INTERACTIONS BETWEEN FERREDOXIN-NADP$^+$ REDUCTASE AND FERREDOXIN AT DIFFERENT REDUCTION LEVELS OF THE TWO PROTEINS

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1. Introduction

Ferredoxin-NADP$^+$ reductase [1] mediates electron-transport, at the level of chloroplast photosystem I, between one-electron donors (ferredoxin) and two-electron acceptors (NADP$^+$). The role of ferredoxin in the 'in vivo' photoreduction of the flavoprotein has been questioned [2]. Besides the evidence showing the essential requirement for ferredoxin in order to photoreduce NADP$^+$ in fragmented chloroplasts [3], it is well documented that the reductase has highly specific binding sites for both ferredoxin and NADP$^+$; indeed spectrally detectable complexes have been shown to form 'in vitro' between FNR and oxidized ferredoxin or NADP$^+$ [4]. Evidence for the ternary complex formation has been reported in [5].

The structure and physiological function of these complexes are still under debate. It is worth noting that most of the ferredoxin-NADP$^+$ reductase appears to be bound to the thylakoid membrane [6], whereas ferredoxin is considered a soluble protein. The reported stoichiometry of 5 Fd:3 FNR:1 P700 [7], does not seem to account for this protein acting like a true substrate in its interaction with the reductase, considering that several other roles have been assigned to ferredoxin. In many photosynthetic organisms, in fact, two molecular forms of ferredoxin have been found [8].

Thus, it is tempting to postulate that ferredoxin could form a stable complex with the flavoprotein, bridging the gap between photosystem I acceptors and NADP$^+$. In this way the soluble protein would be anchored to the thylakoid membrane through a stable interaction with the reductase. Here we have sought to verify 'in vitro' the possible existence of the complex between the two proteins under different reduction states.

Abbreviations: FNR, ferredoxin-NADP$^+$ reductase; Fd, ferredoxin; EDTA, ethylenediaminetetraacetate; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

2. Materials and methods

Ferredoxin-NADP$^+$ reductase and ferredoxin were prepared as in [9]. 3,10-Dimethyl-5-deaza-5-carbaisoalloxazine was a generous gift of Dr V. Massey. Photochemical reductions: Solutions contained in anaerobic spectrophotometer cells equipped with side arms, were made anaerobic by several cycles of evacuation and flushing with nitrogen which had been furtherly made O_2-free by passage through a heated, BASF R3-11 catalyst column. The anaerobic cell was placed in a water, Pirex glass bath maintained at 10°C and illuminated with a 600 W lamp at a total distance of 30 cm. After each period of irradiation, the absorption spectrum was recorded on a Cary 219 spectrophotometer.

3. Results and discussion

3.1. *Photoreduction of ferrodoxin, ferredoxin-NADP$^+$ reductase and their complex*

Light irradiation in the presence of EDTA and 5-deazaflavin as a catalyst [10], reduces, under anaerobiosis, ferredoxin and the flavoprotein on a time-scale of seconds, even at 10°C. Longer times of irradiation should be avoided in the case of Fd, because easily a hyperreduction of the iron–sulfur cluster can bleach the protein irreversibly. Fig.1 shows the photo-

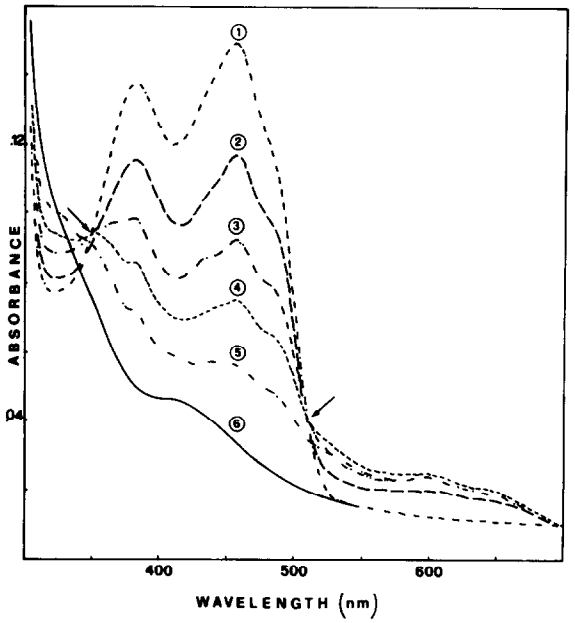

Fig.1. Photochemical reduction of FNR in the presence of EDTA and deazaflavin. Conditions: 12.2 μM FNR, 10 mM Hepes (pH 7), 15 mM EDTA and 1.2 μM deazaflavin, 10°C. Selected spectra only are shown; (1–6) after zero, 25 s, 50 s, 75 s, 105 s and 250 s of illumination, respectively. Isosbestic points at 510 and 355 nm were observed up to 75 s of irradiation. After complete reduction, air was admitted and the original spectrum was regained rapidly.

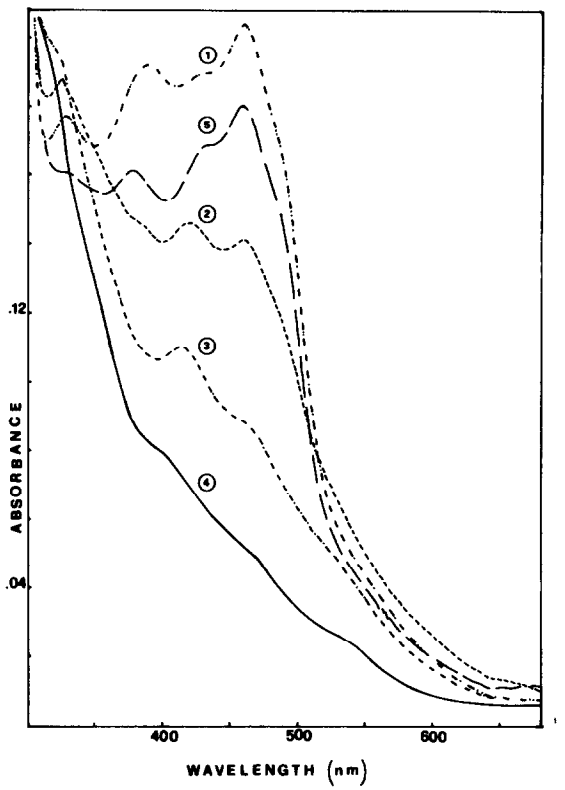

Fig.2. Photochemical reduction of a mixture of FNR and Fd (molar ratio 1:1) in the presence of EDTA and deazaflavin. Conditions were as in fig.1 except that the concentrations of the proteins were 9.3 μM: (1) before illumination; (2–4) after 60 s, 150 s and 330 s of illumination, respectively; (5) after salt addition and full reoxidation by air.

reduction of the reductase. It can be seen that the characteristic absorption spectrum of the blue neutral semiquinone is formed as a transient species during the photoreduction. Within a few minutes of illumination, complete reduction of the enzyme is achieved. In fig.2 it is shown a photoreduction of a mixture of the two proteins. By comparison with the spectrum obtained after addition of 0.5 M NaCl and reoxidation, it is clear that a complex between the two oxidized proteins was formed. The spectrum in the 600 nm region is somewhat difficult to interpret, owing to the behaviour of ferredoxin which decreases its absorbance upon reduction: however, an analysis of the 60 s irradiation spectrum reveals the presence of the semiquinone. Further examination of the 150 s irradiation spectrum indicates that the flavoprotein is being reduced first, followed by ferredoxin. This seems obvious since deazaflavin reduces more rapidly FNR than Fd in separate experiments; furthermore reduced ferredoxin will donate its electron to the reductase. The same reduction pattern is observed when light irradiation is performed on the two proteins after salt addition, although the amount of semiquinone formed seems lower.

3.2. *Complex formation between FNR and ferredoxin at various reduction levels*

Since the two proteins under examination are rapidly autooxidable, we could not use the double-sector cells to obtain the difference spectra. Thus we calculated by algebraic subtraction the difference between the spectra recorded before and after addition of 0.5 M NaCl (as crystals). It is well known that such a high ionic strength brings about a complete dissociation of the complex between the two oxidized proteins; besides, it was ascertained that the spectra of the single proteins were not perturbed by this ionic strength.

To check the inherent rightness of our experimental

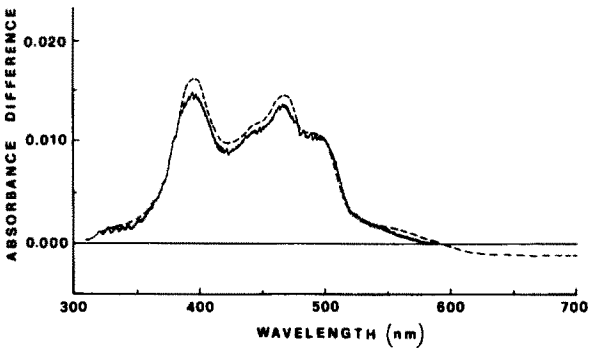

Fig.3. Difference spectrum of the oxidized complex FNR–Fd, obtained by two different methods. (——) Spectrum was recorded by using matched split-compartment cells with a mid-partition and an optical path length of 0.9 cm; (– – –) spectrum was calculated as described in the text from a different experiment. Conditions: FNR and Fd were 5 μM in 10 mM Hepes (pH 7).

system we compared the spectra of the oxidized complex, obtained by the two methods (fig.3). A part from minor details, the agreement is quite good; from both spectra an ϵ_M value at 465 nm of ~2900 for the complex can be calculated, which agrees with the published value [4].

Thus, we photoreduced the two proteins separately in an anaerobic cuvette and mixed them in order to have different amounts of electrons in the two proteins system.

In fig.4, the difference spectra obtained when 0, 1, 2 and 3 electrons are present in the two protein system are reported. To facilitate the comparison, the spectrum with one electron has been shifted upwards. The latter spectrum was obtained either by mixing 1 mol partially reduced FNR with oxidized ferredoxin or by mixing 1 mol reduced ferredoxin with 1 mol oxidized FNR. The two-electron spectrum similarly can be produced either by adding 1 mol oxidized ferredoxin to 1 mol completely reduced FNR or by mixing 2 mol reduced ferredoxin with 1 mol oxidized FNR; whereas the 3-electron spectrum was obtained either by mixing the two proteins completely reduced or by full reduction of the oxidized complex between the two proteins.

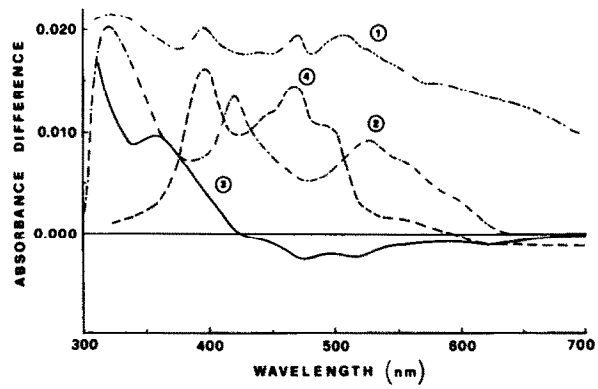

Fig.4. Difference spectra of FNR–Fd complexes obtained at different reduction levels of the two proteins: (1–3) difference spectrum with 1 electron, 2 electrons and 3 electrons in the protein system, respectively. Conditions: 50 mM Hepes (pH 7), FNR and Fd were 10 μM, 10°C. The oxidized difference spectrum is the same of fig.3 (– – –). The one-electron spectrum has been shifted upwards to facilitate the comparison.

Clearly these difference spectra are an indication that a complex between ferredoxin and the reductase, which involves perturbation of the protein chromophores, is present independently of the oxidation–reduction states of the proteins. It can be noted that the one-electron spectrum seems to sum up the features of both the oxidized complex and the two-electron reduced species. This is not unexpected since with one electron on the two proteins system we merely have nearly half of the flavoprotein in the reduced state with a small amount of semiquinone (fig.1); in these conditions, the difference spectrum presents both the contribution of the oxidized complex and that of the complex between oxidized ferredoxin and reduced FNR.

The spectrum of the latter complex should indeed correspond to the difference spectrum obtained at the two-electron reduction stage. The difference spectrum which is observed at the 3 electron reduction stage (fig.4) should be representative of the interactions between reduced ferredoxin and fully reduced flavoprotein.

The oxidized, 2-electron and 3-electron difference spectra show distinct peculiarities; this is not unexpected because the absorption spectra of the two proteins change substantially upon reduction.

4. Conclusions

These results prove that a complex between the ferredoxin-$NADP^+$ reductase and ferredoxin persists even under partial or complete reduction of the two proteins. Thus, it is not unlikely that 'in vivo' such a stable complex could exist and function as a single unit in the photoreduction of $NADP^+$. This hypothesis

would be more consistent with a scheme of the photosystem I segment of the electron-transport chain in the thylakoid membrane, where all the known carriers are membrane bound except for ferredoxin; through its stable interaction with the reductase, this carrier as well, would be anchored to the thylakoid membrane, providing a continuous channel for the flow of electrons to the flavoprotein, which is able to commute between one-electron donors and two-electron acceptors.

Considering the reported stoichiometry between these two proteins [7] it appears that ferredoxin certainly exceeds the 1:1 ratio found for the complex in the 'in vitro' experiments. Accordingly, several other roles have been assigned to ferredoxin, i.e., as electron donor to thioredoxin reductase, sulfite reductase, glutamate synthase, nitrate reductase and presumably to the cyclic photophosphorylation. Thus, it is conceivable that part of the ferredoxin can be sequestered in the complex with FNR for $NADP^+$ photoreduction, which is by far the more conspicuous process in the chloroplast among those cited, leaving enough ferredoxin to accomplish the other roles.

Acknowledgement

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