the first flash, which leads to the reduction of $Q_A$, but not of $Q_B$. It is not accompanied by proton uptake [14]. The NR response (lower trace) was dramatically changed in the presence of DCMU (see Figs. 1, 3 for comparison). The slow phase was suppressed and replaced by a fast (< 1 ms) one with similar (slightly smaller) amplitude. This finding was again fully consistent with the proposed interpretation of the NR response. The fast acidification observed in the presence of DCMU was mainly associated with the $S_1 \rightarrow S_2$ transition. It was not superimposed with a transient alkalinization in the partitions. The slightly smaller amplitude may reflect the loss of PS II activity, e.g., by an increased probability for photochemical misses due to DCMU. It should be noted that the occurrence of a fast acidification response of NR in the presence of DCMU had been reported by Hong et al. [20]. Those authors did not, however, correlate this finding with the slow phase suppression, and were led to invoke a fast PS II cycle.

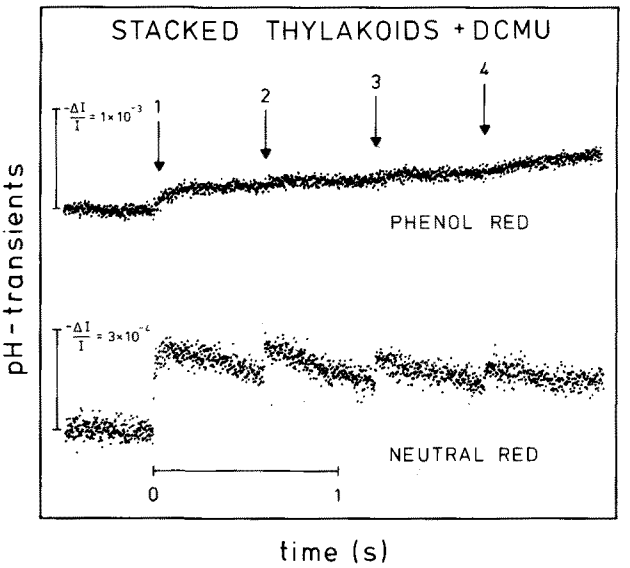


Fig. 4. Absorption changes of PR (top) and NR (bottom) in dark-adapted, stacked thylakoid membranes in the presence of DCMU (5 $\mu$M). Other conditions as in Fig. 3.

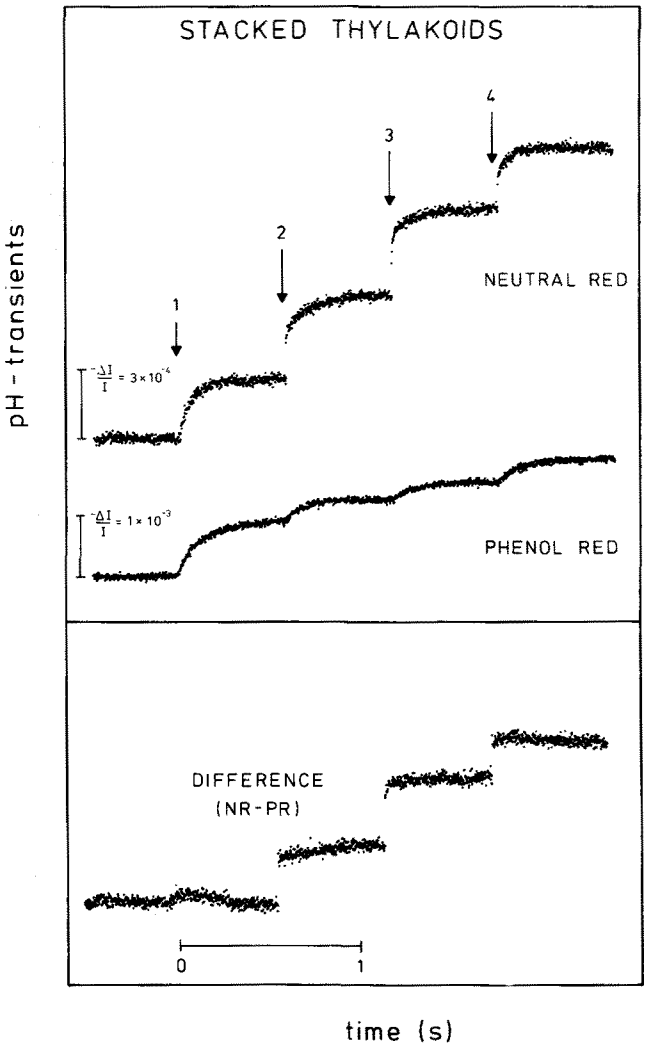


Fig. 3. Absorption changes of Neutral red (NR) at 548 nm (top) and Phenol red (PR) at 559 nm (middle). Four single-turnover flashes, spaced 600 ms apart (arrows), were given to dark-adapted stacked thylakoid membranes. 2.6 mg/ml BSA was present for the measurements with NR, no buffer was added with PR, pH was 7.0. The difference (NR-PR) is shown at the bottom. The signals were normalized to yield the same extent upon the first flash.

Another independent test was the following. One expects that the observed slow NR response should again be converted into a fast one if the PS II acceptor side was more readily accessible to BSA by the unstacking of the membranes. Membranes were destacked under mild EDTA treatment [14]. Fig. 5 shows the absorption changes of NR in destacked (left trace) and stacked thylakoids (right trace). For better comparison of the respective extents, the two curves are presented side by side, horizontally shifted. As expected, with destacked membranes (left traces in Fig. 5) only fast NR responses were detected, meaning that all external contributions were rapidly buffered away by BSA. The amplitudes detected with EDTA-treated membranes were somewhat smaller and therefore normalized (by a factor of 1.35) to the same magnitude as for stacked membranes. The smaller amplitudes were probably due to the altered salt conditions (no $MgCl_2$ present with unstacked membranes). It was previously shown that the apparent p$K$ of membrane-adsorbed NR and the distribution of NR between membrane and bulk phase, as well, depend

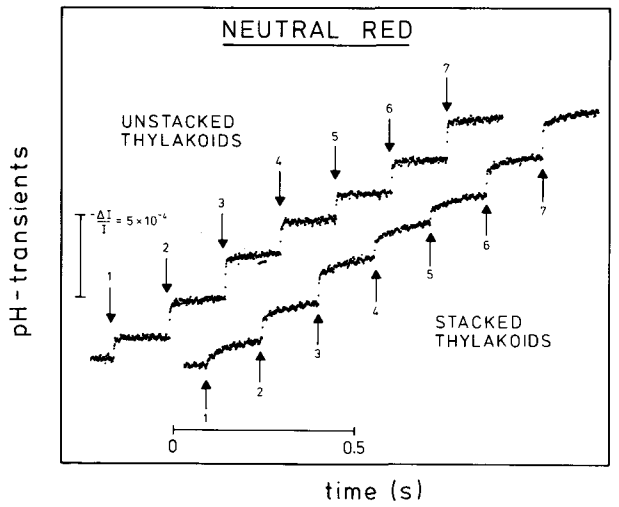


Fig. 5. Absorption changes of NR in dark-adapted unstacked (left trace) and stacked thylakoid membranes (right trace). Excitation with a series of seven flashes, spaced 150 ms apart (arrows). Both experiments in presence of 2.6 mg/ml BSA (pH 7.0).

on the surface potential and thus on the salt conditions [21]. An unchanged activity of PS II and PS I in both preparations was verified by measurements of the electrochromic absorption changes at 515 nm (not documented).

It was obvious from Fig. 5 that the oscillating pattern of the (normalized) response in unstacked membranes matched very well the oscillation of the sum of the fast and slow phases in stacked material. This is also shown in Fig. 6, in which the absorption changes of NR at 5 ms and 150 ms after each flash are replotted.

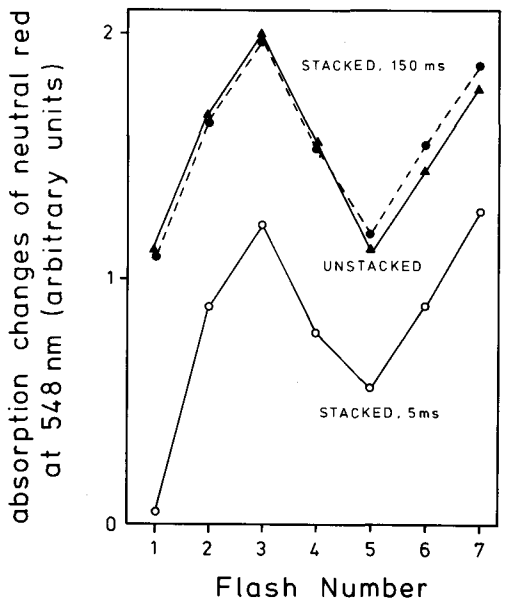


Fig. 6. The relative extent of the absorption changes of Neutral red in dependence of the flash number as derived from the original traces in Fig. 5. For stacked thylakoids, the extent was sampled at 5 ms (open circles) and at 150 ms (closed circles) after each flash. For unstacked thylakoids (triangles) the extent at 5 ms was the same as at 150 ms.

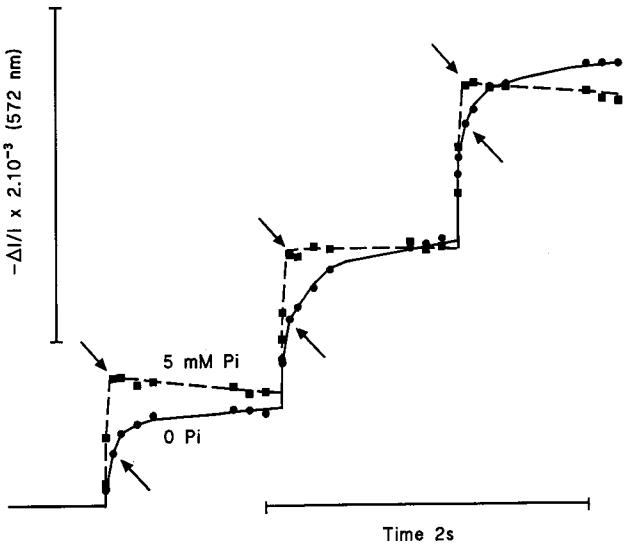


Fig. 7. Absorption changes of NR upon a train of three single turnover flashes, in the absence (circles) or in the presence (squares) of 5 mM phosphate. 2.6 mg/ml BSA pH 7.0 was added in both experiments. The arrows show the absorption changes at 50 ms (the earlier datapoints are at 1 ms and 5 ms).

Correlation between the slow phase of the NR response and lateral proton diffusion in the partitions was also tested in another way. According to theory [22], a hydrophilic mobile buffer such as phosphate should accelerate the relaxation of the transient alkalinization in the partitions. In agreement with this prediction and with the present interpretation of the slow phase, we found a 10-fold acceleration of the slow phase of the NR changes upon addition of 5 mM phosphate, with little change of the total extent (shown in Fig. 7). However, the previous experimental test (monitoring the PR response) has revealed this acceleration with imidazole, but not with phosphate [23]. This is now well understood by the results of Fig. 7. The acceleration by phosphate occurred only at high concentrations (5 mM, see Fig. 7) so that the pH transients in the bulk, that have been monitored in Ref. 23, were quenched. Notice that, on the other hand, the NR experiment with imidazole would be more difficult to interpret, since this buffer penetrates the lumen.

The relative extent of the NR responses arising from alkalinization in the partition and acidifcation in the lumen should depend on the density of adsorbed NR on the respective membrane surface and on the local buffering capacity. Therefore, the fact that this contribution shifted the fast response so as to roughly cancel the acidification signal on the first flash should be coincidental – and particularly misleading since this provides apparent evidence for a wrong (integer) stoichiometry. This coincidental aspect appeared clearly indeed when performing NR experiments outside the pH 7 region. At pH $> 7$ (not shown), the relative contribution from partitions was smaller so that the

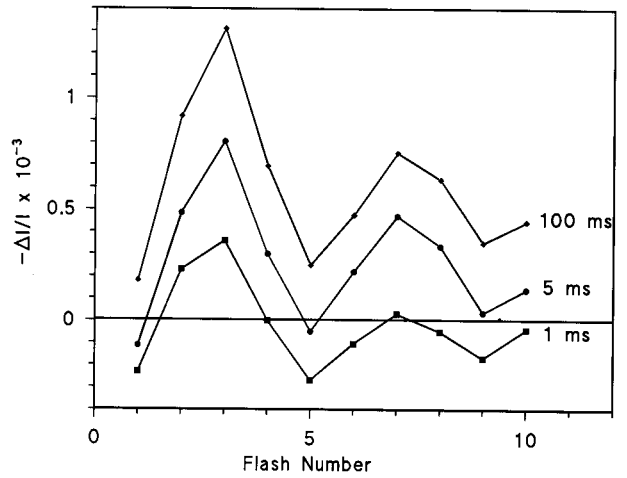


Fig. 8. Plot of the absorption changes of NR at 1 ms, 5 ms and 100 ms after each flash of a series. Same conditions as in Fig. 1, except that the pH was adjusted to 6.5 and no hexacyanoferrate III was present. At this pH, the kinetics of the NR response were faster and reached their full extent at 100 ms.

pattern of the 5 ms changes was shifted upwards with respect to the pH 7 results, closer to the final true acidification signal. Conversely, at pH $< 7$, this relative contribution was larger, meaning a downward shift, as illustrated in Fig. 8 (pH 6.5). The presence of a fast alkalinization signal which can hardly be ascribed to a lumenal event, was here obvious.

## Conclusion

Our data clearly show that the absorption changes of NR in the presence of BSA are perturbed by an alkalinization transient arising from the partition regions, whenever the thylakoids are not completely unstacked. This perturbation modifies quite significantly the determination of proton release during the Kok cycle. The reinterpreted data are no longer consistent with the broadly accepted 1:0:1:2 stoichiometry, that must now be replaced by a non-integer model. Several comments may be added on this point. First, it should be stressed that previous determinations of this stoichiometry using other methods were generally rather qualitative, and strongly biassed by the search for an integer pattern [5,8]. The better resolved data from Fowler [4], on the other hand, should be reexamined, taking into account the abnormally high $H^+/e^-$ overall stoichiometry found by this author (e.g., in the presence of methylviologen as a PS I acceptor). Using a similar approach to that of Saphon and Crofts [5] (difference in the response of an external dye in the absence and presence of a protonophore), Lavergne and Rappaport [15] obtained qualitative agreement with the reinterpreted NR data.

A non-integer proton release pattern was also reported for PS II reaction centre core preparations [24]. The influence of trypsination on the proton release stoichiometry in PS II membrane fragments and the absence of any pattern in some PS II preparations [25] imply an important role of the protein environment of the Mn-centre in the formation of the proton release pattern. It appears that the protolytic events, both, at the $Q_B$-site and at the Mn-centre of the water oxidation do not directly reflect the pairing of electrons and protons at the respective redox cofactor. This is very similar to the situation in bacterial reaction centres [26,27], where non-integer and pH-dependent proton release from the donor side and uptake on the acceptor side have been reported. The interpretation proposed in these papers was that the charge separated by the photochemical reaction cause electrostatic shifts of the p$K$ values of neighbouring protonable groups. When the shifted or unshifted p$K$of such a group falls within, say, one unit of the pH, its contribution to proton exchange will be non-integer. We suggest that such a process is occurring in the oxygen-evolving system, notable upon the $S_1$–$S_2$ transition. Correlative to this view is the prediction that the stoichiometry should be modulated by the ambient pH, affecting the protonation state of the involved groups. A marked pH dependence of the pattern of proton release from the oxygen-evolving system was indeed found as will be reported in a forthcoming paper (Rappaport and Lavergne, unpublished data).

Recently, LHC proteins were suspected to be involved in proton release from the Mn-centre into the thylakoid lumen [28,29]. The role of LHC proteins in the apparent proton release stoichiometry of water oxidation is under further investigation.

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