THE pH INDICATOR PHENOL RED AS AN ARTIFICIAL ELECTRON ACCEPTOR IN SPINACH CHLOROPLASTS

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1. Introduction

The use of the glass electrode for the measurement of changes in pH is limited by its relatively slow response; time resolution of processes with a $t_{1/2}$ down to about 200 msec is possible with the glass electrode. The kinetic analysis of more rapid pH changes, however, is not possible in this way.

Several pH indicators have been used for the study of fast proton movements in energized mitochondria and chloroplasts; absorption indicators such as bromothymol blue (BTB), cresol red, phenol red and bromocresol purple [1–7], and also the fluorescent pH indicator umbelliferon [8] have been used. The significance of absorption changes of BTB has been discussed previously [7,10].

Phenol red has been used in flash experiments [6] as well as under conditions of steady-state illumination in chloroplasts [5]. From the latter experiments a stoichiometry of 3 was calculated between proton flux via the ATPase and ATP synthesis.

In this paper we will demonstrate that phenol red is able to support electron transport and photophosphorylation in chloroplasts. Furthermore, it will be demonstrated that phenol red is bound reversibly to the chloroplasts in the light and finally that the H^+/ATP ratio determined with the glass electrode is consistently lower than that determined with phenol red if measured under identical conditions.

2. Materials and methods

Chloroplasts were prepared as described previously [11]. pH changes and fluorescence of 9-aminoacridine were measured simultaneously in an instrumental set-up as described previously [12]. The response time of the pH-measuring system was characterized by a $t_{1/2}$ of about 0.2 sec upon addition of small aliquots of standard 0.01 N oxalic acid for calibration. The response time of the system to light-induced changes is probably even shorter, because most likely the mixing time was the limiting factor.

Electron transport in the presence of ferricyanide as acceptor was measured as acidification of the medium. Phosphorylation was measured as alkalinization of the medium.

The fluorescence of 9-aminoacridine was excited at 400 nm by a Zeiss monochromator and detected with an RCA photomultiplier (IP28) shielded with 2 Corning filters (9782).

Experiments with phenol red were carried out in an Aminco-Chance Double-Beam spectrophotometer (DW2) in which the photomultiplier was protected against the actinic light by a Corning (9782) glass filter. The binding of phenol red was determined by filtering a chloroplast suspension through a millipore filter in the presence of phenol red in the dark and the light, respectively, and measuring the absorption at 553 nm of the filtrate.

3. Results and discussion

Table 1 summarizes the characteristics of the light-induced proton uptake under phosphorylating and non-phosphorylating conditions measured either by absorption changes of phenol red or with the glass electrode in the presence and absence of 10 μM

Table 1
The H^+/ATP ratio measured either optically with phenol red or with the glass-electrode

Additions	Method of measurement	Proton uptake neq. H^+	ATP synthesis neq. H^+/min	H^+/ATP
None	Electrode	29		1.8
ADP (200 μM)	Electrode	16	50	
Pyocyanine (10 μM)	Electrode	31.5		1.7
Pyocyanine (10 μM) + ADP (200 μM)	Electrode	19.5	85	
None	$\triangle A$ (550–500 nm)	17.5		3.2
ADP (200 μM)	$\triangle A$ (550–500 nm)	9.5	35	
Pyocyanine (10 μM)	$\triangle A$ (550–500 nm)	19.5		1.8
Pyocyanine (10 μM) + ADP (200 μM)	$\triangle A$ (550–500 nm)	13	60	

Incubation medium: 10 mM KCl, 1 mM $MgCl_2$, 1 mM NaP_i, 0.2 mM imidazol, 25 μM anthraquinone, 10^{-7} M valinomycin, 17 μM phenol red, 20 μM ATP and 40 μg chlorophyll at a pH of 8.0. Other additions as indicated in the table. The temperature was 20°C. The final volume was 2.5 ml. H^+/ATP ratios were calculated essentially as described previously [5].

pyocyanine. Calculation of the H^+/ATP ratio was carried out according to Schröder et al. [5]. The discrepancy in the values found between the two methods is apparent. The extent of the H^+ uptake when measured optically is consistently lower than when measured with the glass electrode. This discrepancy might be explained by the fact that phenol red is bound reversibly to the chloroplasts in a light-dependent way, thereby changing its pK_a (cf. ref. 7). Light-induced reversible binding was found indeed: the percentage of phenol red bound in the light increases with increasing chlorophyll concentration as shown in table 2.

It is of importance to note that the value found for the H^+/ATP ratio is equal to that found by Schröder et al. [5] only in the absence of pyocyanine and when the proton movements are measured optically. In other cases this value is found to be less than 2. Moreover, the omission of anthraquinone from the medium did not change the results in any respect, suggesting that anthraquinone does not act as electron acceptor. This leaves the conclusion that phenol red itself is a redox component in illuminated chloroplasts under these conditions.

To test the possible electron-transport function of phenol red the experiment shown in fig.1 was carried out. Energization of the chloroplasts was monitored

Table 2
Light-induced reversible binding of phenol red

Chlorophyll content (μg)	Uptake (%)		
	In the dark	During illumination	In the dark after illumination
25	5	7	5
50	6	16.5	8.5
75	6	25.5	11
100	7	31	14.5

The incubation medium contained in a volume of 1.5 ml 50 mM NaCl, 50 mM KCl, 5mM $MgCl_2$, 2.5 mM KP_i, 10 mM tricine (pH= 8.1), 17 μM phenol red and chlorophyll as indicated in the table. Uptake is expressed as percentage of added phenol red.

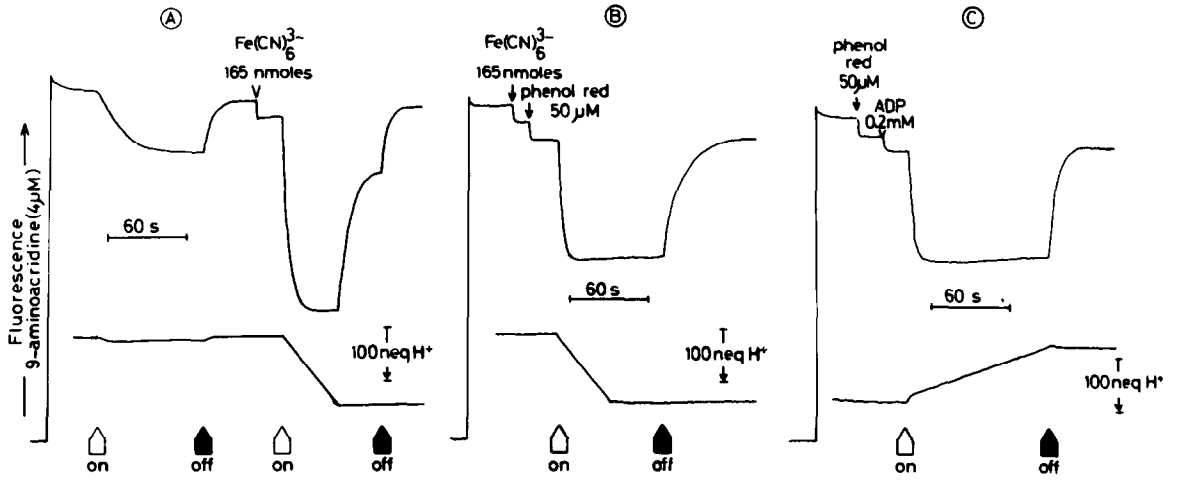

Fig.1. Phenol red as an electron acceptor in illuminated chloroplasts. Incubation medium: 100 mM sucrose, 10 mM KCl, 10 mM NaCl, 3 mM $MgCl_2$, 3 mM KP_i, 10 mM tricine (pH 8.0), 50 μg chlorophyll, in a final volume of 2.5 ml. Other additions as indicated in the figure.

by the light-induced fluorescence quenching of 9-aminoacridine [14,11]. As shown in trace A the chloroplasts are slightly energized upon illumination due to endogenous cyclic electron transport. Upon addition of a low concentration of ferricyanide the fluorescence of 9-aminoacridine is quenched more extensively, concomitant with an acidification of the medium. After complete reduction of the ferricyanide non-cyclic electron transport ceases as shown by the pH trace and the resulting increase in the fluorescence of 9-aminoacridine. In trace B the same experiment is carried out in the presence of 50 μM phenol red. After complete reduction of the ferricyanide acidification of the medium ceases. However, in this case no increase in the 9-aminoacridine fluorescence is observed, indicating that energization still occurs. These observations, combined with the fact that the energization continues for a prolonged period in the presence of 50 μM phenol red indicate that electron transport of a cyclic or pseudocyclic nature is going on. In trace C it is shown that chloroplasts in the presence of phenol red can perform ATP synthesis as well.

Fig. 2A shows the time course of absorption changes of phenol red in the presence and absence of pyocyanine. In the absence of pyocyanine, after an increase in absorption upon illumination a continuous decrease in absorption is found, which is not reversed upon turning off the light. This might indicate that in the redox reactions of phenol red an irreversible step occurs. It may be noted that the absorption band of phenol red at 554 nm completely disappears after

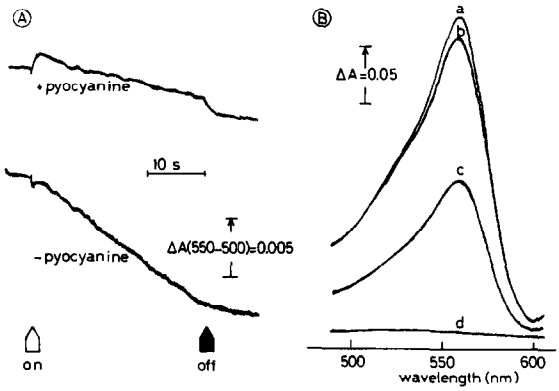

Fig.2. (A) Time course of phenol red reduction in the presence and absence of pyocyanine. Incubation medium: 50 mM NaCl, 50 mM KCl, 3 mM $MgCl_2$, 2 mM KP_i, 1 mM tricine, 15 μM phenol red, 90 μg chlorophyll, in a total volume of 3 ml. (B) Absorption spectra of phenol red. Reaction medium and conditions as in fig.2A, except that 30 mM tricine was present. In the reference cuvette phenol red was absent. a, Absorption spectrum of phenol red in the presence of chloroplasts in the dark; b, idem after 3 min incubation in the light in the presence of pyocyanine; c, idem as in b, but pyocyanine absent; d, base-line and absorption spectrum after addition of dithionite.

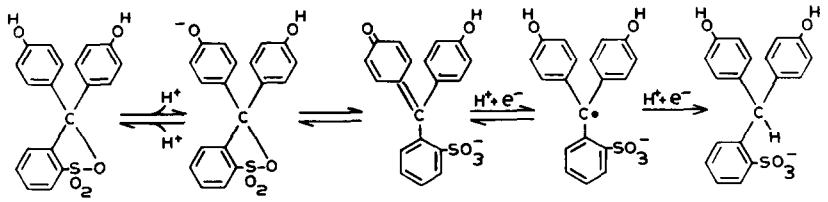

Fig.3. The mechanism of phenol red reduction according to [13].

reduction with dithionite as shown in fig.2B. In the presence of pyocyanine this absorption decrease in the light is much less.

The results might be explained by a mechanism as shown in fig.3 [13]. In this mechanism two separate reduction steps occur, of which the second step is essentially irreversible. The first reduction step is a reversible one. According to this mechanism phenol red is a proton and electron acceptor, which accounts for the absence of pH-changes during phenol red supported electron transport. Apparently, both pyocyanine and ferricyanide are preferentially used by chloroplasts.

Finally, fig.4 shows the sensitivity to DCMU of phosphorylation supported by electron transport with ferricyanide and phenol red, respectively. It appears that the DCMU sensitivity is similar. This indicates

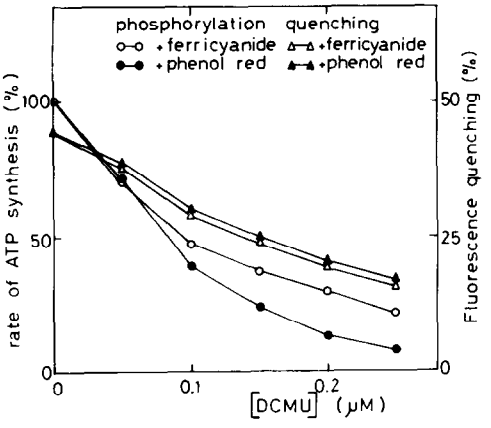

Fig.4. DCMU sensitivity of phenol red supported phosphorylation. The incubation medium contained 50 mM NaCl, 50 mM KCl, 3 mM $MgCl_2$, 2 mM KP_i, 10 mM tricine (pH 8.0), 1 mM ADP, and in addition 0.1 mM $Fe(CN)_6^{3-}$ or 30 μM phenol red as electron acceptor. The chlorophyll content was 53 μg in a final volume of 2.5 ml.

that the phenol red mediated electron transport is most probably pseudocyclic in nature.

It is concluded that phenol red is able to support electron transport in illuminated chloroplasts, especially in the absence of other artificial non-cyclic electron acceptors. The electron transport mediated by phenol red is most probably of a pseudocyclic nature. Absorption changes of phenol red upon illumination may result from pH-changes in the medium, from reversible binding to the chloroplasts and from redox changes. Therefore, H^+/ATP ratios determined by means of absorption changes of phenol red may be used only after correction, especially if determined in the absence of other artificial electron acceptors.

References

[1] Chance, B. and Mela, L. (1966) J. Biol. Chem. 241, 4588–4599.
[2] Schliephake, W., Junge, W. and Witt, H. T. (1968) Z. Naturforsch. 23b, 1571–1578.
[3] Junge, W. and Ausländer, W. (1973) Biochim. Biophys. Acta 333, 59–70.
[4] Ausländer, W. and Junge, W. (1974) Biochim. Biophys. Acta 357, 285–298.
[5] Schröder, H., Muhle, H. and Rumberg, B. (1972) in: Photosynthesis, Two Centuries After Its Discovery by Joseph Priestley (Forti, G., Avron, M. and Melandri, A., eds), Vol. 2, pp. 919–930, Junk, The Hague.
[6] Evans, E. H. and Crofts, A. R. (1973) Biochim. Biophys. Acta 292, 130–139.
[7] Jackson, J. B. and Crofts, A. R. (1969) Eur. J. Biochem. 10, 226–237.
[8] Grünhagen, H. H. and Witt, H. T. (1970) Z. Naturforsch. 25b, 373–386.
[9] Witt, H. T. (1971) Quarterly Reviews of Biophysics 4, 365–477.
[10] Mitchell, P., Moyle, J. and Smith, L. (1968) Europ. J. Biochem. 4, 9–19.

[11] Fiolet, J. W. T. and Van Dam, K. (1973) Biochim. Biophys. Acta 325, 230–239.
[12] Fiolet, J. W. T., Bakker, E. P. and Van Dam, K. (1974) Biochim. Biophys. Acta 368, 432–445.
[13] Zychiewicz–Zajdel, Z. (1962) Ann. Univ. Mariae Curie Sklodowska, Lublin-Polonia, Sect. AA, 17, 103–113.
[14] Schuldiner, S., Rottenberg, H. and Avron, M. (1972) Eur. J. Biochem. 25, 64–70.