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ELECTRON AND PROTON TRANSFERS FROM *P*-430 TO FERREDOXIN-NADP-REDUCTASE IN *CHLORELLA* CELLS

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Summary

After blocking Photosystem II on whole Chlorella cells, we measured the absorption changes between 0°C and —10°C.

The absorption changes measured 2 μs after the beginning of a Xenon Flash are the sum of changes due to P^+-700 and changes due to P^--430 (after the subtraction of the carotenoid triplet change and of the electrochromic effect).

The reduction of P^--430 is not resolved by our technique. Its reoxidation presents a half-time around 1 μs at 0°C and around 2 μs at —10°C.

The reduction and protonation of ferredoxin-NADP-reductase to its neutral semi-quinoid form FNRH° present a half-time of about 3 μs at 0°C and 6 μs at —10°C.

The presence of only one photoreducible ferredoxin-NADP-reductase per Photosystem I center is confirmed. The acceptor preceding ferredoxin-NADP-reductase is not ferredoxin, but is an acceptor X′ the differential extinction coefficients of which are weak or null from 420 nm to 480 nm.

Tentative explanations which would reconcile these results with what was already known about ferredoxin are proposed.

Introduction

In recent years, several researchers have studied Photosystem I acceptors. Experiments done either on Photosystem I particles or at liquid helium temper-

Abbreviations: FNR, ferredoxin-NADP-reductase;Fp, flavin part of a flavoprotein; FNR or Fp, semiquinoid form (neutral or anionic); FNRH° or FpH°, neutral semiquinone; FHR^- or Fp^-, semiquinoid anion; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

ature have yield information on the acceptors closely related to the reaction centers. Four membrane bound acceptors have thus been discovered. According to the terminology of Sauer et al. [1], they are:

A_1, the primary acceptor, is a photoreducible chlorophyll [2—4].

A_2, (or X according to MacIntosh et al. [5]), the secondary acceptor, is an iron sulfur protein [6].

Centre B [7,8] and Centre A [9] are two iron sulfur proteins. The role of centre B in the electron transfer chain is still unclear. Centre A is the final location of the electron at liquid helium temperature or in Photosystem I particles [9,10].

Centre A and Centre B have optical differential spectra with a minimum at 430 nm [10,3] and are also referred to as *P*-430 [10]. Because we will describe experiments of optical spectroscopy, we will use this notation. They are also sometimes named bound-ferredoxins. We will avoid this notation and the term ferredoxin will always refer to the solubilisable protein, the biochemical composition of which is described in Ref. 11 and the spectrum in Ref. 12.

In whole chloroplasts or green algae, the transfer reactions beyond *P*-430 have never been precisely measured. Some solubilisable carriers have been proposed: ferredoxin (Fd) [12], ferredoxin-NADP-reductase (FNR) [13,14] and NADP [15—17], the terminal acceptor of the linear transfer chain. The redox changes of ferredoxin-NADP-reductase have been detected in whole cells [18] by spectroscopic measurements at room temperature. The results show that the semiquinone $\dot{FNR}$ was formed with a half-time inferior or equal to 1 μs [19]. The fully reduced flavin, $FNRH_2$, appears with a half-time varying between 30 μs and 100 μs [19]. In addition, the results presented in Ref. [19] would indicate that the acceptor preceding ferredoxin-NADP-reductase is neither ferredoxin nor *P*-430. The role of ferredoxin in the electron transfer chain has thus become less clear.

In this paper, we have tried to obtain some new information on the electron transfers from *P*-430 to ferredoxin-NADP-reductase, using spectroscopic measurements on whole cells. As all these transfers are temperature dependent, we worked at low temperatures to slow down these reactions which all present a half-time inferior to 1 μs at room temperature [19].

Materials and Methods

Chlorella pyrenoidosa was grown on Knopp medium [20] to which were added Arnon's trace elements A_5 and B_6 [21]; the culture was illuminated by white fluorescent light of 3000 lux. Before use, cells were suspended in 0.1 M phosphate buffer (pH 7) containing 7% Ficoll and 14% ethylene glycol (except for the experiment at $-10°C$ where the concentration of ethylene glycol was 20%). Addition of 14% ethylene glycol did not modify the absorption changes at room temperature. However, after a few hours, ethylene glycol at a concentration of 20% accelerated the decay of the electrochromic effect, probably by changing the permeability of the membranes and only experiments of short duration could be performed at this concentration.

In order to avoid any redox changes due to Photosystem II, hydroxylamine (10^{-4} M) and DCMU (10^{-5} M) were added to the cells which were preilluminated before dark adaptation [22].

Another change due to Photosystem II, the absorption change due to the formation of the triplet state of carotenoid [23,24], is detected very shortly after illumination. The formation of this triplet state depends only on Photosystem II excitation [25]. Thus its contribution 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 [25].

Absorption changes were measured using the flash detector differential spectrophotometer constructed by Joliot et al [26]. With this apparatus, the standard error on $\Delta I/I$ for one cycle is $\sigma = 5 \cdot 10^{-5}$ [26]. This standard error is divided by $\sqrt{n}$ for n cycles. As margin of error, we used $\pm 2\sigma$. Each point on the figures (except Fig. 1) corresponds to the integration of the absorption changes due to 100 to 400 actinic flashes, depending on the precision required. The sample was renewed before each flash (or group of flashes for Fig. 6).

The optical path of the cuvette was 16 mm. The chlorophyll concentration (12 μg/ml) was chosen so that the absorption change, from 400 nm to 600 nm, was a linear function of the concentration of algae. The actinic Xenon flashes (Verrerie Scientifique, type CA972) were filtered through red filters (Corning RG2 + Wratten 70) and presented a half-time of 1 μs. Complementary filters (Corning 4-96) protected the photocells. When we used this combination of filters, the artefact was zero.

Using the absorption change at 515 nm (corrected for carotenoid triplet change) as a linear measurement of the number of photochemical reactions [27], Fig. 1 presents the number of photochemical reactions per Photosystem I center, as a function of the time after the beginning of a flash. This curve is normalized with a saturating laser flash which was assumed to induce one photochemical reaction per Photosystem I center. With the filters and the concentration of algae indicated above, the Xenon actinic flash thus performs a mean value of 0.9 photochemical reaction per Photosystem I center. With this saturation, the probability of double hits is negligible.

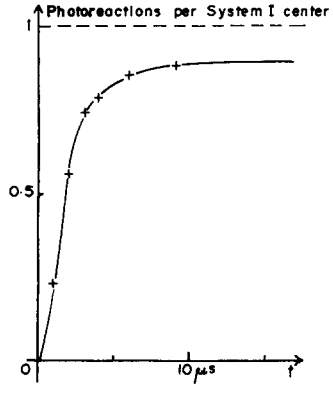

Fig. 1. Number of photochemical reactions per Photosystem I center as a function of the time after the beginning of a flash at -5°C, normalized with a saturating laser flash. Ethylene glycol 14%. Average of 10 flashes.

At any wavelength and at any time, the absorption changes due to the redox reactions of Photosystem I, are mixed with the absorption changes due to the electrochromic effect which thus has to be subtracted. It was shown [28,18] that at room temperature, on dark adapted material, the absorption changes, for times longer than 80 ms after a flash, are only due to the electrochromic effect, from 400 nm to 600 nm. The same results are observed at −5°C but for times longer than 300 ms. In order to subtract the contribution of the electrochromic effect at shorter times, it is necessary to know the kinetics of the electrochromic effect. The kinetics of the absorption changes at 515 nm, the maximum of the electrochromic effect, is a good first approximation of this kinetics. However, at this wavelength, small absorption changes due to P^+-700 and $\dot{F}\dot{N}R$ are also detected [18]. As both transitions P^+-700 − P-700 [29] and $\dot{F}\dot{N}R$—FNR [30] have flat differential absorption spectra between 515 nm and 530 nm, the contribution of these two compounds were avoided by using the absorption change difference $\Delta I/I$ (515 nm) − $\Delta I/I$ (530 nm) which gives the exact kinetics of the electrochromic effect [31]. Once we know the electrochromic effect at 300 ms, at every wavelength, the kinetics of this effect, and assuming that the kinetics of this effect is independent of the wavelength, we can compute the electrochromic effect at everytime and at every wavelength and subtract it from any measurement.

As this method of subtraction has previously been presented and improved [18,19,28,31], we will discuss only the absorption changes corrected for carotenoid triplet and electrochromic effect, in order to simplify the presentation of the results of this paper.

In order to compare 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' [32,33] has to be taken into account. In this case, the distribution of absorbing pigments is not homogeneous and causes a decrease in the absorption changes in cells. We used the differential flattening factor obtained in Ref. 19 for Chlorella cells.

Results and Discussion

Spectra at −5° C

Fig. 2a (open circles) presents the spectrum obtained 2 μs after the beginning of the actinic flash. The sharp minimum at 430 nm is characteristic of P^+-700. The absorption change at 446 nm, an isosbestic point of P^+-700 − P-700 in Chlorella cells, and the low absorption change at 450 nm, indicate that at least one other compound contributes to the absorption change. On the same figure, the differential spectrum of P^+-700 obtained in Chlorella cells [31], normalized at 490 nm, is presented. The difference between these two curves (Fig. 2b) exhibits the differential spectrum of P^--430 [29] corrected for the 'particle flattening effect' [19].

As this deconvolution is somewhat arbitrary, we tried to fit the experimental data with other linear combination of spectra: P^+-700 and Fd^-, or P^+-700 and $\dot{F}\dot{N}R$ (Fig. 2c). The differential spectra of Fd^- [12] and $\dot{F}\dot{N}R$ [30] have been corrected for the 'particle flattening effect', and in addition, the shift of the maximum absorption change of ferredoxin-NADP-reductase due to the forma-

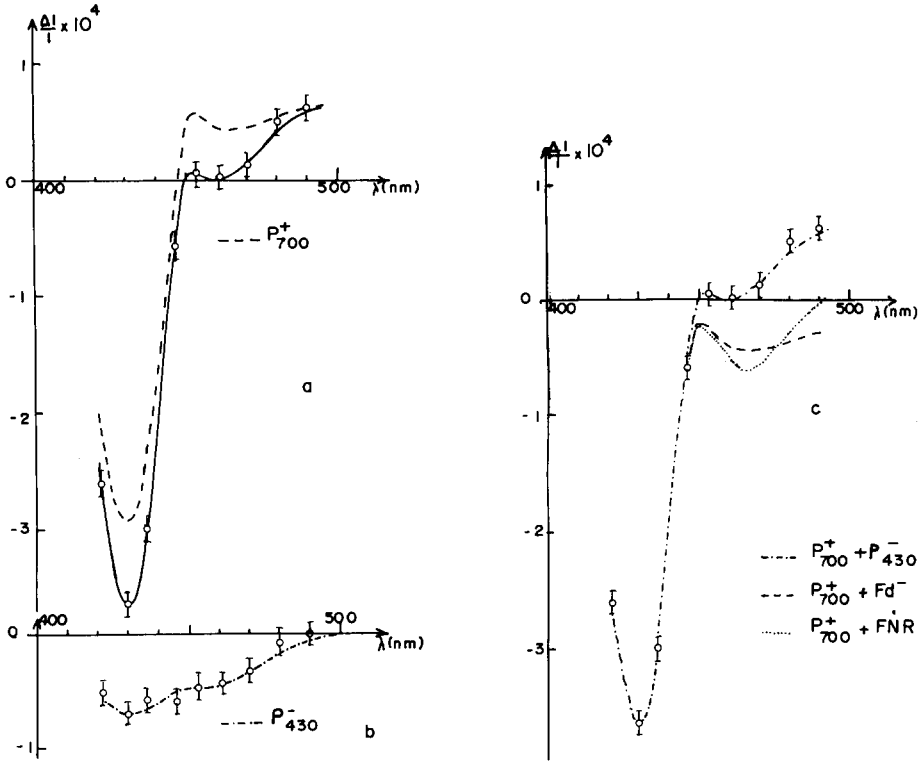

Fig. 2. (a) Open circles: absorption changes 2 μs after the beginning of a flash (after correction of the carotenoid triplet change and of the electrochromic effect) as a function of the wavelength at −5°C. Ethylene glycol 14%. Average of 400 flashes. Dotted line: spectrum of P^+-700 − P-700 drawn from Bouges-Bocquet et al. [19] normalized at 490 nm. (b) Open circles: differences of the two curves of Fig. 2a. Dotted line: spectrum of P^--430 − P-430 from Hiyama and Ke [29]. (c) Open circles: same as Fig. 2a. The curves are computed linear combination of spectra, as indicated in the text. Fd, ferrodoxin.

tion of a complex between ferredoxin-NADP-reductase and ferredoxin [34,35, 18] was taken into account. The coefficients involved in the linear combination of two spectra were computed so that the resulting spectrum coincided with the experimental data at 430 nm and 446 nm (isosbestic point of P^+-700 [31]), but the same results were obtained when the spectra were fitted to other wavelengths: the absorption changes 2 μs after the beginning of the actinic flash is, within the uncertainties of the experiment, a sum of changes due to P^+-700 and changes due to P^--430. None of the other tested spectra could fit the experimental data.

On Fig. 3 (open circles), the absorption changes 9 μs after the beginning of the flash are shown. On this spectrum, the minimum at 470 nm is characteristic of FṄR. The identical absorption changes at 460 nm and 480 nm indicate that the contribution of P^--430 at this time is, at the most, small. Actually, within the margin of error, these absorption changes can be deconvoluted into the sum of changes due to P^+-700 and changes due to FṄR (dotted curves).

Thus, at −5°C, the electron acceptors reduced 2 μs and 9 μs after the beginning of the actinic flash are respectively P^--430 and FṄR. There is no

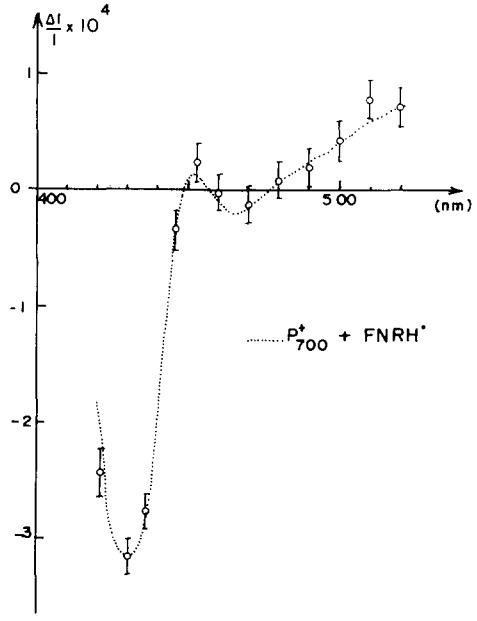

Fig. 3. Open circles: same as Fig. 2a, 9 μs after the beginning of a flash. Dotted line: linear combination of the spectra of P^+-700 — P-700 and of $\dot{F}$NR—FNR drawn from Bouges-Bocquet et al. [19].

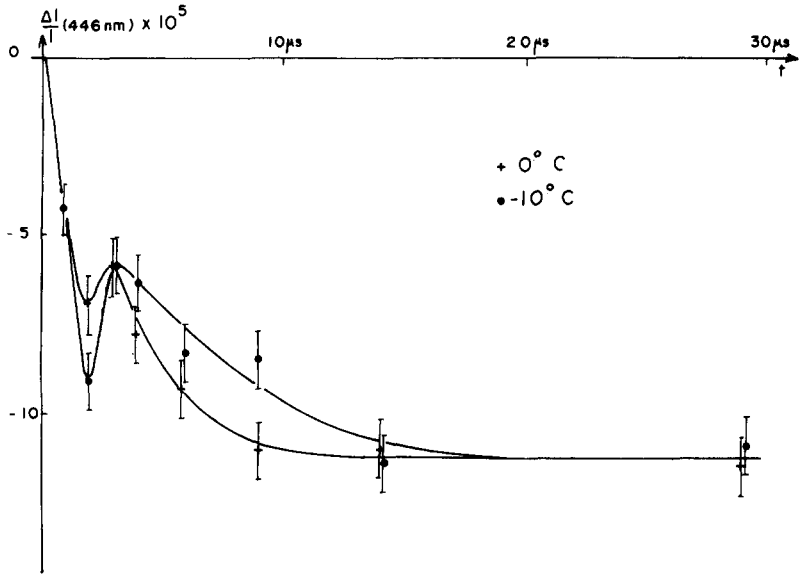

Fig. 4. Absorption changes after a flash at 446 nm (after correction of the carotenoid triplet change and of the electrochromic effect, small at this wavelength), as a function of the time. Ethylene glycol 20%. Temperature indicated on the figure. Average of 300 flashes.

evidence any time for a reduction of ferredoxin, but the uncertainties of these experiments do not allow us to exclude a partial reduction of ferredoxin.

Kinetics of P-430

Fig. 4 shows the kinetics of the absorption changes at 446 nm, the isosbestic point of P^+-700 in Chlorella cells, at −10°C and at 0°C. At this wavelength, there is no contribution on the part of the System I donors. The shape of these kinetics indicates that between 2 μs and 9 μs, at least 3 different acceptors are involved, the intermediate acceptor having the weakest differential extinction coefficient at 446 nm. In particular, the reduced acceptors are different, 2 μs and 9 μs after the flash. This is consistent with the deconvolution of the spectra presented above.

The first decrease in absorption thus corresponds to the reduction of *P*-430. The time of reduction of *P*-430 is the time of distribution of the photons during the actinic flash, and thus the kinetics of reduction of *P*-430 after a photochemical reaction is not resolved. This is consistent with the observation of Ke [36]: *P*-430 is reduced in a time inferior to 100 ns at room temperature.

The following increase of the absorption corresponds to the reoxidation of P^--430. The analysis of the kinetics indicates that the half-time of this reoxidation is around 2 μs at −10°C, 1 μs at 0°C, and may be roughly extrapolated to a value in the order of 200 ns at room temperature.

Kinetics of ferredoxin-NADP-reductase

The flavoproteins present two semiquinoid forms: the semiquinone anion

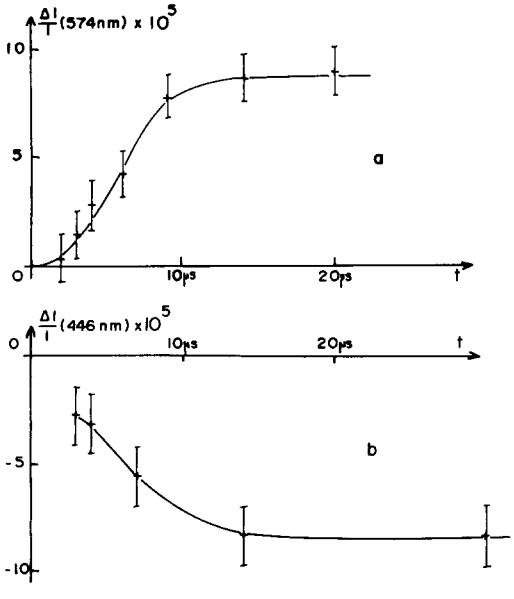

Fig. 5. Absorption changes after a flash (after small correction of the carotenoid triplet change and electrochromic effect at 446 nm and without any corrections at 574 nm where there is no carotenoid triplet change and no electrochromic effect) as a function of the time at −5° C. Ethylene glycol 14%. Average of 100 flashes. (a) 574 nm, (b) 446 nm.

and the neutral semiquinone [37]. Their spectra, almost identical in the blue region, differ above 550 nm. The deconvolution of the spectra performed at room temperature [18,19,31] indicates rather the presence of the neutral semiquinoid form of ferredoxin-NADP-reductase from 15 μs to 40 μs after a flash. However, as we always observed only a sum of the spectra of P^+-700 — P-700 and of FNR—FNR, and error in the spectrum of P^+-700 — P-700 would lead to a wrong conclusion about the semiquinoid form.

In order to solve this problem, Fig. 5 compares the kinetics at 446 nm and at 574 nm, two isosbestic points of P^+-700 — P-700 [31]. The absence of absorption changes at short times (2 μs after beginning of the flash) indicates that 574 nm is indeed the isosbestic point of P^+-700 — P-700. The increase of the absorption changes at 574 nm during the first 10 μs, time during which no oxidation of plastocyanin occurs [31], demonstrates the formation of the neutral semiquinone FNRH$^\bullet$. The identity of the half-times of the increase in absorption change at 574 nm and of the decrease in absorption change at 446 nm, the ratio of their amplitudes identical to the ratio of the extinction coefficients of FNRH$^\bullet$—FNR in Chlorella [19], show that the absorption decrease at 446 nm also reveals the reduction and protonation of FNR to its neutral semiquinoid form.

We thus conclude, on basis of Fig. 4, that the half-time of formation of FNRH$^\bullet$ is about 6 μs at —10°C and 3 μs at 0°C. Extrapolation to 20°C gives a half-time in the order of 700 ns which is consistent with the upper value (1 μs) proposed in Ref. 19. This is only an estimate as we have no information on the temperature coefficient between 0°C and 20°C.

This protonation of ferredoxin-NADP-reductase is too rapid to be a diffusion process at physiological pH. It is necessary therefore to conclude that the proton is already fixed close to the acceptor side before the photochemical reaction.

Acceptors between P-430 and ferredoxin-NADP-reductase

The discrepancy between the rate of reoxidation of P^--430 and the rate of reduction of ferredoxin-NADP-reductase (Fig. 4) clearly indicates that there is at least one intermediate carrier with, at the most, a weak extinction coefficient at 446 nm. Thus, there is at the least an intermediate which is not ferredoxin as the differential extinction coefficient of ferredoxin at 446 nm [38] is similar to that of P-430 [29] and FNRH$^\bullet$ [39]: 5 $mM^{-1} \cdot cm^{-1}$.

After one flash, the analysis of the spectra and of the kinetics of the absorption changes performed above did not allow the detection of some reduced ferredoxin. Thus, either ferredoxin is not in the electron transfer chain or its reduction rate is slower than its reoxidation rate and the amount of reduced ferredoxin remains low.

Further information on the intermediate carrier(s?) between P-430 and ferredoxin-NADP-reductase can be obtained by the following experiment: a first flash forms FNRH$^\bullet$ with a half-time of 3 μs at 0°C (Fig. 4); a second flash, fired 20 μs after the first one will lead to the reduction of the acceptor preceding FNRH$^\bullet$. The absorption changes induced by the second flash are presented on the bottom of Fig. 6 (open circles). They are not consistent with a sum of the spectra of P^+-700 plus Fd^- or FNR when a stoichiometry 1 : 1 is assumed.

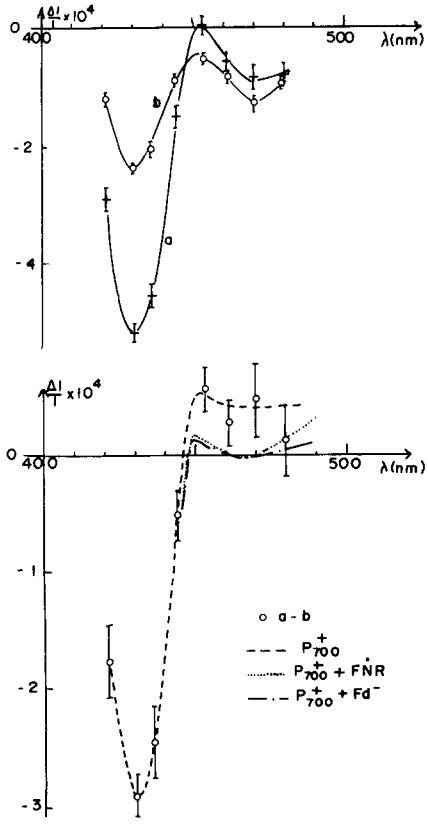

Fig. 6. Absorption changes (corrected for carotenoid triplet change and for electrochromic effect) as a function of the wavelength at 0°C. (Top) 30 μs after the beginning (○) of one flash; (+) of a couple of two flashes (distance 20 μs). Ethylene glycol 14%. Average of 100 to 400 flashes depending on the wavelength. (Bottom) Open circles: difference between the two curves of top figure. The curves are computed spectra as indicated in the text.

On the contrary, they exhibit a pure differential spectrum of P^+-700 — P-700, within the margin of error. Thus, the acceptor preceding FNRH° is neither another ferredoxin-NADP-reductase molecule, nor ferredoxin, nor P-430. This result is consistent with the results of Ref. 19.

The most likely hypothesis is that the transfers from the primary acceptor to ferredoxin-NADP-reductase are the same after two photoreactions and after one photoreaction. According to this hypothesis, the acceptor preceding ferredoxin-NADP-reductase, identical to the acceptor preceding FNRH°, and that we will name X′, has, at the most, weak differential extinction coefficients from 420 nm to 480 nm. It is consistent with the kinetics observed at 446 nm (Fig. 4).

Absence of redox changes of ferredoxin

Since the work of Tagawa and Arnon [12], ferredoxin has been considered the electron acceptor preceding ferredoxin-NADP-reductase into the electron

transfer chain of Photosystem I. The results presented above are not consistent with their assertion.

Two lines of reasoning have been used in favor of ferredoxin as an electron carrier of the chain:

(1) added ferredoxin is reduced by chloroplasts. But ferredoxin, the potential of which is −430 mV [12] could act as an exogenous electron acceptor as methyl-viologen and might receive its electron directly from *P*-430 [40]. The rate of reduction of ferredoxin by *P*-430 in the millisecond range [40] indicates that this reaction is not physiological: in whole cells, ferredoxin-NADP-reductase is reduced in a time inferrior to 1 μs;

(2) ferredoxin is necessary for the transfers to NADP [41]. Our results are consistent with this affirmation. In effect, the spectra observed are those of ferredoxin-NADP-reductase in a complex with ferredoxin [18] and the correct formulation would be that the acceptor is the flavin part of the complex ferredoxin-ferredoxin-NADP-reductase.

However, a question remains: Since this complex can accept three electrons, why does no redox changes of ferredoxin occur? In Ref. 19, we suggested that ferredoxin may already be reduced in the dark. Our attempt to prove this hypothesis was unsuccessful. In the first place, even after dark adaptation in the presence of oxidants entering the membrane, we were unable to detect the formation of photoreduced ferredoxin. Secondly, an in vitro study of the redox levels of the complex ferredoxin-ferredoxin-NADP-reductase showed that Fd^- reduces FNR, in the complex, and thus that the presence of some Fd^- in the dark is unlikely.

In order to account for the absence of redox change of ferredoxin, three other tentative explanations may be proposed:

(1) X′ has a redox potential which is not low enough to reduce ferredoxin.

(2) X′ is already protonated when reduced and transfers a hydrogen rather than an electron.

(3) For steric reasons, the flavin part of the complex is more rapidly accessible to the electron of X'^- than is the iron of the ferredoxin even when the flavin part is already in the semiquinone form.

Conclusions

The results obtained in this paper may be summarized by the following reaction scheme (the rates correspond to a temperature of 0°C, Fp stands for the flavin part of the complex ferredoxin-ferredoxin-NADP-reductase).

$$P\text{-}700 + P\text{-}430 \xrightarrow{h\nu + (t_{1/2} < 500\ \text{ns})} P^+\text{-}700 + P^-\text{-}430$$

$$P^-\text{-}430 + X' \xrightarrow{t_{1/2} \sim 1\ \mu s} P\text{-}430 + X'^- \text{ or } X'H^-$$

$$X' \text{ or } X'H^{\cdot} + Fp \xrightarrow{t_{1/2} \sim 3\ \mu s} X' + FpH^{\cdot}$$

$$2\ FpH^{\cdot} \rightarrow FpH_2 + Fp \text{ (from Ref. 18)}$$

and may be

$$X' \text{ or } X'H^{\cdot} + FpH^{\cdot} \rightarrow X' + FpH_2 \text{ (from Ref. 19)}$$

Nonetheless, sime questions remain: (1) What is the role of ferredoxin? (2) What is the nature of X'? (3) What is the mechanism of protonation of $FpH^{\bullet}$?

Acknowledgements

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