RELATIONSHIP BETWEEN THE REDOX-STATE OF *P*-700 AND PHOTOSYSTEM I-MEDIATED PROTON TRANSLOCATION STUDIED WITH CHLOROPLASTS FROM DARK-GROWN *PINUS NIGRA* SEEDLINGS

E. TYSZKIEWICZ, R. POPOVIC* and E. ROUX

Service de Biophysique, Département de Biologie Centre d'Etudes Nucléaires de Saclay, BP 2, 91190 Gif-sur-Yvette, France

Received 6 June 1977

1. Introduction

It is well known that in illuminated chloroplasts proton uptake is coupled with electron migration along the electron transfer chain [1].

In a short note (2) describing the effects of low temperatures on the formation and conservation of the high energy state (Xe) appearing in spinach chloroplasts during the light step of the two stage phosphorylation, we have also pointed out the parallelism between electron migration and proton uptake. In this previous work the site of photo-induced proton uptake could not be localized or connected to the redox state of a specific component of the electron transfer chain because the simultaneous electron flow from Photosystem II to *P*-700 and from *P*-700 to ferredoxin prevented such a determination, even in the presence of inhibitors such as dichlorophenyldimethylurea which only decrease the amount and the rate of the proton translocation [3].

In order to discriminate between the events which are linked to the electron migration from Photosystem II to *P*-700 (and result in the reduction of *P*-700) and those which are linked to the photo-oxidation of *P*-700 and to the migration of electrons from *P*-700 to ferredoxin, we have worked with chloroplasts isolated from dark grown *Pinus nigra* seedlings.

These chloroplasts are unable to evolve oxygen and to reduce dichlorophenol-indophenol when they are illuminated [4,5] because of an incomplete functional organization of the oxidizing side of Photosystem II. On the other hand, photoreactions associated with Photosystem I, such as the photo-oxidation of reduced cytochrome *c* in the presence of 2,4-dichlorophenyldimethylurea, are operative. Thus, we have found, by utilizing the method described in [6] that the rate of cytochrome *c* oxidation is 3.34 μmol cyt *c* oxid $\times$ min^{-1} $\times$ μmol Chl^{-1} in chloroplasts isolated from dark-grown seedlings. Chloroplasts isolated from light-grown seedlings exhibited a slightly higher oxidation rate (4.3 μmol cyt *c* oxid $\times$ min^{-1} $\times$ μmol Chl^{-1}).

Thus, in chloroplasts isolated from dark grown *Pinus nigra* seedlings, as a result of illumination, Photosystem II (in the absence of any Photosystem II-specific electron donor like diphenylcarbazide) cannot supply Photosystem I with electrons; consequently the redox state of *P*-700 becomes directly dependent on the redox potential of the chloroplast suspension. More precisely, the reduction of photo-oxidized *P*-700 occurs in darkness only when this redox potential is lower than the E_0 of *P*-700 (0.43 V; pH independent) [7]. On the other hand, we have found that the photoinduced proton uptake is reversible in darkness only when the redox potential of the chloroplast suspension is lower than the E_0 of *P*-700. In this paper, from the result that the proton translocation has the same redox potential dependence as the redox state of *P*-700 we conclude that: (1) Proton uptake is linked to the photooxidation of *P*-700. (2) Proton release is linked to the reduction of *P*-700. (3) The site of proton translocation is localized on the reducing side of *P*-700 between *P*-700 and ferredoxin.

* INEP Belgrade (Yugoslavia)

2. Material and methods

Eighteen-day-old *Pinus nigra* seedlings, germinated and grown in darkness at 20°C on vermiculite soaked with water, were harvested and chloroplasts isolated from the cotyledons at 4°C under dim green light following the method described in [8] except that no ascorbic acid was added to the extraction medium. Then, the chloroplasts were washed in 0.1 M KCl, 0.2 M saccharose and resuspended in the same medium.

The chlorophyll concentration was measured according to Arnon [9].

The chloroplast suspension was illuminated by red light (694 nm: Photosystem II and Photosystem I were both excited) in a cuvette (0.1 cm light path) maintained at 4°C. The cuvette was oriented at 45° to the excitation and analysis beams, the latter being used to measure the pH of the suspension according to the method of Chance and Scarpa [10]. The absorption change of Bromo Cresol purple (588–490 nm) was followed in a double beam Aminco DW2 spectrophotometer fitted with a BG 18 3 mm thick Schott filter in front of the photomultiplier tube.

3. Results

The absorption of visible light by an unbuffered suspension of chloroplasts isolated from light grown *Pinus nigra* seedlings induced an increase of the initial

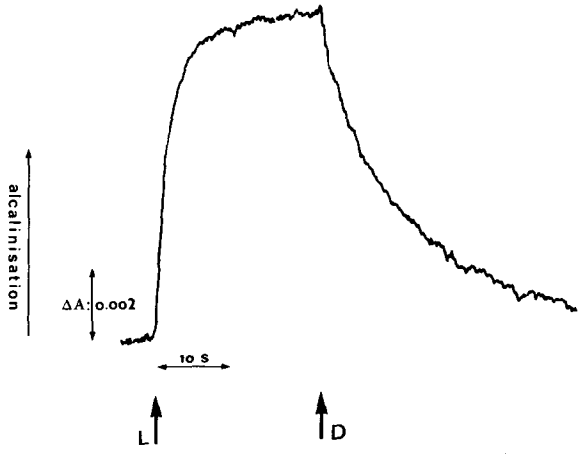

Fig.1. Reversibility in darkness of the light-induced proton uptake in chloroplasts isolated from cotyledons of light-grown *Pinus nigra* seedlings.

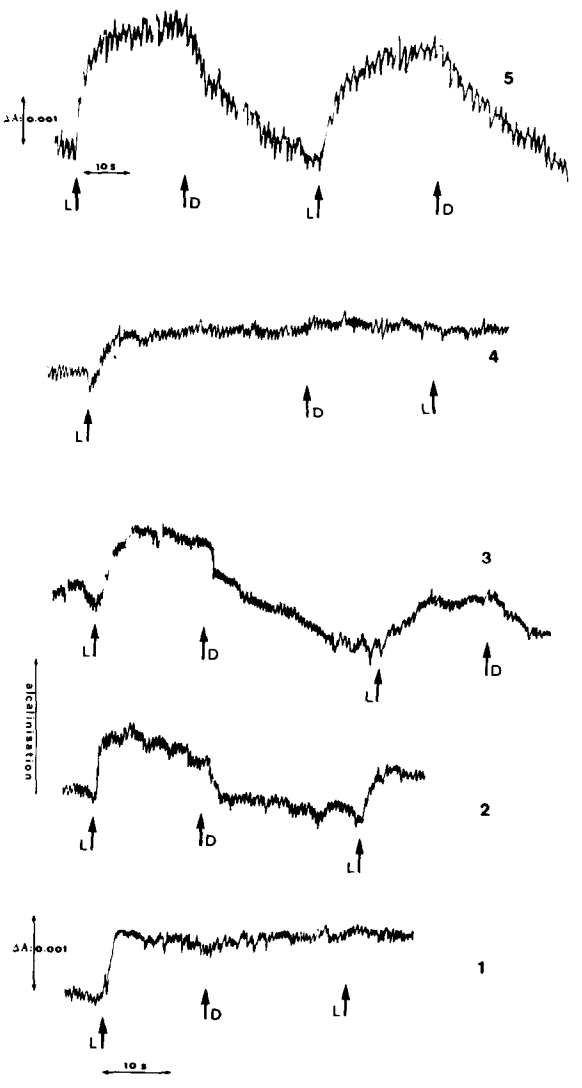

Fig.2. Photoinduced pH-variations in a suspension of chloroplasts from dark grown *Pinus nigra:* the 588–490 nm absorption change was measured. Composition of the suspension medium: saccharose 0.8 M, sodium chloride 0.03 M, bromocresol purple 1.5×10^{-5} M. (1) + chloroplasts (100 μg chlorophyll (a + b)/ml) (2) + chloroplasts + sodium asorbate 10^{-5} M (3) + chloroplasts + diphenyl carbazide 5×10^{-4} M (4) + chloroplasts + diphenyl carbazide 5×10^{-4} M + 3(3,4 dichlorophenyl) 1×1 dimethylurea 5×10^{-6} M (5) + chloroplasts + reduced 2,6 dichlorophenol-indophenol 3×10^{-5} M. (L) light. (D) dark. Scale for time and scale for absorbance are the same for (1), (2), (3), (4). Scales are different for (5) as shown on the graph. Relationship between change in pH and proton translocation was established by titration with 10^{-4} M NaOH.

value of the pH of the suspension (which was equal to 6). At this initial pH value the proton uptake by the chloroplasts was 80×10^{-9} mol protons/mg chlorophyll; this pH variation was completely reversible: in darkness the pH decreased to the initial value (fig.1).

Under the same conditions the light-induced proton uptake by chloroplasts isolated from dark grown *Pinus nigra* cotyledons was ten times less (8×10^{-9} mol protons/mg chlorophyll) but, in contrast to the normal chloroplasts, when the light was off, the pH of the suspension remained constant: dark grown chloroplasts did not release in darkness the protons fixed in light. A second illumination did not induce any additional increase in pH (fig.2/1).

This significant difference between the two types of chloroplasts was used to demonstrate that the release of protons in darkness is connected with the reduction of photooxidized *P*-700.

In darkness the light-induced proton fixation proved to be reversible in the presence of ascorbate (fig.2/2) or diphenylcarbazide, a Photosystem II specific electron donor (fig.2/3). Dichlorophenyldimethylurea suppresses the effect of diphenyl carbazide (fig.2/4).

In the presence of reduced dichlorophenol-indophenol, the light induced proton uptake by chloroplasts isolated from cotyledons of dark-grown *Pinus nigra* seedlings was found to be three times greater than in the absence of reduced dichlorophenol-indophenol at 694 nm as well as at 710 nm (25×10^{-9} mol protons/mg chlorophyll) and proved to be reversible in darkness (fig.2/5).

4. Discussion

The results obtained with chloroplasts isolated from cotyledons of dark-grown *Pinus nigra* seedlings show that:

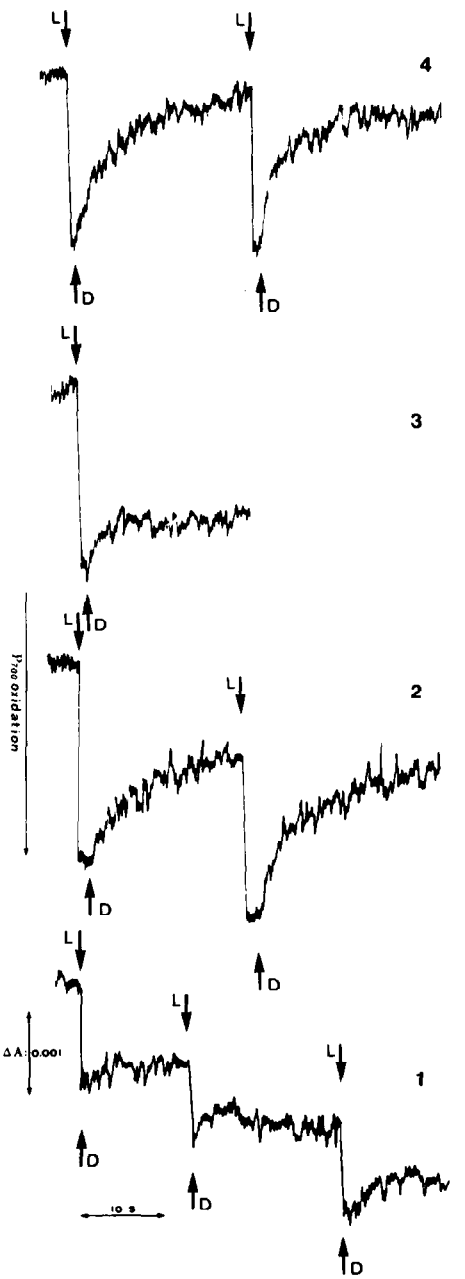

Fig.3. Photoinduced variations of the redox state of *P*-700 in chloroplasts of dark grown *Pinus nigra* seedlings. The 700–740 nm absorption change was measured. Composition of the medium: saccharose 0.8 M, magnesium chloride 10^{-2} M, methyl viologen 2×10^{-7} M (methyl viologen was added to permit the measurement of photooxidized *P*-700). (1) + chloroplasts (22 μg chlorophyll (a + b)/ml). (2) + chloroplasts + diphenyl carbazide 5×10^{-4} M. (3) + chloroplasts + diphenyl carbazide + 3(3,4-dichlorophenyl)1,1-dimethylurea 5×10^{-6} M. (4) + chloroplasts + reduced 2,6-dichlorophenol indophenol 3×10^{-5} M.

(1) The light-induced proton uptake does not depend on the redox state of the primary acceptor of Photosystem II. When chloroplasts were illuminated in the presence of diphenyl carbazide (which permits the charge separation at the level of the trap of Photosystem II and consequently the reduction of the primary acceptor of Photosystem II) + dichlorophenyldimethylurea (which blocks the transfer of electrons between the two photosystems) the primary acceptor of Photosystem II is reduced and remains in the reduced state.

When chloroplasts of the same type were illuminated in the absence of these two compounds, Photosystem II not being functional, its primary acceptor, already oxidized in darkness, remains in the oxidized state. In both cases we observed: (a) The same slight photooxidation of *P*-700, and (b) The same slight photoinduced proton uptake.

(2) When electrons cannot migrate between the two photosystems, as under the two conditions mentioned above: (a) *P*-700 remains in the oxidized state in darkness, and (b) There is no proton release in darkness.

(3) The site of Photosystem I-mediated proton uptake is localized on the reducing side of Photosystem I: when no electron migrates between Photosystem II and *P*-700, the proton uptake, being the direct consequence of the photooxidation of *P*-700, must occur on some negatively charged site(s) on the reducing side of *P*-700 where protons remain fixed as long as *P*-700 remains oxidized.

(4) The proton release takes place in darkness only if the photooxidized *P*-700 is reduced. This reduction is obtained either directly or is mediated by cytochrome *f* or plastocyanin, when the chloroplasts are illuminated in the presence of ascorbate, or diphenyl carbazide or reduced dichlorophenol indophenol.

In normal chloroplasts isolated from light-grown plants this reduction is achieved by electrons derived from photosystem II: in white light the photooxidation of *P*-700 is more rapid than the reduction of oxidized *P*-700 and consequently illumination increases the steady state of the ratio:

$$\frac{\text{Number of photosynthetic units in which } P\text{-}700 \text{ is oxidized}}{\text{Number of photosynthetic units in which } P\text{-}700 \text{ is reduced}}$$

leading to proton uptake, proportional to this ratio. In darkness this ratio approaches zero, and a corresponding release of protons takes place.

Chloroplasts isolated from cotyledons of dark-grown *Pinus nigra* seedlings permit one to discriminate more easily between the events related to *P*-700 reduction and those related to *P*-700 oxidation. Thus, we succeeded in showing that the redox state of *P*-700 governs the Photosystem I-mediated proton translocation.

The hypothesis can be put forward that the molecular arrangement in the very neighbourhood of *P*-700 can exist in two conformations in each photosynthetic unit:

(1) A proton accepting one which is formed during the oxidation of *P*-700.

(2) A proton releasing one which is formed during the reduction of *P*-700.

References

[1] Dilley, R. A. (1971) in: Current topics in Bioenergetics, (Rao-Sanadi, D., ed), Vol. 4, pp. 237–271, Academic Press, New York.
[2] Tyszkiewicz, E. and Roux, E. (1975) Biophys. Biochem. Res. Comm. 65 (4), 1400–1408.
[3] Neumann, J. and Jagendorf, A. T. (1964) Arch. Biochem. Biophys. 107, 109–119.
[4] Farineau, J. and Popovic, R. (1975) Comptes rend. Acad. Sci. Paris, sér. D 281, 1317–1321.
[5] Inoue, Y., Furata, S., Oku, T. and Shibata, K. (1976) Biochim. Biophys. Acta. 449, 357–367.
[6] Plesničar, M., Bendall, D. S. (1970) Biochim. Biophys. Acta 216, 192–199.
[7] Kok, B. and Hoch, G. (1962) in: La Photosynthèse, 94–105, CNRS ed. Paris.
[8] Oku, T., Hayashi, H. and Tomita, G. (1975) Plant Cell Physiol. 15, 175–178.
[9] Arnon, D. I. (1949) Plant. Cell Physiol. 24, 1–15.
[10] Chance, B. and Scarpa, A. (1972) in: Methods in Enzymology (Colowick, S. P. and Kaplan, N. O., eds) Vol. 24, Part B, pp. 336–342, Academic Press, New York.