NEUTRAL RED, A RAPID INDICATOR FOR pH-CHANGES IN THE INNER PHASE OF THYLAKOIDS

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Received 30 September 1975

1. Introduction

During illumination the inner vesicles of chloroplasts (thylakoids) accumulate protons in their internal space. The electrochemical potential thus stored across the thylakoid membrane is very probably used to drive ATP synthesis (for a review, see [1]). Several indirect methods to determine the light induced pH-changes in the internal volume of thylakoids are documented in the literature [2–6]. They were mostly applied to monitor the internal pH under steady illumination. Although widely accepted in practice these methods are subject to methodical criticism (see [7] as to [2], and [8] as to [4–6]). Their common feature is a relatively low time resolution ($t_{1/2} \gg 50$ msec) for transient pH-changes e.g. following a short flash of light. A higher time resolution is desirable to elucidate the mechanism of proton release coupled to water oxidation and plastohydroquinone oxidation respectively, and to follow the kinetics of proton translocation across the ATP-synthetase. The latter kinetics cannot yet be resolved by pH-indicators located in the outer phase of thylakoids, due to a diffusion barrier for protons at the outer side of the membrane (see [9]).

Lynn [10] reported that neutral red, a nitrogenous pH-indicator with a pK of 6.8 [11], responds with spectral shifts which indicate a net acidification to illumination of chloroplasts. He suggested, that neutral red is an indicator for the internal acidification of thylakoids.

In this communication it is shown that Lynn observed a net response of neutral red, which distributes over the inner and the outer phase, to pH-changes in *both aqueous phases*. However, neutral red can be transformed into an indicator specific for pH-changes in the internal phase by selectively buffering away pH-changes in the outer phase by bovine serum albumin (BSA). BSA acts as a nonpenetrating buffer with a broad spectrum of pK values (broad band buffer). When applied together with BSA neutral red is a very sensitive indicator for the internal pH-changes with a response time of at least 0.3 msec. First studies on chloroplasts excited with a single-turnover-flash of light indicate two different kinetic phases of proton release into the inner phase, the more rapid one attributable to the water oxidizing system, the slower one to the oxidation of plastohydroquinone.

2. Experimental

The experiments were carried out with aqueous suspensions of broken chloroplasts. Spinach chloroplasts were prepared as in [12]. The chloroplasts were stored under liquid nitrogen in a medium (pH 8.0) containing: no buffer; sucrose, 0.4 M; NaCl, 10 mM; $MgCl_2$, 1 mM; and dimethylsulphoxide, 5%.

For each measurement thawed samples were suspended in a 20 mm absorption cell at an average chlorophyll concentration of 10 μM in the following standard reaction medium: KCl, 20 mM; $MgCl_2$, 2 mM; benzylviologen, 60 μM; nonactin, 0.3 μM. Further additions to the medium (neutral red, various buffers) are indicated in the figures and their legends.

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Photosynthesis was stimulated by excitation with a saturating single-turnover-flash (halftime 15 μS). Transient absorption changes were recorded with a rapid kinetic spectrophotometer. The signal-to-noise ratio was improved by averaging over several transients induced by repetitive flashes. For details and for specific references, please see our preceding paper [13].

3. Results and discussion

3.1. *A 'proton-release-space' detectable by absorption changes of neutral red*

The absorption changes of the protonated neutral red were measured near its peak wavelength (see fig.1)

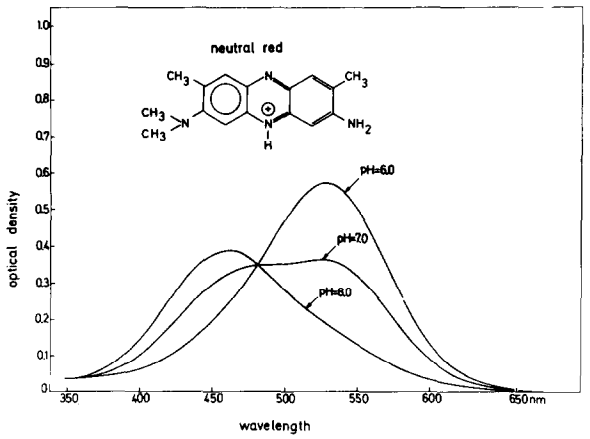

Fig.1. Absorption spectrum of neutral red (30 μM) in aqueous solution at different pH values (optical path length of the cuvette: 10 mm).

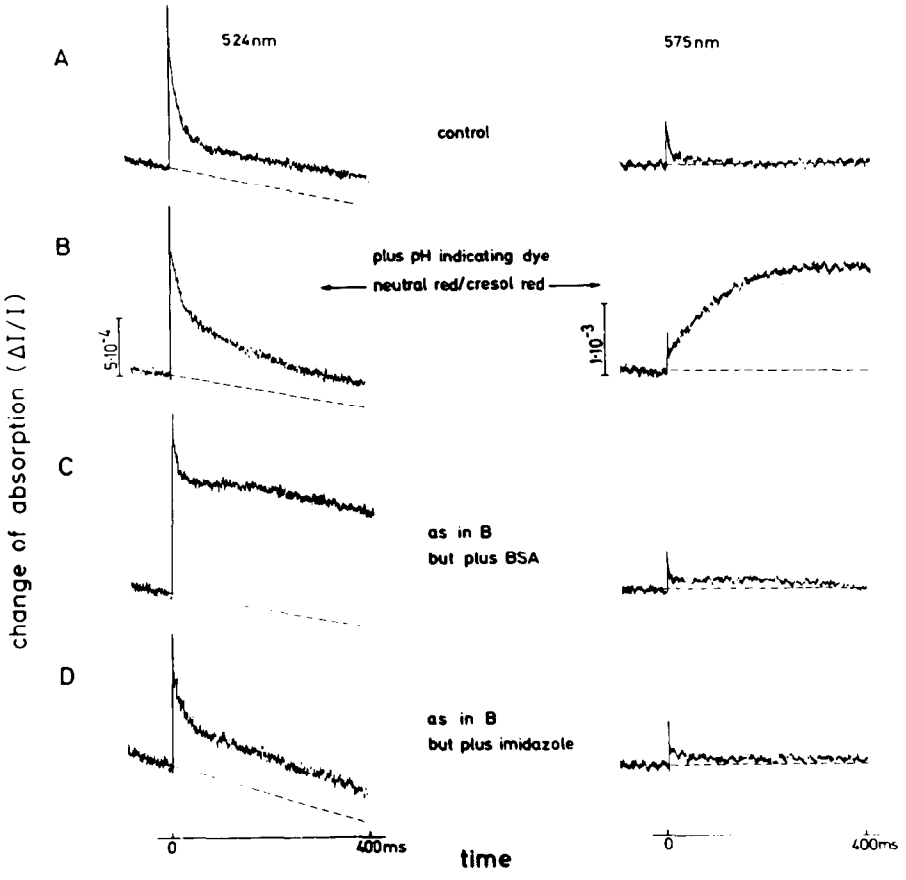

Fig.2. Absorption changes at 524 nm and 574 nm induced by a single-turnover-flash at t=0. (A) standard reaction medium (pH_{out} 7.2 (left) and 7.6 (right)). (B) as in (A) but plus pH-indicating dye (left: neutral red, 10 μM, right: cresol red, 30 μM). (C) as in (B) but plus BSA (1.3 mg/ml). (D) as in (C) but plus imidazole (1 mM). Signals averaged over 30 flashes, repetition rate 0.1 Hz.

at 524 nm. At this wavelength the absorption changes of neutral red are superimposed on the peak of the intrinsic electrochroic carotenoid changes [14,15]. To make the absorption changes of neutral red more obvious, the decay of the electrochroic absorption changes was greatly accelerated by addition of nonactin [16].

Fig.2 shows the response of two pH-indicating dyes to flash excitation of chloroplasts. The left column shows the absorption changes at 524 nm in the presence of the nitrogenous indicator neutral red, the right column absorption changes at 574 nm in the presence of the sulphonic indicator cresol red. The buffering conditions are varied from top to bottom. The background signals (without either indicator) are shown in fig.2A. Addition of the corresponding dyes to the unbuffered chloroplast suspensions produced modified signals which are displayed in fig.2B. In contrast to the deprotonation of cresol red indicated by a flash induced rise in absorption at 574 nm a net protonation of neutral red is obvious from the rise of absorption at 524 nm. The observed absorption changes of both dyes in fig.2B are composite from the respective response of dye molecules located in different environments (external-, internal phase, membrane). Addition of the macromolecular broad-band-buffer BSA caused dramatic changes in the signals at both wavelengths (fig.2C). While the reponse of cresol red at 574 nm is almost completely buffered away (right), the increase in absorption of neutral red at 524 nm is even more pronounced. If this increase indicates an acidification of some internal volume (inaccessible to BSA) it should be reversed by addition of a penetrating buffer, e.g. imidazole [17]. This was in fact observed, as obvious from fig.2D. In contrast to this, imidazole had no further effect on the absorption changes of cresol red at 574 nm. The total buffering capacities in the respective reaction mixtures (vol: 15 ml) in fig.2 were: (A) 0.3 μmol H^+/Δ pH (due to chloroplasts at 10 μM chlorophyll). (B) as in A. (C) 2.5 μmole H^+/Δ pH (mainly due to BSA, 1.3 mg/ml). (D) 11 μmol H^+/Δ pH (mainly due to imidazole, 1 mM).

The experimental evidence presented in fig.2 suggests that the nitrogenous indicator neutral red has access to the external as well as to an internal space of thylakoids, while the sulfonic indicator cresol red to the external space, only. We found that this distinction holds at least for the time range between 1 sec and 5 min after addition of the respective indicator to a chloroplast suspension.

In consequence, the absorption changes of neutral red which Lynn observed in the absence of extrinsic buffers [10] are a superposition of the respective response of the dye to the alkalinisation in the external and the acidification in the internal space. In contrast to this the absorption changes at 524 nm in the presence of BSA represent largely an acidification of an internal space as indicated by neutral red plus a smaller background which is observed after addition of imidazole at sufficiently high concentration (see fig.3C). If the latter signal (imidazole plus BSA) was subtracted from the former (BSA) in the averaging computer we use the term pH_i -indicating absorption

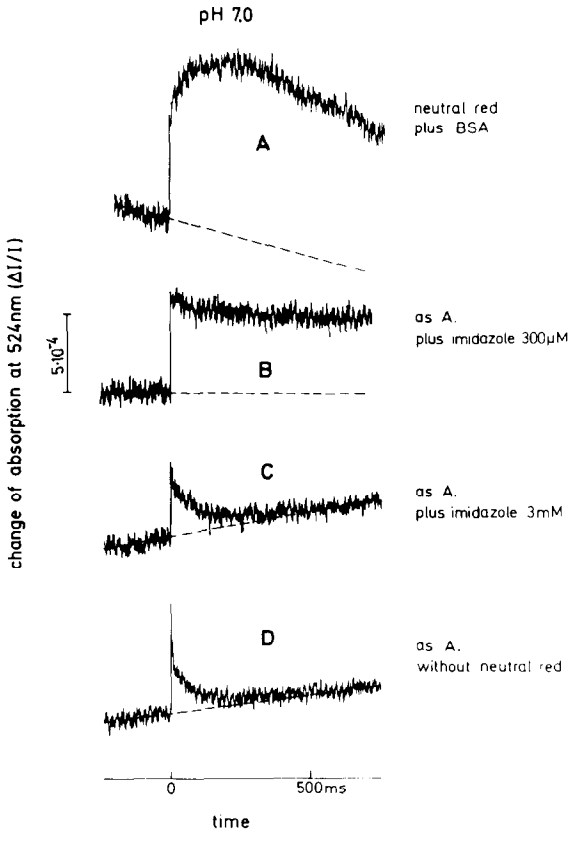

Fig.3. Dependence of the absorption changes of neutral red at 524 nm induced by a single-turnover-flash at t=0 on the concentration of imidazole. Reaction medium and repetition rate, as given in the left of fig.2(B) (deviations are indicated in the figure). Signals averaged over 10 flashes.

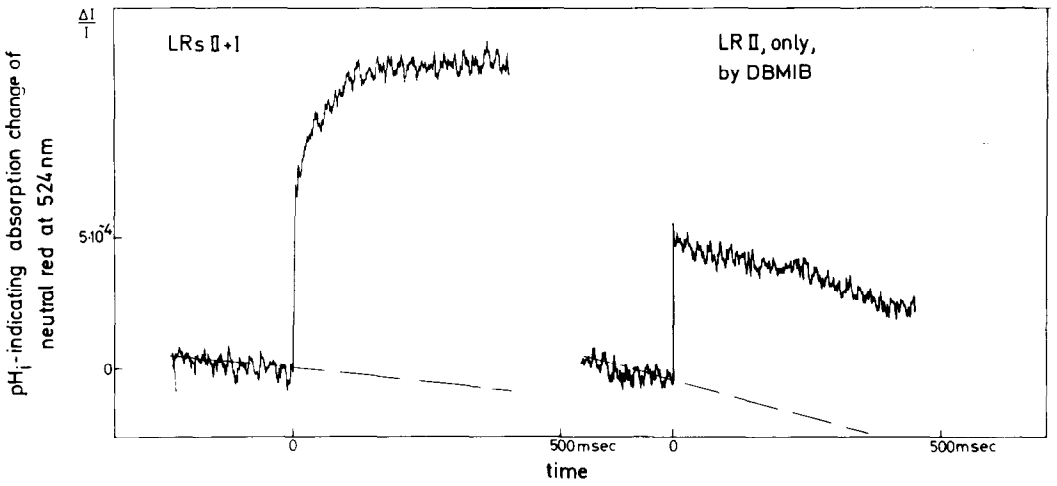

Fig.4. Biphasic kinetic of the proton release into the internal phase as indicated by neutral red. The signals shown in the figure represent a difference between two transient signals. At first the signal in the presence of BSA (1.3 mg/ml) plus neutral red (10 μM) was averaged (10 flashes) and stored. Then imidazole (3 mM) was added and the resulting signal was averaged (10 flashes) and then subtracted from the former by the built-in arithmetics of the averaging computer. (Left) standard reaction medium (pH$_{out}$ 7.2), repetition rate 0.1 Hz. (Right) as in the left, except for electron acceptor (ferricyanide 2 mM) and DBMIB (3 μM).

change of neutral red as indicated in the ordinates of fig.4 and fig.5.

This subtraction eliminates eventual contributions from redox-reactions of neutral red to the resulting difference signal. However, it is already obvious from comparison of fig.3C with fig.3D that eventual redox contributions to the signal at 524 nm are negligible. The same holds for eventual directly stimulated photochemical reactions of the indicator dye. Fig.3C shows the absorption change at 524 nm of a strongly buffered

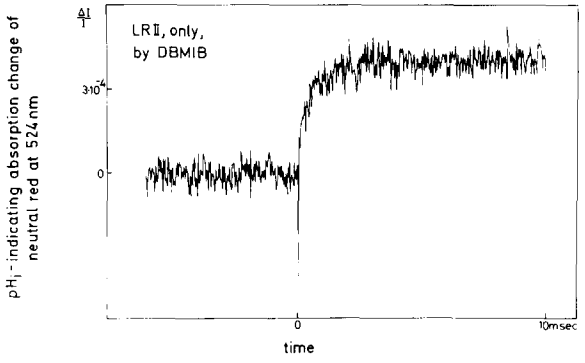

Fig.5. Proton release attributable to LR II at higher time resolution. Difference signal as indicated in fig.4. Reaction medium as in fig.4 (right). Average over 800 flashes, repetition rate 0.3 Hz. New samples were used after 200 flashes.

chloroplast suspension in the presence of neutral red, fig.3D the signal in the absence of neutral red. These signals are practically equal.

The precise location of the imidazole accessible, BSA inaccessible space, where neutral red records a light induced acidification is not known, yet. We have evidence that neutral red is bound or adsorbed at the inner side of the thylakoid membrane rather than just being soluble in the aqueous (or icy) internal phase. This will be evaluated in a subsequent paper. The same evidence indicates that the diffusion of neutral red driven by the light induced pH-difference does not markedly alter the signal amplitude, due to the fact that this diffusion does not appreciably change the total amount of dye in the BSA inaccessible inner space. Thus the indicator mechanism of neutral red is entirely different from the one of the diffusible acridine dyes [4–6].

3.2. *An estimate for the pH$_i$-change following a single-turnover-flash*

A rigorous calibration of the pH$_i$ - indicating absorption changes of neutral red necessitates information on the shape of the response curve of the internally located dye and on the internal buffering capacity. In this communication we will neglect non-

linearities resulting from the former and just give a semiquantitive estimate based on the latter. To evaluate the internal buffering capacity under the assumption of a linear response of the dye in terms of absorbancy over pH_i we measured the dependence of the absorption changes at 524 nm (plus BSA) on the concentration of imidazole (see fig.3). To approximate the linearity assumption best the experiments were carried out at an outer pH value of 7.0, closely above the pK of neutral red in water. At a concentration of imidazole of 300 μM the pH_i -indicating absorption change of neutral red were just halfed (compare fig.3B and fig.3A). This implies that the internal buffering capacity is doubled by addition of the given amount of imidazole, the buffer capacity of which is then just equal to the one of the intrinsic buffering groups. The buffering capacity of 1 litre 300 μM imidazole at a pH of 7.0 is 172.5 μmol H^+/Δ pH. We assume that imidazole does not bind to the membrane but just is soluble in the internal aqueous phase. This assumption is justified by the finding that an equivalent amount of other penetrating buffers e.g. tricine yielded the same signal reduction.

Our prior calibration of the protolytic reactions at both sides of the membrane yielded that two protons are released into the internal space per electron transport chain on excitation with a single-turnover-flash [13]. The internal volume per electron transport chain follows from the subsequent data: an electron transport chain comprises about 600 chlorophyll molecules [18], the internal volume of chloroplasts similar to ours is 50 l/mole chl [5,17]. From these data we conclude that the pH_i change on excitation of a single-turnover-flash is 0.33 pH units at pH 7.0.

This estimate involves assumptions as to the linearity of the indicator response at pH_{out} 7.0 and the internal volume. An evaluation and correction of these assumptions will be presented in a subsequent paper.

3.3. *Kinetics of proton release into the internal space*

In a preceding paper we presented evidence for two sites of proton release into the internal phase [13]. One of these was attributed to the oxidation of water by LR II* [13,19], the other one to the oxidation of plastohydroquinone by LR I [13]. A direct attribution of one site to the water oxidizing system was presented by Fowler and Kok [20].

We have furthermore shown that the kinetics of proton binding from the outer phase kinetically match the reduction of plastoquinone (except for some time lag) by LR II and of the terminal acceptor by LR I [9]. A similar kinetic comparison between protolytic reactions and redox reactions at the inner side of the membrane was desirable.

Fig.4 shows the pH_i -indicating absorption change neutral red under conditions where both light reactions were operative (left) and where only LR II released one proton in the presence of DBMIB (right). The deactivation of the proton release site associated with LR I by DBMIB (on the action of DBMIB, see [21]) is documented in the literature [19,22]. As obvious from fig.4, the kinetics of proton release is biphasic, the rapid phase being attributable to LR II, and thus according to Fowler and Kok [20] to the water oxidizing system. The halftimes come out as 20 msec and 0.3 msec, respectively. The rapid phase at higher time resolution is documented in fig.5. The observed kinetic constants for the internal proton release are in good agreement with the kinetic constants for the water oxidation by LR II [23,24] and plastohydroquinone oxidation by LR I [25] under flash excitation conditions.

It has to be mentioned, that a 1:1 ratio between the fast and the slower phase of proton release was not always observed. Usually the ratio was smaller, in about 30% of the chloroplast preparations no fast phase was observed at all. We did not yet detect the parameters of growth, preparation or storage determining this reasonable varation in the results. The absence of a fast proton release in some preparations might imply that the water-proton is not always directly released into the same space as the plastohydroquinone one. This item is subject to further studies.

4. Conclusion

It is shown that a pH-indicating dye with sulphonic groups (cresol red) does not penetrate the thylakoid membrane, while a nitrogenous one (neutral red) does. The latter dye together with the non-penetrating

Abbreviations: DBMIB, dibromothymoquinone. LR I, light reaction I. LR II, light reaction II.

macromolecular buffer BSA, can be used to obtain rapic read-out of pH-transients in some internal phase of thylakoids. This method should be applicable to pH-measurements in the submicroscopic volumes of mitochondria and retinal rods as well.

It is shown that the pH rise in the internal phase of thylakoids which follows excitation with a single-turnover-flash is in the order of 0.3–0.4 pH units. Two distinct kinetic phases of proton release are identified, a rapid one (half time 0.3 msec) attributable to the oxidation of water, and a slower one (half-time 20 msec) attributable to the oxidation of plastohydroquinone.

These results together with those from our prior studies [9,13,19,26] give almost conclusive evidence that the protolytic reactions at both sides of the thylakoid membrane are a direct consequence of a vectorial electron transport system as postulated by Mitchell [27].

Acknowledgements

We wish to thank Mrs Columbus for technical assistance. This work has been supported by a grant form the Deutsche Forschungsgemeinschaft.

References

[1] Jagendorf, A. T. (1975) in: Bioenergetics of Photosynthesis (Govindjee, R., ed.), pp. 414–494, Academic Press, New York.
[2] Rumberg, B. and Siggel, U. (1969) Naturwissenschaften 56, 130–132.
[3] Rottenberg, H., Grunwald, T. and Avron, M. (1971) FEBS Lett. 13, 41–44.
[4] Rottenberg, H., Grunwald, T. and Avron, M. (1972) Eur. J. Biochem. 25, 54–63.
[5] Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.
[6] Schuldiner, S. and Avron, M. (1971) FEBS Lett. 14, 233–236.
[7] Bamberger, H. S., Rottenberg, H. and Avron, M. (1973) Eur. J. Biochem. 34, 557–563.
[8] Fiolet, J. W., Bakker, E. P. and van Dam, K. (1974) Biochim. Biophys. Acta 368, 432–445.
[9] Ausländer, W. and Junge, W. (1974) Biochim. Biophys. Acta 357, 285–298.
[10] Lynn, W. S. (1968) Journal of Biol. Chem. 243, 1060–1064.
[11] Bishop, E. (1972) Indicators, p. 473, Pergamon Press, Braunschweig.
[12] Siggel, U., Renger, G., Stiehl, H. H. and Rumberg, B. (1972) Biochim. Biophys. Acta 256, 328–335.
[13] Junge, W. and Ausländer, W. (1974) Biochim. Biophys. Acta 333, 59–70.
[14] Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 244–254.
[15] Emrich, H. M., Junge, W. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1144–1146.
[16] Schmid, R. and Junge, W. (1975) Biochim. Biophys. Acta 394, 76–92.
[17] Reinwald, E. (1970) Thesis, TU Berlin.
[18] Boardman, N. K. (1968b) Advan. Enzymol. 30, 1–79.
[19] Ausländer, W., Heathcote, P. and Junge, W. (1974) FEBS Lett. 47, 229–235.
[20] Fowler, Ch. F. and Kok, B. (1974) Biochim. Biophys. Acta 357, 299–307.
[21] Trebst, A. and Reimer, S. (1973) Biochim. Biophys. Acta 305, 129–139.
[22] Gould, J. M. and Izawa, S. (1974) Biochim. Biophys. Acta 333, 509–524.
[23] Joliot, P. (1966) in: Energy conversion by the photosynthetic apparatus pp. 418–433, Brookhaven Symp. Biol. 19.
[24] Vater, J., Renger, G., Stiehl, H. H. and Witt, H. T. (1968) Naturwissenschaften 55, 220–221.
[25] Stiehl, H. H. and Witt, H. T. (1969) Z. Naturforsch. 24b, 1588–1599.
[26] Schliephake, W., Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 1571–1578.
[27] Mitchell, P. (1966) Biol. Rev. 41, 445–502.