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Characterization of the cross-linked complex formed between ferredoxin-NADP$^+$ reductase and flavodoxin from *Anabaena* PCC 7119

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A covalent complex between ferredoxin-NADP$^+$ reductase and flavodoxin, two flavoproteins isolated from the nitrogen-fixing cyanobacterium *Anabaena*, has been formed by a cross-linking reaction with a water-soluble carbodiimide. The complex has a 1:1 stoichiometry and an absorption spectrum similar to that of the mixture of the free proteins. Both proteins can be detected immunochemically when they are in the complex, since they react with antisera raised against the isolated proteins. The complex is shown to be active in the NADPH–cytochrome *c* reductase reaction, although with a lower turnover number than FNR when ferredoxin is used as the electron carrier. Stopped-flow experiments have shown a lower rate of electron transfer (approx. 20-fold) from the semiquinone form of flavodoxin, when bound into the complex, to cytochrome *c*, as compared to the free protein. Anaerobic titration of the reduced complex with NADP$^+$ also indicates that there is transfer of electrons between the reductase and flavodoxin in the complex. Nevertheless, the covalent complex is found to be unable by itself to mediate the transfer of electrons from photosynthetic particles to NADP$^+$. This indicates that the pathway for the entrance of electrons into the complex is partially or completely blocked depending on the degree of cross-linkage. However, some activity is shown when ferredoxin is added to the system. The covalent complex shows a limited ability to act as an electron transfer protein between photosynthetic membranes and either exogenous FNR or cytochrome *c*. The concentration of complex required in this case is also much higher than when free ferredoxin or flavodoxin are present. It is concluded that the covalent complex formed by ferredoxin-NADP$^+$ reductase and flavodoxin from the cyanobacterium *Anabaena* PCC 7119 can be used as a simplified model for the study of electron transfer reactions between two flavoproteins. It can not be used for the study of reactions involving reduction of NADP$^+$ by photosynthetic particles.

Introduction

Electron transfer reactions between flavoproteins are fundamental in many biochemical pathways such as mitochondrial fatty acid degradation, microsomal cytochrome *P*-450 reduction or photosynthetic electron transfer. Progress has recently been made on the knowledge of the structural and functional features of certain specific proteins such as the enzyme ferredoxin-NADP$^+$ reductase (EC 1.18.1.2 (FNR)) [1,2] and the low-molecular-weight protein flavodoxin [3,4], providing a suitable system for the study of electron transfer reactions through flavin-flavin interaction. The reductase isolated from higher plants and cyanobacteria [5,6] is an FAD containing redox protein that participates in the reductive side of the photosynthetic chain transferring electrons from reduced ferredoxin to NADP$^+$. Flavodoxin is an FMN-containing low- molecular-weight protein that is present in some species of microorganisms in conditions which prevent synthesis of ferredoxin as a consequence of the low concentration of iron in the medium, and has been proposed to replace ferredoxin in all reactions in which this redox

Abbreviations: FNR, ferredoxin-NADP$^+$ reductase; Fld, flavodoxin; Fd, ferredoxin; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; cyt *c*, cytochrome *c*.

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protein participates [3]. This includes the reductase-dependent reduction of $NADP^+$ which occurs in the light in the presence of spinach chloroplasts and also the NADPH–cytochrome *c* reductase activity, which are described as the typical reactions for ferredoxin determination. In both of these reactions the reductase forms stable electrostatic complexes with flavodoxin [7]. It has also been established that cyanobacterial flavodoxin acts as an efficient electron donor to nitrogenase with a very low K_m value, suggesting a role for this protein in the nitrogen-fixing pathway in these organisms [8].

There is some three-dimensional structure information available for both ferredoxin-$NADP^+$ reductase from spinach [2] and flavodoxin from different origins, including the cyanobacterium *Synechococcus* PCC 6031 (*Anacystis nidulans*) for which high-resolution X-ray structure has been reported [9]. The reductase and flavodoxin from the nitrogen-fixing *Anabaena* PCC 7119 have been isolated and partially characterized [8] and have been found to be a very appropriate system for the study of the electron-transfer reactions from which general mechanistic conclusions could be drawn. Both proteins have been found to be very stable, they are water-soluble and can be obtained with rather high yields. Moreover, both proteins from *Anabaena* have been cloned [10,11] and could be genetically manipulated to produce specific mutants. The formation of the electrostatic complex between the two proteins from the same organism, which is proposed to be the first step in the electron-transfer reaction, probably involves some negative groups in flavodoxin interacting with positively charged groups present in ferredoxin-$NADP^+$ reductase [7]. Chemical cross-linking of these redox proteins would provide a way to stabilize the otherwise transient kinetic intermediate, allowing the amino acids involved in the complex to be identified. The cross-linked complex could be also a useful system for the study of the structural and functional requirements for the efficient electron transfer in flavoproteins. We recently described the formation of a covalent cross-linked complex between *Anabaena* FNR and *Azotobacter* flavodoxin [12]. Very low activities were obtained for both the NADPH–cytochrome *c* reductase activity and the photoreduction of $NADP^+$ in the covalent complex. It was of interest to determine whether the complex formed by proteins from the same organism, which could be involved in physiological reactions, would show higher activities and could then be used to study the mechanism of electron transfer among flavoproteins.

Materials and Methods

All proteins, i.e., ferredoxin-$NADP^+$ reductase, flavodoxin and ferredoxin, were purified from *Anabaena* PCC 7119 as described previously [6,8] from cells that were grown autotrophycally on nitrate, either under low iron concentration (2.6 μM) to allow synthesis of flavodoxin, or at 17 μM of ammonium ferric citrate, for the isolation of ferredoxin. All reagents were purchased from commercial sources and were of analytical grade. Cross-linking reactions were performed in 25 mM phosphate buffer (pH 7.0) and 20°C using 30 μM concentrations of each protein. EDC was added to a final concentration of 2 mM and aliquots taken at various intervals to either assay the activity or to quench the reaction by the addition of dissociation buffer (1% SDS, 1% 2-mercaptoethanol, 40% sucrose in 0.4 M sodium phosphate buffer (pH 3)) before application to the electrophoresis gel. SDS-polyacrylamide gel electrophoresis was performed according to Hames [13] using a Pharmacia Phast System apparatus. Rabbit antisera for ferredoxin-$NADP^+$ reductase and for flavodoxin were obtained by standard procedures after injection of approx. 300 μg of each homogeneous protein. Enzymatic assays were performed as described earlier [6] and photoreduction reactions of $NADP^+$ (or cytochrome *c*) were recorded continuously while illuminating with a 50 W halogen lamp at room temperature using a Hewlett-Packard diode array spectrophotometer. Washed chloroplasts were prepared as described by Walker [14]. The reductase was released from the membranes by incubation at room temperature for 24 h and eliminated by centrifugation. Stopped-flow kinetic studies were performed anaerobically using a Kinetic Instruments apparatus as described by De Francesco et al. [15]. Anaerobic photoreduction and reoxidation by $NADP^+$ addition were performed under an argon atmosphere using a cell similar to that of Burleigh et al. [16]. 5-Deazariboflavin was a gift from Professor D.E. Edmondson of Emory University.

Results

Cross-linking reaction between ferredoxin-$NADP^+$ reductase and flavodoxin

Treatment of the reductase and flavodoxin with the water-soluble carbodiimide EDC results in the formation of a cross-linked complex between the two proteins as evidenced by SDS polyacrylamide gel electrophoresis (Fig. 1). The reaction proceeds quite rapidly, since it is almost complete in 60 min, producing a rather clean band for the complex. The molecular weight of the species formed was found to be approx. 56000 as corresponds to the 1:1 complex between the enzyme (M_r 36000) and flavodoxin (M_r 20000), although an amount of a higher molecular weight complex, probably formed by the cross-linking of a flavodoxin dimer with the reductase, is also formed (see below). The slight heterogeneity that is observed in the

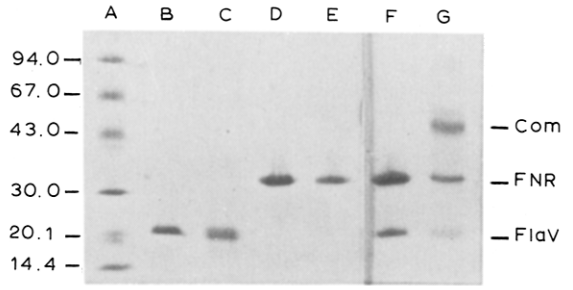

Fig. 1. SDS-polyacrylamide gel electrophoresis of reaction products after treatment of flavodoxin and ferredoxin-NADP$^+$ reductase with EDC. Com, complex; Flav, flavodoxin. Lane A: molecular weight standard proteins: phosphorylase *b* (94000), bovine serum albumin (67000), ovoalbumin (43000), carbonic anhydrase (30000), soybean trypsin inhibitor (20000) and α-lactalbumin (14000). Lane B: flavodoxin. Lane C: flavodoxin treated with EDC. Lane D: reductase treated with EDC. Lane E: reductase. Lane F: flavodoxin and reductase without EDC. Lane G: flavodoxin and reductase treated with EDC. The concentration of the reactants were: FNR and flavodoxin 30 μM and EDC 2 mM.

band corresponding to the complex can be attributed to internal cross-linking of flavodoxin which makes it interact with SDS differently from the native protein and explains the diffuse band that this protein produces when incubated with EDC alone (cf. lanes B and C in Fig. 1).

Molecular characterization of the cross-linked complex

In order to identify and characterize the reductase-flavodoxin cross-linked complex, sufficient amount of the two proteins were treated with EDC and separated from the unreacted proteins and the excess of reagent using a Sephadex G-100 superfine column. Fig. 2 shows the elution profile of this chromatographic process in which the larger peak corresponds to the 1:1 complex, but a significant amount of the higher molecular weight complex is also formed in these conditions in which the

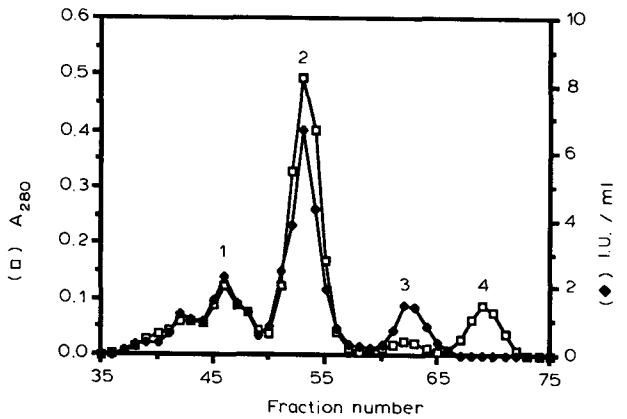

Fig. 2. Purification of FNR-Fld covalent complex by gel filtration in a Sephadex G-100 superfine column (2.5 × 150 cm). Peaks were located by the absorbance at 280 nm and the diaphorase activity with dichlorophenol indophenol. (1) High-molecular-weight complex; (2) FNR-Fld complex; (3) unreacted FNR; (4) unreacted flavodoxin.

flavodoxin concentration is rather high. The isolated complex showed an absorption spectrum with similar characteristics to those of the individual proteins, although the extinction coefficient, calculated after extracting the cofactors by boiling the protein solution, was shown to be 21.4 mM^{-1} cm^{-1} at 461 nm, while the sum of those in the proteins is 18.8 mM^{-1} cm^{-1}. This hyperchromic effect has been described to occur on electrostatic complex formation between ferredoxin-NADP$^+$ reductase and several electron transferring proteins such as flavodoxin [7] and has been used as a measure of the extent of complex formation in titration experiments [17]. Both flavodoxin and ferredoxin-NADP$^+$ reductase present in the complex could be detected immunochemically, since they both show precipitation lines when exposed to antisera raised against ferredoxin-NADP$^+$ reductase and flavodoxin (not shown).

Kinetic and functional characterization of the cross-linked complex

Once the covalent complex between ferredoxin-NADP$^+$ reductase and flavodoxin had been formed it was essential to probe its functionality, since this would indicate that the interaction between the two proteins is similar to that in the "natural" electrostatic complex. For this reason we investigated the ability of the complex to participate in the reactions in which the individual components show activity.

Diaphorase activity. Incubation of the reductase with EDC alone had no effect on the diaphorase activity, while the covalent complex lost approx. 50% of both the ferricyanide and DCPIP reductase activity. These results indicate that the covalently bound flavodoxin interferes with the accessiility of the electron acceptor to the flavin group in ferredoxin-NADP$^+$ reductase.

NADPH–cytochrome c reductase activity. Table I shows the cytochrome *c* reductase activity of ferredoxin-NADP$^+$ reductase in the free and complexed

TABLE I

NADPH-cytochrome c reductase activity of ferredoxin-NADP$^+$ reductase and the reductase-flavodoxin covalent complex

The assay mixture contained 0.75 mg/ml cytochrome *c* and 0.2 mM NADPH in 50 mM Tris-HCl (pH 8.0). FNR concentration was 9 nM and complex concentration was 69 nM. Flavodoxin and ferredoxin were 20 μM.

System	NADPH-cyt *c* reductase activity T.N. (min^{-1})
FNR	30
FNR + Fd	5231
FNR + Fld	4500
Complex	751
Complex + Fd	639
Complex + Fld	625

state. An increase of 175-fold is observed when the assay is carried out in the presence of saturating concentrations of ferredoxin, which indicates the suitability of this assay for the rapid detection of ferredoxin in biological preparations. Flavodoxin also produces an important increase in the reductase activity, which is close to the value obtained with ferredoxin.

The NADPH–cytochrome *c* reductase activity of the reductase-flavodoxin covalent complex is found to be approx. 17% of that of the non covalent complex in the presence of saturating concentrations of flavodoxin. It is also interesting to note that the addition of large amounts of either ferredoxin or flavodoxin to an enzymatic assay mixture in which the complex is present does not produce any stimulatory effect on the activity, indicating that the binding site for ferredoxin on the reductase is already occupied by flavodoxin in the covalent complex.

Two possible reasons could be inferred for the decreased NADPH–cytochrome *c* reductase activity of the covalent complex with respect to that of the free proteins: (1) that the intramolecular (FNR-Fld) electron transfer is diminished as a consequence of the cross-linking reaction; and (2) that the interaction between reduced flavodoxin and cytochrome *c* is impaired in the complex. While progress has been made with respect to the first possibility by using laser-flash photolysis techniques [18] we have investigated the rate of electron transfer between reduced flavodoxin in the complex and cytochrome *c* by stopped-flow kinetic studies. The neutral semiquinone species of flavodoxin can be prepared by illuminating the complex in the presence of 5-deazariboflavin and EDTA. This is the most stable partially reduced species in the complex, according to the midpoint potentials determined for both flavin groups [6,19]. Fig. 3A shows one of the traces obtained during the oxidation of complexed flavodoxin semiquinone by cytochrome *c*. Two different kinetic processes seem to be taking place. There is a very rapid pseudo-first-order reaction that shows a non-linear dependence with respect to cytochrome *c* concentration, as Fig. 3B indicates. This behaviour is also observed with free flavodoxin (not shown) and has been previously attributed to an intermediate complex formation according to the mechanism proposed by Simondsen et al. [20]:

$$FldH^{\cdot} + cytc_{ox} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [FldH^{\cdot}\text{----}cytc_{ox}] \overset{k_2}{\rightarrow} Fld_{ox} + cytc_{red}$$

The rate constants for this mechanism were evaluated from these data by a non-linear squares computer-fitting procedure as described previously [20]. The results are given in Table II, where the value of k_1, which reflects the rate of complex formation, is 10-times lower for the covalent complex than for the free flavodoxin. The value of k_2, which is a measure of the rate of the electron-transfer step, is also decreased approx. 20-fold, indicating that both the interaction of flavodoxin with cytochrome *c* and the electron transfer are slowed down by the formation of the complex. The stability of the complex between flavodoxin and cytochrome *c* is higher in the covalent complex, as K_D in Table II indicates, probably as a consequence of the compensating effect in the k_{-1} value that is also observed. It is suggested then, that the productive complex between flavodoxin and cytochrome *c* forms more slowly when the flavoprotein is cross-linked to

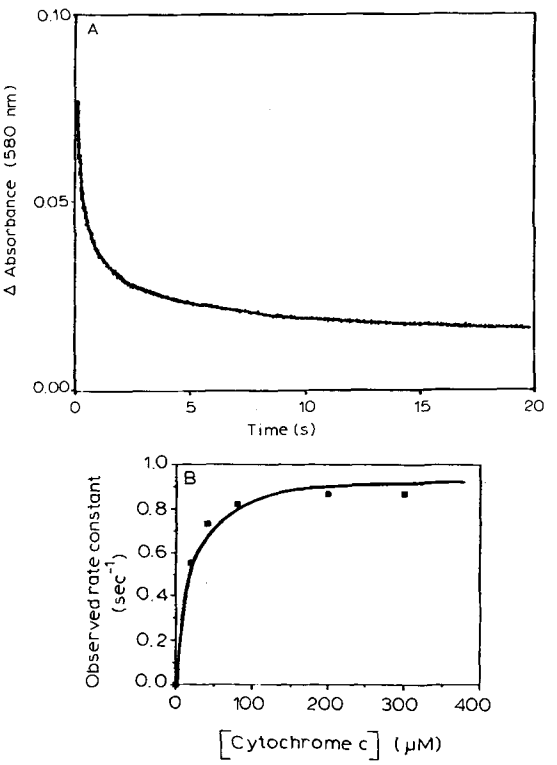

Fig. 3. Stopped-flow absorbance kinetic trace of the reduction of horse heart cytochrome *c* by cross-linked complex flavodoxin semiquinone. The reaction was monitored at 580 nm and 25°C under anaerobic conditions. Flavodoxin semiquinone was generated by illumination in the presence of 1 μM 5-deazariboflavin and 1 mM EDTA. The buffer used was 5 mM Hepes, 45 mM KCl, 1 mM EDTA (pH 7.25). The reactant concentrations (after mixing) were 8.4 μM cross-linked complex and 200 μM cytochrome *c*. (B) Plot of observed pseudo-first-order rate constant for cross-linked complex flavodoxin semiquinone oxidation vs. cytochrome *c* concentration.

TABLE II

Kinetic constants for the transfer of electrons between free and FNR-bound flavodoxin and cytochrome c

	k_1 ($M^{-1}\,s^{-1}$)	k_{-1} (s^{-1})	k_2 (s^{-1})	K_D (M)
Flavodoxin	5.2×10^5	13	18.75	2.5×10^{-5}
Complex	6.5×10^4	0.55	0.90	8.4×10^{-6}

the reductase but, once formed, it dissociates more slowly. The slower component observed in Fig. 3 could be attributed to non-specific long-range electron-transfer processes. The conclusion is, then, that the binding site for cytochrome in flavodoxin is less accessible when it is cross-linked to ferredoxin-NADP$^+$ reductase, the transfer of electrons between the flavin and the heme groups being less efficient.

Photoreduction of NADP$^+$

When we compared the NADP$^+$photoreductase activity of both the reductase and the cross-linked complex, significant results were obtained which indicate the limited functionality of the covalent adduct. For this assay it is necessary to prepare spinach chloroplasts, completely devoid of intrinsic reductase, so that addition of excess ferredoxin does not produce any observable amount of NADPH when they are illuminated. Fig. 4 shows that addition to this mixture of *Anabaena* ferredoxin-NADP$^+$ reductase as well as either flavodoxin or ferredoxin yields a very substantial reduction of NADP$^+$ (47–52 nmol NADP$^+$/min). Incubation of the chloroplast preparation with excess of the reductase-flavodoxin complex in the presence of saturating concentrations of ferredoxin produces the reduction of 17 nmol of NADP$^+$/min, i.e., roughly one third of that produced by the free reductase. It is important to state that, as Fig. 4 shows, the concentration of the cross-linked complex required to reach saturation in this assay is almost 10-times that required

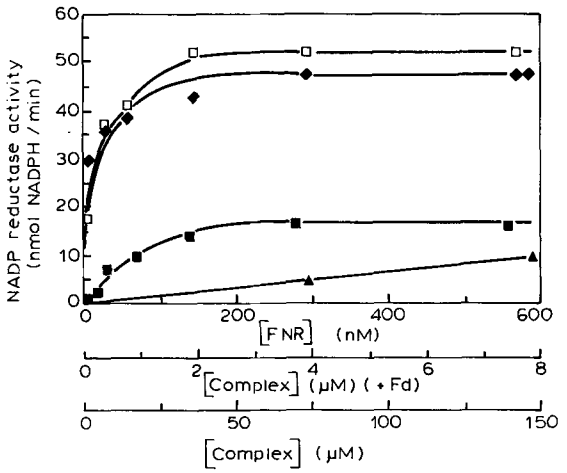

Fig. 4. Photoreduction of NADP$^+$ using washed spinach chloroplasts devoid of ferredoxin-NADP$^+$ reductase. A cuvette containing spinach chloroplasts (26 μg chlorophyll/ml), 100 mM ascorbate, 19 μM dichlorophenol indophenol, 1mM NADP$^+$ and ferredoxin, flavodoxin, the reductase or the complex at concentrations indicated, in 0.1 M Tris-HCl (pH 8.0), in a total volume of 1.35 ml, was illuminated with a 50 W halogen lamp at room temperature. The formation of NADPH was followed by the change in absorbance at 340 nm. (□) FNR in the presence of 7.7 μM ferredoxin. (♦) FNR in the presence of 8.1 μM flavodoxin. (■) Complex in the presence of 7.7 μM ferredoxin. (▲) Complex.

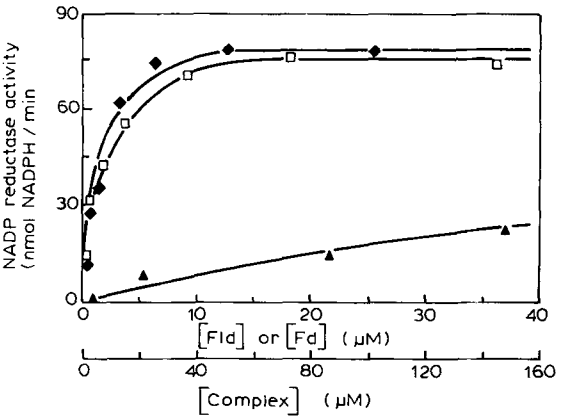

Fig. 5. Photoreduction of NADP$^+$ using washed spinach chloroplasts. A cuvette containing spinach chloroplasts (30 μg chlorophyll/ml) 1 mM NADP$^+$ and ferredoxin, flavodoxin or complex at the concentrations indicated, in 0.1 M Tris-HCl (pH 8.0), in a total volume of 1.15 ml, was illuminated with a 50 W halogen lamp at room temperature. The formation of NADPH was followed by the change in absorbance at 340 nm. (□) Ferredoxin. (♦) Flavodoxin. (▲) Complex.

when free reductase is present. This figure also presents data on the ability of the complex alone (without any additional ferredoxin or flavodoxin) to transfer electrons from the photosynthetic particles to NADP$^+$. Significantly low values (8 nmol/min) are obtained and only after a very large concentration of the complex is present in the preparation. This is a clear indication of the incompetence of the covalent complex to transfer electrons from the photosynthetic membranes to NADP$^+$ and suggests that it can not substitute for both the reductase and the electron-transfer protein in the NADP$^+$ photoreduction assay. The presence of flavodoxin did not stimulate the activity of the covalent complex.

We have also examined the ability of the complex to act as an electron carrier protein mediating the transfer of electrons between the photosynthetic particles and the reductase present in the spinach chloroplast preparation. Fig. 5 shows that the complex yields some 25% of the total activity observed with either ferredoxin or flavodoxin which, on the other hand, are almost identical. Also in this case the concentration of the complex required to produce saturation of the reaction is approx. 3–4-times that for the free proteins.

Interaction of the cross-linked complex with NADP$^+$

The cross-linked complex formed by the reductase and flavodoxin could be photoreduced under anaerobic conditions and in the presence of 5-deazariboflavin and EDTA. Curve B in Fig. 6 shows the absorption spectrum of the fully reduced species of the covalent complex formed upon illumination. Both FMN and FAD appear to be in their hydroquinone form. Anaerobic addition of NADP$^+$produces the immediate reoxi-

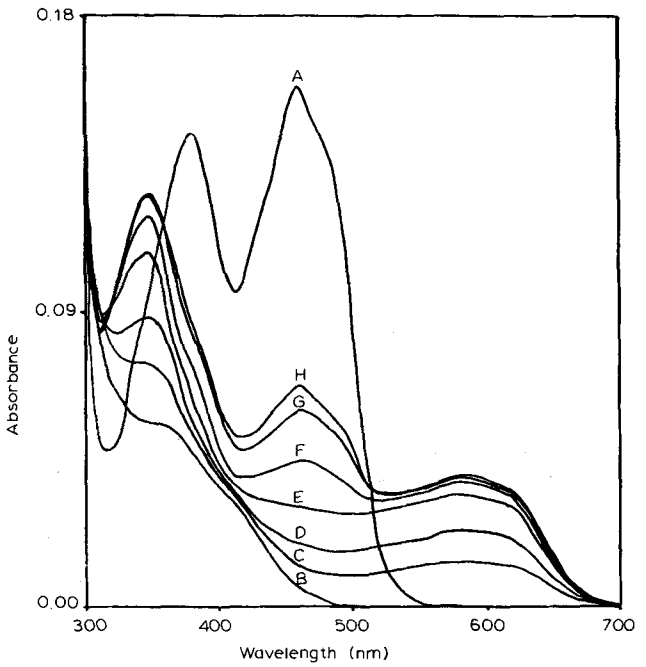

Fig. 6. Absorbance spectrum changes occurring after addition of $NADP^+$ to reduced cross-linked complex under anaerobic conditions. The complex was reduced by illumination in the presence of 2 μM 5-deazariboflavin and 1 mM EDTA. The buffer used was 50 mM Tris-HCl (pH 8.0). The initial volume was 950 μl and the initial complex concentration was 7.2 μM. Added $NADP^+$ was 95.2 μM. (A) Spectrum before reduction; (B) reduced complex; (C–H) spectra after addition of 30 μl (C), 60 μl (D), 90 μl (E), 130 μl (F), 190 μl (G), 300 μl (H) of $NADP^+$ solution and mixing by hand.

dation (during the mixing time) of the complex, as evidenced by the increase in absorbance in the 450 and 600 nm regions. The first transition observed corresponds to the reoxidation of flavodoxin hydroquinone to the semiquinone form ($E'_o = -418$ mV, Ref. 19), as is indicated by the appearence of the absorption band in the 600 nm region (lines B to E). The neutral blue semiquinone radical can not be attributed to the reductase, since very limited amounts of this are observed during titration of this enzyme [6,18]. Since $NADP^+$ can not interact directly with flavodoxin, the appearance of a partially oxidized form of this protein would be an indication of the electron transfer between the two flavoproteins. One could argue, nevertheless, that $NADP^+$ takes up two electrons simultaneously so that the presence of the semiquinone form, which has lost only one electron implyes that there is a reorganization of the electrons among different molecules of the complex leading to the formation of two molecules of partially reduced complex bearing three electrons each. Further addition of anaerobic $NADP^+$ produces the reoxidation of the reductase in the complex ($E'_o = -320$ mV, Ref. 6) (lines E to H) until all the molecules of the complex are in the form $FNR_{ox} - Fld_{sq}$. This species can not be further reoxidized by $NADP^+$ due to the difference in redox potentials between the species involved ($E'_{o\ NADP/NADPH} = -320$ mV; $E'_{o\ Fldox\ hq/Fldsq} = -255$ mV, Ref. 19).

Direct electron transfer between the pyridine nucelotide and the complex was also demonstrated by another experiment (not shown) in which NADPH was added to an anaerobic solution of the covalent complex and the reduction of the flavins in the complex was monitored spectrophotometrically. The decrease in the absorbance in the 460 nm region and the increase in the 600 nm region were indicative of the reduction of the complex by NADPH and, moreover, of the existence of internal transfer of electrons between reduced FAD and FMN, since the broad band in the 600 nm region that appears during the first minutes of the incubation is attributed to the FMN semiquinone form, which is only formed with electrons taken from NADPH through the reductase.

Photoreduction of cytochrome c

Finally, we investigated the possibility of using the cross-linked complex to mediate the reduction of cytochrome *c* with electrons generated in the photosynthetic membranes of broken spinach chloroplasts. The presence of cytochrome c in this assay mixture requires that the photosynthetic membranes use water as the final electron donor involving both Phosystems I and II. Ascorbate, the reductant used for $NADP^+$ photoreduction, can not be used in this system, since it would produce the spontaneous reduction of cytochrome *c*. Fig. 7 shows that the complex behaves similarly to both ferredoxin and flavodoxin producing a significant reduction of cytochrome on illumination of the mixture. The cross-linked complex produces the same rate of cytochrome reduction in these conditions,

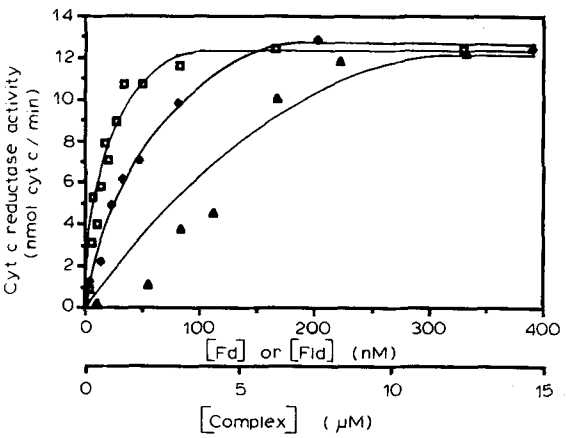

Fig. 7. Photoreduction of cytochrome *c* using washed spinach chloroplasts. A cuvette containing washed spinach chloroplasts (29 μg chlorophyll/ml) 0.75 mg/ml cytochrome *c* and ferredoxin, flavodoxin or the complex at the concentrations indicated, in 0.1 M Tris-HCl (pH 8.0) buffer in a total volume of 1.2 ml, was illuminated with a 50 W halogen lamp at room temperature. The reduction of cytochrome *c* was followed by the change of absorbance at 550 nm. (□) Ferredoxin. (♦) Flavodoxin. (▲) Complex.

although the concentration required to yield these values is much higher than when the free proteins are acting as electron carriers, indicating a partial steric hindrance is introduced into the flavodoxin molecule when is covalently bound to the reductase.

Discussion

The results presented here indicate that a covalent complex can be formed between the two flavoproteins ferredoxin-$NADP^+$ reductase and flavodoxin isolated from the cyanobacterium *Anabaena* PCC 7119. The molecular weight determined for the adduct indicates that there is one molecule of each protein in the isolated complex, although binding of the reductase with a flavodoxin dimer is also possible, especially at high concentrations of flavodoxin, as Fig. 2 indicates.

The formation of a cross-linked complex between the spinach reductase and ferredoxin has been reported by two different groups [21,22] and we have described the preparation of a complex between *Anabaena* ferredoxin-$NADP^+$ reductase and Azotobacter flavodoxin [12]. Their data fully agree with ours in that the diaphorase activity in the covalent adduct falls approx. 40–50%, while incubation of the reductase with only EDC does not produce any inhibitory effect. We propose, then, that the reductase, when covalently bound to flavodoxin, can not interact efficiently with the electron acceptor compounds used in the diaphorase reaction. This indicates a certain degree of interaction between the binding site for flavodoxin and that for the substrates of the diaphorase reaction.

All previous data referring to the complex formed between flavodoxin or ferredoxin and the reductase from *Anabaena* [12] or spinach [21,22] indicate that electron transport occurs between both proteins, although at substantially lower rate than in the non-covalently stabilized complex. Our present data indicate that electrons can be transferred from NADPH to cytochrome *c* through the FNR-Fld covalent complex (Table I). Significant values were obtained for the NADPH–cytochrome *c* reductase activity when the covalent complex was used. This indicates that there is transfer of electrons from the reduced reductase to flavodoxin in the complex and viceversa (cf. Fig 6). The lower activity obtained with the covalent complex as compared to the free proteins could be due to the decrease in the rate of reduction of cytochrome *c* by flavodoxin when bound to the reductase as is indicated in Table II. It could also be attributed to a diminished rate of intramolecular electron transfer between the two flavins in the complex, as has been investigated by rapid kinetic techniques using laser flash photolysis [18]. When the reaction is assayed in the oposite direction, i.e. from PS I to $NADP^+$, it apears that the complex alone is completely incapable of promoting the reduction of $NADP^+$ (Fig. 4). Since we have shown (Fig. 6) that $NADP^+$ can be reduced by the cross-linked complex, the most reasonable interpretation would be to assume that the pathway for the entrance of electrons into the complex is, at least in some complex molecules, partially, blocked. Since the carbodiimide reagent can react with proteins in many places, there is the possibility that some molecules of the complex, which are relatively loose, allow flavodoxin to interact with the photosynthetic membranes. Other complex molecules, with more cross-links, would not be able to interact with the membranes at all. The covalent complex also shows a limited ability to act as electron-transfer protein between the photosynthetic membranes and either exogenous FNR (Fig. 5) or cytochrome *c* (Fig. 7). Both experiments show that there is reduction of either $NADP^+$ or cytochrome *c* upon illumination of the spinach chloroplast preparation only after addition of the covalent complex or ferredoxin (or flavodoxin). The requirement of very high concentrations of the covalent complex to produce a significant effect on the activity supports the hypothesis of the limited accessibility of bound flavodoxin to the photosynthetic membranes. The increase in activity observed in the presence of saturating concentrations of ferredoxin could be due to an nonespecific interaction between reduced ferredoxin and the complex. These results would indicate that flavodoxin performs both the electron accepting and donating reactions, through the same site, in such a way that once it is bound to FNR, it can not expose its FMN group to receive electrons from bulky reductants such as the tylakoidal membranes. This model opposes the other alternative in which the cofactor would shuttle from the acceptor to the donor sites, thereby mediating the transfer of electrons. The relatively high rate of electron transfer obtained in the NADPH–cytochrome *c* reductase activity could be a consequence of the very large difference in redox potential between flavodoxin and cytochrome *c*.

We can conclude, therefore, that the covalent complex formed between the enzyme ferredoxin-$NADP^+$ reductase and flavodoxin from the cyanobacterium *Anabaena* PCC 7119 can be used as a simplified model system for the study of electron-transfer reactions between flavins. This system would be very useful for evaluating the effect of the ionic strength in the medium on the rate of electron transfer, independently of the interaction between the proteins. Construction of complexes of flavoproteins with different redox potentials, generated either chemically or through site-directed mutagenesis, could be used to correlate these thermodynamic properties with the rates of electron transport. Finally, it could be useful to determine the peptide fragments in the two proteins which are involved in the binding between the proteins, as has been done with

other systems [1,23–26]. This complex can not be used, nevertheless, for the study of the electron-transfer reaction from photoynthetic membranes to $NADP^+$.

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References

1 Zanetti, G., Morelli, D., Ronchi, S. Negri, A., Aliverti, A. and Curti, B. (1988) Biochemistry 27, 3753–3759.
2 Karplus, P.A., Daniels, M.J. and Herriot, J.R. (1991) Science 251, 60–66.
3 Mayhew, S.G. and Ludwig, M.L. (1975) in The Enzymes, 3rd Edn. (Boyer, ed.), pp. 57–118, Academic Press, London.
4 Rogers, L.J. (1987) in The Cyanobacteria (Fay, P. and Van Baalen, C., eds.), pp. 52–61, Elsevier, Amsterdam.
5 Zanetti, G.and Curti, B. (1980) Methods Enzymol. 69, 250–253.
6 Sancho, J., Peleato, M.L., Gómez-Moreno, C. and Edmondson, D.E. (1988) Arch. Biochem. Biophys. 260, 200–207.
7 Gómez-Moreno, C., Sancho, J., Fillat, M.F. Pueyo, J.J. and Edmondson, D.E. (1987) in Flavins and Flavoproteins (Edmondson, D.E. and McCormick, D.B., eds.), pp. 335–339, W. de Gruyter, Berlin.
8 Fillat, M.F., Sandmann, G. and Gómez-Moreno, C. (1988) Arch. Microbiol. 150, 160–164.
9 Smith, W.W., Pattridge, K.A. Ludwig, M.L. Petsko, G.A. Tsernoglou, D. Tanaka, M. and Yosunobu, K.T. (1983) J. Mol. Biol. 165, 737–755.
10 Fillat, M.F., Borias, W.E. and Weisbeek, P.J. (1991) Biochem. J. in press.
11 Fillat, M.F., Bakker, H.A.C. and Weisbeek, P.J. (1990) Nucleic Acids Res. in press.
12 Pueyo J.J., Sancho, J., Edmondson, D.E. and Gómez-Moreno, C. (1989) Eur. J. Biochem. 183, 539–544.
13 Hames, B.D. (1981) in Gel electrophoresis of proteins: a practical approach (Hames, B.D. and Richwood, D., eds.), pp. 6–14 IRL, London.
14 Walker, D.A. (1980) Methods Enzymol. 69, 94–104.
15 De Francesco, R., Tollin, G. and Edmondson, D.E. (1987) Biochemistry, 26, 5036–5042.
16 Burleigh, B.D., Jr., Foust, G.P. and Williams, C.H., Jr. (1969) Anal. Biochem. 27, 536–544.
17 Foust, G.P., Mayhew, S.G. and Massey, V. (1969) J. Biol. Chem. 244, 964–970.
18 Walker, M.C., Pueyo, J.J., Gomez-Moreno, C. and Tollin, G. (1990) Arch. Biochem. Biophys. 281, 76–83.
19 Fillat, M.F., Edmondson, D.E. and Gomez-Moreno, C. (1990) Biochim. Biophys. Acta 1040, 301–307.
20 Simondsen R.P., Weber, P.C. Salemme, F.R. and Tollin, G. (1982) Biochemistry 21, 6366–6375.
21 Zanetti, G., Aliverti, A. and Curti, B. (1984) J. Biol. Chem. 259, 6153–6157.
22 Colvert, K.K. and Davis, D.J. (1988) Photosynth. Res. 17, 231–245.
23 Waldmeyer, B. and Bossard, H.R. (1985) J. Biol. Chem. 260, 5184–5190.
24 Nisimoto, Y. and Otsuka-Murakami, H. (1988) Biochemistry 27, 5869–5876.
25 Morand, L.Z., Frame, M.K. Colvert, K.K. Johnson D.A. Krogmann, D.W. and Davis, D.J. (1989) Biochemistry 28, 8039–8047.
26 Geren, L.M., O'Brien P., Stonehuerner, J. and Millet, F. (1984) J. Biol. Chem. 259, 2155–2160.