The Relation of pH and Oxidation-Reduction Potential to the Association State of the Ferredoxin-Ferredoxin:NADP⁺ Reductase Complex*

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The interaction between spinach ferredoxin and ferredoxin:NADP⁺ reductase was studied by varying pH and oxidation-reduction state. The \( K_d \) of the oxidized ferredoxin-ferredoxin:NADP⁺ reductase complex increases with increasing pH in the range from pH 6 to pH 8; the \( K_d \) is pH-independent above pH 9. These data are interpreted as showing 1 proton binding/complex at neutral pH. The extent of association of the complex was also varied by manipulation of salt concentration, and of concentration of the individual proteins. Increased association accompanied a negative shift in potential of ferredoxin relative to that of ferredoxin: NADP⁺ reductase. Oxidation-reduction titrations showed that the oxidation-reduction potential of ferredoxin is reduced to above −510 mV when in complex with ferredoxin:NADP⁺ reductase, a change of about −90 mV; the potential of ferredoxin:NADP⁺ reductase is changed little (no more than +20 mV). Conversely, these data also showed that the oxidation-reduction state of ferredoxin strongly affected its association with the flavoprotein, increasing the \( K_d \) at least 30-fold on reduction of ferredoxin.

Iron-sulfur proteins serve as electron donors in a variety of systems, interacting with flavoproteins, hemoproteins, and other iron-sulfur proteins, or with iron-sulfur moieties of more complex electron transport enzymes. In green plants, the iron-sulfur protein, ferredoxin, is normally reduced by photosystem I (1, 2); two systems which serve to transfer the low potential electrons of reduced Fe⁺ (\( E_{\text{m}}(\text{Fe}) = -420 \text{ mV} \)) to the metabolic pathways of the chloroplast are the NADP⁺ reductase and the assimilatory nitrate and sulfate reductases. Thus, ferredoxin can donate electrons 1) to the flavoprotein, ferredoxin: NADP⁺ reductase, which reduces NADP⁺ to NADPH, which in turn serves as a source of reducing equivalents in the general reductive metabolism of the chloroplast (3, 4); and 2) presumably via Fe₃S₄⁺ groupings to the assimilatory enzymes, nitrite reductase and sulfate reductase, which catalyze the conversion of nitrite to ammonia and sulfate to sulfide. In both of these 6-electron assimilatory reductions, a novel heme, siroheme (5, 6), serves as the substrate-binding site; a closely interacting iron-sulfur moiety (7) probably accepts electrons from ferredoxin and transfers them via the heme to the substrate (8).

Our laboratory has long been interested in electron transfer between iron-sulfur centers and other oxidation-reduction-active groups, and we have reported detailed studies of the interaction between the flavin, iron-sulfur protein, and hemoprotein moieties of the adrenal mitochondrial steroid hydroxylation system (9–17); in the latter, electrons from NADPH are channeled by a flavoprotein (adrenodoxin reductase) and an iron-sulfur protein (adrenodoxin) to a reaction-specific cytochrome P-450. Many features of the mechanism of electron transfer from pyridine nucleotide to cytochrome P-450 in the adrenal systems depend for their mechanism on close interaction, sometimes with complex formation, between the flavoprotein and the iron-sulfur protein (12, 16, 17). The dissociation constants of these complexes vary with the oxidation-reduction states of the oxidation-reduction-active components; there is thus an interplay between the association constants of the two enzymes and the oxidation-reduction potentials of the system (10).

In the case of chloroplast NADP⁺ reduction, the system is formally analogous to the adrenal system in that it also comprises NADPH, a mono-FAD flavoprotein, and an acidic Fe₃S₄⁺ protein. The systems differ in the oxidation-reduction potentials of their components, the plant system being much more negative in potential (10, 18, 19), and in the thermodynamically favored and biological direction of electron flow: in the adrenal, electrons flow from metabolism to NADPH to the iron-sulfur protein; in the plant, electrons flow from photosystem I, through ferredoxin to the flavin, to NADPH.

Our laboratory has initiated a study of the plant FNR-Fd system in the light of the work on the adrenal adrenodoxin-reductase system. The present report deals with the oxidation-reduction states and the oxidation-reduction potentials of the flavin and iron-sulfur components, and with the effects of these parameters upon the association of the flavoprotein with the iron-sulfur protein.

**EXPERIMENTAL PROCEDURES**

**Materials**

NADPH and NADP⁺ were obtained from P-L Biochemicals, benzyl viologen and methyl viologen were from Sigma, neutral red was from Pfaltz and Bauer, and quinhydrone was from Eastman Chemicals.

**Methods**

**Enzyme Purification**—Ferredoxin and ferredoxin:NADP⁺ reductase were purified from spinach along with nitrite reductase, using the procedure reported for the latter enzyme (20). Ferredoxin from 2 bushels of spinach (12–18 kg) was collected on a 2-liter bed of DE52 cellulose (10 mM Tris, pH 8.0, 0.2 mM NaCl), which did not retain the nitrite and NADP⁺ reductases. The dark brown layer was scraped off, resuspended in the same buffer, and poured into a column. The

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The abbreviations used are: Fd, spinach ferredoxin; FNR, ferredoxin:NADP⁺ reductase; Tris, tris(hydroxymethyl)aminomethane; Tricine, N-tris(hydroxymethyl)methylglycine.
Ferredoxin: Ferredoxin:NADP⁺ Reductase Complex

Ferredoxin was then eluted with 1 mM NaCl, 30 mM Tris, pH 8.0, into a small (30-100 ml) concentrated fraction. After dialysis versus 0.2 mM NaCl, 30 mM Tris, pH 8.0, the ferredoxin was applied to a DE52 column (2.5 x 75 cm) equilibrated with the same buffer, washed with at least 2 volumes of buffer, and then eluted with 0.26 mM NaCl, 30 mM Tris, pH 8.0. Fractions with a ratio of A₂₈₀ to A₂₆₀ > 0.4 were then pooled and concentrated using a Amicon DM5 or Amicon PM10. Fractions with a ratio of A₄₅₀ to A₂₈₀ > 0.46 were used for spectrophotometric experiments, pure ferredoxin having a ratio of 0.49 (21).

Ferredoxin:NADP⁺ reductase separates from nitrite reductase at the first DEAE-cellulose column in the nitrite reductase purification scheme (20), eluting in 0.1 mM NaCl, 10 mM Tris, pH 8.0. Fractions containing NADPH:ferricyanide reductase activity were pooled and applied to a column (2.5 x 40 cm) of reactive blue Sepharose equilibrated with the same buffer. The column was washed with 0.2 mM NaCl, 30 mM Tris, pH 6.0. The flavoprotein was eluted with a salt gradient from 0.2 to 0.35 M (or occasionally to 0.4 M NaCl) in the Tris buffer, containing NADPH:ferricyanide reductase activity were pooled and then concentrated using an Amicon DM5 or PM10. Fractions with a ratio of A₄₅₀ to A₂₈₀ > 0.13 (for pure enzyme (22)) and a ratio of A₄₆₀ to A₂₈₀ > 4 (our pure samples had a ratio of 0.5) were used for spectrophotometric experiments. Other fractions were further purified using a Fd-Sepharose affinity column made and used as reported by Shin and Oshino (22).

Spectrophotometric data were obtained using a Varian Cary 14,000 UV-visible spectrophotometer. pH was measured with a Radiometer pH meter and a Radiometer 2322c combination electrode.

Analysis of Spectra—We determined the concentration of oxidized and reduced species in complex mixtures by using a data analysis program developed in our laboratory by Lambeth et al. (20) and applied to similar problems in the adrenal system. We adapted this program for use on the MINC-11 computer. Table I gives the values for reduced minus oxidized extinction coefficients at 20 wavelengths for each species present in our titrations. The values for neutral and anionic flavin semiquinones are those of Azobacter flavoprotein and t-amino acid oxidase, respectively (40), minus the extinction coefficients for oxidized ferredoxin.NADP⁺ reductase; we could not produce sufficient quantities of either radical form of ferredoxin:NADP⁺ reductase to calculate such values. Spectra from a titration were analyzed by determining the absorbance at selected wavelengths. Generally, the first 15 wavelengths in Table I were used, although equivalent results were calculated using all 20 wavelengths. We corrected for dilution, and subtracted from the initial, oxidized spectrum to generate difference spectra. The program determined the best fit of the absorbance curve in Table I to the experimental spectra, reporting the concentration of each reduced species. The concentration of oxidized species was taken to be the total concentration minus the concentration of reduced species.

RESULTS

Effects of Salt and pH upon Dissociation Constants

The dissociation constant of the Fd-FNR complex was found to be modified by two variables: pH and salt concentration; the salt dependence has previously been described by Foust et al. (30). The dissociation constant of the Fd-FNR complex was studied over a pH range from 5.5 to 11, using the characteristic binding spectrum (mixed versus the sum of absorbance of ferredoxin plus ferredoxin:NADP⁺ reductase) of the Fd-FNR complex as a measure of the concentration of complex (30-32). Over this range of pH, the binding spectrum made a rapid change at fixed values of the dissociation constant.

<table>
<thead>
<tr>
<th>Wave-</th>
<th>Extinction coefficients used in data analysis of titrations</th>
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<tr>
<td>length (nm)</td>
<td>Fd⁺</td>
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<td>388</td>
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<td>-545</td>
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<td>675</td>
<td>-210</td>
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* Spectrum of the reduced species minus that of the oxidized species.
* Spectrum of reduced minus that of oxidized ferredoxin:NADP⁺ reductase.
* Spectrum of Azobacter flavoprotein neutral semiquinone (40) minus that of oxidized ferredoxin.NADP⁺ reductase.
* Spectrum of t-amino acid oxidase anionic semiquinone (40) minus that of oxidized ferredoxin:NADP⁺ reductase.
did not change in shape; the ratio of extinction coefficients at 420, 465, and 540 nm did not change appreciably with pH.

The relation of \( K_d \) to pH and salt concentration was studied by measuring absorbance changes while titrating NaCl ("salt titrations") into a fixed concentration of ferredoxin plus ferredoxin:NADP\(^+\) reductase, and by titrating ferredoxin into ferredoxin:NADP\(^+\) reductase solutions ("ferredoxin titrations") at several salt concentrations. These two procedures, each performed at several pH levels, were:

**Salt Titrations**—A solution of 2 mM NaCl (see the legend to Fig. 1) was titrated into 1:1 mixtures of ferredoxin and ferredoxin:NADP\(^+\) reductase originally at \( [\mu] = 0.01 \) M. At pH values lower than 8 and at the concentrations used, nearly all ferredoxin:NADP\(^+\) reductase is in complex at \( [\mu] \) relative to its high, the maximum absorbance for association was established by extrapolation of data points from ferredoxin titrations (see below). This maximum absorbance was found to be essentially identical (within 10%) with the absorbance of oxidized ferredoxin and oxidized ferredoxin:NADP\(^+\) reductase did not change as pH was varied from 6 to 11, or as salt was added in the range 0.001-0.5 M NaCl. Thus, within these ranges, changes in absorbance can be ascribed uniquely to changes in the concentration of FD-FNR complex, and not to changes in the absorbance of either component of the complex.

**Ferredoxin Titrations**—Ferredoxin was titrated into reference and sample cells until it was in 3-fold molar excess to ferredoxin:NADP\(^+\) reductase. Extrapolations to infinite ferredoxin concentration gave the values for the difference extinction coefficients (465 m\(^{-1}\) cm\(^{-1}\) for \( \Delta A_{460} - \Delta A_{420} \) and 1790 m\(^{-1}\) cm\(^{-1}\) for \( \Delta A_{490} - \Delta A_{430} \)) previously mentioned; extrapolated absorbances were independent of salt concentration and pH. A computer program was used to calculate the best fit between the data from ferredoxin titrations and the theoretical curve predicted by the \( K_d \). Best fit was considered to be the \( K_d \) which gave the least sum of squared error.

Fig. 1 presents the \( K_d \) values obtained by titrations with NaCl and ferredoxin, carried out at pH 7.5 and 8.5. As previously described, salt addition was found to increase the \( K_d \). Fig. 1 shows that \( K_d \) is also larger at the higher pH. \( K_d \) values obtained by ferredoxin titration gave excellent agreement with those obtained by NaCl titration (Fig. 1, open versus solid points).

Fig. 2 shows the relationship between pH and p\( K_d \) of the Fd-FNR complex. Each point (3 different buffers; see legend) was obtained from a series of lines obtained as in Fig. 1, by NaCl and/or ferredoxin titrations at the indicated pH (Fig. 2), using the \( K_d \) values obtained at 90 mM salt concentration. It is apparent that \( K_d \) increases (p\( K_d \) decreases) with increasing pH. For pH values between 6.5 and 8.5, plotting p\( K_d \) versus pH gave straight and parallel lines, but this may be fortuitous, as small variations in the value for maximal absorbance (100% binding) will markedly affect the value calculated for \( K_d \) at low ionic strength.

An increase in \( K_d \) with increasing pH can be explained most easily by the binding of a proton when ferredoxin and ferredoxin:NADP\(^+\) reductase form a complex. We can fit these data to a theoretical line derived from this model; we assume that one of the proteins has an ionizable group which is affected by complex formation, described by Equation 1, derived from the relationships shown below (Pr designates protein and L designates ligand):

\[
K_d = [\text{Pr}H^+][L]/[\text{Pr}H^+]L \\
K_d = [\text{Pr}][\text{H}^+]/[\text{Pr}H^+] \\
K_d = [\text{Pr}][\text{L}]/[\text{Pr}L] \\
K_d = K_d(0) + (1 + [\text{H}^+]K_{\alpha})(1 + [\text{H}^+]K_{\alpha})^{-1} \tag{1}
\]

where \( K_d \) is the limiting dissociation constant approached at high pH, \( K_d \) is the ionization constant for the free protein, and \( K_{\alpha} \) is the ionization constant for that group in the protein-protein complex. We find the best fit to the data using \( K_d = 60 \mu M \), p\( K_{\alpha} = 6.0 \), and p\( K_d = 8.2 \). These data predict that the fully protonated form should have a dissociation constant (\( K_d \)) of about 0.4 \( \mu M \). If more than one proton were bound, the absolute value of the slope would be greater than 1 between pH 6 and pH 9, and a good fit could not be obtained using Equation 1. As \( K_d \) (measured) decreases with increasing pH...

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**Fig. 1. Binding of ferredoxin and ferredoxin:NADP\(^+\) reductase as a function of ionic strength.** All titrations used 20 \( \mu M \) ferredoxin:NADP\(^+\) reductase in 10 mM Tris buffer. The difference between the absorbance at 460 nm and that at 450 nm in the binding spectrum was used as a measure of complex formation. All used 4 cuvettes, 2 on the reference side, 2 on the sample side. Those marked with solid symbols were titrated by adding equal quantities of ferredoxin and ferredoxin:NADP\(^+\) reductase to the sample and reference cells. The 2 electron carriers were added to the same cell on the sample side, and to different cells on the reference side. Spectra were taken initially and after each addition of 2 mM NaCl to all cells. \( K_d \) values were calculated assuming complete association in the initial spectrum. The points labeled by open symbols were obtained by titrating the front cells with approximately 20 \( \mu M \) ferredoxin:NADP\(^+\) reductase and the rear cells with buffer, then titrating in equal amounts of 200 \( \mu M \) ferredoxin to the front sample and rear reference cells, mixing, and taking spectra after each addition. We extrapolated to infinite ferredoxin concentration to obtain the maximal change in absorbance. \( K_d \) values were obtained by fitting the theoretical curve to the experimental points.

**Fig. 2. Variation in \( K_d \) with pH.** All points were derived from lines obtained as in Fig. 1 at the point of \( \phi = 0.3 \), with the exception that subsequent calculations used a difference extinction coefficient of 473 m\(^{-1}\) cm\(^{-1}\) for the difference in absorbance between 464 and 418 nm to calculate \( K_d \) values. Buffers used were: 10 mM sodium phosphate buffer (\( \bullet \)), 10 mM potassium carbonate buffer (\( \mathcal{O} \)), or 10 mM Tricine (\( \mathcal{M} \)). All buffers had an initial ionic strength of 0.03 M. The curve was calculated assuming a group which is protonated with a p\( K_d \) of 6.0 in uncomplexed protein and a p\( K_d \) of 8.2 in the FD-FNR complex, limiting \( K_d \) in basic conditions of 56 \( \mu M \).
pH, we can conclude that at intermediate pH values, a proton is taken up when ferredoxin and ferredoxin:NADP+ reductase form a complex; at pH greater than 8.5, the proteins are no longer protonated and the strength of the complex is no longer pH-dependent.

Oxidation-Reduction Titrations of Fd-FNR Mixtures

The previous data describe the properties of the fully oxidized Fd-FNR complex. If reduction alters the $K_d$, then the oxidation-reduction behavior of one or both proteins must change. Our laboratory has studied analogous relationships in the adrenal system; these studies have established that the $E_m$ of adrenodoxin becomes more negative when bound to adrenodoxin reductase, indicating that the reduced adrenodoxin binds the flavoprotein with less affinity than does oxidized adrenodoxin (10).

Preliminary experiments indicated that fully reduced and semiquinone forms of ferredoxin:NADP+ reductase did indeed form complexes with reduced ferredoxin. Thus, the paramagnetic probe, dysprosium, when chelated by either o-phenanthroline or EDTA, was found to broaden and relax the EPR signals of reduced ferredoxin and ferredoxin:NADP+ reductase neutral semiquinone. Each protein was substantially shielded when the other was added under conditions favoring complex formation. Addition of NaCl could restore the Dy$^{3+}$ effect.

We investigated the possibility that these reduced complexes might differ in $K_d$ from the oxidized complex by carrying out oxidation-reduction titrations of 1:1 mixtures of ferredoxin and ferredoxin:NADP+ reductase, changing salt or protein concentration to vary the degree of association of ferredoxin and ferredoxin:NADP+ reductase. We plotted the fraction of reduced ferredoxin versus $30 \log((\text{FNR}_o)/\text{FNR}_{red})$ to measure $E_m(Fd) - E_m(FNR)$.

![Fig. 3. Photoreduction of 1:1 Fd:FNR at high ionic strength. A 1-ml reaction mixture containing 56.6 μM ferredoxin:NADP+ reductase, 38.8 μM ferredoxin, 1 mM EDTA, and 1.5 μM 5-deazaflavin in 100 mM potassium carbonate buffer, 0.19 M NaCl, μ = 0.3 M, pH 9.0, was evacuated and regassed with Ar 7 times to make the solution anaerobic. The mixture was mixed with the gas after each exchange to equilibrate the gas with any dissolved gases. The mixture was illuminated for periods of 5 min by a 500-Watt slide projector at 12 inches; during illumination, the anaerobic cuvette was immersed in ice water to keep it cool. Spectra were taken before illumination and after each period of illumination. Following complete reduction, the cuvette was opened and allowed to reoxidize. The spectrum of the reoxidized mixture was recorded to check for the retention of intact ferredoxin and ferredoxin:NADP+ reductase.](image-url)

![Fig. 4. Photoreduction of Fd+FNR complex. A 1-ml reaction mixture of 36.7 μM ferredoxin:NADP+ reductase, 35.3 μM ferredoxin, 10 mM glycine, pH 9.0, 10 mM EDTA, 1.5 μM 5-deazaflavin was rendered anaerobic and reduced in the same manner as described in the legend to Fig. 3. Curve 1 was recorded before illumination, and each successive curve was recorded after illumination for increasing periods of time.](image-url)

We first titrated 1:1 mixtures of oxidized ferredoxin and ferredoxin:NADP+ reductase at low and at high ionic strengths, using three reduction systems: light-deazaflavin-EDTA (photoreduction), dithionite, and dithionite-methyl viologen. Figs. 3 and 4 show the spectra from two such titrations, photoreducing the electron carriers. Fig. 3 displays spectra from several time points in a photoreduction at $μ = 0.3$ M, where the Fd-FNR complex is completely dissociated. Both proteins can be reduced fully, and the $E_m$ values are similar; bleaching occurs both at long wavelengths (>500 nm), where ferredoxin is the primary chromophore, and in the region between 400 and 480 nm, where ferredoxin:NADP+ reductase is bleached on reduction.

Fig. 4 shows some of the spectra from a similar photoreduction of 1:1 Fd-FNR, but in 10 mM buffer, pH 9. Under these conditions ($K_d = 0.2$ μM), the oxidized proteins are >95% associated in the Fd-FNR complex. The intermediate spectra are markedly different from those recorded at high ionic strength (Fig. 3). Thus, spectrum 5 (Fig. 4) resembles that of the sum of oxidized ferredoxin plus reduced ferredoxin: NADP+ reductase; it is clear that the difference between the midpoint potentials of the two proteins has markedly increased under those conditions of ionic strength which favor association.

The spectra shown in Figs. 3 and 4 were analyzed for the concentrations of the oxidation-reduction forms of the components by the method of Lambeth et al. (10) (see also under "Methods"). Fig. 5 shows the results of a series of such analyses, plotting the percentage of reduced ferredoxin versus $30 \log((\text{FNR}_o)/\text{FNR}_{red})$. Such a plot allows calculation of the separation of the midpoint potentials of ferredoxin and ferredoxin:NADP+ reductase. For $μ = 0.3$ M (if we assume that the two proteins are fully dissociated in all oxidation-reduction states, and that ferredoxin:NADP+ reductase obeys simple Nernst equation $n = 2$ behavior), we find that ferredoxin has an $E_m$ 20 mV positive to that of ferredoxin:NADP+ reductase, a finding compatible with earlier studies (19). In this case, the curve for ferredoxin deviates somewhat from the predicted $n = 1$ curve; the plotted data (Fig. 5, dashed line) fit an $n = 1.4$
Ferredoxin·Ferredoxin:NADP⁺ Reductase Complex

Fig. 5. Relative potentials of ferredoxin and ferredoxin: NADP⁺ reductase at 2 salt concentrations. The spectra from Figs. 3 and 4 were analyzed using all 20 wavelengths of Table 1 to yield concentrations of oxidized and reduced enzymes. Open circles (Fig. 3), closed circles (Fig. 4).

curve better, an observation we do not attempt to explain.

At low ionic strength, where the Fd·FNR complex is essentially fully associated, the spectra (from Fig. 4) gave the titration curve shown in open circles in Fig. 5. In this case, the ferredoxin titration fits an n = 1 curve better, but is shifted considerably to the negative of the free ferredoxin:NADP⁺ reductase potential. The curve drawn through the data points is that predicted for a $E_m$, bound ferredoxin 70 mV negative to the $E_m$ of bound ferredoxin:NADP⁺ reductase. Reduced titration using dithionite and methyl viologen as well as black-ferriyanide as reductants. We found that $E_m$, oxidized proteins were associated to about 0.3, data from Fig. 3; $\mu$ = 0.011, data from Fig. 4.

We also varied the association of ferredoxin and ferredoxin: NADP⁺ reductase in oxidation-reduction titrations by using different concentrations of the 1:1 Fd·FNR mixture. We carried out 5 reductive titrations of 1:1 Fd·FNR at concentrations from 1 to 50 μM (each) using dithionite and methyl viologen as reductants. We found that $E_m$(Fd) – $E_m$(FNR) changed from +15 mV at 1 μM (a concentration at which 65% of the proteins are associated) to about –60 mV at 50 μM (complete association of the oxidized proteins). At higher concentrations, the data deviated from ideality; too much ferredoxin was reduced at high potential compared to the point at which it was 50% reduced. In this experiment, as before, $E_m$(Fd) – $E_m$(FNR) is more negative when the electron carriers are more associated.

The changes in $E_m$(Fd) – $E_m$(FNR) observed with varying association could be explained as: 1) a negative change in the $E_m$ of ferredoxin, 2) a positive change in the $E_m$ of ferredoxin: NADP⁺ reductase, or 3) both a negative change in ferredoxin potential and a positive change in ferredoxin:NADP⁺ reductase potential (a combination of the above). The following treatment attempts to distinguish among these possibilities.

The possible complexes between oxidized and reduced forms of ferredoxin:NADP⁺ reductase are shown in Equations 2–5. We neglect states with the semiquinone form of ferredoxin:NADP⁺ reductase, as it was a minor species at equilibrium in all experiments.

\[
\begin{align*}
K_{d1} &= \frac{[Fd_{\text{ox}}][FNR_{\text{red}}]}{[Fd_{\text{red}}][FNR_{\text{ox}}]} \\
K_{d2} &= \frac{[Fd_{\text{red}}][FNR_{\text{ox}}]}{[Fd_{\text{ox}}][FNR_{\text{red}}]} \\
K_{d3} &= \frac{[Fd_{\text{ox}}][FNR_{\text{red}}]}{[Fd_{\text{red}}][FNR_{\text{ox}}]} \\
K_{d4} &= \frac{[Fd_{\text{red}}][FNR_{\text{ox}}]}{[Fd_{\text{ox}}][FNR_{\text{red}}]}
\end{align*}
\]

For case 1 (a change of $E_m$(Fd) to a more negative value when bound to FNR), the $K_d$ varies only with the oxidation-reduction state of ferredoxin ($K_{d1} = K_{d3} > K_{d2} = K_{d4}$). In such a system, the midpoint potential would vary with uncomplexed ferredoxin:NADP⁺ reductase concentration [FNR] as in Equation 6:

\[
E_{\text{app}} = E_m(Fd) + \frac{(RT/F)\ln}{(1 + [FNR]/K_{d0})/(1 + [FNR]/K_{d0})}
\]

If this explanation were correct, then, at saturating ferredoxin: NADP⁺ reductase, this equation would collapse to Equation 7:

\[
E_{\text{app}}(Fd) = E_m(Fd) + (RT/F)\ln(K_{d0}/K_{d0})
\]

Equation 7 predicts an $n = 1$ titration curve displaced 60 log($K_{d0}/K_{d0}$) mV from that of free ferredoxin.

If $E_{\text{app}}(Fd) - E_m(Fd) = -70$ mV, then $K_d$ "reduced" ($K_{d2}$, $K_{d3}$) is at least 15 times $K_d$ "oxidized" ($K_{d1}$, $K_{d4}$). This predicts that reduced ferredoxin would be less associated with ferredoxin:NADP⁺ reductase than oxidized ferredoxin, and that $E_m(Fd) - E_m(FNR)$ should become more negative as the concentration of protein is increased, until that concentration is much greater than the largest $K_d$. Such an interpretation is compatible with the data supporting case 1.

An equivalent treatment for case 2 (a positive change in $E_m(FNR)$ reveals that $K_{d1}$ ($K_{d2}$, $K_{d3}$) would be about 215 times $K_{d0}$ ($K_{d1}$, $K_{d4}$). This would predict a large change at 1 μM, where nearly all reduced ferredoxin:NADP⁺ reductase and 65% of the oxidized ferredoxin:NADP⁺ reductase would be complexed to ferredoxin. The data are inconsistent with this interpretation; the change observed in Fig. 5 cannot be attributed to a change in the potential of FNR alone. A small contribution to the total change by ferredoxin:NADP⁺ reductase as in case 3 cannot be ruled out.

We then carried out preliminary experiments using oxidation-reduction-active dyes or excess ferredoxin:NADP⁺ reductase as indicators of $E_m$ (see Equation 9), to provide independent measurements of the effect of complexation upon the oxidation-reduction behavior of each of the two enzymes. We first used neutral red as oxidation-reduction indicator ($E_m = -440$ mV at pH 9 (33), confirmed by potentiometric titration). Titration of each protein separately with neutral red gave $E_m(Fd) = -430$ mV and $E_m(FNR) = -445$ mV. When we titrated a mixture of ferredoxin and ferredoxin:NADP⁺ reductase with neutral red as the oxidation-reduction indicator using spectral analysis to quantify the oxidation-reduction states of each component, the $E_m$ of ferredoxin:NADP⁺ reductase was indistinguishable from that measured in the absence of ferredoxin.

In complex, the potential of ferredoxin was changed by −75 mV to −905 mV. Use of methyl viologen gave similar results. In each case, the potential at which ferredoxin is largely reduced is subject to considerable error; only a small portion of the indicator remained oxidized through these points.

A different approach was to titrate a 10:1 mixture of ferredoxin:NADP⁺ reductase and ferredoxin using the excess free ferredoxin:NADP⁺ reductase as a oxidation-reduction indicator. Here also, we found that the potential of ferredoxin in complex was about 75 mV lower in potential than the sum of the complexed and the free ferredoxin:NADP⁺ reductase. The data in this experiment deviated from a Nerstian line as described earlier, so we considered possible contributions to the $K_d$ from the oxidation-reduction state of ferredoxin: NADP⁺ reductase in constructing a simulated line which would fit the data. Using Equations 2–5 and the Nerst equation, we substitute into Equation 8:

\[
\frac{\% Fd_{\text{ox}}}{100} = 100 \left[ \frac{[Fd_{\text{ox}}][FNR_{\text{red}}] + [Fd_{\text{ox}}][FNR_{\text{ox}}]}{[Fd_{\text{ox}}]} \right]
\]

to derive Equation 9, which describes the percentage of ferredoxin reduction as a function of the dissociation constants, the oxidation-reduction potentials of free ferredoxin and ferredoxin:NADP⁺ reductase, and the concentration of free fer-
redoxin:NADP$^+$ reductase, oxidized and reduced.

$$\% \text{Fd}_{\text{red}} = 100 \left[ 1 + \frac{[\text{FNR}_{\text{red}}]}{K_{d1} + [\text{FNR}_{\text{red}}]} \times \frac{K_{d1} + [\text{FNR}_{\text{red}}]}{K_{d1} + [\text{FNR}_{\text{red}}]} \right]$$

where $E_a = E_m(\text{FNR}) + 30 \log([\text{FNR}_{\text{red}}]/[\text{FNR}_{\text{red}}])$.

Using Equation 9 and considering the concentration of bound ferredoxin:NADP$^+$ reductase to be negligible, we can simulate oxidation-reduction titrations. By varying the values for $K_{d1}$, $K_{d2}$, and $K_{d3}$, we fit the predicted titration curve to the data. We used $K_{d1} = 0.2 \mu M$, $K_{d2} = 4 \mu M$, $K_{d3} = 20 \mu M$, and $K_{d4} = 0.4 \mu M$, $K_{d5}$ determined by the methods of Fig. 1. Using that value for $K_{d4}$, reasonable fits to the data can be made of $K_{d5}$ between 5 and 20 $\mu M$; $K_{d6} = 2-4 \mu M$; and $K_{d7} = 0.1-0.4 \mu M$. In all cases, $K_{d8}$ must be about 4 times greater than $K_{d7}$, and $K_{d9}$ in turn must be at least 10-fold greater than $K_{d7}$ or $K_{d8}$. Other arrangements of values predict differently shaped curves. When we simulated the titration curve for bound ferredoxin:NADP$^+$ reductase using $K_{d3}$ values which give a good fit for the ferredoxin reduction, that curve varied little from that of free ferredoxin:NADP$^+$ reductase.

Potentiometric Titration of Ferredoxin and Ferredoxin: NADP$^+$ reductase

We also made simultaneous spectrophotometric and electropotential measurements during titrations of ferredoxin: NADP$^+$ reductase and ferredoxin, first each protein separately, then in complex. Titrations were carried out in a Dutton cell (34), and benzyl viologen and methyl viologen in a ratio of 1:1:10 to enzyme were used as mediators. The system used was calibrated against systems of known potential: quinhydrone, benzyl viologen, and ferricyanide. Separate titrations of ferredoxin and ferredoxin:NADP$^+$ reductase showed similar $E_a$ values; $E_m(\text{Fd})$ (pH 8.4) = -240 mV, and $E_m(\text{FNR})$ (pH 8.4) = -442 mV. Each titration conformed to an $n = 1$ and $n = 2$ titration curve, respectively.

We then titrated a 1:1:3 mixture of ferredoxin and ferredoxin:NADP$^+$ reductase with both dithionite and ferricyanide (Fig. 6). Benzyl viologen and methyl viologen, the mediators, were present at a ratio of 1:10 to ferredoxin:NADP$^+$ reductase. The data from Fig. 6 support the conclusions of the preliminary experiments. A marked negative potential shift is evident, about 90 mV (corresponding to at least a 30-fold change in $K_{d9}$ values), but a small change (about +20 mV) is also seen in the potential of ferredoxin:NADP$^+$ reductase. The data points for percentage of reduced ferredoxin:NADP$^+$ reductase in the reductive experiment best fit an $n = 1.8$ curve. This might indicate some dependence of $K_{d9}$ on the oxidation-reduction state of ferredoxin:NADP$^+$ reductase but a smaller one; about 4-5-fold.

We cannot calculate a ratio of $K_{d9}$ values for oxidized and reduced complexes with much confidence from Fig. 6, since the reduced ferredoxin was probably not entirely associated with ferredoxin:NADP$^+$ reductase; nevertheless, from a potential change of -90 mV, we can calculate that reduced ferredoxin would form a complex with ferredoxin:NADP$^+$ reductase with a $K_{d9}$ at least 30 times that of the oxidized ferredoxin, giving a $K_{d9}$ of at least 5 $\mu M$, a value indicating some dissociation under our experimental conditions. If we assume some dissociation, the values of $K_{d9}$ and $K_{d10}$ could easily be 50-100 times that of $K_{d9}$.

As observed earlier, the data points for ferredoxin reduction varied significantly from a Nasrasten n = 1 curve; a small amount of ferredoxin was reduced (~6%) at a potential of -380 mV, and the concentration of reduced ferredoxin decreased as ferredoxin:NADP$^+$ reductase was reduced. After nearly complete reduction of ferredoxin:NADP$^+$ reductase, reduction of ferredoxin proceeded to completion. The reduction of ferredoxin at very low potential followed a curve steeper than that predicted by a simple $n = 1$ curve, but ferredoxin was 50% reduced at a potential about 90 mV more negative than in the titration of ferredoxin alone. Back-titration with ferricyanide reversed the sequence. These data confirm the conclusions drawn earlier; in complex, ferredoxin is more negative than the uncomplexed ferredoxin, and thus serves as a stronger reductant for ferredoxin:NADP$^+$ reductase, and reduced ferredoxin dissociates more readily from ferredoxin:NADP$^+$ reductase than does oxidized ferredoxin.

DISCUSSION

We find that the strength of the 1:1 complex between ferredoxin and ferredoxin:NADP$^+$ reductase is affected not only by salt concentration (30), but also by pH and the oxidation-reduction state of the proteins. Upon formation of the Fd:FNR complex, ferredoxin becomes at least 90 mV more negative in potential, and the $K_{d9}$ of the FNR-reduced Fd complex is at least 30 times the $K_{d9}$ of the oxidized Fd:FNR complex; ferredoxin:NADP$^+$ reductase may also change in potential, but to a lesser degree, perhaps 20 mV positive. The variation of the dissociation constant of the oxidized proteins with pH suggests the existence of a group on either ferredoxin or ferredoxin:NADP$^+$ reductase with a $pK_a$ of about 6 on the free protein. Protonation of this group seems to favor complexation; the $pK_a$ of this group appears to be about 8 when the two proteins form a complex. The slope of the line in Fig. 2, which is never more than -1, shows that only one group is protonated per complex, if two or more residues were protonated on formation of the protein-protein complex, the slope should be nearly -2 at pH 7.

Foust et al. (30) reported a similar variation of $K_{d9}$ with pH (larger $K_{d9}$ at higher pH) in the analogous 1:1 complex between the 1-electron carrier, flavodoxin, and ferredoxin:NADP$^+$ reductase (30). As ferredoxin:NADP$^+$ reductase forms pH-sensitive complexes with 2 different small electron carriers, the same group on ferredoxin:NADP$^+$ reductase may be involved.
in forming both complexes, protonation favoring complexation.

Comparison of the Fd-FNR system with the analogous system from the adrenal cortical mitochondrion reveals interesting similarities and differences (Table II). The adrenal proteins physiologically carry electrons in a direction opposite to that of ferredoxin and ferredoxin:NADP⁺ reductase, and are also much more positive in potential. In both systems, the reduced iron-sulfur protein is less associated with the flavoprotein, and is thus more negative in midpoint potential in complex. In the adrenal system, this seemed to facilitate release of the reduced adrenodoxin so that it could shuttle electrons to a cytochrome P-450 (15). Tight binding of oxidized ferredoxin might seem likely to inhibit electron transfer from adrenodoxin reductase complex. They reported a rapid association between ferredoxin and ferredoxin:NADP⁺ reductase, and we have no data to indicate preferential binding to either of the oxidation states of ferredoxin.

Consideration of our results within the context of the chloroplast yields two possible physiological consequences. 1) The increase in stromal pH, from pH 7 to pH 8, observed when chloroplasts are illuminated (38) should be accompanied by a decrease in the association of oxidized ferredoxin and ferredoxin:NADP⁺ reductase, perhaps freeing ferredoxin to shuttle electrons. We do not know whether comparable changes would occur in more reduced complexes. 2) Since complex formation between ferredoxin and ferredoxin:NADP⁺ reductase causes the E₅₀ of ferredoxin to become about 90 mV more negative, association of the proteins will cause the reduction of ferredoxin:NADP⁺ reductase by ferredoxin to be greatly favored, even though at pH 8, each protein has a similar E₅₀ when measured separately. This would facilitate the physiological direction of electron flow. Evaluation of these possibilities will require greater understanding of the milieu of the chloroplast.

**REFERENCES**


**Table II**

<table>
<thead>
<tr>
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<th>Kinetic &amp; Thermodynamic Properties of the Fd-FNR Complex and the Adrenodoxin-Adrenodoxin Reductase Complex</th>
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<tr>
<td></td>
<td>kₐ,₁₅</td>
</tr>
<tr>
<td></td>
<td>mV</td>
</tr>
<tr>
<td>Adrenal⁺</td>
<td>~2.5 x 10⁻⁴ m</td>
</tr>
<tr>
<td>Spinach</td>
<td>1 x 10⁻⁴ M</td>
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<td>(pH 7, μ = 0.05)</td>
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* Determined by potentiometric titration.
* Calculated assuming that kₐ,₁₅ > 10⁶ m⁻¹ s⁻¹ and kₐ,₁₅ = 1 x 10⁻⁴ m.


39. Deleted in proof