REACTIONS LEADING TO THE PHOTO-INDUCED REDUCTION OF FERREDOXIN-NADP-REDUCTASE (FNR) IN *CHLORELLA* CELLS

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Received 20 June 1978
Revised version received 10 August 1978

1. Introduction

Spectroscopy between 400 nm and 550 nm is an effective method for the study of the kinetics of the acceptors of photosystem I. In a preceding paper [1], we observed that the main acceptor forms are half-reduced $\dot{F}\dot{N}R$ 40 μs after a flash, and totally reduced $\dot{F}\dot{N}R$ 320 μs after a flash. A dismutation process

$$2\ \dot{F}\dot{N}R \longrightarrow FNR + \dot{F}\dot{N}R \qquad (1)$$

with a half-time of 30 μs was proposed.

In this paper, we have tried to identify the precursor of $\dot{F}\dot{N}R$, firstly by means of a better time resolution, secondly by means of a more powerful flash which produced a mean value of 1.9 photochemical reactions/system I center [2] and thus produced photochemical reactions in centers where FNR was already in the half-reduced form $\dot{F}\dot{N}R$.

2. Materials and methods

Chlorella pyrenoidosa was grown on Knop medium [1a] to which were added Arnon's trace elements A_5 and B_6 [1b]; the preparation was illuminated by white fluorescent light of 3000 lux. Before use, cells were resuspended in 0.1 M phosphate buffer (pH 7.0) containing 7% Ficoll.

Photosystem II was blocked by a pre-illumination in the presence of hydroxylamine (10^{-4} M) and DCMU (10^{-5} M) [3].

Absorption changes were measured using the flash detector differential spectrophotometer in [4] whose sensitivity was improved by P. Joliot in collaboration with D. Béal and B. Frilley. The actinic flashes (General Radio Sroboslave with high capacity) were filtered through red filters (Wratten 29) and had 7 μs as half-time of photon distribution.

Complementary filters (Schott BG 38 + Wratten 34 between 400 nm and 450 nm, + Wratten 44A between 450 nm and 550 nm, + Schott VG3 between 560 nm and 580 nm) protected the photoelectric cells from the actinic light.

Each point corresponded to the integration of the absorption changes of 100 actinic flashes. The sample was renewed before each flash. Under these conditions, the changes $\Delta I/I$ were measured with an error margin of $\pm 5 \times 10^{-6}$.

The chlorophyll concentration (10 μg/ml) was chosen so that the absorption changes in the wavelength range studied depended linearly on the concentration of algae.

At short times, an absorption change due to the formation of a triplet state of carotenoid was detected [5,6]. The formation of this triplet state depends only on photosystem II excitation [7]. Thus, the contribution of the carotenoid triplet (and of the eventual artefact of the actinic flash) can be eliminated by subtracting the absorption change obtained when both photoreactions are blocked, that is, when the actinic flash is fired in the presence of a strong background of continuous illumination with hydroxylamine and DCMU [7].

Abbreviations: FNR, ferredoxin-NADP-reductase; Fd, ferredoxin; DCMU, 3-(3,4-dichlorophenyl)1,1-dimethylurea; DAD, 2,3,5,6, tetramethyl-*p*-phenylenediamine

At every wavelength, an electrochromic effect interferes with the absorption changes due to the donors and to the acceptors. In order to subtract this electrochromic effect from our measurements, its spectrum and kinetics have been determined by the following method: when the sample is renewed between each flash, the absorption changes for times > 80 ms after a flash are due to the electrochromic effect [1,8]. Thus, the spectrum of the electrochromic effect can be obtained. At 515 nm, the wavelength where the maximal electrochromic effect is observed, absorption changes due to P^+-700 and $\dot{F}NR$ are also detected [1]. In order to avoid any contribution of P^+-700 and of $\dot{F}NR$, the kinetics of the electrochromic effect were obtained by the absorption change difference $(\Delta I/I)$ 515 nm$-(\Delta I/I)$ 530 nm: in effect, both transitions, P-700 $\rightarrow$ P^+-700 [9] and FNR $\rightarrow$ $\dot{F}NR$ [10] have flat differential absorption spectra between 515 nm and 530 nm. Knowing the electrochromic effect at 80 ms at every wavelength, the kinetics of this effect, and assuming that the kinetics of this effect are independent of the wavelength, we can compute the electrochromic effect at every time and at every wavelength and subtract it from any measurement.

In order to permit a comparison of the absorption changes measured on whole *Chlorella* cells with the absorption changes (spectra and extinction coefficients) observed for molecules in solution, the 'particle flattening effect' [11] has to be taken into account: the distribution of absorbing pigments is not homogeneous and causes a decrease in the absorption changes in cells. Figure 1 presents the differential flattening factor (the ratio between the absorption change in solution and the absorption change in cells), obtained, as a function of wavelength for *Chlorella* cells, in our apparatus, according to the method developed in [12].

The version of the spectrophotometer built by P. Joliot, on which the following experiments, were performed, uses light pipes to collect scattered light and thus the correction for light scattered losses are unnecessary.

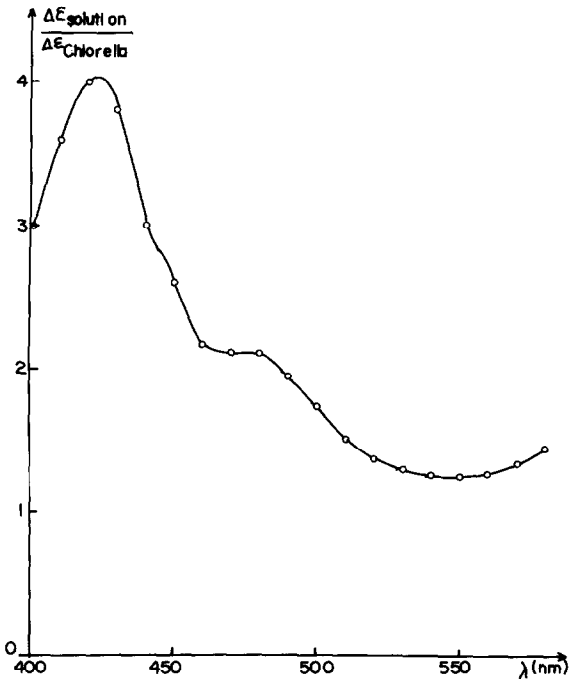

Fig.1. Differential flattening factor as a function of wavelength in *Chlorella* cells, calculated according to the method proposed in [12].

3. Results

In order to study the precursors of $\dot{F}NR$, we performed experiments with a a short time resolution (around 1 μs). We then observed that $\dot{F}NR$ is already formed 1 μs after a photochemical reaction. Thus, the investigation of the precursors of $\dot{F}NR$ would need a time resolution below 1 μs which was not obtainable by our technique.

Another method for forming the precursor of $\dot{F}NR$ would be the use of strong flashes which produce more than 1 photochemical reaction/system I center. It has been shown that the regeneration of photosystem I after a single photoreaction takes but a few microseconds [13] and that during the flash used for the experiments presented in this paper, a mean value of 1.9 photochemical reactions/system I center occurred [2]. The first photochemical reaction leads to the formation of some $\dot{F}NR$ in less than 1 μs (see above) and the second photochemical reaction thus takes place in centers where some $\dot{F}NR$ is already in the half-reduced form $\dot{F}NR$.

Figure 2a presents the spectrum of the absorption changes (corrected for the electrochromic effect and the carotenoid triplet changes as indicated in section 2)

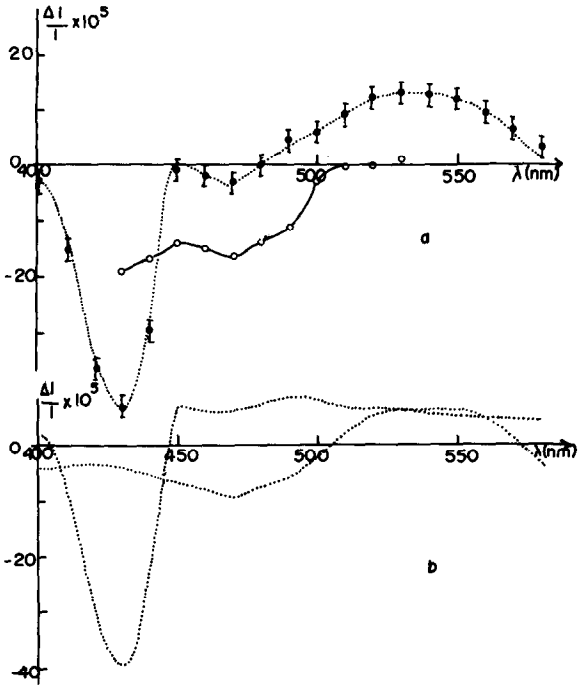

Fig.2. (a) Absorption changes at 15 μs (closed circles) and at 320 μs (open circles) (after correction for the carotenoid triplet change and for the electrochromic effect), as a function of the wavelength. Dotted lines show the sum of the absorption changes presented on fig.2b. (b) Contribution of P^+-700 (the spectrum of P^+-700–P-700 in whole *Chlorella* cells is drawn from [2]) and of FN̈R (the spectrum of FN̈R–FNR is drawn from [10]) taking into account the 'particle flattening effect' and the displacement of FNR absorption maximum.

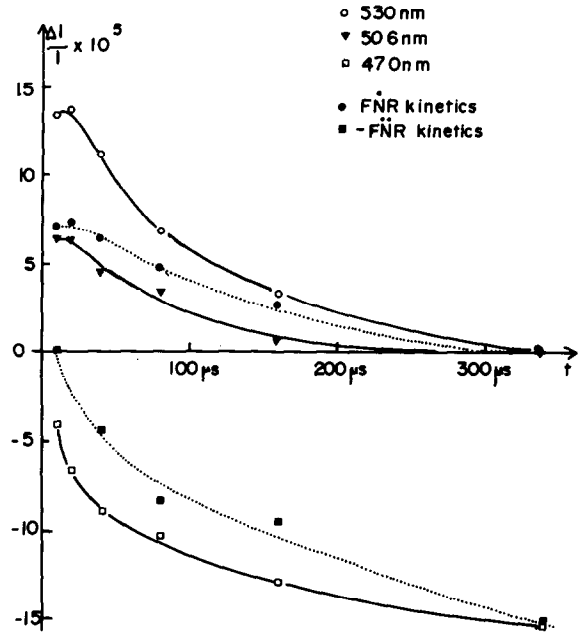

Fig.3. Absorption changes (after subtraction of the carotenoid triplet change and of the electrochromic effect) as a function of the time. (○) ($\Delta I/I$) 530 nm; (▼) ($\Delta I/I$) 506 nm; (▫) ($\Delta I/I$) 470 nm; (●) ($\Delta I/I$) 530 nm–($\Delta I/I$) 506 nm (kinetics of FN̈R); (▪) ($\Delta I/I$) 470 nm + 1.5 ($\Delta I/I$) 530 nm–2.5 ($\Delta I/I$) 506 nm (kinetics of –FN̈R).

15 μs and 320 μs after this double-hitting flash. At 15 μs the sharp trough at 430 nm is characteristic of the spectrum of (P^+-700–P-700) [9] while the minimum at 470 nm and the maximum at 530 nm are characteristic of the spectrum of (FN̈R–FNR) [1,10]. Figure 2b shows that the spectrum obtained at 15 μs is the sum, within the margin of error, of the spectra of these two compounds (taking in account the particle flattening effect). At 320 μs, the absorption change which is maximal at 470 nm and zero over 510 nm, is characteristic of the spectrum of (FN̈R–FNR) [1,10]; a small contribution of P^+-700 is observed below 450 nm.

The kinetics of FN̈R and FN̈R after the double-hitting flash are reported in fig.3.. FN̈R kinetics are obtained from the difference in absorption change ($\Delta I/I$) 530 nm–($\Delta I/I$) 506 nm [1]: in effect, the differential absorption of (FN̈R–FNR) is zero over 506 nm, and the spectrum of (P^+-700–P-700) is almost flat between 506 nm and 530 nm. FN̈R kinetics cannot be obtained by a single difference of absorption change. The difference of absorption changes ($\Delta I/I$) 530 nm–($\Delta I/I$) 470 nm after correction of the electrochromic effect detect a sum of the kinetics of FN̈R and of FN̈R: in effect, the contribution of P^+-700 is negligible in this difference (fig.2b). Using the spectrum of (FN̈R–FNR) (fig.2b) one can deduce that, if $\Delta I'/I$ are the absorption changes due to the transition FNR→FN̈R, and $\Delta I/I$ the measured absorption changes, corrected from the electrochromic effect, the contribution of FN̈R to the absorption change ($\Delta I/I$) 530 nm–($\Delta I/I$) 470 nm is: ($\Delta I'/I$) 530 nm–($\Delta I'/I$) 470 nm = 2.5 [($\Delta I'/I'$) 530 nm–($\Delta I'/I'$) 506 nm] = 2.5 [($\Delta I/I$) 530 nm–($\Delta I/I$) 506 nm]. Thus, the expression 2.5 ($\Delta I/I$)

506 nm–1.5 ($\Delta I/I$) 530 nm–($\Delta I/I$) 470 nm is proportional to the amount of $\dot{F}\dot{N}R$.

The plateau at the beginning of the kinetics of $\dot{F}\dot{N}R$ may be explained by the competition between its disappearance and its formation by the photoreactions taking place more than 10 μs after the flash (due to the tail of the flash [13]). After this lag, the kinetics of the formation of $\dot{F}\dot{N}R$ follows the kinetics of the disappearance of $\dot{F}\dot{N}R$: both present a half-time of around 100 μs.

Using the measurements in [14], the differential flattening factor (fig.1) and taking into account the displacement of the maximal absorption of FNR due to the formation of a complex between FNR and ferredoxin [15–17], we obtained the following extinction coefficients for FNR in *Chlorella* cells: 1.45 $mM^{-1}cm^{-1}$ at 530 nm and 0 at 506 nm for ($\dot{F}\dot{N}R$–FNR), 4.5 $mM^{-1}cm^{-1}$ at 470 nm for ($\dot{F}\dot{N}R$–FNR). Using these extinction coefficients, one can compute from fig.3 (after the lag of the kinetics of $\dot{F}\dot{N}R$) that the ratio between the amount of disappearing $\dot{F}\dot{N}R$ and the amount of formed $\dot{F}\dot{N}R$ is ~1.5. This ratio is significantly smaller than 2.

We measured the amounts of $\dot{F}\dot{N}R$ and $\dot{F}\dot{N}R$ produced during a series of flashes performed on dark-adapted cells. No oscillations could be observed even in the presence of oxidants which enter the membrane (ferricyanide 2×10^{-4} M + DAD 5×10^{-5} M [18]), and which would presumably oxidize any $\dot{F}\dot{N}R$ molecule during the dark adaptation. Thus, a cooperation of different chains for the electron transfer between the system I acceptors has to be assumed. This cooperation could explain why the half-time of $\dot{F}\dot{N}R$ disappearance was around 30 μs when non-saturating flashes were used [1] and around 100 μs with double-hitting flashes (fig.3).

4. Discussion

The ratio between the amount of disappearing $\dot{F}\dot{N}R$ and formed $\dot{F}\dot{N}R$ after a double-hitting flash, significantly smaller than 2, is not compatible with reaction (1). We must admit that with double-hitting flashes, some electrons are stored on an additional acceptor that we shall call X′, and that $\dot{F}\dot{N}R$ is partly formed by reaction (2):

$$\dot{F}\dot{N}R + X'^{-} \longrightarrow \dot{F}\dot{N}R + X' \qquad (2)$$

This implies that there is only 1 FNR molecule/system I center. It is consistent with the fact that we could never detect more than 1 photo-reduced FNR molecule/system I center. The first photochemical reaction would lead to the formation of $\dot{F}\dot{N}R$ and the second one to the formation of X'^{-}.

There would then be a mean value of 0.6 X'^{-} formed/system I center 15 μs after a flash (1.6 photochemical reactions/system I center have occured at this time). Despite this, the spectrum observed (fig.2a) presents no evidence for an absorption due to X'^{-}: its eventual contribution would be within the margin of error. Thus, after correction for the 'particle flattening effect', the differential extinction coefficient of the transition $X' \rightarrow X'^{-}$ ought to be inferior to 1.7 $mM^{-1}cm^{-1}$ at 460 nm. This information about X′ would indicate that it would be neither X-430 ($\Delta\epsilon$ around 5 $mM^{-1}\,cm^{-1}$ at 460 nm [9]) nor ferredoxin ($\Delta\epsilon$ around 5 $mM^{-1}cm^{-1}$ at 460 nm [19]).

This information seems to contradict the results of biochemical experiments. In effect, ferredoxin-NADP-reductase which forms complexes with both ferredoxin [15–17] and $NADP^{+}$ [17,20] transfers the electrons relatively specifically from reduced ferredoxin to NADP in vitro [21]. In addition, ferredoxin was shown to be necessary for the photochemical electron transfer to FNR in chloroplasts [22].

This contradiction may be resolved if one assumes that there is one electron stored on the complex ferredoxin–ferredoxin-NADP-reductase in the dark. The electron transfers at the level of the system I acceptors would then written:

$$\begin{array}{l} X'\ \overline{\dot{F}d\ \dot{F}\dot{N}R} \xrightarrow{h\nu} X'^{-}\ \overline{\dot{F}d\ \dot{F}\dot{N}R} \xrightarrow{t_{1/2} \leqslant 1\ \mu s} \\ X'\ \dot{F}d\ \dot{F}\dot{N}R \xrightarrow{h\nu} X'^{-}\ \dot{F}d\ \dot{F}\dot{N}R \\ \quad \downarrow\uparrow \qquad\qquad \uparrow\downarrow \\ X'\ \dot{F}d\ \dot{F}\dot{N}R \xrightarrow{h\nu} X'^{-}\ \dot{F}d\ \dot{F}\dot{N}R \\ \qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\quad X'\ \ \dot{F}d\ \dot{F}\dot{N}R \end{array} \qquad (3)$$

and

$$\left.\begin{array}{l} \dot{F}d\ \dot{F}\dot{N}R \\ \quad \text{or} \\ \dot{F}d\ \dot{F}\dot{N}R \end{array}\right\} + \dot{F}d\ \dot{F}\dot{N}R \longrightarrow \overline{\dot{F}d\ \dot{F}\dot{N}R} + \dot{F}d\ \dot{F}\dot{N}R \qquad (4)$$

Reaction (4) which is a new way of writing reaction (1) implies that two complexes $\dot{Fd}$ $\dot{FNR}$ or Fd $\ddot{FNR}$ may cooperate to form an $\dot{Fd}$ $\dot{FNR}$ complex. If, as we have proposed, there is only 1 FNR molecule/system I center, a cooperation between different system I chains is necessary for reaction (4) to take place. The cooperation presented in the last paragraph of section 3 is consistent with the reactions proposed. In addition, the possibility of both reactions (3) and (4) taking place would explain why the stoichiometry between the disappearing $\dot{FNR}$ and the formed $\dot{FNR}$ may vary between 1 and 2.

In conclusion, it is interesting to note that these reactions resemble those of the complex adrenoxin–adrenoxin-reductase (a complex similar to the complex ferredoxin–ferredoxin-NADP-reductase, from the biochemical point of view) observed in vitro [23].

Acknowledgements

The author wishes to thank Pierre Joliot for the construction of the spectrophotometer on which the above experiments are performed. This work was supported by CNRS and by the Commission of European Communities.

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