Fixation of CO₂ by green plants requires transport of electrons from water to NADP⁺, the last step in photosynthetic electron flow. 

Ferredoxin:NADP⁺ oxidoreductase (ferredoxin: NADP⁺ reductase, EC 1.18.1.2) was shown to form a ternary complex with its substrates ferredoxin (Fd) and NADP⁺, but the ternary complex was less stable than the separate binary complexes. $K_d$ for oxidized binary Fd-ferredoxin NADP⁺ reductase complex was less than 50 nM; $K_{NADP⁺}$ increased with NADP⁺ concentration, approaching 0.5–0.6 μM when the flavoprotein was saturated with NADP⁺. $K_{NADP⁺}$ also increased from about 14 μM to about 310 μM on addition of excess Fd. The changes in $K_d$ were consistent with negative cooperativity between the associations of Fd and NADP⁺ and with our unpublished observations which suggest that product dissociation is rate-limiting in the reaction mechanism. Similar interference in binding was observed in more reduced states; NADPH released from the ternary complex with its substrates ferredoxin:NADP⁺ reductase may facilitate the overall reaction. Complexation between Fd and ferredoxin: NADP⁺ reductase was found to shield each center from paramagnetic probes; charge specificity suggested that the active sites of Fd and ferredoxin:NADP⁺ reductase were, respectively, negatively and positively charged.

Fixation of CO₂ by green plants requires transport of electrons from water to NADP⁺, the last step in photosynthetic electron flow. The transfer of electrons from ferredoxin to NADP⁺ via an FAD-containing enzyme, ferredoxin:NADP⁺ oxidoreductase (ferredoxin:NADP⁺ reductase) (1). Ferredoxin:NADP⁺ reductase may also catalyze the reverse reaction, electron flow from NADPH to Fd in the dark, thus facilitating reduction of nitrite and sulfite (2, 3).

The stoichiometry of the Fd to NADP⁺ electron transfer requires that the reductase oxidize two Fd molecules (one electron/Fd) before passing two electrons to NADP⁺ (1). One might envision a ping-pong mechanism in which the reductase reacts with each substrate, utilizing binary ferredoxin:NADP⁺ reductase-substrate complexes; however, steady state kinetic data suggest participation of a ternary complex (4). Demonstration of formation of a ternary complex would strengthen this conclusion.

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$^a$ The abbreviations used are: Fd, ferredoxin; 2',5'-ADP, adenosine 2',5'-diphosphate; FNR, ferredoxin:NADP⁺ reductase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Dy-EDTA, Dy⁺ chelated by EDTA; Dy-o-phenanthrolne, Dy⁺ chelated by o-phenanthrolne.

Workers from several laboratories have identified binary Fd-FNR and FNR-NADP⁺ (H) complexes (5–7). Equilibrium studies have shown that complexation alters the oxidation-reduction properties of ferredoxin:NADP⁺ reductase and its substrates; electron transfer from Fd to ferredoxin:NADP⁺ reductase and from ferredoxin:NADP⁺ reductase to NADP⁺ is more highly favored in complex than would be predicted from the mid-point potentials of the separate components (8, 9, 24).

Identification of a ternary complex is less certain. Ricard et al. (10) reported (on the basis of difference mixing spectra) that the associations of Fd and NADP⁺ with the flavoprotein were independent, and a ternary complex formed. In contrast, we reported preliminary experiments indicating competition between Fd and NADP⁺ for complex with ferredoxin:NADP⁺ reductase (11); Davis (12) reported similar results. The latter findings tend to argue against a ternary complex.

We report here a series of experiments designed to determine whether any Fd-FNR-NADP⁺ complex forms, and if so, whether $K_a$ values are different than in the binary Fd-FNR and FNR-NADP⁺ complexes. The data indicate that ferredoxin:NADP⁺ reductase will allow simultaneous complexation by Fd and NADP⁺; however, addition of one substrate decreased the association of the other in a pattern of negative cooperativity.

We will report, separately, rapid kinetic studies which indicate that a ternary complex must participate in NADP⁺ reduction. The kinetic data suggest that the rate of electron transport may be limited by dissociation of oxidized Fd from ferredoxin:NADP⁺ reductase; destabilization of Fd-FNR complex by NADP⁺ may facilitate the overall reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**

NADP⁺, 2',5'-ADP, 2',5'-ADP-Sepharose, CNBr-activated Sepharose 4B, o-phenanthrolne, methyl viologen, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma. NAD and NADPH were from P-L Biochemicals. Tris base was purchased from Schwarz/Mann, and Hepes was from Calbiochem-Behring or Research Organics, Inc. Dithionite (sodium hydrosulfite) was purchased from Fisher. EDTA was from Mallinckrodt Chemical Works, and DyCl₃ was from Alpha Products. CF-25, PM-30, and DM-5 ultrafiltration membranes were purchased from Amicon. 5'-Deazaflavin was a gift from Dr. David Seybert of Duquesne University.

Fd and ferredoxin:NADP⁺ reductase were purified as reported previously (9, 13), with the exception of the last step in ferredoxin:NADP⁺ reductase purification. After chromatography on DE52 cellulose, we applied the ferredoxin:NADP⁺ reductase preparation to a 2',5'-ADP-Sepharose column; enzyme and column were in 50 mM Tris, pH 8.0. The column-bound flavoprotein was eluted with the same buffer plus 10 mM Na₂P₂O₇. Fractions in which $A_{480}/A_{390}$ exceeded 0.12 were pooled, dialyzed versa 50 mM Hepes, pH 8.0, and stored under liquid N₂. Sodium dodecyl sulfate-polycrylamide gel electrophoresis revealed only one major band in these preparations.
Methods

All experiments, unless otherwise noted, were conducted in 50 mM Hepes buffer, pH 8.0, at room temperature. Fd used had an A_{420}/A_{276} ratio \( \geq 0.46 \); ferredoxin:NADP\(^+\) reductase had an A_{420}/A_{230} \geq 0.12. Concentrations of Fd, ferredoxin:NADP\(^+\) reductase, NADP\(^+\), and NADPH were determined using extinction coefficients reported previously (9); we assumed an extinction coefficient of 15,400 cm\(^{-1}\) M\(^{-1}\) at 259 nm for 2',5'-ADP (16). Absorbance spectra were recorded using a Cary 219 UV-VIS spectrophotometer linked to a Minc-11 computer. EPR spectra were recorded using a Varian E-9 spectrometer equipped with an Air Products gas-transfer line. Oxygen-free Ar was produced by passing prepurified Ar through a BASF column, then through a bubbler filled with methyl viologen photo-reduced by 5-deazafavin and EDTA as described by Massey and Hemmerich (35).

Assays—Ferredoxin:NADP\(^+\) reductase was assayed by its ferricyanide reductase activity (16). Ferredoxin:NADP\(^+\) reductase was added to a solution containing 1 mM K\(_3\)Fe(CN)\(_6\), 1 mM glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, and 50 \( \mu \)M NADPH in a 1-ml total volume. Ferricyanide reduction was followed by the decrease in A_{420}.

NADP\(^+\)(H) was also assayed by stimulation of ferricyanide reduction. The assay contained the same concentrations of ferricyanide, glucose-6-phosphate, and glucose-6-phosphate dehydrogenase as above, but with 1.06 \( \mu \)M ferredoxin:NADP\(^+\) reductase and no NADPH. Standard curves were linear from 0.08 to 2 nmol NADP\(^+\)(H).

Spectrophotometric Binding Titrations—Two matched cells were used; ferredoxin:NADP\(^+\) reductase was in the sample cell, and an equal volume of buffer was in the reference cell. Spectra were recorded of the initial solutions and again after each addition of aliquots of ligand to sample and reference cells; mixing was by "plumper" (Calbiochem-Behring). Difference mixing spectra were calculated by subtracting the absorbance at each wavelength of the initial ferredoxin:NADP\(^+\) reductase solution from the dilution-corrected absorbance of the mixed ligand/ferredoxin:NADP\(^+\) reductase solution.

In some cases, difference mixing spectra were determined by use of tandem cells. Ferredoxin:NADP\(^+\) reductase was placed in the front compartment of the cell; an equal volume of ligand was in the rear. The reference cell contained only buffer. Spectra were recorded of the components before and after mixing the contents of the two compartments. Difference spectra were calculated arithmetically.

Calculation of \( K_d \) Values—\( K_d \) values were calculated either by a computer program adapted from Duggleby (17) or by a program developed in our laboratory for fitting binding curves to data by a nonlinear least squares procedure. The method of Duggleby (17) uses a Gauss-Newton method to approximate \( K_d \) and \( \Delta A_{\text{max}} \) (absorbance change observed at saturating ligand), and best fit was determined by least squared error; \( K_d \) values reported with standard errors were determined by this method. The other procedure was to vary \( \Delta A_{\text{max}} \) manually and calculate a "best" \( K_d \) for each \( \Delta A_{\text{max}} \); in each case, best fit was determined by minimization of the sum of squared error. Both programs used the following theoretical relation:

\[
\alpha = \frac{\Delta A}{\Delta A_{\text{max}}} = \frac{E \cdot L}{E - B} = \frac{B - \sqrt{B^2 - 4E \cdot L}}{2E}
\]

where \( E \cdot L = \) concentration of enzyme-ligand complex, \( E = \) total enzyme concentration, \( L = \) total ligand concentration, \( K_d = \) dissociation constant of 1:1 E-L complex, and \( B = E + L + K_d \).

This relation accounts for the fraction of ligand and enzyme in complex and was the correct expression for experiments in which a significant fraction of ligand was bound to the enzyme. The two procedures gave the roughly equivalent values for \( K_d \) and \( \Delta A_{\text{max}} \).

RESULTS

Demonstration of Ternary Complex Formation—We used difference mixing spectra to investigate the simultaneous association of Fd and NADP\(^+\) with ferredoxin:NADP\(^+\) reductase. First, Fd was added in a small molar excess to a ferredoxin:NADP\(^+\) reductase solution (Fig. 1A); spectrum a is the difference mixing spectrum\(^2\) characteristic of the Fd-FNR complex. Under these conditions, nearly all ferredoxin:NADP\(^+\) reductase molecules were bound to Fd (\( K_d < 50 \) nM, see also Ref. 7). NADP\(^+\) was then titrated into the Fd-FNR mixture, producing additional perturbations (spectrum b–d) in the absorbance spectra without obvious loss of the Fd-FNR mixing spectrum. Mixing Fd and NADP\(^+\) (no ferredoxin:NADP\(^+\) reductase) caused no spectral perturbation. Thus, it is likely that the spectral changes in spectra b–d correspond to formation of the ternary complex Fd-FNR-NADP\(^+\).

We found that the complex spectra (b–d) could be resolved into perturbations due to Fd-FNR and FNR-NADP\(^+\) association; subtraction of the spectrum of the Fd-FNR mixture from succeeding spectra (Fig. 1B) removes the initial Fd-FNR mixing spectrum, giving the perturbations caused by NADP\(^+\) in the presence of Fd. The shapes of the difference spectra were essentially the same as those observed in the absence of Fd.\(^3\) The lack of any absorbance changes indicating dissociation of Fd from ferredoxin:NADP\(^+\) reductase indicates that NADP\(^+\) is able to occupy its site on ferredoxin:NADP\(^+\) reductase and produce the FNR-NADP\(^+\) difference mixing spectrum without displacing Fd. Hence an Fd-FNR-NADP\(^+\) complex must be forming.

\(^2\)The absorbance of the mixture of ferredoxin:NADP\(^+\) reductase and ligands minus the sum of the separate absorbances of ferredoxin:NADP\(^+\) reductase plus ligand(s).

\(^3\)In Fig. 1B, the FNR\(^+\) binding spectra have an upward deflection from 550 to 350 nm as compared to the spectra observed in the absence of Fd (6); this deflection was not observed if mixing spectra were produced by use of tandem cells. Such a deflection might result from small errors in corrections made for dilution.
We also observed apparent formation of ternary complex when Fd was titrated into FNR-NADP* complex (0.6 μM ferredoxin:NADP* reductase, 2 mM NADP*). As Fd was added, we observed absorbance changes corresponding to the appearance of the Fd-FNR complex, but could not detect significant disappearance of FNR-NADP* complex; thus, order of addition of substrate was not important in our equilibrium experiments.

In view of data (presented below) suggesting interference between Fd and NADP* in complex formation with the NADP* reductase, we used ultrafiltration to determine whether Fd remained associated with ferredoxin:NADP* reductase under the conditions of Fig. 1. Amicon PM-30 membranes retained ferredoxin:NADP* reductase entirely, but Fd only slightly (<20% by absorption spectra of the filtrate). After addition of equimolar ferredoxin:NADP* reductase to a 25 μM Fd solution, essentially no Fd was found in the filtrate. Addition of NADP* (up to 1.6 mM) did not cause any measurable appearance of Fd in the filtrate; however, after the solution was brought to 1 M NaCl by addition of solid NaCl (Fd-FNR complex is salt-dissociable (7, 14)), Fd was again found in the filtrate. Thus, at concentrations of NADP* which result in formation of NADP* binding spectra, most Fd remained bound to ferredoxin:NADP* reductase, confirming the conclusions drawn on the basis of difference mixing spectra.

Evidence for Interaction—Although Fig. 1 demonstrates ternary complex formation, we found that the associations of Fd and NADP* with ferredoxin:NADP* reductase were not independent. Higher NADP* concentrations were required to form the FNR-NADP* difference mixing spectrum in the presence of Fd than in its absence (Fig. 2). The Kd of the complex between ferredoxin:NADP* reductase and NADP* was only about 14 μM in the absence of Fd (in agreement with Dykes and Davis (18), who presented evidence suggesting that association of a second NADP* at a low affinity site (Kd ≥ 1 mM) caused overestimation of Kd in previous studies (5, 7)). Use of changes in A300-A200 as an indication of FNR-NADP* complex obviates the need to consider contributions from the low affinity site (13). When Fd was present, Kd for FNR-NADP* complex was much greater than when Fd was absent (Fig. 2B and Table I); no absorbance changes were observed which would indicate association of a second NADP* (data not shown).

Kd values in Table I were calculated as described under "Methods" assuming formation of 1:1 complex. The theoretical curves generally fit the data well; there was no indication of stoichiometries >1:1. We cannot rule out some association of a second NADP* with ferredoxin:NADP* reductase, but no absorbance changes corresponding to such complex were observed in the presence of Fd. The titration of NADP* into 1.94 μM ferredoxin:NADP* reductase probably provides the most accurate determination of K(G(NADP*)), for binary FNR-NADP* complex; error in ΔA300 or ferredoxin:NADP* reductase concentration would cause only small changes in Kd. Those titrations in which [ferredoxin:NADP* reductase] > Kd are more sensitive to errors. We do have some variation in Kd, but it seems to be approaching 310 μM at high Fd:FNR ratios. If the molar ratio of Fd:FNR was 0.5, NADP* binding was not monophasic. We could simulate the data by assuming high and low affinity phases: 45% Kd = 35 μM, 55% Kd = 350 μM. These phases probably correspond to binding to free ferredoxin:NADP* reductase and Fd-FNR, respectively; thus, Fd apparently lowers the affinity of ferredoxin:NADP* reductase for NADP*.

The increase in K(G(NADP*)) on inclusion of Fd might be explained by Scheme I. This model is governed by the following relations:

K(G(NADP*)) = K(FN)K(FN)K(FN)K(FN)K(FN)

(1)

Apparent K(G(NADP*)) = K(FN) + [Fd]/K(FN)

(2)

Apparent K(FN) = K(FN) + [NADP*]K(FN)

(3)

From the data of Table I, we find that K(G(NADP*)) increased about 23-fold when excess Fd was present. If the increase results from interactions as those in Scheme I, then K(FN) should increase with NADP*, but approach a saturating value of K(FN) when [NADP*] ≈ 320 μM (K(FN) = 100 μM).

Effect of NADP* on Kd on Fd-FNR—We tested this prediction by determining Kd of the Fd-FNR complexation as a function of NADP* concentration (Fig. 3). Fd-FNR complex was determined by A300-A200 (A300 is at or near an isosbestic point in the FNR-NADP* difference mixing spectrum; thus, changes in the association of ferredoxin:NADP* reductase and NADP* should not interfere. With no NADP* present, Fd-FNR complex was essentially fully formed at 1:1 Fd-FNR; hence K(G(NADP*)) was calculated as (FNR-NADP*) + (Fd-FNR).

We did not distinguish between binary Fd-FNR and ternary Fd-FNR-NADP* complexes; we assumed that both complexes give identical absorbance changes. Hence K(FN) was ([FNR-NADP*] + [FNR])/[Fd]/[Fd-FNR-NADP*] + [Fd-FNR]).
were determined by titrating Fd into ferredoxin:NADP+ reductase in the absence of FNR. Volumes were 27-28 ml; 10-cm cells were used. Changes in absorbance were small under "Methods." The noise in these determinations results from variations as the result of the increased relative to changes in absorbance at 0.6 NADP+. Kd was found to be 7 μM (−Fd) and 156 μM (+Fd); these values are in reasonable agreement (given the greater uncertainty of the titration experiment) with those determined using difference mixing spectra (see Table I). At the higher NADP+ concentrations, bound NADP+ was evaluated as the difference between two larger numbers.

Ferredoxin:NADP+ reductase binds to 2',5'-ADP-Sepharose (11, 19) and Fd-Sepharose (14); we have previously reported using these immobilized ligands to investigate interactions between Fd and NADP+ (or NADP+ analogues) in their associations with ferredoxin:NADP+ reductase. Ferredoxin:NADP+ reductase bound to 2',5'-ADP-Sepharose, but was eluted by 5 μM Fd in a sharp band; the first fraction contained almost 1:1 Fd:FNR (11). Analogously, 3 mM NADP+, but not NADP+, was found to elute ferredoxin:NADP+ reductase from Fd-Sepharose (11). In more recent work, we have found (data not shown) that 2',5'-ADP would elute most Fd bound to FNR-Sepharose (ferredoxin:NADP+ reductase was covalently linked to cyano gen bromide-activated Sepharose). These observations, while qualitative, corroborate our conclusion that occupation of the NADP+-binding site decreases the association of ferredoxin:NADP+ reductase and Fd.

The Association of Fd, Ferredoxin:NADP+ Reductase, and NADP+ in Reduced States: EPR Studies—The preceding experiments all examine the interactions of Fd, ferredoxin:NADP+ reductase, and NADP+ in the fully oxidized state. We extended our studies to more reduced (and thus possibly more catalytically relevant) states. Paramagnetic probes were used to establish the existence of reduced Fd:FNR complexes and conversely, to investigate the effect of complexation on the proximity of the redox centers of ferredoxin:NADP+ reductase, and NADP+ in the fully oxidized state (11). Analogously, 3 mM NADP+, but not NADP+, was found to elute ferredoxin:NADP+ reductase from Fd-Sepharose (11). In more recent work, we have found (data not shown) that 2',5'-ADP would elute most Fd bound to FNR-Sepharose (ferredoxin:NADP+ reductase was covalently linked to cyano gen bromide-activated Sepharose). These observations, while qualitative, corroborate our conclusion that occupation of the NADP+-binding site decreases the association of ferredoxin:NADP+ reductase and Fd.

Kd cannot be accurately determined from such a titration (best fit was 20 nM), but Kd must be less than 50 nM to give a sharp break at 1:1 Fd:FNR.

As NADP+ was increased, Kd increased, becoming measurable. The variation of Kd versus [NADP+] showed saturation above 1 mM NADP+, as predicted by Equation 3. The curve drawn through the data is that predicted in Kd(Fd(FN) = 20 nM, Kd(FAD) = 14 μM, and Kd(NADP+ = 320 μM. Hence, Kd(Fd(FN) = 0.55 μM. The data are in fair agreement with the theoretical line, but the wide data scatter permits only rough quantitation. The noise in these determinations results from use of low ferredoxin:NADP+ reductase concentrations which yield small absorbance changes upon complex formation. Certainly, Kd increased with NADP+ as we would expect; in addition, Kd seemed to become fairly constant at very high NADP+ concentrations.

In an earlier report, we concluded that ferredoxin:NADP+ reductase formed only binary complexes with its substrates (11). This conclusion was based on preliminary difference mixing spectra and upon elution of ferredoxin:NADP+ reductase from affinity columns (see below).

We found that titration of Fd into 100 μM NADP+ + 32 μM ferredoxin:NADP+ reductase produced perturbations in A666 relative to changes in A600, suggesting at least partial loss of FNR:NADP+ complex. We would now explain these observations as the result of the increased Kd for ternary complex; Kd(FN) = 14 μM < 100 μM < 310 μM = Kd(NADP+ = 320 μM). Hence, much of the FNR:NADP+ complex should dissociate upon addition of excess Fd. At high salt concentration (100 mM NaCl), 10 mM NADP+ could cause loss of Fd:FNR mixing spectrum. Apparent Kd(FN) has been found to increase with NADP+ at this ionic strength (data not shown); this probably explains at least part of the dissociation.

Other Measures of Binding—We used several other measures of binding to assure that the observed interaction was not an artifact of difference mixing spectra. We titrated NADP+ into 53 μM ferredoxin:NADP+ reductase ± 75 μM Fd; free unbound NADP+ was determined as the concentration of NADP+ passing through an Amicon CF-25 membrane. Bound NADP+ was determined as the difference between total NADP+ added and free NADP+. Kd values were found to be 7 μM (−Fd) and 156 μM (+Fd); these values are in reasonable agreement (given the greater uncertainty of the filtration experiment) with those determined using difference mixing spectra (see Table I). At the higher NADP+ concentrations, bound NADP+ was evaluated as the difference between two larger numbers.

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Rapidly relaxing paramagnetic species (such as Dy3+, a rare earth) will strongly affect neighboring paramagnetic species through dipolar interactions (20-22). These interactions can cause the EPR signals of the affected centers to broaden and also to relax more rapidly (thus relieving power saturation); the closer the probe to the redox center, the stronger the interaction (20, 21). Thus, interaction is strongest when the redox center is exposed to solvent or when the probe binds to the protein. In this study, we used two probes: Dy3+ chelated by EDTA, a negatively charged probe, and Dy3+ chelated by o-phenanthroline, a positively charged probe.

Fig. 4A presents the following EPR spectra: reduced Fd alone (−−), reduced Fd + Dy-o-phenanthroline (−−−), and reduced Fd + ferredoxin:NADP+ reductase + Dy-o-phenanthroline (−−−−). Excess dithionite was present in all samples; thus, ferredoxin:NADP+ reductase was also reduced. Dy-o-

1 The CF-25 Centrifo membranes were found to retain ferredoxin:NADP+ reductase and Fd completely, no retention of NADP+ was observed in the absence of ferredoxin:NADP+ reductase.
phenanthroline broadened the Fd signal considerably, and also relaxed the center (Fd + Dy-o-phenanthroline) was not saturated at 10 K and 200 milliwatts. 2 mM Dy-EDTA also broadened the Fd signal, but to a lesser degree (data not shown). The difference in effectiveness of the two probes is consistent with the net negative charge of Fd.

Inclusion of ferredoxin:NADP+ reductase in a parallel sample greatly reduced the line broadening caused by both probes. This indicates that Fd and ferredoxin:NADP+ reductase form a fully reduced complex; if ferredoxin:NADP+ reductase were instead binding Dy-o-phenanthroline, removing it from Fd, we would expect a large effect of Dy-o-phenanthroline on ferredoxin:NADP+ reductase semiquinone.

The neutral ferredoxin:NADP+ reductase semiquinone, produced by aerobic addition of NADPH (23), was also accessible to Dy (Fig. 4B). Ferredoxin:NADP+ reductase alone gave a 19 G line (---); Dy-o-phenanthroline (-----) gave a slight broadening of the signal, but Dy-EDTA (-----) broadened the signal to a great degree (to 39 G). The greater effectiveness of the negatively charged probe would be expected if ferredoxin:NADP+ reductase had positive charges clustered about an Fd-binding site. Equimolar Fd (-----) narrowed the Dy-broadened line to 32 G, suggesting complex formation (Fd\textsubscript{oxidized} + FNR\textsubscript{semiquinone}). We could not determine whether or not NADP(H) was also bound to ferredoxin:NADP+ reductase.

**Fig. 4.** Complex formation and the effect of spin probes on Fd and ferredoxin:NADP+ reductase. A, accessibility of Fd to Dy\textsuperscript{3+}. 0.4-ml samples were prepared containing 100 μM Fd reduced by excess dithionite. --- Fd alone; ----, Fd + 1 mM Dy\textsuperscript{3+} chelated with 2 mM o-phenanthroline; ---, Fd + 1 mM Dy-o-phenanthroline and 100 μM ferredoxin:NADP+ reductase. EPR spectra were recorded at 10 K and 20 milliwatts. B, accessibility of ferredoxin:NADP+ reductase to Dy. Samples were prepared by addition of 500 μM NADPH to 100 μM ferredoxin:NADP+ reductase (final concentrations). ---, ferredoxin:NADP+ reductase alone; ---, ferredoxin:NADP+ reductase + 1 mM Dy-o-phenanthroline; ----, ferredoxin:NADP+ reductase + 2 mM Dy-EDTA; ---, ferredoxin:NADP+ reductase + 2 mM Dy-o-phenanthroline and 100 μM Fd. Spectra were recorded at 20 K and 1 milliwatt.

**Fig. 5.** Ferredoxin:NADP+ reductase release from Fd-Sepharose by NADPH. Ferredoxin:NADP+ reductase was applied to about 1 ml of Fd-Sepharose contained in a small column. After removing excess ferredoxin:NADP+ reductase by washing with buffer, the enzyme-bearing Sepharose was transferred to an anaerobic titration cell and stirred under Ar for 20 min in 5 ml of buffer. Anaerobic NADPH was titrated into the cell; after each addition, the gel was then mixed with the buffer and allowed to settle. The clear solution over the Sepharose beads was then assayed for ferredoxin:NADP+ reductase and NADPH as described under "Methods." Following the titration, solid NaCl was added (final [NaCl] = 0.5 M) to release the ferredoxin:NADP+ reductase from the Fd. Temperature was 4 °C. O, FNR\textsubscript{oxidized} + Fd\textsubscript{oxidized} - Sepharose; ●, FNR\textsubscript{reduced} + Fd\textsubscript{reduced} - Sepharose. The beginning of the titration, 500 μM NADPH released much of the ferredoxin:NADP+ reductase; however, addition of solid NaCl (bringing the solution to 0.5 M NaCl) released additional
NADPH decreases the association of initially oxidized Fd and ferredoxin:NADP+ reductase. Thus, NADPH decreases the association of initially oxidized Fd and ferredoxin:NADP+ reductase.

We next titrated NADPH into dithionite-reduced Fd-FNR-Sepharose (Fig. 5, ●). As expected, reduction of the proteins decreased Fd-FNR complexation (9); more ferredoxin:NADP+ reductase was free in solution initially. Less NADPH was required to release ferredoxin:NADP+ reductase than in the previous titration; as before, some ferredoxin:NADP+ reductase remained bound to Fd-Sepharose even at very high NADPH.

These data can be simulated by assuming partial competition between NADPH and Fd; however, we do not present theoretical lines for negative cooperativity because too many variables are unknown to allow much confidence in such a calculation. We cannot be sure that all Fd molecules were bound to the Sepharose in the same manner; thus, ferredoxin:NADP+ reductase-binding sites might be heterogeneous. We could also not determine the extent of reduction of Fd and ferredoxin:NADP+ reductase by NADPH in the first titration. Changes in redox state have significant effects on Kd values of both Fd-FNR and FNR-NADP(H) complexes (9, 24).

 Nonetheless, we can conclude that association of NADPH with ferredoxin:NADP+ reductase reduces the affinity of the flavoprotein for Fd-Sepharose. Some ferredoxin:NADP+ reductase remained associated with Fd-Sepharose at very high NADPH concentrations; this is compatible with formation of a ternary Fd-FNR-NADP+ complex. It seems likely that in the reduced states, NADP(H) and Fd interact in the same fashion as in the oxidized state.

In a complementary experiment, we mixed Fd with a mixture of ferredoxin:NADP+ reductase and NADPH. Ferredoxin:NADP+ reductase and NADPH form a stable two-electron reduced charge-transfer species; addition of oxidized anaerobic Fd from a side arm of the anaerobic cuvette caused loss of the long wavelength band diagnostic of the charge-transfer complex. In addition, absorbance changes at 456 nm suggested some electron transfer from NADPH to ferredoxin:NADP+ reductase. Thus, Fd altered the relationship between the flavin and pyridine nucleotide centers of ferredoxin:NADP+ reductase and NADPH; we could not determine whether the NADP(H) dissociated from the flavoprotein or whether Fd merely abolished the charge-transfer interactions.

Stopped flow studies were conducted to investigate the rates at which Fd and NADP+ associate or dissociate with ferredoxin:NADP+ reductase. We sought to determine whether the changes in Kd resulted from increases in the rate of dissociation (k0) or decreases in the rate of association (k∞) (Kd = k0/k∞). The association of NADPH with ferredoxin:NADP+ reductase was too fast to be observed (complete within 3 ms). But, we did observe a very fast increase in A410 (k > 1000 s⁻¹) when we mixed NADPH with preformed Fd-FNR complex (data not shown). This suggests that k∞ decreases when Fd is bound to ferredoxin:NADP+ reductase, consistent with the increase in Kd, as the observed reaction was at the limit of observation, this conclusion is very tentative.

Association of Fd with ferredoxin:NADP+ reductase or with FNR-NADP+ was too fast to be observed. Similarly, the salt-induced dissociation ofFd-FNR complex was also complete within 3 ms. Thus, our data do not permit us to explain the changes in Kd in terms of changes in association and dissociation rates; we can be sure, however, that the tight association of Fd and ferredoxin:NADP+ reductase is not the result of a very slow dissociation of Fd.

**Discussion**

Ferredoxin:NADP+ reductase has long been known to have binding sites for Fd and NADP+ (6-7); Ricard et al. (10) have also reported formation of ternary complex. The present data support the conclusion that both substrates can associate simultaneously with ferredoxin:NADP+ reductase; there are at least two substrate-binding sites. This conclusion is supported by the simultaneous appearance of Fd-FNR and FNR-NADP+ mixing spectra when both ligands are at high concentration (Fd > ferredoxin:NADP+ reductase > 20 μM and NADP+ > 2 mM). Ultrafiltration binding studies confirm Fd-FNR association at high NADP+ concentrations.

Although separate sites for Fd and NADP+ were found, binding of the two substrates to ferredoxin:NADP+ reductase was not independent; association of one substrate (i.e. Fd) with ferredoxin:NADP+ reductase inhibited the binding of the other substrate (NADP+), as manifested in an increase in the apparent Kd for FNR-NADP+ or Fd-FNR complex. Specifically, Fd was found to increase the Kd of the FNR-NADP+ association by about 23-fold; conversely, Kd for Fd-FNR complexation increased with NADP+ from less than 50 nM to a saturating level of about 500 nM.

The changes in apparent Kd,Fd probably do not result from two classes of Fd-binding sites. No spectral perturbations were observed, indicating an Fd:FNR stoichiometry greater than 1:1. In addition, the spectral changes associated with complex formation were the same whether or not NADP+ was present; thus, Fd was probably occupying the same site. Similar arguments make it unlikely that changes in apparent Kd,FNADP+ result from multiple binding sites. We could explain the data fairly well by a model of cooperative interactions (Scheme I); thus, although ternary complex (Fd-FNR-NADP(H)) can form, enzyme-substrate interactions are weaker than in binary enzyme-substrate complex.

Titration ofNADPH into ferredoxin:NADP+ reductase bound to Fd-Sepharose was found to release most, but not all, of the ferredoxin:NADP+ reductase from the Fd-Sepharose. Ferredoxin:NADP+ reductase was released whether the proteins were initially oxidized or dithionite-reduced. Hence, we conclude that the partial competition observed in the oxidized states probably applies as well to reduced (and presumably more catalytically relevant) states. The dithionite-reduced Fd-FNR complex was disrupted at lower NADPH concentrations than the initially oxidized Fd-FNR complex; this is probably a reflection of increase in Kd for Fd-FNR complex upon reduction of Fd (9).

Our conclusions are different from those of Ricard et al. (10), who reported that the Kd values for Fd-FNR or FNR-NADP+ complex (determined by difference mixing spectra) did not change if the other substrate was present. In light of their report, we also used binding assays in other than mixing spectra. Kd values for FNR-NADP+ complex were similar whether binding was assayed by difference mixing spectra or ultrafiltration. In addition, qualitative studies using immobilized ligands confirmed that NADP+ (or its analogue, 2',5'-ADP) caused partial dissociation of Fd-FNR complex. We cannot explain the differences between the studies; in our hands, mixing experiments designed to meet the conditions of the studies of Ricard et al. (10) indicated that Fd interfered with FNR-NADP+ association.

The two studies also differ with regard to the effect of Fd on the absorbance changes associated with FNR-NADP+

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6 C. J. Batie and H. Kamin, unpublished observations.
binding. Ricard et al. (10) found that Fd caused a large decrease in maximal absorbance changes, but with no change in shape of the FNR-NADP* difference mixing spectrum. We, however, found that at saturating NADP*, the absorbance perturbations were as large (possibly even somewhat larger) when Fd was present than when Fd was absent.

Two paramagnetic probes differing in charge (positively charged Dy-o-phenanthroline and negatively charged Dy-EDTA) were used to perturb the EPR spectra of ferredoxin:NADP+ reductase and Fd (semiquinone and reduced, respectively). The positively charged probe broadened the signal of the acidic (negatively charged) ferredoxin; Dy-EDTA did so also, but to a lesser extent. On the other hand, Dy-EDTA was much more effective in perturbing the radial signal of ferredoxin:NADP+ reductase than was Dy-o-phenanthroline. Mixing Fd and ferredoxin:NADP+ reductase substantially decreased the line broadening by either agent; thus, one-electron reduced (Fdredox/NFNRredox) and three-electron reduced (Fdthree/NFNRthree) complexes form.

The present data indicate that the flavin of ferredoxin:NADP+ reductase is exposed at the 8-position but not the pyrimidine ring. One-electron reduced ferredoxin:NADP+ reductase, and NADP+ (these data will be reported in another paper in this series) indicate that ternary complexes may facilitate photosynthetic NADP+ reduction.

Chemically analogous electron transfer systems are found in adrenal mitochondria and in Pseudomonas bacteria; they catalyze electron transfer from NAD(P)H to cytochromes P-450 via an FAD-containing protein and an FeS* protein (22, 31). Ternary NADP(H)-adrenodoxin reductase-adrenodoxin complex has been identified; it has been shown that electron transfer from NADPH to adrenodoxin proceeds via the ternary complex (22, 32). Lambeth et al. (22, 32) did not test for interactions between NADP(H) and adrenodoxin in formation of ternary complex with the reductase. The mechanism of the putidaredoxin/putidaredoxin reductase system (the bacterial iron-sulfur protein and flavoprotein (31)) has not yet been described, nor has ternary complex been reported. Thus, it is not clear whether a destabilized ternary complex facilitates other electron transfers among pyridine nucleotides, flavoproteins, and iron-sulfur proteins.

The decrease in NADP(H) binding caused by Fd may explain earlier observations that Fd inhibits the diaphorase and transhydrogenase activities of ferredoxin:NADP+ reductase (33, 34). Nakamura and Kimura (33) described the inhibition of diaphorase activity (electron transfer from NADPH to dichlorophenol-indophenol) as being partially competitive with respect to NADPH. We have also observed inhibition of ferricyanide reductase activity by Fd, competitively with respect to NADPH (data not shown). Nelson and Neumann (34) reported that Fd inhibited electron transfer from NADPH to NAD. Decreased NADPH binding caused by association of Fd with ferredoxin:NADP+ reductase could explain both results.

These studies do not address the question of the mechanism of interaction between Fd and NADP+. Steric hindrance between Fd and NADP+ could provide an explanation, or, alternately, occupation of one site could cause changes in ferredoxin:NADP+ reductase conformation which decrease the association of the other substrate with ferredoxin:NADP+ reductase. Resolution of these possibilities may have to wait upon further clarification of the structure of ferredoxin:NADP+ reductase.

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