Complex Formation between Ferredoxin Triphosphopyridine Nucleotide Reductase and Electron Transfer Proteins*

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SUMMARY

Ferredoxin-triphosphopyridine nucleotide reductase (FTR) forms complexes with spinach ferredoxin, bacterial ferredoxin, rubredoxin, and flavodoxin. The protein-protein interactions are complete within 3 msec after mixing and the resulting complex contains 1 molecule of FTR and 1 molecule of electron carrier. Complex formation causes changes in the visible spectrum. The complexes are sensitive to ionic strength; they are completely dissociated at high ionic strength. The effect of ionic strength on a catalytic assay involving FTR and these electron carriers suggests that the complexes are important catalytically. The calculated values for $\Delta G'$, $\Delta H'$, and $\Delta S'$ and the sensitivity of the complexes to ionic strength suggest that the interactions are mainly hydrophilic in nature.

TPN also causes perturbations in the visible spectrum of FTR. The changes are qualitatively and quantitatively different from those obtained with the protein electron carriers. Formation of the difference spectrum is affected by ionic strength, although the effect is less marked than in the protein-protein complexes. The initial changes, which occur rapidly, are followed by a slower first order decay to a second species.

Ferredoxin-TPN reductase is a flavoprotein isolated from plant chloroplasts (1-4). It functions in System I of the photosynthetic electron transport chain transferring electrons from the iron sulfur protein, ferredoxin, to TPN (2). The purified enzyme has a molecular weight of about 40,000, contains 1 molecule of FAD per molecule of protein, and shows a broad specificity for electron acceptors when TPNH is the electron donor (1, 5-10). It reduces both one and two electron acceptors including DPN, DPNH and TPN analogues, cytochrome f, plastocyanin, artificial dyes, and non-heme iron proteins. The mechanism of action is not understood for any of these reactions; at least two different mechanisms have been proposed to explain steady state kinetic data obtained with different protein substrates (5, 6).

This paper reports experiments which show that oxidized FTR forms 1:1 complexes with spinach ferredoxin and with TPN. Complexes are also formed between FTR and bacterial ferredoxin, rubredoxin, and flavodoxin. These complexes between a flavoprotein and either a non-heme iron protein or another flavoprotein are of interest in that they may provide useful information regarding the interactions between the oxidation-reduction chromophores in the more complex flavoproteins and metalloflavoproteins. A preliminary report of some of these findings has appeared (11). The spectral evidence for a complex between FTR and spinach ferredoxin has recently been confirmed (12, 13), and evidence for a TPN-FTR interaction has been presented (13).

MATERIALS AND METHODS

Ferredoxin-TPN reductase was prepared from spinach leaves by a method similar to that of Shin, Tagawa, and Arnon (2). The experimentally determined extinction coefficient at 458 nm was 10,300 M$^{-1}$ cm$^{-1}$.

Spinach ferredoxin was the generous gift of D. E. Petering and G. Palmer. The concentration of this protein was estimated from the absorbance at 420 nm and an extinction coefficient$^2$ of 9400 M$^{-1}$ cm$^{-1}$.

Clostridium pasteurianum ferredoxin was prepared as described by Mortenson (14). Concentrations were estimated from the absorbance at 390 nm with an extinction coefficient$^3$ of 24,500 M$^{-1}$ cm$^{-1}$ at this wave length.

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Peptostreptococcus elsdenii rubredoxin and flavodoxin were prepared by methods described elsewhere (15, 16). Extinction coefficients of 7,630 M$^{-1}$ cm$^{-1}$ at 490 nm (17) and 10,200 M$^{-1}$ cm$^{-1}$ at 445 nm (16) were used for rubredoxin and flavodoxin, respectively.

Measurements of Difference Spectra—All spectrophotometric measurements were made with a thermostatted Cary model 14 spectrophotometer. The spectrophotometer cells used for difference spectra measurements were dual compartment silica cells.

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$^1$ The abbreviation used is FTR, ferredoxin-TPN reductase.

$^2$ D. Petering and G. Palmer, personal communication.

$^3$ J. C. Rabinowitz, personal communication.
Fig. 1. The visible absorption spectra of spinach ferredoxin-TPN reductase, spinach ferredoxin, C. pasteurianum ferredoxin, P. elsdensii flavodoxin, and P. elsdensii rubredoxin. Samples were dissolved in 0.03 M Tris-HCl buffer, pH 7.5. Curve 1, $4.6 \times 10^{-6}$ M P. elsdensii flavodoxin; Curve 2, $4.5 \times 10^{-6}$ M spinach ferredoxin; Curve 3, $3.54 \times 10^{-5}$ M ferredoxin-TPN reductase; Curve 4, $1.18 \times 10^{-5}$ M C. pasteurianum ferredoxin; Curve 5, $1.68 \times 10^{-5}$ M P. elsdensii rubredoxin.

Fig. 2. Difference spectra of FTR-electron transfer protein complexes. Experiments were done at 12° in dual compartment spectrophotometer cells as described under “Materials and Methods.” Solutions of FTR were titrated with an electron transfer protein to give maximum difference spectra. The experiment with spinach ferredoxin was done in 0.005 M phosphate buffer, pH 7.0; the FTR concentration was $3.07 \times 10^{-6}$ M. The experiments with C. pasteurianum ferredoxin and rubredoxin were done in 0.03 M Tris-HCl buffer, pH 7.5; the FTR concentrations were $2.89 \times 10^{-5}$ M and $3.11 \times 10^{-5}$ M, respectively. The experiment with flavodoxin was done in 0.003 M sodium phosphate buffer, pH 7.6; the FTR concentration was $2.70 \times 10^{-5}$ M.

RESULTS

Spectrophotometric Titration of Ferredoxin-TPN Reductase with Electron Transfer Proteins—The visible absorption spectra of ferredoxin-TPN reductase and of the electron transfer proteins used in these experiments are shown in Fig. 1.

When ferredoxin-TPN reductase was mixed with spinach ferredoxin, bacterial ferredoxin, flavodoxin, or rubredoxin, as described under “Materials and Methods,” positive difference spectra were observed between the mixture and its separated components (Fig. 2). In control experiments in which bovine serum albumin, horse heart cytochrome c, or potassium ferricyanide was mixed with ferredoxin-TPN reductase no difference spectra occurred. Similarly, no difference spectra were found when flavodoxin was mixed with bacterial ferredoxin or rubredoxin or when bacterial ferredoxin was mixed with rubredoxin. The observed changes were evidently specific for mixtures of ferredoxin-TPN reductase and electron transfer proteins with which this enzyme reacts catalytically.
The difference spectrum was too great to measure with the stopped flow apparatus of Gibson and Milnes (15). When equal amounts of ferredoxin-TPN reductase and spinach ferredoxin \((3 \times 10^{-4} \text{M})\) were mixed at 15°C, spectral changes were complete within the dead time (3 msec) of the instrument. The magnitude of the difference spectrum and the shapes of the titration curves were strongly influenced by ionic strength. The effect of ionic strength of the complex was investigated further. FTR and electron carrier were mixed at low ionic strength at which almost full complex formation occurred. The ionic strength of the mixture was then slowly raised by adding increments of a concentrated salt solution. Difference spectra were recorded after each addition. In all experiments, the difference spectrum decreased with increasing ionic strength; in the presence of excess salt difference spectra completely disappeared indicating complete dissociation of the complex. Similar results were obtained when the salt was sodium chloride, ammonium sulfate, or sodium phosphate, indicating that this was not a specific ion effect but rather an effect of ionic strength. Values for the dissociation constant for the complex were calculated at each ionic strength during a titration. The results of these experiments are summarized in Fig. 4, where \(-\log_{10} K\) is plotted vs. ionic strength.

Certain regions of the difference spectra were qualitatively similar for the four electron carriers tested; all showed maxima near 395 mµ and 465 mµ. However, marked differences are apparent in other regions, particularly toward longer wavelengths. Formation of the difference spectrum was rapid in all cases. Changes in these static experiments were complete before measurements could be made. The rate of appearance of the difference spectrum was too great to measure with the stopped flow apparatus of Gibson and Milnes (15). When equal amounts of ferredoxin-TPN reductase and spinach ferredoxin \((3 \times 10^{-4} \text{M})\) were mixed at 15°C, spectral changes were complete within the dead time (3 msec) of the instrument. The minimum second order rate constant consistent with this observation is approximately \(10^4 \text{M}^{-1} \text{sec}^{-1}\).

Experiments were done to determine the amount of electron carrier required for maximum spectral change. Samples of ferredoxin-TPN reductase were titrated with carrier and difference spectra were recorded after each addition. Titrations were followed by measuring the absorbance changes at a wave length where the total change was large. All four electron carriers gave similar results. The absorbance increased with increasing amounts of electron carrier until a maximum was reached (Fig. 3). The shape of the titration curves suggested that under ideal conditions maximum changes occurred when FTR and electron carrier were present in equimolar concentrations (the observed deviations from perfect stoichiometry are probably due to combined errors in the estimation of protein concentrations or to incomplete complex formation at the ionic strength used). The results indicated that 1 molecule of FTR interacts with 1 molecule of protein substrate. The upper curve in Fig. 3 was used to obtain a difference extinction coefficient for the FTR-spinach ferredoxin complex. The value calculated was \(2.8 \times 10^4 \text{M}^{-1} \text{cm}^{-1}\) at 465 mµ.

The effect of temperature on the difference spectrum was examined with a mixture (1:1) of spinach ferredoxin and ferredoxin-TPN reductase in 0.05 M phosphate buffer, pH 7.0. No significant changes in the difference spectrum were detected when the temperature was varied between 5°C and 30°C.

The effect of ionic strength of the complex was investigated further. FTR and electron carrier were mixed at low ionic strength at which almost full complex formation occurred. The ionic strength of the mixture was then slowly raised by adding increments of a concentrated salt solution. Difference spectra were recorded after each addition. In all experiments, the difference spectrum decreased with increasing ionic strength; in the presence of excess salt difference spectra completely disappeared indicating complete dissociation of the complex. Similar results were obtained when the salt was sodium chloride, ammonium sulfate, or sodium phosphate, indicating that this was not a specific ion effect but rather an effect of ionic strength. Values for the dissociation constant for the complex were calculated at each ionic strength during a titration. The results of these experiments are summarized in Fig. 4, where \(-\log_{10} K\) is plotted vs. ionic strength.
against $\sqrt{I}$ (ionic strength) for titrations done with complexes containing spinach ferredoxin, rubredoxin, or flavodoxin (bacterial ferredoxin was excluded from this series of experiments because this protein was found to be unstable at low ionic strength). Plots for complexes of flavodoxin or rubredoxin with FTR were similar when the titrations were done at pH 7.5. They were different from the plot obtained for spinach ferredoxin at pH 7. Thus in this pH region, the effect of ionic strength of the flavodoxin-FTR and rubredoxin-FTR complexes is greater than the effect on the spinach ferredoxin-FTR complex. The effect of pH on this dissociation by salt was examined in the case of flavodoxin. The effect of ionic strength on the dissociation was significantly smaller at pH 5.2 than at pH 7.5, indicating a stronger interaction of flavodoxin and FTR at lower pH. The experimental curves in Fig. 4 were extrapolated to give values for $K$ at ionic strength of zero. The values obtained were similar for all three protein substrates. $K$ was estimated as approximately $5 \times 10^{-4}$ M at ionic strength of zero. In preliminary experiments done with K. Suzuki, J. A. Peterson, and R. W. Estabrook, Johnson Foundation, FTR has been shown also to form a complex with the adrenal iron protein adrenodoxin.

Sedimentation Velocity Experiments—Further evidence for complex formation between FTR and electron transfer proteins was obtained with the ultracentrifuge. The molecular weights of rubredoxin, spinach ferredoxin, and FTR are approximately 6,000 (15), 11,000 (19), and 40,000 (6), respectively. Because of the low molecular weights of ferredoxin and rubredoxin their contribution to the refractive index gradient (when mixed in equimolar quantities with FTR at concentrations comparable to those used in the spectral experiments) is very small. In addition, the difference in sedimentation coefficients between FTR and the 1:1 complex is also small, especially in the case of the rubredoxin-FTR complex. These difficulties were overcome by following independently the sedimentation of the total protein and the iron protein. This was achieved by making light absorption measurements (iron protein) and refractive index gradient measurements (total protein) from the schlieren plates obtained in studies with the ultracentrifuge modified as described under “Materials and Methods.” Only ferredoxin and rubredoxin (as shown in Fig. 1) have appreciable light absorption in the spectral region of the light passing through the Kodak No. 16 Wratten filter to the camera (i.e. wave lengths greater than 520 mp). Fig. 5 shows schlieren photographs for the rubredoxin-FTR system. Fig. 5A is FTR alone, Fig. 5B is rubredoxin alone, and Fig. 5C is a mixture of the two. The light areas on the plates where rubredoxin is present and the absence of this effect in the case of FTR alone shows that light absorption is due to the iron protein. This absorption was measured on the photographic plates in the region above the schlieren peaks by scanning with a microdensitometer.

Results of experiments in which spinach ferredoxin and FTR were first sedimented separately and then together in a molar ratio of two FTR to one ferredoxin are given in Table I. The experiments were done at intermediate ionic strength at which significant complex formation was expected. A single boundary was observed with both schlieren and absorption measurements. The data of Table I show that the sedimentation coefficient of the mixture is greater than that of either component and, further, that ferredoxin sediments with the total protein.

Similar experiments done with rubredoxin show the effect of ionic strength on the complex (Table I). Rubredoxin and FTR were first run separately at low ionic strength, at which tight binding was observed in the spectral experiments ($K \sim 8 \times 10^{-7}$ M). An equimolar mixture of the two was then centrifuged under the same conditions. The light absorption data again indicated that in the mixture rubredoxin sedimented with the total protein. The experiment was then repeated at an ionic strength (0.3) at which no complex formation was observed in the spectral experiments. Under these conditions rubredoxin and FTR sedimented independently with sedimentation coefficients very similar to those obtained at low ionic strength when the individual components were run separately. These exper-
Sedimentation velocity experiments showing complex formation between FTR and spinach ferredoxin or rubredoxin, and effect of ionic strength on complex with rubredoxin

Experiments were carried out at 12°C in sodium phosphate buffer, pH 7.0, at the indicated ionic strength.

<table>
<thead>
<tr>
<th>Experiment and protein components</th>
<th>Ionic strength</th>
<th>Sedimentation coefficient</th>
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<tbody>
<tr>
<td></td>
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<td>Schlieren</td>
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<tr>
<td>A. 1. FTR (7 × 10⁻⁴ M)...........</td>
<td>0.12</td>
<td>2.6</td>
</tr>
<tr>
<td>2. Spinach ferredoxin (5 × 10⁻⁴ M)</td>
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<td>1.3</td>
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<tr>
<td>3. FTR (1 × 10⁻⁴ M) plus spinach</td>
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<td>2.9</td>
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<td>ferredoxin (5 × 10⁻⁸ M)...........</td>
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<tr>
<td>B. 1. FTR (8 × 10⁻⁵ M)...........</td>
<td>0.01</td>
<td>2.5</td>
</tr>
<tr>
<td>2. Rubredoxin (1 × 10⁻⁴ M)........</td>
<td>0.01</td>
<td>1.1</td>
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<tr>
<td>3. FTR (1 × 10⁻⁸ M) plus rubredoxin</td>
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<tr>
<td>4. FTR (1 × 10⁻⁴ M) plus rubredoxin</td>
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*The ionic strength was raised from 0.01 to 0.30 by the addition of potassium chloride.

FIG. 6. FTR-TPN difference spectra. FTR, 0.8 ml, 8.5 × 10⁻⁴ M, in 0.075 M phosphate buffer, pH 7.0, was pipetted into each of two 1-cm light path micro-cells. The spectrophotometer was balanced and 20 µl of 6 × 10⁻⁷ M TPN (neutralized to pH 7.0) were added to the sample cell and 25 µl of buffer were added to the reference. Difference spectra were then recorded with time until equilibrium was reached. Curve 1 was the first recorded spectrum. Curves 2 through 5 were spectra recorded 20, 80, 170, and 250 min after mixing.

The experiments clearly show that, at low ionic strength, rubredoxin and FTR form a complex and that the complex dissociates when the ionic strength is raised.

TPN Difference Spectra—Perturbations in the visible spectrum of FTR are also caused by TPN. When a 20-fold molar excess of TPN was mixed with FTR, the difference spectrum shown by Curve 1 in Fig. 6 was obtained. This difference spectrum is different from those obtained with the protein substrates in the following respects. First, the spectrum is qualitatively different and has both positive and negative regions. Second, the perturbations are quantitatively much smaller. Third, the rapidly formed species is unstable and slowly reacts to form a second species (Fig. 6). The four isosbestic points in the family of difference spectra in Fig. 6 indicated that only two species were present during this slow phase. The decay of the rapidly formed species followed first order kinetics. The rate of this slow reaction varied between experiments. There was some evidence that it was affected by the TPN concentration and by ionic strength.

Titration experiments similar to those carried out with the protein substrates were not done with TPN because measurements were complicated by these secondary changes. The stoichiometry of TPN binding, the dissociation constants, and the extinction coefficients for the complex were therefore obtained as follows. Different concentrations of TPN were mixed with FTR and absorbance changes at 512 mµ were followed with time. First order plots for the decay of the rapidly formed species were made and values for the change in absorption due to this species at time zero were obtained by extrapolation. Experiments were done at four different concentrations of TPN and three different ionic strengths.

The data were treated for 1:1, 2:1, and 3:1 complexes of TPN:FTR. The 1:1 model was the only one to give consistent values for the dissociation constant of the complex. The data were therefore plotted for a 1:1 model as described by Benesi and Hildebrand (20). Results are summarized in Fig. 7.

A value for the difference extinction coefficient of the complex at 512 mµ was obtained by extrapolation of the curves in Fig. 7 to infinite TPN concentration. The difference extinction coefficient calculated was 700 m⁻¹ cm⁻¹. The complex was sensitive to ionic strength although the effect was much less marked than that with complexes of FTR and protein substrates (Fig. 4).

No significant perturbations were observed when FTR was mixed with excess DPN, 2'-AMP, or nicotinamide mononucleotide.

Effect of Ionic Strength on Catalytic Activity of FTR—The experiments described previously show that complexes are formed between FTR and certain protein electron carriers. There is indirect evidence that these complexes are important catalytically. The assay used in these experiments was a coupled system in which catalytic amounts of FTR plus an electron carrier protein...
were incubated with TPNH and cytochrome c. This system, which is modified from Davenport and Hill (21), has been used previously to assay rubredoxin (22) and in kinetic studies with FTR and spinach ferredoxin (5). The reaction was followed by measuring the rate of cytochrome c reduction. The rate depends on the concentration of both FTR and the electron transfer protein. A standard amount of FTR ($7.5 \times 10^{-8} \text{M}$) was therefore used in all experiments and under these conditions the rate was proportional to the amount of electron carrier added. The preparation of FTR used showed some blank reduction of cytochrome c in the absence of electron carrier. Control assays were therefore run in which the electron carrier was omitted. The blank rate, which increased with pH, was subtracted from the rate measured in the presence of carrier.

Spinach ferredoxin, bacterial ferredoxin, rubredoxin, and flavodoxin all mediated the reduction of cytochrome c by TPNH in the presence of FTR. The apparent pH optimum for the reaction varied with the electron carrier (Fig. 8). Spinach ferredoxin showed a broad optimum between pH 6.5 and pH 7.5; the bacterial proteins all had optima near pH 5.0. In all cases the rate of reaction was influenced by ionic strength. This effect was examined with spinach ferredoxin at pH 7 and with flavodoxin at pH 5.2 (Fig. 9). In both cases the reaction was strongly inhibited by increased ionic strength. These effects may be related to those observed earlier in which complex formation between FTR and electron transfer proteins was found to depend on the ionic strength. However, there are quantitative differences between these two observations. The FTR-flavodoxin complex was somewhat less sensitive to ionic strength than the catalytic assay; 50% inhibition of activity and complex formation was achieved at ionic strengths of 0.055 and 0.085, respectively. The FTR-spinach ferredoxin complex was more sensitive to ionic strength than the assay. In this case, complex formation was 50% inhibited at an ionic strength of 0.12, while an ionic strength of 0.2 was required for 50% inhibition of activity.

Complete agreement between these two measurements would be surprising because (a) catalytic amounts of FTR and carrier were used in the assay while substrate amounts were required for the difference spectra; (b) both components were in the oxidized form in the spectral experiments but in the assay they are alternately oxidized and reduced; (c) in the assay system factors other than complex formation between FTR and electron carrier may be affected by ionic strength. Thus, for example, it is known that the binding of TPN to FTR is influenced by ionic strength.

**DISCUSSION**

Ferredoxin-TPN reductase catalyzes the reaction of TPNH with a wide range of one and two electron acceptors ranging from artificial dyes to electron transfer proteins containing iron or
flavin. The enzyme has been implicated in the physiological catalysis of two independent reactions in the spinach chloroplast.

These are the reduction of TPN by ferredoxin (2) and the reduction of cytochrome f (6). Different mechanisms have been proposed for these two reactions (5, 6). The mechanism of the reaction with plant ferredoxin is particularly interesting since it involves a crossover from one-electron to two-electron transfer in the electron transfer chain. This reaction may be similar to electron transfer reactions occurring in more complex proteins containing iron, labile sulfide, and flavin.

The results reported here show that ferredoxin-TPN reductase forms 1:1 complexes with spinach ferredoxin, bacterial ferredoxin, rubredoxin, and flavodoxin. The spectral experiments show that complex formation causes perturbations in the oxidation-reduction chromophores. Difference spectra for all four complexes show major peaks in the 395 m\(\mu\) and 465 m\(\mu\) regions. These peaks are probably due in part to changes in the environment of the flavin at the FTR-active site. They are not due simply to a change in flavin environment approaching that of free flavin. FTR has a resolved spectrum with an absorption maximum at 458 m\(\mu\), and the extinction coefficient for enzyme bound FAD at this wave length is 10,300 M\(^{-1}\) cm\(^{-1}\). Free FAD in aqueous solution has an absorption maximum at 450 m\(\mu\) and an extinction coefficient of 11,300 M\(^{-1}\) cm\(^{-1}\) at this wave length (23). Thus any changes in the flavin environment in FTR toward that of free flavin would result in a net blue shift in the difference spectrum. The observed shifts were red. In addition, the magnitude of the observed perturbations was greater than would be predicted for a change in environment in FTR to that of free flavin. Each complex gave a characteristic difference spectrum, suggesting that the spectral changes were at least partly caused by perturbations in the iron or flavin chromophore of the electron carrier protein joined in complex with FTR. The ultracentrifuge experiments clearly show that complexes are indeed formed between FTR and the electron transfer proteins studied. Furthermore, they show that the effect of increasing ionic strength is to disrupt such complexes.

The dissociation constant for the protein complexes obtained by extrapolation to zero ionic strength was 5 \times 10^{-8} M. The value for \(\Delta F'\) obtained from these data is \(\sim 10\) keal per mole. From the observed lack of temperature effects we can assume a \(\Delta H'\) approximately equal to zero. The entropy change obtained from these data is \(\sim 30\) e.u. These values and the effects of ionic strength and pH on the dissociation of the protein-protein complexes strongly suggest that the forces responsible for binding are primarily electrostatic in nature (24).

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