ABSORPTION CHANGES FROM 437 nm TO 530 nm IN *CHLORELLA PYRENOIDOSA* UNDER FLASH EXCITATION

Probable detection of ferredoxin–NADP-reductase

Bernadette BOUGES-BOCQUET

Institut de Biologie Physico-Chimique, 13 Rue Pierre et Marie Curie, 75005 Paris, France

Received 23 November 1977

1. Introduction

Four different electron acceptors of photosystem I have been observed:

1. X_{430}, shown to be the primary acceptor [1], has $\Delta A_{430\ max}$ and estimated redox potential −500 mV [1].
2. *Ferredoxin,* located in the electron transfer chain [2], has a large $\Delta A_{400-500\ nm}$ and redox potential −430 mV [2].
3. *Ferredoxin–NADP-reductase (FNR),* discovered and localized in the electron transfer chain [3,4], a flavoprotein accepting two electrons [5]. The absorption spectra of the three redox states are presented in fig.1a, drawn from [6]. The half-reduced form, FNR, is obtained by reduction with $NADPH_2$ [5] and the totally reduced form, FNR, by reduction with stronger reductants. The oxidized form exhibits a broad $A_{456\ max}$, the half-reduced form shows a continuous $A_{400-650}$, and the reduced form a decreasing $A_{400-550}$. A redox potential approx. −380 mV was observed [7].
4. *NADP,* shown to be the terminal acceptor of the electron transfer chain [8–10], presents no absorption in the visible range and a redox potential of −320 mV.

No information has been reported on the rate of the reactions of these components. The spectra which have been quoted above indicate that spectroscopy between 400 nm and 550 nm could be a good method to study the kinetics of the acceptors of photosystem I. Some new results obtained by this method are presented in this paper.

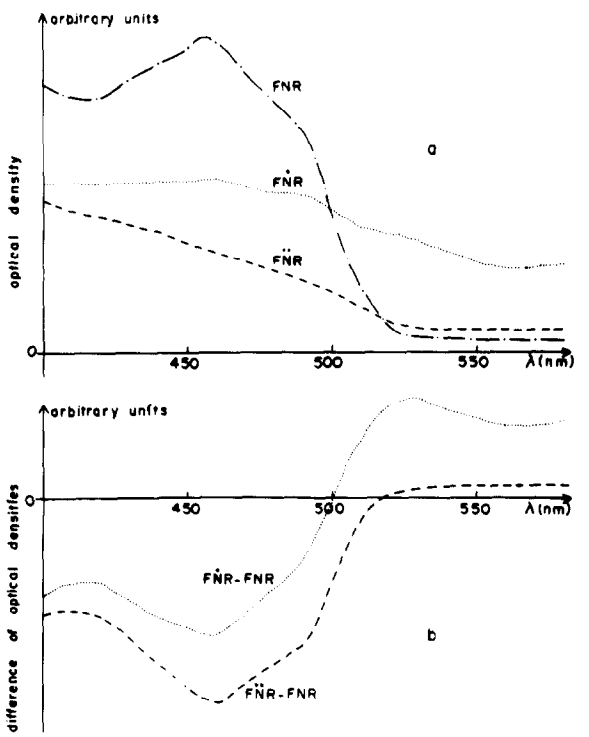

Fig.1. (1a). Spectra of FNR (– · -), FNR (– – –) and FNR (. . . . .) from [6]. (1b.) Difference spectra FNR–FNR (– – – –) and FNR–FNR (. . . . .) computed form fig.1a.

Abbreviations: NADP, nicotinamide-adenine-dinucleotide-phosphate; FNR, ferredoxin-NADP-reductase; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea

2. Materials and methods

Chlorella pyrenoidosa were grown in Knop medium with Arnon's trace elements A_5 and B_6. Before use cells were resuspended in 0.1 M phosphate buffer (pH 7) containing 7% Ficoll. Dark-adapted cells were placed into the cuvette before each measure.

Absorption changes were measured using the flash detector differential spectrophotometer described [11] and modified to increase its sensitivity by P. Joliot with D. Beal and B. Frilley. The actinic flashes (General Radio Stroboslave, with medium capacity) had half-time 3 μs and were filtered by filter Wratten 24 (red). Complementary filters (Schott BG 38 + Wratten 34 between 430 nm and 450 nm, Schott BG 38 + Wratten 44A between 450 nm and 530 nm) were placed in front of the photoelectric cells. Each point corresponded to the integration of the absorption changes of 100 actinic flashes fired with a frequency of 0.5 Hz. Under these conditions, the absorption changes were measured with an error margin ± 5 × 10^{-6}. The chlorophyll concentration (10 μg/ml) was chosen in a concentration range where the absorption changes between 430 nm and 530 nm depended linearly on the concentration of algae.

We limited this study to the wavelengths between 437 nm and 530 nm in order to avoid the contribution of cytochrome *f* below 437 nm (see spectrum [12]) and of plastocyanin above 530 nm (see spectrum [13]). The contribution of cytochrome *f* around 520 nm is close to the error margin of our method. Photosystem II was blocked by a preillumination in the presence of hydroxylamine (10^{-4} M) and DCMU (10^{-5} M) [14]. The changes due to P700 (see spectrum in [15]) and to the electrochromic effect [16–18] were unavoidable.

3. Results and discussion

3.1. *Analysis of* Δ A *at 320 μs*

Figure 2a presents the absorption changes at 320 μs, 40 ms and 200 ms after the flash, which are normalized to the same value at 515 nm. The changes at 40 ms and 200 ms exhibit the same spectra in the observed wavelength range. The spectrum of the 320 μs change is identical with the other spectra over 507 nm, but a clear discrepancy appears at shorter wavelengths. The difference between the changes at 320 μs and the normalized changes at 40 ms and 200 ms has a spectrum (fig.2b) which resembles the difference spectrum (FN̆R – FNR) (fig.1b) with a maximum observed at 470 nm instead of 460 nm.

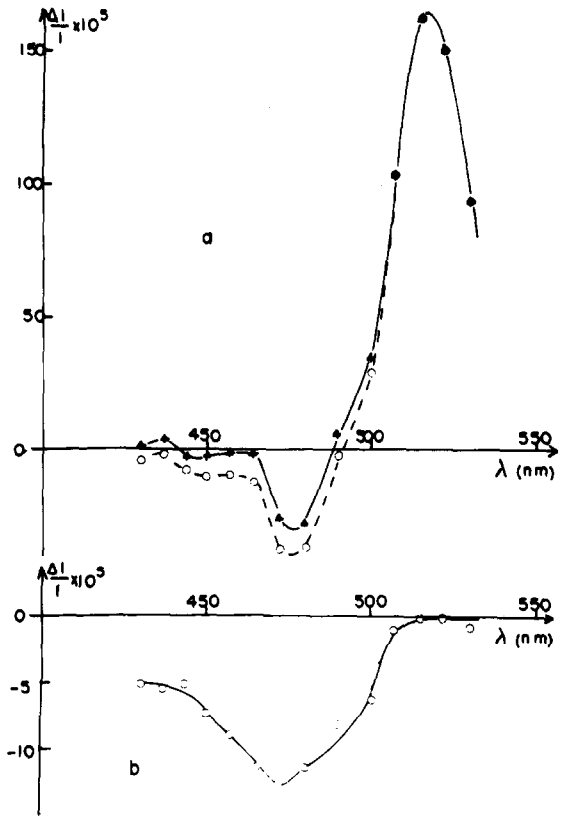

Fig.2. Absorption changes as a function of wavelength. (a), (○) 320 μs after flash. (+) 40 ms after flash. (△) 200 ms after flash. (+,△) with amplitude normalized such that the normalized change is equal to the change at 320 μs for λ 515 nm. (b) Difference between the two curves of fig.2a.

In view of these results, it is reasonable to assume that for times greater than 40 ms, the absorption changes are due to the electrochromic effect. As no known donor or acceptor of system I presents a sharp peak at 515 nm, we will admit that at 320 μs, the changes from 507 nm to 530 nm are also due to electrochromic effect. At shorter wavelengths, at 320 μs, we observe a change due to FN̆R in addition to the electrochromic effect. No other reduced acceptors are detectable at this time. The fact that no

$P700^+$ is observed indicates that under our experimental conditions (rather short, non-saturating flashes), the reduction of $P700^+$ is over at 320 μs.

A shift of the FNR absorption maximum has been observed in vitro when FNR forms a complex with ferredoxin [19–21]. The presence of a maximum at 470 nm in *Chlorella* cells indicates that the in vivo ferredoxin–NADP-reductase forms a complex with ferredoxin.

3.2. *Analysis of Δ A at 40 μs*

An increase of the electrochromic effect with a half-time of 10 ms (phase b [11]) can be observed at 515 nm for times greater than 320 μs. An extrapolation for times shorter than 320 μs indicates that phase b produces a 3% increase of the electrochromic effect from 40 μs to 320 μs. Due to the tail of the flash, another small increase of the electrochromic effect is observed after 40 μs [22]. This increase is estimated to be 2% of the electrochromic effect. Using the above electrochromic effect at 320 μs, we have drawn the electrochromic effect at 40 μs (fig.3a crosses), assuming a 5% increase from 40 μs to 320 μs. The difference between the observed absorption changes at 40 μs (fig.3a open circles) and the electrochromic effect are reported on fig.3b (open circles).

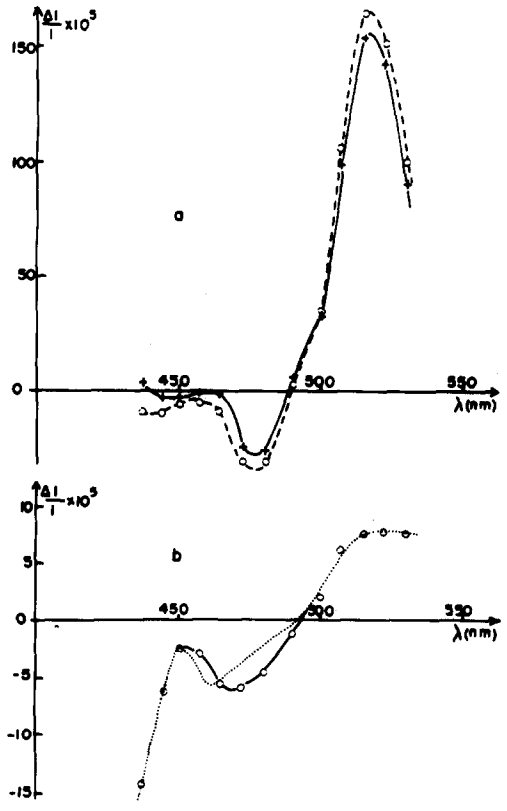

Fig.3. Absorption changes as a function of wavelength. (3a), (o) 40 μs after flash. (+) Electrochromic effect at 40 μs (see text). (3b), (o) Difference between the two curves of fig.3a. (· · · ·) Linear combination of the spectra ($P700^+$–P700) [15] and (FNR–FNR) (fig.1b).

The minimum at 470 nm and the maximum at 525 nm resembles the difference spectrum (FNR – FNR) (fig.1b). The maximum at 450 nm and the strong decrease at 437 nm indicate that some $P700^+$ is present at 40 μs. In order to test if only $P700^+$ and FNR are present at 40 μs in addition to the electrochromic effect, we have combined linearly the spectrum of ($P700^+$–P700) [15] and of (FNR – FNR) [6] so that the sum coincides with the experimental curve at 450 nm and 530 nm (fig.3b dotted line). The discrepancy between the computed and the experimental curves originates in the shift of the FNR-absorption maximum which already been noted from the spectrum of FNR – FNR (fig.2b). When taking into account this shift, the fact that the two curves are identical within the wavelength range studied, indicates that indeed the absorption changes at 40 μs are due to $P700^+$, FNR and electrochromic effect.

Hence the main acceptor forms are FNR at 40 μs and FNR at 320 μs.

3.3. *FNR reoxidation kinetics*

The information on FNR reoxidation kinetics was obtained at 464 nm. At this wavelength, the contribution of the electrochromic effect is negligeable. In addition, we concluded from fig.2 that $P700^+$ reduction is over at 320 μs and that FNR is no longer observed at 320 μs. Thus, the kinetics of the absorption changes, at 464 nm, after 320 μs (fig.4a) reflects the kinetics of FNR reoxidation: an exponential curve with a half-time of 4 ms.

When illuminated with a series of flashes at a frequency of 0.5 Hz, in the presence of hydroxylamine and DCMU, the algae necessarily transfer the electrons in a cyclic pathway around photosystem I. In addition, the above results (spectra and kinetics) are also observed at a higher flash frequency. Thus we

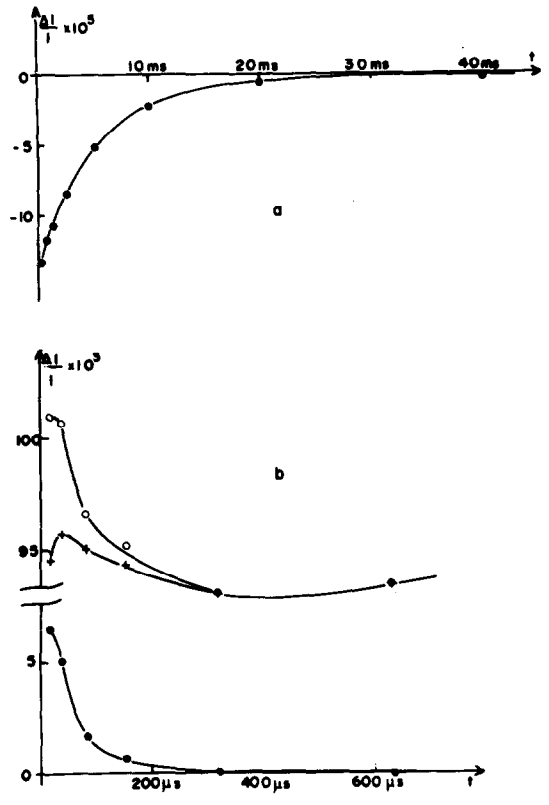

Fig.4. ΔA as a function of time. (4a), (•) at 464 nm. (4b), (○) at 530 nm. (+) at 506 nm. (•) Difference between the last two changes.

conclude that FNR is involved in the cyclic pathway around photosystem I, as suggested [23]. The nature of its oxidant is not yet clear. It cannot be cytochrome *f* whose reduction in cyclic conditions has a half-time of 1 s [24]. A likely candidate could be cytochrome b_6 which is a two electron acceptor [25] involved in the cyclic pathway around photosystem I [26], with a half-reduction time of a few milliseconds [27].

3.4. *FṄR – FṄR transformation kinetics*

From the spectrum of the electrochromic effect (fig.2a) we noted that the contribution of the electrochromic effect is identical at 506 nm and 530 nm. The contribution of $P700^+$ is almost identical at these two wavelengths [15]; in this wavelength range, FNR does not absorb, and the absorption due to FṄR is small. Thus, the kinetics of the absorption changes difference (530 nm – 506 nm) (fig.4b closed circles) reflects the kinetics of FṄR. The concentration of FṄR decreases with a half-time of 30 µs. According to the spectra drawn at 40 µs and 320 µs (fig.2,3) we know that FṄR becomes transformed in FN̈R.

From the ratio of the extinction coefficients of the transitions FNR → FṄR and FNR → FN̈R (fig.1b) we compute that for one FN̈R produced at 320 µs (fig.2b), 1.2 FṄR was present at 40 µs (from fig.3b) and 1.5 at 20 µs (data not shown on a figure). This ratio significantly higher than 1, is in favor of a dismutation process.

$$2\ \text{FṄR} \xrightarrow{t_{1/2}\ 30\ \mu s} \text{FNR} + \text{FN̈R}$$

3.5. *FNR reduction kinetics*

The kinetics of the disappearance of FṄR are not exponential. The inflexion point between 20 µs and 40 µs, though close to the error margin of the method, is reproducible and indicates that the maximum amount of FNR is near the value reached at 20 µs. This maximum amount then corresponds about to 1.5 FṄR per FṄR instead of 2 as would be observed if the rate of formation of FṄR was infinitely high compared to its rate of disappearance. A ratio of 0.75 between the observed maximum amount and the stochiometric amount indicates a ratio in the order of 10 between the rates of formation and disappearance of FṄR. A half-time of FṄR formation around 3 µs estimated in this way would lead to a maximum amount of FṄR at 15 µs, a time close to 20 µs. This half-time, almost identical to the half-time of the photon distribution by the flash indicates that the reactions leading to the formation of FṄR are performed with a half-time inferior or equal to 3 µs. Further experiments with shorter flashes would have to be performed to obtain further informations.

Acknowledgements

The author wishes to thank Pierre Joliot for the construction of the spectrophotometer on which the above experiments are performed.

References

[1] Hiyama, T. and Ke, B. (1971) Arch. Biochim. Biophys. 147, 99–108.
[2] Tagawa, K. and Arnon, D. I. (1962) Nature 195, 537–543.

[3] Lazzarini, R. A. and San Pietro, A. (1962) Biochim. Biophys. Acta 62, 417–420.
[4] Shin, M., Tagawa, K. and Arnon, D. I. (1963) Biochem. Z. 338, 84–96.
[5] Forti, G., Melandri, B. A., San Pietro, A. and Ke, B. (1970) Arch. Biochem. Biophys. 140, 107–112.
[6] Shin, M. and Arnon, D. I. (1965) J. Biol. Chem. 240, 1405–1411.
[7] Massey, V., Matthews, R. G., Foust, G. P., Howell, L. G., Williams, C. H., Zanetti, G. and Rondri, S. (1970) in: Pyridine Nucleotide Dependent Dehydrogenases, (Sund, H. ed) pp. 393–409, Springer.
[8] Vishmiac, W. and Ochoa, S. (1951) Nature 167, 768–769.
[9] Tolmach, L. J. (1951) Nature 167, 946–948.
[10] Arnon, D. I. (1951) Nature 167, 1008–1010.
[11] Joliot, P. and Delosme, R. (1974) Biochim. Biophys. Acta 357, 267–284.
[12] Gorman, D. S. and Levine, R. P. (1966) Plant. Physiol. 41, 1648–1656.
[13] Katoh, S., Shiratori, I. and Takamiya, A. (1962) J. Biochem. 51, 32–40.
[14] Bennoun, P. (1970) Biochim. Biophys. Acta 216, 357–363.
[15] Hiyama, T. and Ke, B. (1972) Biochim. Biophys. Acta 267, 160–171.
[16] Witt, H. T., Müller, A. and Rumberg, B. (1961) Nature 191, 194–195.
[17] Rumberg, B. (1964) Nature 204, 860–861.
[18] De Kouchkovsky, Y. (1969) in: Progress in Photosynthesis Research (Metzner, H. ed) pp. 959–970, Tübingen.
[19] Foust, G. P. and Massey, V. (1967) Fed. Proc. Fed. Am. Soc. Exp. Biol. 26, 732.
[20] Nelson, N. and Neumann, J. (1968) Biochem. Biophys. Res. Commun. 30, 142–147.
[21] Shin, M. and San Pietro, A. (1968) Biochem. Biophys. Res. Commun. 33, 38–42.
[22] Joliot, P., Delosme, R. and Zickler, A. (1978) submitted to Biochim. Biophys. Acta.
[23] Forti, G. and Zanetti, G. (1969) in: Progress in Photosynthesis Research (Metzner, M. ed) pp. 1213–1216, Tübingen.
[24] Bouges-Bocquet, B. (1977) Biochim. Biophys. Acta 462, 371–379.
[25] Böhme, H. and Cramer, W. A. (1973) Biochim. Biophys. Acta 325, 275–283.
[26] Hind, G. and Olson, J. M. (1966) Brookhaven Symp. Biol. 19, 188–194.
[27] Dolan, E. and Hind, G. (1974) Biochim. Biophys. Acta 357, 380–385.