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ELECTROCHEMICAL TITRATIONS OF A FERREDOXIN-FERREDOXIN:NADP$^+$ OXIDOREDUCTASE COMPLEX

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Summary

Potentiometric titrations employing an electrochemical thin-layer cell indicate that complex formation between ferredoxin and ferredoxin:NADP$^+$ oxidoreductase alters the midpoint oxidation-reduction potentials of both proteins. The midpoint potential of ferredoxin in the complex becomes 22 ± 6 mV more negative compared to ferredoxin alone while the midpoint potential of ferredoxin:NADP$^+$ oxidoreductase becomes 23 ± 4 mV more positive on complex formation.

Introduction

Reduced ferredoxin is known to be the source of electrons for a wide variety of reductive metabolic pathways in plants and bacteria [1]. Among the most important of these in plants is the reduction of NADP$^+$ [2,3], a reaction catalyzed by the FAD-containing enzyme ferredoxin:NADP$^+$ oxidoreductase (EC 1.18.1.2). Ferredoxin and ferredoxin:NADP$^+$ oxidoreductase are known to form a 1 : 1 complex [4—8] and evidence suggests that the complex is the catalytically active species in NADP$^+$ reduction [5,6,9]. The complex (K_d = $5 \cdot 10^{-8}$ at zero ionic strength) is held together largely by electrostatic forces [5] and appears to involve extensive surface areas of the proteins [10].

Circular dichroism measurements suggest that significant conformational changes accompany complex formation [8,11,12]. It seemed possible that these changes in conformation, or other aspects of protein-protein interaction in the complex, might alter the oxidation-reduction properties of the Fe_2S_2

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Abbreviations: E_m, oxidation-reduction midpoint potential; CD, circular dichroism.

center of ferredoxin or the FAD group of ferredoxin:$NADP^+$ oxidoreductase. This possibility was important to explore because although the oxidation-reduction properties of the individual proteins have been extensively investigated [1,13,14], nothing was known about the oxidation-reduction properties of the catalytically active ferredoxin-ferredoxin:$NADP^+$ oxidoreductase complex. To that end, we have determined the midpoint potentials of both proteins in the 1 : 1 complex and compared these values to those obtained for the separate proteins.

Methods

Ferredoxin and ferredoxin:$NADP^+$ oxidoreductase were isolated from spinach leaves as described previously [12]. The proteins had absorbance ratios of $A_{420} : A_{276} = 0.43$ and $A_{546} : A_{275} = 0.115$ for ferredoxin and ferredoxin:$NADP^+$ oxidoreductase, respectively. Protein concentrations were determined using extinction coefficients of 9.7 $mM^{-1} \cdot cm^{-1}$ at 420 nm for ferredoxin [15] and 10.7 $mM^{-1} \cdot cm^{-1}$ at 456 nm for ferredoxin:$NADP^+$ oxidoreductase [16]. Ferredoxin:$NADP^+$ oxidoreductase was passed through a Sephadex G-25 column immediately before use to remove any free FAD [8,12].

Absorbance spectra were measured using an Aminco DW-2a spectrophotometer and circular dichroism (CD) spectra were obtained as described previously [12].

Potentiometric titrations were performed essentially as described by Hawkridge and Ke [17] using a Wenking potentiostat and a quartz thin-layer cell containing an optically transparent gold mesh electrode (60% transmittance, Buckbee-Mears Co.). The cell optical pathlength was 0.33 mm. The Ag|AgCl reference electrode contained 0.1 M KCl as the electrolyte solution. At higher KCl concentrations, leakage of KCl through the porous frit in the electrode raised the sample ionic strength to the point where dissociation of the ferredoxin-ferredoxin:$NADP^+$ oxidoreductase complex occurred. The reference electrode was calibrated against a standard calomel electrode for each titration. The titration apparatus was initially checked for accuracy and freedom from O_2 leaks by titrating FAD, methyl viologen and benzyl viologen. All titrations were fully reversible and gave E_m values within 5 mV of literature values. A mixture of benzyl and methyl viologens (at the same concentrations used for the protein titrations) was titrated and the absorbance at 460 nm due to the viologens alone was subtracted from the total absorbance at 460 nm observed at each value of E_h in order to calculate the oxidized:reduced ferredoxin:$NADP^+$ oxidoreductase ratios for Nernst plots. The viologens have no CD signal [18], so no correction was necessary.

All titrations were performed at 5—7°C because it was found that ferredoxin:$NADP^+$ oxidoreductase lost substantial amounts of FAD at higher temperatures during the 1—2 h required for the titrations. After all titrations involving ferredoxin:$NADP^+$ oxidoreductase, the titrated sample was passed over a Sephadex G-25 column and only experiments in which no free FAD was detected were utilized. To insure that the complexes being titrated would not dissociate during the course of the titration, a sample was left in the cell for 2.5 h under conditions identical to those used during a titration. Addition of

NaCl to raise the ionic strength produced the absorbance change expected for complete dissociation of the complex indicating that at least 95% of the protein was still in the form of a complex after this time. The titration data shown below were all obtained in the reductive direction. However, after complete reduction had occurred, the ambient potential was made more positive and spectra obtained at several potentials in the oxidative direction to check for reversibility. All the titrations were completely reversible. Midpoint potentials and n values were calculated by fitting the experimental points to the Nernst equation using the least-squares program on a Texas Instruments Model TI-58C programmable calculator.

Results and Discussion

Fig. 1 shows the absorbance spectra of the ferredoxin-ferredoxin:$NADP^+$ oxidoreductase complex at several ambient potentials during the course of a potentiometric titration of the complex. Fig. 2 shows the data from the experiment of Fig. 1 plotted according to the Nernst equation for a two electron carrier (the actual least-squares n value was 1.95). The concentrations of oxidized and reduced ferredoxin:$NADP^+$ oxidoreductase were calculated from the absorbance at 460 nm as described in Methods (460 nm was chosen as the wavelength where benzyl and methyl viologen, added to facilitate equilibrium between the FAD group of ferredoxin:$NADP^+$ oxidoreductase and the electrode [13], gave the minimum spectral interference). An average value (two titrations) of -349 ± 3 mV was obtained for the E_m value of ferredoxin:$NADP^+$ oxidoreductase in the complex.

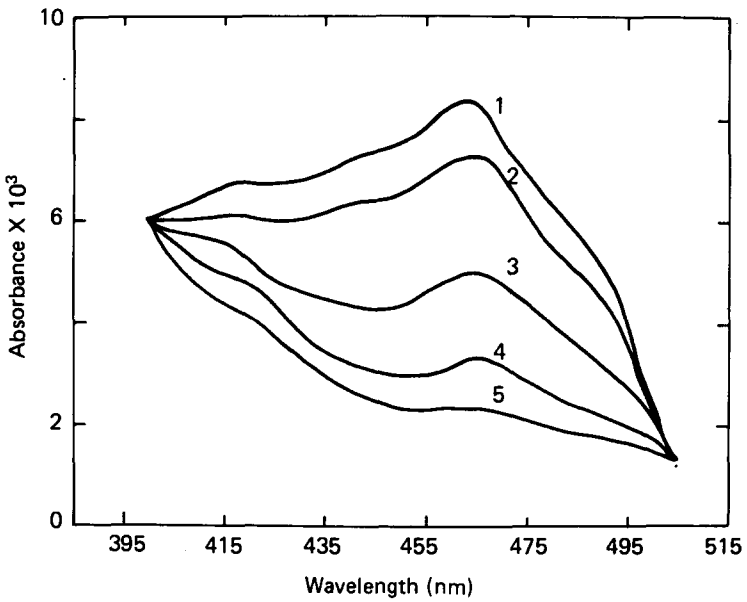

Fig. 1. Absorbance spectra of the ferredoxin-ferredoxin:$NADP^+$ oxidoreductase (FNR) complex at defined ambient potentials. The sample (1.3 ml total volume) contained 37.4 μM FNR, 10 μM methyl viologen, 10 μM benzyl viologen in 30 mM Tris-HCl buffer (pH 7.3) under a N_2 atmosphere. Spectra 1—5 were recorded at ambient E_h values of —120, —330, —350, —370 and —420 mV, respectively.

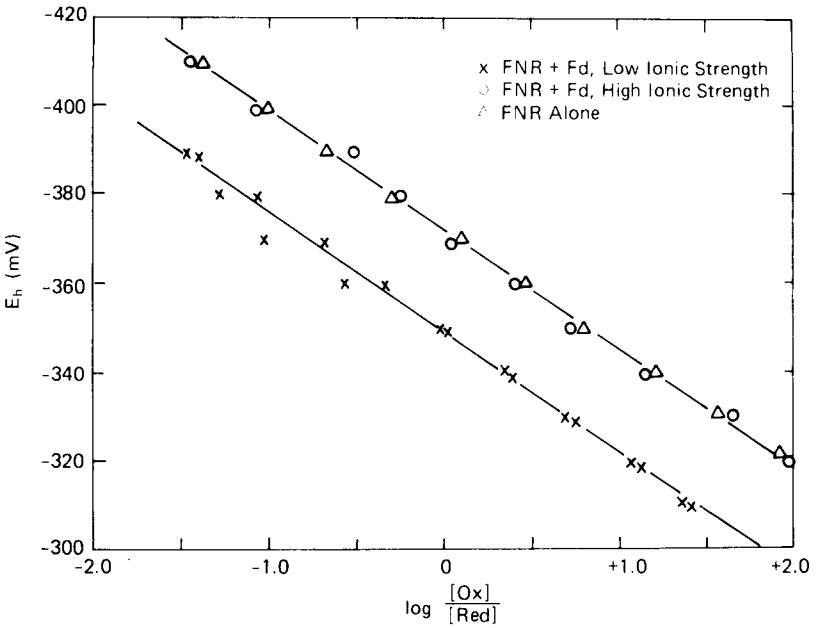

Fig. 2. Oxidation-reduction potentials for ferredoxin:NADP$^+$ oxidoreductase (FNR). Data from titrations performed under conditions identical to those described in Fig. 1 were plotted according to the Nernst equation (n = 2) for FNR alone (△) or the ferredoxin-FNR complex (X). Identical experiments were performed for the ferredoxin/FNR mixture (○) except that NaCl (final concentration 0.5 M) was added to raise the ionic strength and dissociate the complex. Least-squares E_m values were —349 ± 3 mV for FNR in the complex and —372 ± 2 mV for FNR alone or in the presence of ferredoxin at high ionic strength.

Fig. 2 also shows the results of titrations of ferredoxin:NADP$^+$ oxidoreductase alone or of a 1 : 1 mixture of ferredoxin:NADP$^+$ oxidoreductase and ferredoxin at high ionic strength where no complex exists [4—8]. Ferredoxin:NADP$^+$ oxidoreductase alone had a midpoint of —372 ± 2 mV (pH 7.3), essentially identical to the value of —0.37 V reported previously by Keirns and Wang at this pH [13]. The E_m value of ferredoxin:NADP$^+$ oxidoreductase in the presence of equimolar ferredoxin but under conditions (high ionic strength) that preclude complex formation was identical (E_m = —372 ± 2 mV, average of two titrations) to that obtained for ferredoxin:NADP$^+$ oxidoreductase alone (the least-squares n values for ferredoxin:NADP$^+$ oxidoreductase alone and for the ferredoxin:NADP$^+$ oxidoreductase/ferredoxin mixture at high ionic strength were 2.01 and 1.99, respectively.) Thus, complex formation with ferredoxin increased the E_m value for ferredoxin:NADP$^+$ oxidoreductase by 23 ± 4 mV.

Fig. 3 shows CD spectra of an equimolar mixture of ferredoxin:NADP$^+$ oxidoreductase and ferredoxin (at high ionic strength) at several ambient potentials during the course of a potentiometric titration. Accurate monitoring of the oxidation state of ferredoxin by absorbance measurements is precluded by the large absorbance changes of the oxidation-reduction mediators but ferredoxin can be readily monitored without interference using CD detection [17,18]. Ferredoxin has a larger CD signal than ferredoxin:NADP$^+$ oxidoreductase and the CD signal at 426 nm can be conveniently used to follow ferredoxin

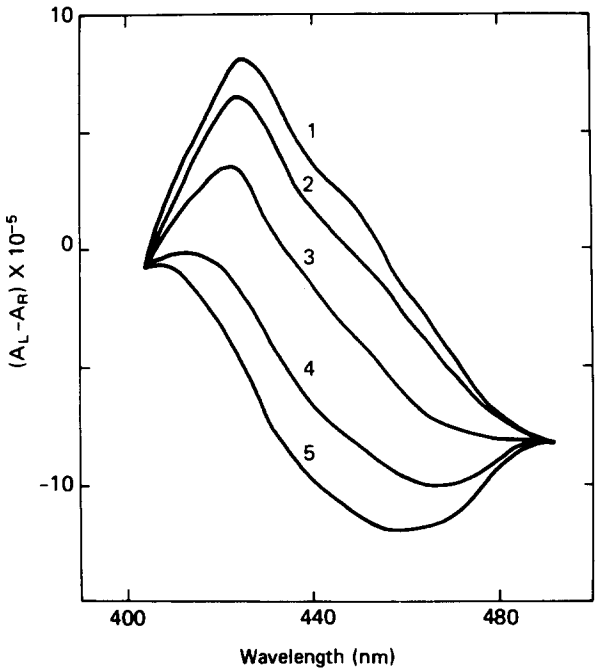

Fig. 3. CD spectra of a ferredoxin/ferredoxin:$NADP^+$ oxidoreductase (FNR) mixture at defined ambient potentials. Reaction conditions as in Fig. 1 except that ferredoxin and FNR were each present at a concentration of 82.3 μM. Spectra 1—5 were recorded at ambient E_h value of —330, —380, —420, —460 and —500 mV, respectively.

with little contribution from ferredoxin:$NADP^+$ oxidoreductase [8,17—19]. The results of the experiment shown in Fig. 3 are plotted in Fig. 4 according to the Nernst equation for a one electron carrier along with the results of similar titrations of ferredoxin alone and the ferredoxin-ferredoxin:$NADP^+$ oxidoreductase complex (at low ionic strength). The values of E_m for ferredoxin alone and ferredoxin in the presence of equimolar ferredoxin:$NADP^+$ oxidoreductase at high ionic strength were both —429 ± 3 mV (average of two titrations). This value is similar to values that have been obtained previously [15,17,18]. The E_m value for ferredoxin in the ferredoxin-ferredoxin:$NADP^+$ oxidoreductase complex was —451 ± 3 mV (average of two titrations), significantly lower than that for ferredoxin alone. (Least-squares n values for ferredoxin alone, the ferredoxin/ferredoxin:$NADP^+$ oxidoreductase mixture at high ionic strength and the ferredoxin-ferredoxin:$NADP^+$ oxidoreductase complex were 0.99, 1.1 and 0.91, respectively.) Thus complex formation with ferredoxin:$NADP^+$ oxidoreductase lowers the E_m value of ferredoxin by 22 ± 6 mV.

Although the titrations of ferredoxin/ferredoxin:$NADP^+$ oxidoreductase mixtures at high ionic strength provide useful controls for interfering absorbance or CD signals, there is a danger in using them to calculate shifts in E_m values because E_m values are a function of ionic strength for oxidation-reduction couples in which the oxidized and reduced species differ in net charge [20]. The fact that the high ionic strength titrations gave the same E_m values as

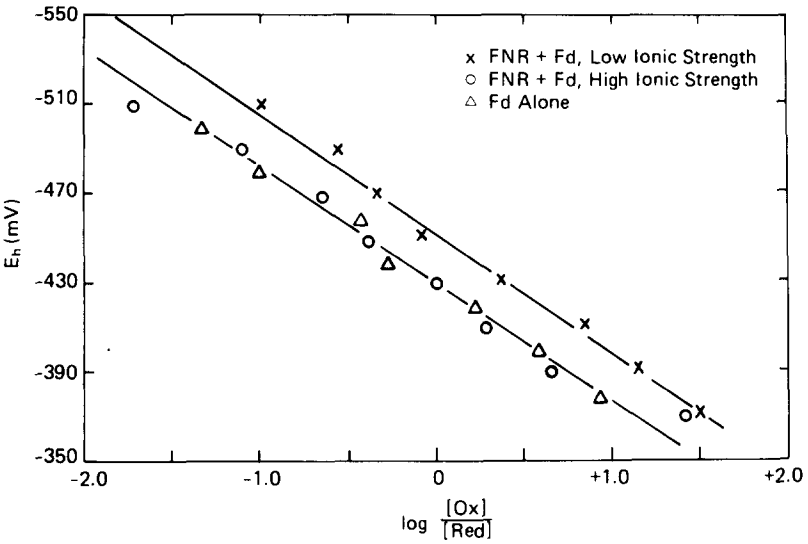

Fig. 4. Oxidation-reduction potentials for ferredoxin. Data from titrations of ferredoxin alone (△) or the ferredoxin-ferredoxin:NADP$^+$ oxidoreductase (FNR) complex in 30 mM Tris buffer (X) were plotted according to the Nernst equation (n = 1). Data from the experiment in Fig. 3 were plotted for the ferredoxin/FNR mixture (○). Least-squares E_m values were −451 ± 3 mV for ferredoxin in the complex (X) and −429 ± 3 mV for ferredoxin alone (△) or in the presence of FNR at high ionic strength (0.5 M NaCl).

titrations of the individual proteins at low ionic strength suggests that for both proteins such charge effects are not significant. It should be pointed out that the E_m value shifts reported above were calculated using titrations of the individual proteins at the same ionic strength (as well as pH and temperature) used during titrations of the complex.

The [2Fe-2S] cluster of ferredoxin and the FAD group of ferredoxin:NADP$^+$ oxidoreductase titrate as separate one electron and two-electron groups, respectively, in the ferredoxin-ferredoxin:NADP$^+$ oxidoreductase complex, just as they do in the isolated proteins. No evidence was obtained for any cooperative electron transfer in this study. The n = 2 value obtained for ferredoxin:NADP$^+$ oxidoreductase in the complex indicates that the oxidation-reduction reaction involves the fully oxidized and fully reduced FAD group of the enzyme, with little contribution from the semiquinone state. A semiquinone oxidation state with an absorbance maximum near 600 nm has been reported for ferredoxin:NADP$^+$ oxidoreductase [2,13,16,21] but we observed no absorbance changes in the 600 nm region above that expected from the reduced viologen oxidation-reduction mediators. Furthermore, no CD feature was observed in the 600 nm region during titrations of the ferredoxin-ferredoxin:NADP$^+$ oxidoreductase complex using CD detection. It must be pointed out that the high absorbance of the mediators in this spectral region limited the accuracy of our measurements and FAD semiquinone present at concentrations below 5% of the total FAD would have escaped detection. It also should be noted that the mechanism of FAD reduction by mediators in the electrochemical cell may be significantly different from that in vivo. As ferredoxin is a one electron carrier,

one would expect that reduction of ferredoxin:$NADP^+$ oxidoreductase in vivo must involve the FAD semiquinone while a two electron reduction can occur under the conditions of our experiments.

Although the changes in E_m values reported above are not large, the 45 mV difference in thermodynamic driving force (ΔE_m) for electron transfer from ferredoxin to ferredoxin:$NADP^+$ oxidoreductase that accompanies complex formation is hardly insignificant. The observation that complex formation between ferredoxin and an enzyme that uses ferredoxin as an electron donor causes significant changes in oxidation-reduction properties raises the possibility that similar effects may be observed in other ferredoxin-protein complexes. Such complexes appear to be widespread, having been reported for ferredoxin and the plant enzyme ferredoxin:nitrite oxidoreductase [12,22] and for ferredoxins from sources as diverse as the sulfate-reducing bacterium *Desulphovibrio gigas* [23] and the mammalian adrenal cortex [24,25].

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