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ON THE CONFORMATION OF RECONSTITUTED FERREDOXIN:NADP$^+$ OXIDOREDUCTASE IN THE THYLAKOID MEMBRANE

STUDIES VIA TRIPLET LIFETIME AND ROTATIONAL DIFFUSION WITH EOSIN ISOTHIOCYANATE AS LABEL

RICHARD WAGNER [a], NESTOR CARRILLO [b], WOLFGANG JUNGE [a] and RUBEN H. VALLEJOS [b]

[a] *Schwerpunkt Biophysik, Universität Osnabrück, Postfach 4469, D-4500 Osnabrück (F.R.G.) and* [b] *Centro de Estudios Fotosinteticos y Bioquimicos, CONICET, Fundacion Miguel Lillo, Universidad Nacional de Rosario, 2000 Rosario (Argentina)*

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Eosin isothiocyanate was covalently bound to isolated ferredoxin-NADP$^+$ reductase under protection of the NADP-binding domain. The bound label did not impair the functional reconstitution of the enzyme into depleted thylakoid membranes. Laser spectrophotometric experiments were carried out on thylakoids which were reconstituted with labeled ferredoxin-NADP$^+$ reductase. Bound eosin isothiocyanate was used as a spectroscopic probe for conformational changes of ferredoxin-NADP$^+$ reductase in either of two ways: We studied the rotational diffusion of labeled ferredoxin-NADP$^+$ reductase in the membrane by the photoselection technique, and we studied the triplet lifetime of bound eosin, which measures polypeptide chain flexibility (via access of oxygen) around the binding site. The latter technique was complemented by measurements of the librational motion of bound dye. We observed: (1) When ferredoxin is absent, ferredoxin-NADP$^+$ reductase undergoes very rapid rotational diffusion in the thylakoid membrane (correlation time less than 1 μs at 10°C). This is drastically slowed down (40 μs) upon addition of water-soluble ferredoxin. We propose that ferredoxin mediates the formation of a ternary complex with ferredoxin-NADP$^+$ reductase and the Photosystem I complex. According to our data, this complex would live longer than required for the photoreduction of ferredoxin-NADP$^+$ reductase by Photosystem I via ferredoxin. (2) Under the given incubation conditions, the binding sites for eosin isothiocyanate were located in the FAD domain of ferredoxin-NADP$^+$ reductase. We found increased chain flexiblity in this domain upon addition of NADP. This suggests induced fit for the binding of NADP and allosteric control of the FAD domain by the remote NADP domain. (3) Acidification of the internal phase of thylakoids decreased the chain flexibility in the FAD domain. This is of particular interest, since ferredoxin-NADP$^+$ reductase is a peripheral external membrane protein. It suggests the existence of a binding protein for the oxidoreductase which spans the membrane and senses the internal pH.

Introduction

Ferredoxin-NADP$^+$ reductase (ferredoxin : NADP$^+$ oxidoreductase, EC 1.18.1.2) is a flavoprotein which operates as the terminal elec-

Abbreviations: Tricine, *N*-tris(hydroxymethyl)methylglycine; DCCD, *N*,*N'*-dicyclohexylcarbodiimide; DCIP, 2,6-dichlorophenolindophenol.

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tron-carrier protein in the photosynthetic electron-transport chain [1,2]. X-ray diffraction analysis at 3.7 Å resolution revealed two distinct domains of this enzyme, one containing ferredoxin-$NADP^+$ reductase, the other binding NADP [46]. The enzyme was shown to be located on the outer side of the thylakoid membrane in a wide variety of photosynthetic organisms including higher plants [3–6], eucaryotic algae [7,8], diatoms [9] and cyanobacteria [10,11]. Studies on the inhibition of the membrane-bound enzyme by monospecific antibodies with stacked and non-stacked thylakoids suggested that it is located preferentially in the stroma lamella. Reconstitution of the soluble reductase into thylakoids which were depleted either by aging [10] or by EDTA treatment [13] resulted in restoration of the reductase-dependent NADP photoreduction activity upon addition of excess purified flavoprotein. Recently, it was shown that functional reconstitution required the presence of a suitable amount of cations in the incubation medium [14]. Little or no specifity was observed between cations of the same valence group, the order of effectiveness being $C^{3+} > C^{2+} > C^{+}$. These results were interpreted in terms of the Gouy-Chapman double-layer theory, as applied to biological membranes by Barber [15] and Sculley et al. [16]. Analogous to the behavior of the coupling factor for photophosphorylation (CF_1), the ferredoxin-$NADP^+$ reductase seems to be bound (perhaps via an intrinsic membrane protein) to the outer side of the membrane by van der Waals' forces in competition with electrostatic repulsion of (negative) fixed charges. Carrillo et al. [17,19] have recently shown that the activity of ferredoxin-$NADP^+$ reductase is modulated by the energetic state of the thylakoid membrane. Thus, we deemed it desirable to obtain more detailed information on the interaction of this enzyme with the membrane and other membrane proteins. To this end, we covalently attached the spectroscopic probe, eosin isothiocyanate, to the isolated oxidoreductase. We then monitored the amount and mode of rebinding of the labeled enzyme to depleted membranes via the absorption changes of the eosin isothiocyanate probe.

Eosin isothiocyanate is a valuable tool for studies on the binding of proteins to membranes and on conformational changes of proteins. Upon excitation with a flash from a frequency-doubled Nd-YAG laser, the dye is efficiently transformed into a relatively long-lived triplet state. This gives rise to absorption changes which can be used to monitor the binding state and the conformation of the host potein in either of two ways: (1) The triplet lifetime depends on the access of O_2 to a given bindig site of eosin isothiocyanate in the host protein. Broadly speaking, the triplet lifetime reflects the proximity of a binding site to the bulk medium and/or the flexibility of the polypeptide chains which cover a binding site. We have previously used this property to follow the conformational changes of both the isolated [20,22] and membrane-bound coupling factor of photophosphorylation (CF_1) [21] and of the isolated ferredoxin-$NADP^+$ reductase [23]. (2) The decay of the linear dichroism of the absorption changes of eosin isothiocyanate (in photoselection experiments) in part reflects the rotational diffusion of the host protein (see Ref. 31). We have previously studied the rotational diffusion of the coupling factor for photophosphorylation in the thylakoid membrane by this technique [21].

In this paper we report on the rotational mobility and on conformational changes of reconstituted oxidoreductase as function of its redox state and of the proton-motive force across the thylakoid membrane.

Materials and Methods

1. Isolation and treatment of chloroplasts. Chloroplasts were isolated from fresh spinach leaves (*Spinacea oleracea*) as described previously [24] and suspended in a medium containing 2 mM Tricine-Tris (pH 8), 330 mM sorbitol, 1 mM dithiothreitol, bovine serum albumin (0.1 mg/ml) and 5 μM $CaCl_2$ [25]. After centrifugation (10 min at 10000 × *g*), two successive incubations (15 min) were carried out with the same medium at 4°C and 0.1 mM Chl. After this treatment, the thylakoids with their diaphorase activity reduced to 15–20% were washed once with 50 mM Tricine-NaOH (pH 8), 5 mM $MgCl_2$ and 250 mM sucrose, and finally resuspended in the same medium at a concentration of 2 mM Chl. Total chlorophyll was determined as described in Ref. 26.

2. Labeling of solubilized ferredoxin-NADP$^+$ reductase by eosin isothiocyanate and reconstitution of the labeled enzyme. Ferredoxin-NADP$^+$ reductase [26] and eosin isothiocyanate [27] were prepared according to previously published procedures. The enzyme was labeled with eosin isothiocyanate as described elsewhere [22]. The conditions of labeling ferredoxin-NADP$^+$ reductase with eosin isothiocyanate without impairing the diaphorase activity are also described in Ref. 22. The ferredoxin-NADP$^+$ reductase concentration was determined either spectrophotometrically, using $\epsilon_{456} = 10.7\ mM^{-1} \cdot cm^{-1}$ [26] or with the Coomasie brilliant blue Method [28]. The amount of eosin isothiocyanate bound to the protein was measured as described in Ref. 22.

Reconstitution experiments were carried out essentially as described in Ref. 14. The concentration of soluble eosin-ferredoxin-NADP$^+$ reductase was routinely 10 μM in a medium containing 50 mM Tricine-NaOH (pH 8), 5 mM $MgCl_2$, 250 mM sucrose and thylakoids corresponding to 0.3 mM Chl. After 5 min of incubation at room tempeature, the samples were washed once and finally resuspended in the same buffer at 20–30 μM Chl.

The amount of eosin-ferredoxin-NADP$^+$ reductase reassociated with the membranes was estimated by treating the samples with pure acetone to a final concentration of 80% (v/v). After centrifugation (10 min at $10000 \times g$), the supernatant was discarded and the small pellet dried (60°C, 1h) and redissolved in 0.6 ml buffer containing 2% SDS. Controls were run in parallel with the corresponding amount of nonlabeled enzyme and processed in the same manner. Absorbance of eosin isothiocyanate at 532 nm was recorded against a blank of the control sample.

3. Enzyme assays. Diaphorase [17] and ferredoxin-dependent cytochrome *c* reductase activities [26] of both the soluble and membrane-bound ferredoxin-NADP$^+$ reductase, as well as light-driven proton uptake by thylakoid membranes with phenazine methosulfate as cofactor, were measured with a pH electrode as described previously [30].

4. Laser flash spectrometry with reconstituted eosin-ferredoxin-NADP$^+$ reductase. The principle of the laser flash spectrophotometry for photoselection and the instrumental setup are given in more detail elsewhere [20]. Briefly, the sample was placed in an optical absorption cell (1 cm path, 1 ml volume) which was thermostatically controlled. The composition of the sample is described in part 2 of Materials and Methods and in the legends to figures. Excitation was provided by a frequency-doubled (wavelength 532 mm) Q-switched Nd-YAG Laser with a pulse duration of 10 ns (full-width at half-maximum) at typically 20 $mJ \cdot cm^{-2}$ pulse energy. The photomultiplier was protected from the scattered flash by a special cutoff filter (manufacturer Dr. Hugo Anders), with 70% transmission at the measuring wavelength (545 nm) but $A > 7$ at the wavelength of excitation. Transients of the output voltage of the photomultiplier were amplified, digitized (Biomation 6500) and averaged (Tracor TN-1500). The triplet state of the ferredoxin-NADP$^+$ reductase-bound eosin was measured indirecly via the ground state depletion (545 mm). The time resolution of the apparatus was limited by the blank-out caused by the residual flash burst artefact (200 ns). The interpretation of signals after 1 μs was unambiguous.

5. Photoselection experiments and analysis of the data. The principles and geometrical features for measuring rotational diffusion of macromolecules with extrinsic probes by photoselection are given in detail elsewhere [30,31]. After measuring the saturation behavior of the eosin absorption changes in chloroplast suspensions reconstituted with eosin-ferredoxin-NADP$^+$ reductase, the excitation energy of the intrinsically vertically polarized laser (E vector) was adjusted to yield less than 20% saturation of the eosin absorption changes.

The measuring light was polarized by a rotatable Glan-Thomson prism. In the following, $\Delta A_{\parallel}(t)$ and $\Delta A_{\perp}(t)$, respectively, denote the transient absorption changes which were recorded under parallel and perpendicular polarization of the E vector of the exciting laser flash and of the measuring light. These two observables were combined to yield two interesting parameters:

$$\Delta A_t(t) = \Delta A_{\parallel} + 2\Delta A_{\perp} \quad \text{(total absorption change)}$$

and

$$r(t) = \frac{\Delta A_{\parallel} - \Delta A_{\perp}}{\Delta A + 2\Delta A_{\perp}} \quad \text{(absorption anisotropy)}$$

As so defined, the total absorption change reflects the formation and decay of the triplet state without interference of rotational diffusion, while the absorption anisotropy reflects rotational diffusion without interference of the decay of the triplet state. The time dependence of ΔA_t and of r was determined by a least-squares analysis of the experimental points at times $t + n\Delta t$.

Results

1. Labeling of isolated oxidoreductase with eosin isothiocyanate and its effects (brief account of previous results)

We have previously reported on the different modes of binding of eosin isothiocyanate to the isolated oxidoreductase [22]. The results are summarized in the schematic drawing shown in Fig. 1. We found at least two different types of binding sites for eosin isothiocyanate, one of which (the A site) is in close proximity to the catalytic site for NADP reduction. Binding of eosin isothiocyanate to this site can be prevented if the enzyme is incubated with eosin isothiocyanate in the presence of sufficient amounts of NADP. As documented in Table I, this (protected) incubation mode has the advantage of leaving the diaphorase activity of the enzyme practically unaffected.

Table I also shows that modification of the additional sites (namely, B and C in Fig. 1) decreased the rate of ferredoxin-dependent cytochrome *c* reduction mediated by the flavoprotein. The addi-

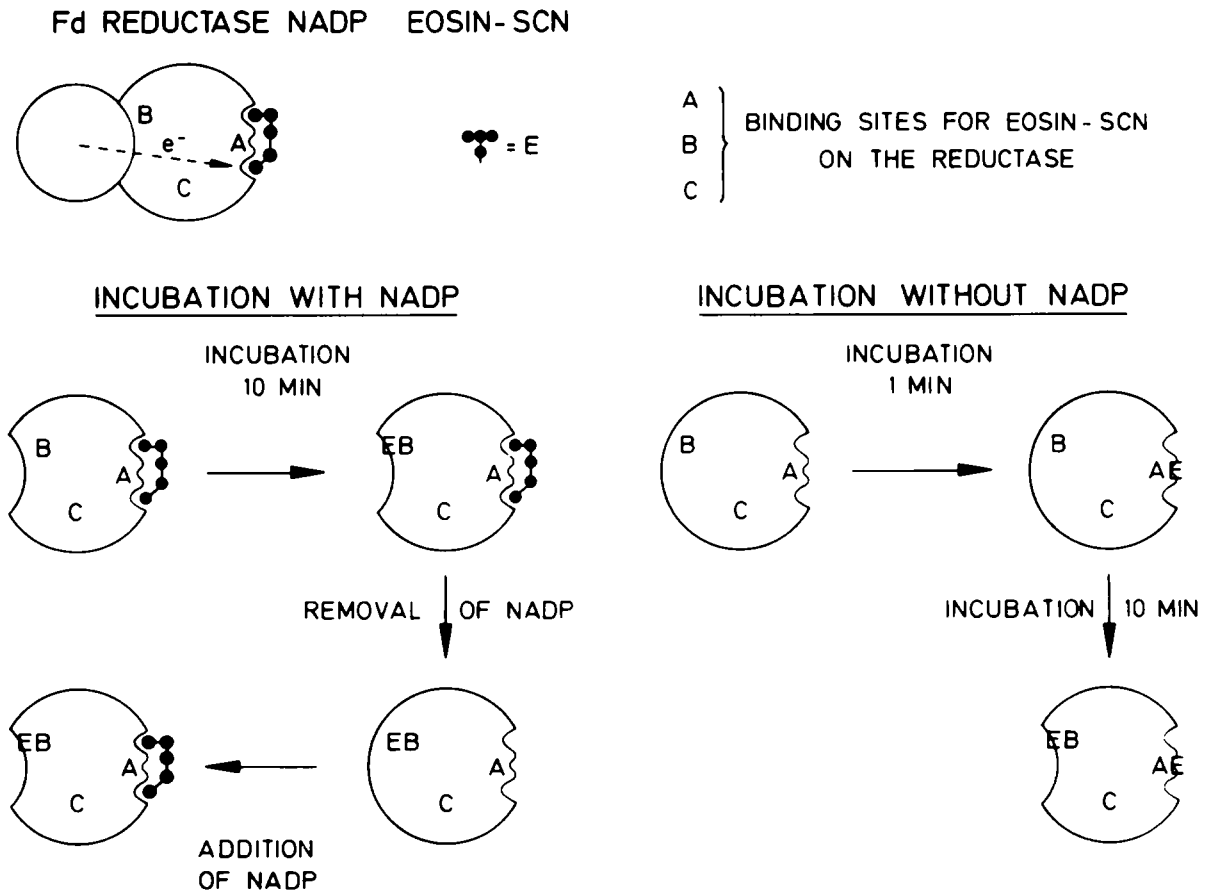

Fig. 1. Schematic picture of the labeling of the soluble ferredoxin-NADP$^+$ reductase (Fd reductase NADP) by eosin isothiocyanate (eosin-SCN) during incubation, either in the presence or in the absence of NADP. The distinction between labeling sites A–C is based on triplet lifetime data and on the effect of labeling on the activities of the enzyme [22]. The conformational change in the neighborhood of site B upon binding of NADP was also established in previous work [22].

TABLE I

EFFECT OF EOSIN ISOTHIOCYANATE ON THE CATALYTIC ACTIVITIES OF FERREDOXIN-NADP$^+$ REDUCTASE

Ferredoxin-NADP$^+$ reductase (5 μM) was incubated with 0.5 mM eosin isothiocyanate for 5 min. The reaction was stopped by the addition of glycine up to a concentration of 0.1 M. Excess of reagent was removed as described in Ref. 22. NADP$^+$ and ferredoxin, when present, were at 5 mM and 32 μM, respectively. Enzymatic activities and eosin load of the oxidoreductase were measured as described under Materials and Methods. Concentrations: eosin isothiocyanate, 0.5 mM; NADP, 5 mM; ferredoxin, 32 μM. Incubation time: 5 min.

Incubation medium	Diaphorase activity (μmol DCIP/mg per min)	Cytochrome *c* reductase (μmol cytochrome *c*/mg per min)	Eosin ferredoxin-NADP$^+$ reductase (mol/mol)
Control	14.8	10.5	-
+ eosin	3.0	2.1	2.80
+ eosin + NADP	14.5	3.1	1.97
+ eosin + NADP + ferredoxin	14.4	3.7	1.70

tion of high amounts of ferredoxin to the incubation medium afforded only slight protection against both deactivation and incorporation of the reagent into the protein (Table I, row 4). Conversely, it appears that blocking of sites B and C hardly affects the ability of the enzyme to interact with ferredoxin. We observed that addition of catalytic amounts of ferredoxin to soluble eosin-ferredoxin-NADP$^+$ reductase (labeled only at sites B and C) resulted in significant alterations in the environment of those sites, as monitored by a change in triplet lifetime of bound eosin (data not shown). This result is in line with previous reports of conformational changes in ferredoxin-NADP$^+$ reductase which accompany complex formation with ferredoxin [32,33]. Thus, the inhibitory effect on the ferredoxin-dependent cytochrome *c* reduction documented in Table I was probably not due to an inability of ferredoxin to complex with ferredoxin-NADP$^+$ reductase which was labeled at sites B and C with eosin. Therefore, such inhibition can be tentatively attributed to action of eosin on the electron pathway from ferredoxin through the ferredoxin-NADP$^+$ reductase polypeptide to the flavin moiety of ferredoxin-NADP$^+$ reductase. Site A on the one hand, and site B on the other, could be distinguished by the triplet lifetime of the eosin which was respectively bound to them [22].

2. Reconstitution of eosin isothiocyanate-labled ferredoxin-NADP$^+$ reductase into depleted membranes

Fig. 2 shows the diaphorase activity of reconstituted membranes as a function of the amount of labeled oxidoreductase in the reconstitution medium. The amount of labeled enzyme on membranes was determined via the absorption of eosin isothiocyanate at 532 nm (see Materials and Methods). We used enzymes which were labeled only at the B or C sites, with site A unmodified. As the figure indicates, the diaphorase activity of

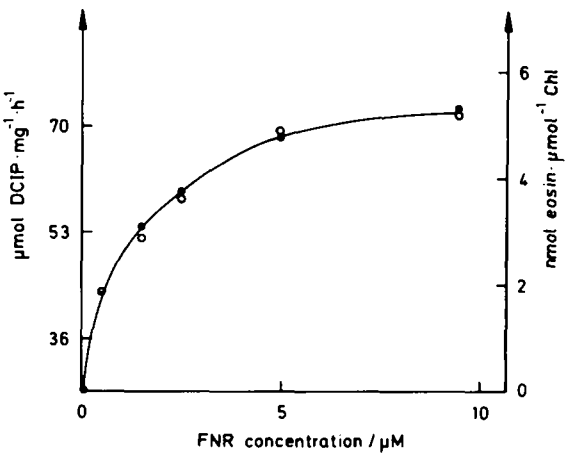

Fig. 2. Reconstitution of ferredoxin-NADP$^+$ reductase (FNR) into depleted membranes. $CaCl_2$ particles (diaphorase activity 27.7 μmol DCIP/mg per h) were incubated for 5 min at room temperature with the indicated amounts of eosin isothiocyanate-labeled ferredoxin-NADP$^+$ reductase (1.97 eosin/FAD). After centrifugation and washing of the pellet, diaphorase activity (●) and eosin concentration (○) in the particulate fraction were determined as described in Material and Methods.

thylakoids increased in parallel with the amount of labeled enzyme on thylakoid membranes.

Considering a ratio of 2 eosin molecules/ferredoxin-NADP$^+$ reductase (see Table I), the maximal reassociation attained was 2.6 nmol ferredoxin-NADP$^+$ reductase/μmol Chl, in good agreement with the results obtained for the reconstitution of the nonlabeled enzyme [14]. These results suggest that binding of eosin isothiocyanate at sites B and C in ferredoxin-NADP$^+$ reductase does not affect the functional reconstitution of the flavoprotein into depleted membranes.

3. Laser flash spectrophotometry of thylakoids which were reconstituted with eosin isothiocyanate-labeled ferredoxin-NADP$^+$ reductase

Fig. 3a shows the time course of absorption changes at 545 nm after flash excitation of a chloroplast suspension which was reconstituted with eosin isothiocyanate-labeled ferredoxin-NADP$^+$ reductase. Fig. 3b shows analogous measurements using chloroplasts which were reconstituted with unlabeled enzyme.

The extent of the observed absorption changes $\Delta I/I$ was $1.0 \cdot 10^{-2}$ in Fig. 3a, and less than $5 \cdot 10^{-4}$ in Fig. 3b. The smaller absorption changes

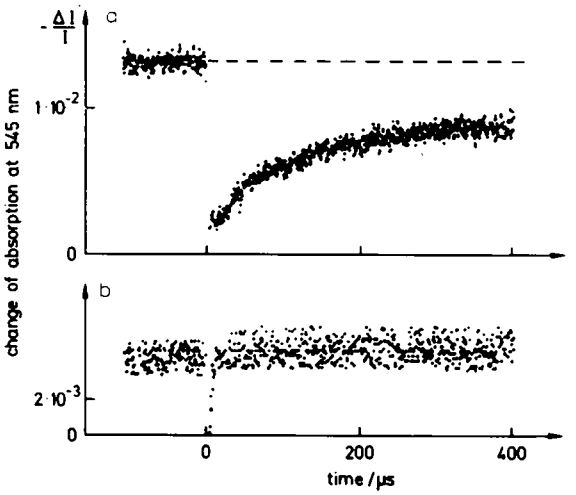

Fig. 3. Time course of absorption changes at 545 nm after flash excitation of a chloroplast suspension reconstituted with ferredoxin-NADP$^+$ reductase. (a) Reconstitution was carried out with eosin isothiocyanate-labeled enzyme (1.97 eosin/FAD). (b) With nonlabeled ferredoxin-NADP$^+$ reductase. Experimental details concerning reconstitution are given in Materials and Methods.

in the unlabeled sample were mainly caused by cytochromes [35]. They can be neglected in the following for their smallness and for the fact that they are virtually independent of time (see also Appendix).

With the extinction coefficient of eosin at 545 nm ($4.6 \cdot 10^4$ M$^{-1} \cdot$ cm^{-1}), one calculates the concentration of 'excited' eosin from the extent of the absorption changes in Fig. 3a:

$$\Delta A = \epsilon \Delta c d = 1.0 \cdot 10^{-2}/2.3$$

For this experiment we obtained Δc(excited) = 9.4 nM. The overall content of eosin isothiocyanate in the sample was determined in a static absorption experiment (see Materials and Methods) to be 80 nM, which is approx. 8-times greater. This discrepancy was probably caused by both lack of saturation of the sample by the laser flash and only partial intersystem crossing of the singlet excited dye.

4. Rotational diffusion of eosin-labeled-ferredoxin-NADP$^+$ reductase after reconstitution into the membrane

Rotational diffusion of reconstituted ferredoxin-NADP$^+$ reductase was measured via the absorption changes of eosin isothiocyanate by the photoselection technique. Fig. 4 documents the time course of the absorption changes at 545 nm for parallel and for perpendicular polarization of the exciting light relative to the polarization of he measuring light beam (Fig. 4a). Fig. 4b shows the time course of the total absorption change (ΔA_t) and Fig. 4c that of the absorption anisotropy (r), both in semilogarithmic form. The experiment was carried out at 10°C. The sample contained 32 μM ferredoxin, and white light (0.1 μW/cm^2) was applied from 105 ms until 5 ms before firing of the laser flash. The linear dichroism of the absorption changes is apparent. The total absorption change decays (seemingly monoexponentially) much slower than the anisotropy. The decay of the anisotropy is at least biphasic.

We measured the decay of the anisotropy at different redox state of the enzyme. The results and reaction conditions are documented in Fig. 5 and its accompanying legend. The following features are apparent:

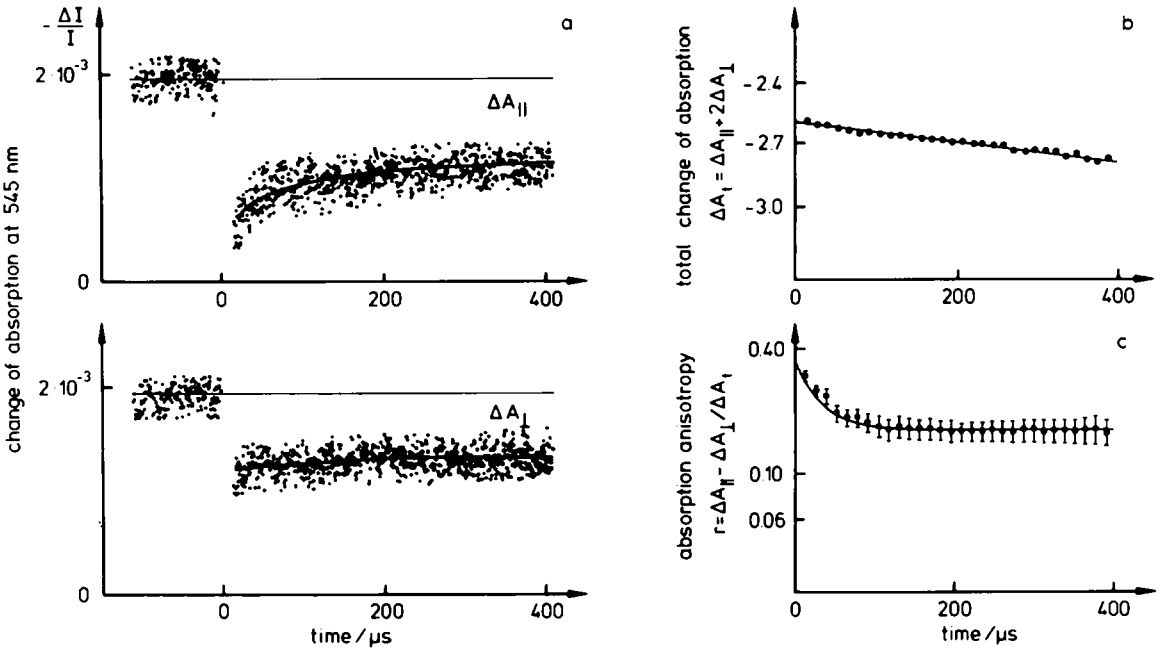

Fig. 4. Absorption changes of eosin at 545 nm in thylakoids which were reconstituted with eosin isothiocyanate-labeled ferredoxin-NADP$^+$ reductase. (a) Left: under parallel and right, under perpendicular polarization of the exciting and the measuring light. Semilogarithmic plot of (b) the total absorption change and (c) of the absorption anisotropy.

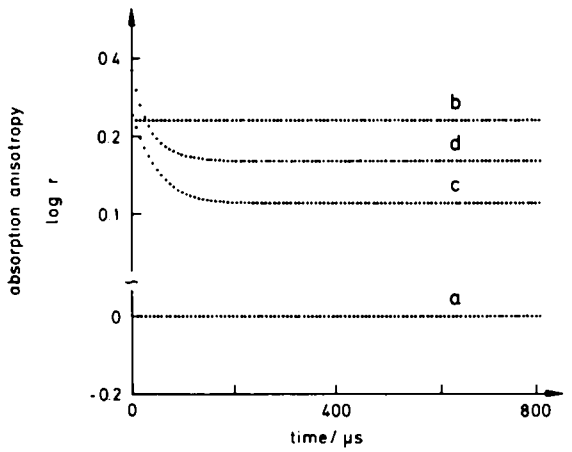

Fig. 5. Semilogarithmic plot of the time course of the absorption anisotropy at 545 nm under different conditions of eosin isothiocyanate-labeled ferredoxin-NADP$^+$ reductase in the thylakoid membrane. (a) Reconstituted membranes without further additions and without strong preillumination. (b) Reconstituted membranes which were preincubated with 2% glutaraldehyde for 15 min. (c) Reconstituted membranes in the presence of 32 µM ferredoxin. (d) Same conditions as in c, but after strong preillumination of the sample (0.1 W/cm^2 from 105 to 5 ms before firing of the laser flash).

Trace a: When the labeled ferredoxin-NADP$^+$ reductase was reconstituted without further treatments, no dichroism was detectable ($r = 0$) in the time domain from 1 µs to 1 ms. This implies a more rapid rotational motion (less than 1 µs) of the enzyme and/or of the dye relative to the enzyme. It is worth noting that the labeled ferredoxin-NADP$^+$ reductase was indeed bound to the membrane inasmuch as it could not be removed by multiple washings in buffer.

Trace b: When reconstituted membranes were preincubated with glutaraldehyde (2% for 15 min) linear dichroism appeared at an absorption anisotropy of $r = 0.23$, and did not decay within 1 ms. The time independence of the anisotropy conforms with the expectation that glutaraldehyde cross-links membranes proteins and hence prevents their rapid rotation (see Ref. 41). The limited extent of the anisotropy (0.23 instead of a theoretical upper limit of 0.4) was not caused by instrument imperfections as we previously demonstrated (with an 'eosin-candy') that anisotropies as high as $r = 0.37$ are obtainable with immobilized eosin in

our instrument (see Ref. 20). The low anisotropy value is therefore attributable to residual mobility of the chromophore in the oxidoreductase even after cross-linking with glutaraldehyde. It is noteworthy that the same lower value of the absorption anisotropy was obtained with isolated labeled ferredoxin-NADP$^+$ reductase which had been immobilized by attachment to DEAE-Sephadex (data not shown).

Trace c: When 32 μM ferredoxin was added to reconstituted membranes (no glutaraldehyde fixation) greater linear dichroism was induced. The extent of the anisotropy at the beginning of the record is similar to that in trace b, but now there was a partial decay and a much smaller irreversible component. The time course of the anisotropy corresponded to:

$$r_c(t) = 0.14 \exp(-t/40\,\mu s) + 0.11$$

Trace d: Preillumination of chloroplasts with white light (0.1 μW from 105 to 5 ms before the laser flash) in the presence of ferredoxin yielded a higher initial anisotropy (0.35). Its time course was:

$$r_d(t) = 0.09 \exp(-t/15\,\mu s) + 0.1 \exp(-t/40\,\mu s) + 0.16$$

In the light of the foregoing it seems that both protein rotation and chromophore mobility were affected by illumination. It is noteworthy that the relatively short preillumination in the absence of a cofactor for cyclic electron transport had not produced a significant proton-motive force in these chloroplasts.

5. Changes in the triplet lifetime of reconstituted eosin-ferredoxin-NADP$^+$ reductase

The triplet lifetime of reconstituted eosin-ferredoxin-NADP$^+$ reductase was measured under conditions in which the enzyme was supposed to be in different redox states. Additionally, these measurements were performed in the absence of other soluble electron carriers, i.e., ferredoxin or NADP, but under energization of the thylakoid membrane (via the formation of a light-driven proton gradient across the membrane during rapid cyclic electron flow).

Fig. 6 shows a typical time course of the absorption changes at 545 nm after flash excitation of a chloroplast suspension reconstituted with eosin-ferredoxin-NADP$^+$ reductase. The absorption changes reflect the ground state depletion of eosin

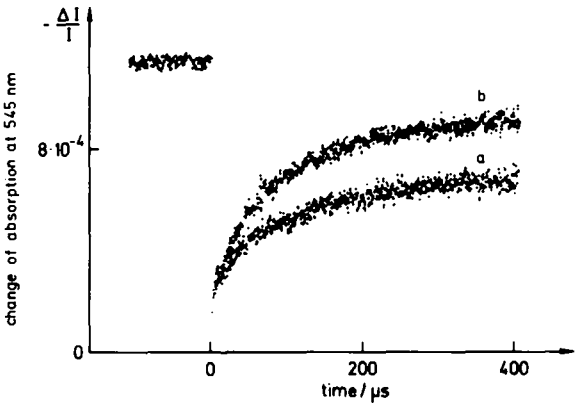

Fig. 6. Time course of absorption changes of eosin at 545 nm in thylakoids which were reconstituted with eosin isothiocyanate-labeled ferredoxin-NADP$^+$ reductase. Negatively directed changes (upon firing of the laser flash at $t = 0$) indicate population of the triplet state, positively directed changes the decay of the triplet state. (a) In the absence of NADP, (b) in the presence of NADP (0.2 mM). For other conditions, see text.

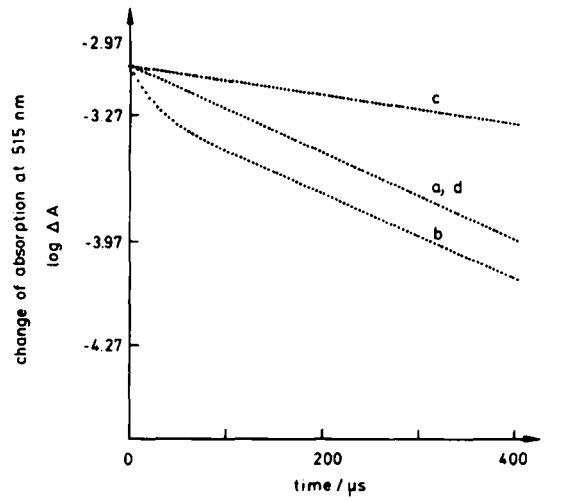

Fig. 7. Semilogarithmic plot of the eoxin triplet decay in thylakoids which were reconstituted with eosin isothiocyanate-labeled ferredoxin-NADP$^+$ reductase, based on experiments as documented in Fig. 6. (a) Standard sample with ferredoxin (32 μM), (b) as a but NADP (0.2 mM) added, (c) same as a but 100 ms preillumination (0.1 W/cm^2), (d) same as b but with preillumination. For further conditions, see text.

and its subsequent repopulation from the triplet state. The sample, partially deoxygenated (see Materials and Methods), always contained ferredoxin (32 μM) and in trace b also NADP (0.2 mM). The experiment was carried out at 10°C. The results of triplet lifetime measurements with the reconstituted eosin-ferredoxin-NADP$^+$ reductase under different conditions of the enzyme (corresponding in principle to different redox states) are summarized in Fig. 7. The time course of the eosin absorption changes shown in this figure was recorded from measurements as in Fig. 6.

Comparison of traces a and c in Fig. 7 reveals that the decay of eosin absorption changes was slowed by a factor of 3 when the sample was preilluminated, and that the addition of NADP leads to a biphasic decay (appearance of a rapid decay component with a half-time of 20 μs, trace b). This decay was reversed upon preillumination of the sample (trace d). Since the decay of the measured eosin absorption changes reflects the accessibility of the eosin binding sites (B and C sites) to O_2, the results can be explained as follows: The binding of NADP of the enzyme exposes one part of the eosin-binding sites to the bulk medium. This was previously shown to occur with isolated ferredoxin-NADP$^+$ reductase [22] with the same sites labeled and is schematically illustrated in Fig. 1.

Discussion

According to the results of several authors [3–12], ferredoxin-NADP$^+$ reductase seems to be located in the nonstacked and fringe portions of thylakoid membranes. It thus resides preferentially in proximity to Photosystem I and to the coupling factor for photophosphorylation (CF_1). Since the enzyme can be detached from the membrane by washing in low-salt media [13,14,26], it is probably not an intrinsic membrane protein. Its binding seems to occur when the ionic repulsion between negatively charged groups on the enzyme and on its counterpart in the membrane falls behind van der Waals' attraction under ionic shielding of the repulsive charges. The enzyme mediates the reduction of NADP by Photosystem I via soluble ferredoxin acting as an essential intermediary electron carrier.

The isolated and purified enzyme can be reconstituted into depleted membranes [14]. We found that, when labeled with eosin isothiocyanate, the modified enzyme can be reconstituted also. If the enzyme was labeled in the protected mode, i.e., when 'site A' was protected by the presence of NADP during incubation with eosin isothiocyanate (see Fig. 1 and Ref. 22), the reconstituted ferredoxin-NADP$^+$ reductase had full diaphorase activity (up to a load of 2 eosin isothiocyanate/ferredoxin-NADP$^+$ reductase). This suggests that the modified and reconstituted enzyme which we used was functionally competent both in its activity and its ability to rebind to the membrane.

Photoselection experiments on rotational mobility of chromophore in the protein and of enzyme in the membrane

We would like to discuss first the absence of any detectable dichroism with the reconstituted enzyme in the absence of either ferredoxin or NADP at a time resolution of 1 μs (Fig. 5a). Does rotational diffusion of the protein in the membrane and librational motion of the dye around its binding axis in the protein account for the rapid (less than 1 μs) dissipation of the photoinduced linear dichroism? We already mentioned in Results that the theoretical upper limit for photoselection experiments with a totally immobilized linear chromophore ($r_{lim} = 0.4$) was experimentally well approximated in our instrument ($r_{max}^{exp} = 0.37$). When eosin isothiocyanate was bound to ferredoxin-NADP$^+$ reductase, and the enzyme was immobilized either by cross-linking the membrane-bound enzyme with glutaraldehyde (Fig. 5b) or by attaching the isolated enzyme to DEAE-Sephadex (data not documented), we obtained $r = 0.26$ for the initial absorption anisotropy. The most probable interpretation is that librational motion of the dye around its binding axis rapidly dissipated the difference between 0.37 and 0.26 of the anisotropy, while rotational diffusion of the enzyme in the membrane dissipated the remainder (0.26). This holds for the enzyme in the absence of its substrates (see Fig. 5a).

That the enzyme may carry out rotational diffusion which is as rapid as 1 μs is not surprising even

if it were an intrinsic membrane protein. Assuming an apparent viscosity of 1 P for the thylakoid membrane, one would expect rotational correlation times of 3 μs for this relatively small enzyme (35–40 kDa; see also related studies on rhodopsin in the disk membrane [41]). It is striking, however, that the rapid rotational diffusion is slowed by orders of magnitude upon the addition of soluble ferredoxin to the suspension (see trace c in Fig. 5). We consider the following interpretation probable. Ferredoxin itself is a water-soluble protein and thus would not be affected by changes in the membrane viscosity. Furthermore, it is too small (12 kDa) to account alone for such slowing of the rotational diffusion of the ferredoxin-$NADP^+$ reductase. Hence, we propose that the slowing is due to the linkage of ferredoxin-$NADP^+$ reductase to a larger, more slowly rotating component, possibly the Photosystem I complex. The observed rotational correlation time of 40 μs would conform with a complex size of 200–300 kDa residing in a membrane with a viscosity of 1 P.

In contrast with the situation in the absence of ferredoxin, there remained a nondecaying portion of the absorption anisotropy at $r_{steady} = 0.11$ when ferredoxin was added to the suspension medium (see Fig. 5c). This may indicate that the orientation of the chromophore axis to the normal of the membrane plane (which is the axis of admissible protein rotation) was changed by the addition of ferredoxin. For instance, if it were inclined at the magic angle in the absence of ferredoxin, with ferrodoxin it would be more inclined towards the plane.

Even more dramatic changes in the rotational mobilities occurred upon illumination of thylakoids in the presence of ferredoxin (see Fig. 5d). Here the initial extent of the anisotropy increased up to almost 0.4, which was followed by an apparently biphasic decay (15 and 40 μs decay times) and an irreversible component as large as 0.16. This is attributable only to the input of electrons into the enzyme complex and not to the energization of the membrane by a proton-motive force (see Results). We interpret this as follows: (1) The reduction of the ferredoxin-$NADP^+$ reductase in the presence of ferredoxin altered that region in the enzyme where eosin isothiocyanate can bind to sites B and C (see Fig. 1 for an illustration). The alteration must have been such that the librational motion of the chromophore in the protein was drastically slowed and possibly also restricted to a more limited angular domain. In this communication we have not attempted to quantitate these statements because of the as yet unresolved partition between chromophore motion and protein motion in the decay of the anisotropy. (2) Under illumination, either the protein rotation was also limited to a certain angular domain, which we regard as improbable, or the orientation of the chromophore axis was further changed in the direction of the membrane plane. In a previous paper [22] we presented evidence that binding sites B and C for eosin isothiocyanate on the ferredoxin-$NADP^+$ reductase are located near the electron pathway from ferredoxin to the flavin moiety. The influence that electrons residing on the flavin exert on the mobility of eosin isothiocyanate is compatible with such a location.

It is worth mentioning that the initial value of the anisotropy in the presence of ferredoxin and light is close to the theoretical limit for a situation in which the same transition moment is both excited and interrogated. This again demonstrates the absence of any major experimental imperfections in our setup. The finding that the flavoprotein is more rigidly held in the membrane in the presence of ferredoxin can be accounted for by two possible mechanisms (or a combination of both): (i) Complex formation occurred between ferredoxin and the membrane-bound ferredoxin-$NADP^+$ reductase. This was probably accompanied by conformational changes of the flavoprotein. As a result of those changes the interaction of the enzyme with some larger membrane protein became stronger. (ii) Ferredoxin not only interacted with the membrane-bound ferredoxin-$NADP^+$ reductase, but also with a larger membrane protein and in so doing acted as a bridge between the oxidoreductase and this larger protein.

The interaction of ferredoxin-$NADP^+$ reductase with ferredoxin has been previously studied in solution [32,33,38]. The available information concerning the complex forming properties of membrane-bound oxidoreductase is as follows: Addition of purified ferredoxin to a chloroplast suspension prevents the reaction of the membrane-bound ferredoxin-$NADP^+$ reductase

with monospecific antibodies [39]. More recently, it was reported that in whole *Chlorella* cells the flavin absorption peak is shifted in the same way as observed during complex formation with ferredoxin in vitro [35]. Finally, strong evidence was presented showing that the conformation of the ferredoxin-flavoprotein complex is important for the catalytic reduction of NADP by chloroplasts [39].

Changes of the triplet lifetime of bound eosin isothiocyanate

The triplet lifetime of bound eosin isothiocyanate indicates the accessibility of a binding site to the quencher oxygen. Studies on the diffusion of another small molecule, CO, out of and into the heme pocket of myoglobin, by Frauenfelder and Petsko [43] have shown that the diffusion of this small molecule is largely controlled by the flexibility of the chain structure covering the binding site. By analogy, shortening of the triplet lifetime of bound eosin isothiocyanate by some operation on the ferredoxin-$NADP^-$ reductase could be caused by either or both of the following: the respective binding site is transferred from the interior towards the surface of the protein, or constraints on the flexibility of the covering chain elements are removed. With the B- and C-labeled and reconstituted ferredoxin-NAD^- reductase, and in the absence of substrates and light, we obtained a monoexponential decay of the triplet state with a lifetime of 200 μs (see Fig. 7a) in the partially deoxygenated samples. The triplet decay was partially accelerated (decay faster than 20 μs) upon binding of $NADP^+$ to the reconstituted ferredoxin-$NADP^+$ reductase. This paralleled the behavior that we previously reported for the isolated enzyme [22]. We had speculated that the binding of $NADP^+$ causes an allosteric effect upon the region where ferredoxin binds, possibly for facilitated transfer of electrons to the flavin moiety. It is noteworthy that in both cases (with and without $NADP^+$ in Fig. 7a and b), the enzyme was in its oxidized state.

A drastic prolongation of the triplet lifetime occurred if the fully reduced state of the enzyme was generated by preillumination in the absence of $NADP^+$ but in the presence of ferredoxin (Fig. 7c). The triplet lifetime was then 0.6 ms. It can be assumed that the enzyme was in its fully reduced state under the given conditions [35]. Prolonged triplet lifetime in the fully reduced state of the flavin can be interpreted as follows: It is known that the chromophore undergoes a conformational change from a planar to a roof conformation (folding like a book between N-5 and N-10 with 145° between the benzene and the pyrimidine plane) when the enzyme is transferred from the oxidized into the fully reduced state [39]. This conformational change is transmitted to the region of eosin isothiocyanate to sites B and C. It makes the protein chain elements which cover these sites more rigid and/or it buries sites B and C deeply in the interior.

Fig. 7d documents how the conformational change upon reduction interferes with the other conformational change which occurs upon binding of $NADP^+$. The triplet lifetime was again shortened, however, it became monophasic with the same decay time as observed for the oxidized enzyme in the absence of ferredoxin. Under these conditions, the ferredoxin-$NADP^+$ reductase was in its half-reduced (semi-quinonoid) form [33] with rapid turnover of $NADP^+$/NADPH.

The disappearence of the very rapid phase of triplet quenching which appeared upon binding of $NADP^+$ to the oxidized ferredoxin-$NADP^+$ reductase after transformation of the enzyme into the half-reduced state by light can be attributed to the transition of the flavin moiety back into its planar conformation [39]. We note again that preillumination did not create a considerable pH difference under our experimental conditions, but acted on the redox state only.

We generated a proton-motive force by 'plugging' the holes in the thylakoid membrane with DCCD and inducing rapid cyclic electron flow and proton pumping via the cofactor phenazine methosulfate. In the absence of ferredoxin this prolonged the triplet lifetime of eosin isothiocyanate from 100 to 250 μs. The reversibility of this effect upon addition of the uncoupler NH_4Cl showed that it was indeed an effect of the electrochemical energization of the membrane and not due to an altered redox state of ferredoxin-$NADP^+$ reductase. These observations are in line with previous reports on the effect of a proton-motive force on the binding of inhibitors to the oxidore-

ductase [17], and on the kinetic parameters of the enzyme [18].

Summary and Conclusions

We observed four types of structural alterations of the eosin isothiocyanate-labeled membrane-bound ferredoxin-NADP$^+$ reductase (see Table II):

(1) Addition of soluble ferredoxin to thylakoids drastically slowed the rotational diffusion of ferredoxin-NADP$^+$ reductase in the membrane. It also altered the orientation of the label and/or the angular domain of rotational mobility for more than 1 ms.

(2) The binding sites for eosin isothiocyanate under our incubation conditions were probably located in the FAD domain of the protein. Reduction of the flavin reduced the flexibility of polypeptide chain elements, which cover these sites.

(3) Binding of NADP to ferredoxin-NADP$^+$ reductase increased the flexibility of these chain elements.

(4) Under conditions where the rotational mobility of ferredoxin-NADP$^+$ reductase is high, i.e., in the absence of ferredoxin, the acidification of the internal phase of thylakoids (with the outer phase well buffered) decreased the flexibility of chain elements covering the eosin isothiocyanate label.

This must be correlated with the known information concerning the function and structure of ferredoxin-NADP$^+$ reductase:

(a) Ferredoxin-NADP$^+$ reductase mediates electron transfer in the sequence: Photosystem I complex → ferredoxin → ferredoxin-NADP$^+$ reductase → NADP$^+$. Its reduction occurs in 300 μs under flash excitation of Photosystem I [35].

(b) Full reduction (but not half reduction) causes the transition of the flavin from a planar into a roof conformation [39].

(c) Ferredoxin-NADP$^+$ reductase consists of two domains, one contains the FAD and the other binds NADP at its surface [46].

(d) Ferredoxin-NADP$^+$ reductase is located at the outer side of the thylakoid membrane. It is not a transmembrane protein (see Introduction).

Taking this together, we would like to present the following tentative interpretation of our data:

(i) Ferredoxin links ferredoxin-NADP$^+$ reductase to the Photosystem I complex. The ternary complex lives long enough (1 ms) for the reduction of ferredoxin-NADP$^+$ reductase to occur (300 μs). We plan to test this with antibodies directed against Photosystem I.

(ii) The planar-to-roof transformation upon full reduction of the flavin induces strain in the FAD domain of the protein which decreases chain flexibility.

(iii) NADP binds via induced fit, since its binding causes conformational changes even in one other domain of ferredoxin-NADP$^+$ reductase.

TABLE II

SUMMARY OF EXPERIMENTAL RESULTS

Correlation times are given for the rotation of bound eosin isothiocyanae relative to the protein ('librational motion') and for the rotational diffusion of ferredoxin-NADP$^+$ reductase in the membrane as well as eosin triplet lifetimes of reconstituted eosin-ferredoxin-NADP$^+$ reductase under various experimental conditions. Results are expressed in μs.

Conditions	Eosin isothiocyanate librational motion in ferredoxin-NADP$^+$ reductase	Triplet lifetime of bound eosin isothiocyanate (65 μM O_2 in medium)	Ferredoxin-NADP$^+$ reductase rotational diffusion in membrane
Standard medium	<1	100	<1
+ ferredoxin	<1	200	40
+ ferredoxin + light	15	600	40
+ ferredoxin + NADP$^+$	<1	200	40
+ ferredoxin + NADP$^+$ + light	<1	200	40
+ proton-motive force	-	250	-

Whether increased chain flexibility in the FAD domain is favorable for electron transfer from ferredoxin to FAD remains to be investigated.

(iv) Ferredoxin-$NADP^+$ reductase, which is located at the outer side of the thylakoid membrane, 'senses' the pH in the thylakoid interior. Possibly, this could be due to a specific binding protein for ferredoxin-$NADP^+$ reductase, which traverses the membrane.

Appendix

In order to give a semiquantitative treatment of the data obtained from measurements of the rotational diffusion of reconstituted eosin-ferredoxin-$NADP^+$ reductase by applying the photoselection technique, it is useful to simplify the experimental situation in a first approximation as described below.

The measurable absorption anisotropy is, in principle, affected by the following factors:

(a) Symmetry properties of the chromophore. According to our previous work [20], eosin is a very good approximation for a linear oscillator.

(b) Librational motion of the dye around its binding axis.

(c) Rotational diffusion of the enzyme around the axis perpendicular to the membrane plane.

(d) Instrumental factors and experimental imperfections such as depolarization in the sample or at the interfaces of optical filters, excessive excitation energy, etc.

For the interpretation of the time course of the absorption anisotropy, r, it is important to distinguish between the above-described contributions. In a first approximation this can be achieved in the following way:

(a) The instrumental factors can be checked by measuring the maximum obtainable value for r with a reference sample in the measuring light path. The preparation of a sample with totally immobilized eosin (eosin-candy) which is useful for the calibration of the light path is described elsewhere [20]. For our light path we obtained a maximal value of $r_{max} = 0.37$, which is quite close to the theoretical value of $r_{max} = 0.4$ for one linear absorber [20].

(b) The contribution of librational motion of the dye in the protein to the decay of the absorption anisotropy can be determined by immobilizing the protein in a Sephadex matrix. Thus, even when the decay of the absorption anisotropy due to the very fast motion of the dye cannot be resolved in time, it can be inferred from the difference between the maximal $r = 0.37$ and the value observed under these conditions. For eosin-ferredoxin-$NADP^+$ reductase immobilized on Sephadex we obtained $r(0) = 0.21$. Thus, the restricted dye motion dissipates a value of $\Delta r = 0.16$. From this we calculate an amplitude for uniaxial librational motion of the dye of $\Delta\phi = 115°$ [30].

(c) The decay of the absorption anisotropy due to uniaxial rotational diffusion of the eosin-ferredoxin-$NADP^+$ reductase reconstituted in the membrane can be described by the following expression [31]:

$$r(t) = a_1 \cdot \exp(-D_{\parallel} t) + a_2 \cdot \exp(-4D_{\parallel} t) + a_3$$

where $a_1 = (6/5)(\sin^2\phi \cdot \cos^2\phi)$, $a_2 = (3/10)(\sin^4\phi)$, $a_3 = (1/10)(3\cos^2\phi - 1)^2$, and where ϕ denotes the angle between the transition moment and the normal to the membrane. This expression holds if the protein can only rotate around the normal to the membrane ($D_{\perp} = 0$). It is noteworthy that the absorption anisotropy (r) does not necessarily decay to zero, but to a time-independent value a_3 (for a plot of $a_i = f(\phi)$, see Ref. 45).

It is noteworthy that the time course of the absorption anisotropy as calculated from the measured dichroic absorption changes at 545 nm (as shown, for example, in Fig. 3a) is not affected by intrinsic absorption changes of chloroplasts at this wavelength. This can be explained as follows: The absorption anisotropy observed at a given wavelength which is attributed to several different transition dipoles can be described by:

$$r = \sum_{i=1}^{k} \sum_{j=1}^{m} p_i q_j r_{i,j}$$

where p_i is the relative absorption at the wavelength of excitation and q_j the relative change of absorption at the wavelength of observation. Since there is no interaction of the transition dipoles in our case, the $r_{i,j}$ values become 0 for $i \neq j$. This means that excitation of chloroplast antennae does not cause energy transfer into bound eosin and

vice versa. Taking into account that $r_{intrinsic} = 0$ for chloroplasts at 545 nm [34] the expression reduces to:

$$r_{obs} = q_{eos} r_{eos}$$

With $q_{eos} = 19/20$ (from Fig. 3) and $r_{eos}^{ideal} = 0.4$ (the theoretical value for a linear transition dipole). We finally obtain $r_{obs} = 19/20 \cdot 0.4 = 0.38$. This deviation is negligible relative to the noise in our experimental curves.

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