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Oxidation-reduction potentials of ferredoxin-NADP$^+$ reductase and flavodoxin from *Anabaena* PCC 7119 and their electrostatic and covalent complexes

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The oxidation-reduction potentials of ferredoxin-NADP$^+$ reductase and flavodoxin from the cyanobacterium *Anabaena* PCC 7119 were determined by potentiometry. The potentials at pH 7 for the oxidized flavodoxin/flavodoxin semiquinone couple (E_2) and the flavodoxin semiquinone/hydroquinone couple (E_1) were −212 mV and −436 mV, respectively. E_1 was independent of pH above about pH 7, but changed by approximately −60 mV/pH below about pH 6, suggesting that the fully reduced protein has a redox-linked pK_a at about 6.1, similar to those of certain other flavodoxins. E_2 varied by −50 mV/pH in the range pH 5–8. The redox potential for the two-electron reduction of ferredoxin-NADP$^+$ reductase was −344 mV at pH 7 (ΔE_m = −30 mV/pH).

In the 1:1 electrostatic complex of the two proteins titrated at pH 7, E_2 was shifted by +8 mV and E_1 was shifted by −25 mV; the shift in potential for the reductase was +4 mV. The potentials again shifted following treatment of the electrostatic complex with a carbodiimide, to covalently link the two proteins. By comparison with the separate proteins at pH 7, E_2 for flavodoxin shifted by −21 mV and E_1 shifted by +20 mV; the reductase potential shifted by +2 mV. The potentials of the proteins in the electrostatic and covalent complexes showed similar pH dependencies to those of the individual proteins. Qualitatively similar changes occurred when ferredoxin-NADP$^+$ reductase from *Anabaena variabilis* was complexed with flavodoxin from *Azotobacter vinelandii*.

The shifts in redox potential for the complexes were used with previously determined values for the dissociation constant (K_d) of the electrostatic complex of the two oxidised proteins, in order to estimate K_d values for the interaction of the different redox forms of the proteins. The calculations showed that the electrostatic complexes, formed when the proteins differ in their redox states, are stronger than those formed when both proteins are fully oxidized or fully reduced.

Ferredoxin-NADP$^+$ reductase (FNR) is an FAD-containing flavoenzyme that functions on the reductive side of photosynthesis, transferring reducing equivalents from an electron-transfer protein to NADP$^+$ [1]. The enzyme usually uses the iron-sulphur protein, ferredoxin, as the electron carrier, but it can also use the FMN-containing protein, flavodoxin (Fld). In *Cyanobacteria* [2], as in microorganisms of several other groups [3], flavodoxin is synthesized as a functional replacement for ferredoxin when the organism is grown under iron-deficient conditions. Ferredoxin-NADP$^+$ reductase is known to interact with its electron carrier to form a 1:1 complex that is required for catalytic activity [4–6]. The two proteins in the complex are linked non-covalently under physiological conditions, but chemical cross-linking can be used *in vitro* to stabilize the complex in a covalent, but catalytically active, form [7–9], thus facilitating the study of electron transfer between FNR and its electron carriers. Laser flash photolysis experiments have shown that electron transfer is very fast in both the electrostatic and the covalent complexes of FNR and flavodoxin from *Anabaena* PCC 7119 [10].

The complexes between FNR and flavodoxin have now been characterized further by the determination of their oxidation-reduction potentials. This paper reports on the redox potentials for the *Anabaena* PCC 7119 proteins, both free and in their electrostatic or covalent complexes. The potentials of the complexes formed between FNR from *Anabaena variabilis* and *Azotobacter vinelandii* flavodoxin have been measured for comparison.

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Abbreviations. FNR, ferredoxin-NADP$^+$ reductase; Fld, flavodoxin; ox, oxidized; sq, semiquinone; red, reduced; PDQ^{2+}, 1,1′-propylene-2,2′-bipyridylium^{2+}; E_1, potential at pH 7 for couple flavodoxin semiquinone/hydroquinone; E_2, potential at pH 7 for couple flavodoxin flavodoxin/flavodoxin; E_h, potential versus the standard hydrogen electrode.

Enzyme. Ferredoxin-NADP$^+$ reductase (EC 1.18.1.2).

MATERIALS AND METHODS

Anabaena PCC 7119 and *A. variabilis* (strain 1403.4b) FNR and *Anabaena* PCC 7119 flavodoxin were purified as described previously [11, 12] from cells that were grown autotrophically on nitrate and, in the case of flavodoxin, using a low iron concentration (2.6 μM) to allow synthesis of this protein. Iron was provided to the cultures as ammonium ferric citrate. The culture was aerated with a mixture of 5% (by vol.) CO_2 and air and illuminated with fluorescent-light tubes that delivered an intensity of 4000 lux at the surface of the cultures.

A. vinelandii flavodoxin was a gift of Dr D.E. Edmondson, Emory University, Atlanta, USA and was purified from cells (strain OP) grown under nitrogen-fixing conditions [13].

The covalent complexes between *A. variabilis* FNR and *A. vinelandii* flavodoxin and between *Anabaena* PCC 7119 FNR and flavodoxin were prepared using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide as cross-linking reagent, as described previously [8, 9].

Oxidation-reduction potentials were measured by potentiometry using a spectroelectrochemical cell similar to that described by Stankovich [14] with a gold working electrode, a platinum secondary electrode, a saturated calomel electrode as reference and a Sycopel Ltd. DP 301/S potentiostat to introduce reducing equivalents into the cell and monitor the redox potential. The calomel and secondary electrodes were connected to the cell contents via salt bridges that consisted of capillary tubes filled with agar and saturated KCl; the rate of diffusion of air through the salt bridges was so low that additional precautions, such as soaking the bridges in sodium dithionite [14], were not required to prevent the ingression of oxygen. A Perkin-Elmer 550S spectrophotometer, equipped with a cell-stirring attachment and thermostatted cell holder at 25°C, was used to monitor reduction of proteins in the cuvette. The cuvette contained, in a final volume of 2.8 ml, 18–50 μM protein (free FNR or flavodoxin, a 1:1 mixture of FNR and flavodoxin or a covalent complex of FNR with flavodoxin), 50 mM sodium acetate, pH 5, 50 mM potassium phosphate, pH 6 or pH 7, 50 mM Tris/HCl, pH 8, 15% (by vol.) glycerol (included to prevent the appearance of turbidity [11]), 100 μM 1,1′-propylene-2,2′-bipyridylium^{2+} (PDQ^{2+}; $E_{m,7} = -550$ mV; kindly provided by ICI Ltd., Jealotts Hill) to ensure electron transfer from the gold electrode to the protein during electrolysis in the cell and at least one additional mediator dye, at a concentration of 1 μM, to poise the gold electrode in the appropriate range of redox potential for potentiometric measurement of the redox potential(s) of the protein(s). The following mediator dyes were used: methyl viologen ($E_{m,7} = -446$ mV; Sigma), benzyl viologen ($E_{m,7} = -359$ mV; Sigma), neutral red ($E_{m,7} = -325$ mV; Merck); rosinduline ($E_{m,7} = -281$ mV; BDH), phenosafranine ($E_{m,7} = -273$ mV; BDH), anthraquinone-2-sulphonate ($E_{m,7} = -225$ mV; Fluka), anthraquinone-2,6-disulphonate ($E_{m,7} = -184$ mV; Aldrich) and 2-hydroxy-1,4-naphthoquinone ($E_{m,7} = -149$ mV; Koch Light). A side arm on the cuvette contained a mixture of EDTA (1 mM), Tris/HCl (0.1 M, pH 8), methyl viologen (0.2 mM) and 5-deazariboflavin (2 μM). The cell and contents, with only the gold electrode in place, was made anaerobic by several cycles of evacuation using a vacuum pump filled with argon, which was purified by passage over a BASF catalyst (RG3-11) at 120°C. The salt bridges to the reference and secondary electrodes were then added under a stream of argon and the methyl viologen in the sidearm photoreduced with 5-deazariboflavin to provide a sink for any residual traces of oxygen in the cell. The protein in the cell was then reduced stepwise by electrolysis. The extent of reduction was monitored spectrophotometrically during each period of electrolysis and the cell contents were allowed to equilibrate after electrolysis until the redox potential stabilized. The period required for stabilization varied with the protein(s) used and with the extent of their reduction, but was usually in the range 10–30 min. At least eight points were obtained during each reductive titration.

The concentrations of the oxidized and reduced proteins were determined at each point in the titration from measurements of the absorption spectra and the following absorption coefficients of the oxidized proteins were used: *Anabaena* PCC 7119 FNR, $\varepsilon_{458} = 9.4\ mM^{-1} \cdot cm^{-1}$ (J.J. Pueyo and C. Gomez-Moreno, unpublished result); flavodoxin, $\varepsilon_{464} = 9.4\ mM^{-1} \cdot cm^{-1}$ [12]; *A. variabilis* FNR, $\varepsilon_{459} = 9.7\ mM^{-1} \cdot cm^{-1}$ [11]; *A. vinelandii* flavodoxin, $\varepsilon_{450} = 10.6\ mM^{-1} \cdot cm^{-1}$ [15]. Values for the reduced proteins were calculated from redox experiments on each protein, in which the protein was completely converted to the hydroquinone. The values calculated were as follows: hydroquinone of FNR from *Anabaena* PCC 7119, $\varepsilon_{458\,nm} = 0.9\ mM^{-1} \cdot cm^{-1}$; hydroquinone of FNR from *A. variabilis*, $\varepsilon_{459\,nm} = 0.6\ mM^{-1} \cdot cm^{-1}$; semiquinones of flavodoxins from *Anabaena* PCC 7119 and *A. vinelandii*, $\varepsilon_{580\,nm} = 5.1\ mM^{-1} \cdot cm^{-1}$ and $5.4\ mM^{-1} \cdot cm^{-1}$, respectively; hydroquinones of the flavodoxins, $\varepsilon_{580\,nm} = 0\ mM^{-1} \cdot cm^{-1}$. Absorbance measurements during the redox titrations of the electrostatic and covalent complexes of FNR and flavodoxin, were made as follows: the absorbance was first measured at the maximum in the visible region to determine the concentration of the oxidized complex; measurements were made at 580 nm to determine the concentration of flavodoxin semiquinone during the addition of one reducing equivalent/mol complex; measurements of the change in absorbance at 458 nm (*Anabaena* PCC 7119) or 459 nm (*A. variabilis*) were then made during the addition of two further reducing equivalents to determine the extent of reduction of FNR to the hydroquinone; the absorbance at 580 nm was measured during the addition of a fourth reducing equivalent to determine the extent of reduction of flavodoxin semiquinone to the hydroquinone.

Plots were made of E_h, the potential relative to the standard hydrogen electrode, versus log [(oxidized protein)/(reduced protein)]. A straight line was drawn through the points in this Nernst plot to obtain the value of the midpoint redox potential, E_m, when log [(oxidized protein)/(reduced protein)] is equal to zero. The redox potentials are reported with reference to the potential of the standard hydrogen electrode. As noted later, the data in the Nernst plots conformed well to straight lines and the slopes of the plots were as expected for one-electron or two-electron redox couples. Redox titrations of indigodisulfonate (K & K Laboratories) gave a value for the midpoint potential in agreement with the published value ($E_{m,7} = -116$ V) [16].

RESULTS AND DISCUSSION

Redox potentials of Anabaena *PCC 7119 proteins*

Flavodoxin. The semiquinone of FMN is highly stable in this protein, so that close to 100% of the flavin is in the form of the semiquinone after the addition of one electron. This facilitates measurements of the redox potentials of the two one-electron couples for the protein, oxidized flavodoxin/flavodoxin semiquinone (E_2) and flavodoxin semiquinone/fully reduced flavodoxin (E_1). Values for E_1 and E_2 were determined over the pH range 5–8. Equilibration of this protein with the mediators and gold electrode occurred readily throughout the titrations and plots of E_h versus log [(oxidized flavodoxin)/(flavodoxin semiquinone)] or log [(flavodoxin semiquinone)/(flavodoxin hydroquinone)] were linear with slopes of 0.059 ± 0.003 V (Fig. 1). The midpoint potentials at pH 7 were -212 mV (E_2) and -436 mV (E_1), with the difference (E_2-E_1 = 224 mV) in keeping with the percentage of the FMN in the semiquinone form at half reduction (98.8%). The potentials for both steps in the reduction were

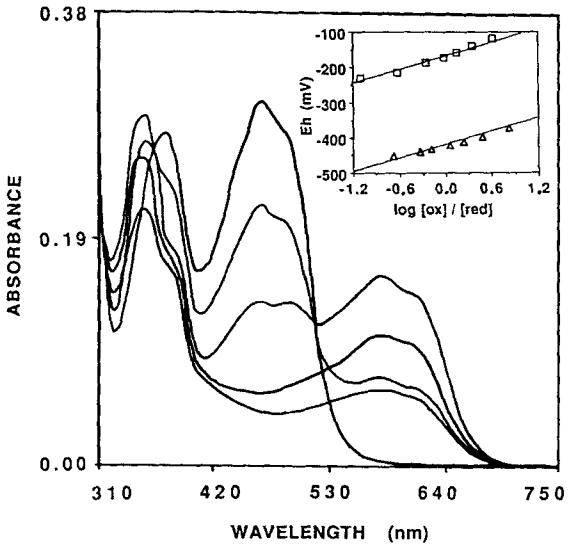

Fig. 1. *Potentiometric titration of* Anabaena *PCC 7119 flavodoxin at pH 6.0 and spectra of the reductive titration.* Inset: Nernst plots of the data. (□) Fld_{ox}/Fld_{sq}; (△) Fld_{sq}/Fld_{red}. The protein concentration was 33.19 μM in 50 mM potassium phosphate, pH 6.0, containing 15% (by vol.) glycerol. Also present were 100 μM PDQ^{2+}, 1 μM methyl viologen and 1 μM anthraquinone-2,6-disulfonate

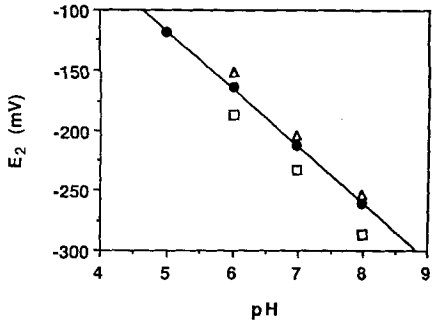

Fig. 2. *Effect of pH on* E_2 *of* Anabaena *PCC 7119 flavodoxin.* (●) Flavodoxin alone, (△) electrostatic and (□) covalent complexes of FNR and flavodoxin

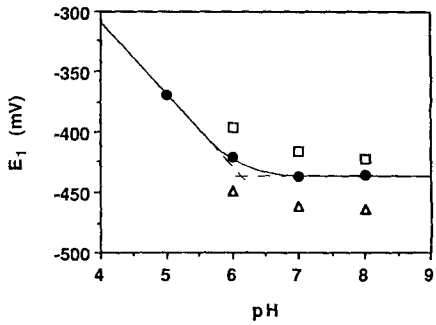

Fig. 3. *Effect of pH on* E_1 *of* Anabaena *PCC 7119 flavodoxin.* (●) Flavodoxin alone, (△) electrostatic and (□) covalent complexes of FNR and flavodoxin. The solid line represents the theoretical curve relating E_1 to pH for a one-electron oxidation-reduction reaction in which the reduced species has a $pK_a = 6.1$

found to vary with pH. The slope of E_2 versus pH is −0.05 V over the range pH 5−8 (Fig. 2). This is less than the theoretical value (−0.059 V) predicted for the addition of an electron and a proton to the neutral oxidized FMN to form the neutral flavin semiquinone and it may indicate that there is a redox-linked pK_a associated with the semiquinone. However, there was no evidence from the absorption spectra for the formation of the anion of FMN semiquinone. The presence of other ionizable groups could also affect the pH dependence of E_2, altering the environment of the FMN and varying its redox properties.

The values for E_1 are pH dependent at low pH, but they become pH independent with increasing pH up to pH 8; they fall on a curve that is consistent with a theoretical line in which the reductant has a redox-linked pK_a at 6.1 (Fig. 3). Similar pH dependencies have been observed for E_1 for certain other flavodoxins, although with somewhat different pK_a values [17−19]. The pK_a was originally ascribed to the N1 H of the flavin [17] which, in protein-free FMN, has a value of 6.5 [20]; however, NMR measurements have shown that protonation of the hydroquinone of FMN in flavodoxins does not occur in this pH range [21−23] and recent measurements on *Megasphaera elsdenii* and *Clostridium beijerinckii* flavodoxins have suggested that the redox-linked pK_a is associated with a group on the protein [24]. Ludwig et al. [24] observed that the spectrum of the hydroquinone of *M. elsdenii* flavodoxin was independent of pH in the range pH 4.8−8.5, in contrast to the corresponding spectrum of FMN which changes in accordance with the pK_a at 6.5; it was not possible to determine whether the spectrum of the hydroquinone of *Anabaena* PCC 7119 flavodoxin changes between pH 5 and pH 8 from the redox titrations, because the spectrum is obscured by absorption due to reduced PDQ^{2+}, which begins to form when flavodoxin is almost fully reduced.

Values for the potentials of *Anabaena* PCC 7119 flavodoxin have been reported recently under conditions similar to those reported here [25]. The values for E_1 and E_2 were −390 mV and −195 mV, respectively, at pH 7 and −418 mV and −255 mV, respectively, at pH 8. These values are less negative than the corresponding values of Table 1. We cannot account for these differences, especially the large difference in E_1 at pH 7 (46 mV). Based on the change in E_1 between pH 7 and pH 8, it was suggested that the pK_a associated with the hydroquinone is between pH 7 and pH 7.5 [25], rather than at pH 6.1 (Fig. 3).

An extensive study has also been made of the redox potentials of flavodoxin from a different strain of *Anabaena* (PCC 7120) [26]. The buffers used to study the effects of pH on the potentials of this protein were not specified and chemical and photochemical reduction was used instead of the electrochemical method used in the present work. Nevertheless, the potentials reported from that study are not too different from those of Table 1 and Figs 2 and 3, with the notable exception that the values of E_1 for the PCC 7120 strain did not show a clear pH dependence in the pH range studied (pH 6−8.5). The slope of E_2 versus pH was −0.052 V and therefore similar to the value reported above for the protein from the PCC 7119 strain.

Ferredoxin-NADP$^+$ reductase

Redox titrations of FNR from *Anabaena* PCC 7119 showed that this protein also stabilizes the flavin semiquinone in its neutral form. However, the proportion of the total FAD in the form of the semiquinone at equilibrium during the titrations in the pH range 6−8 was very low (29−12%) and it was not possible to measure the redox potentials for the two one-electron steps directly. Therefore, values for the potentials

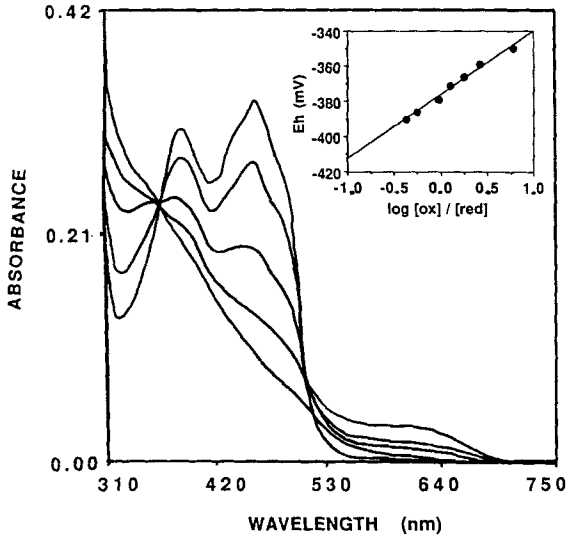

Fig. 4. *Potentiometric titration of* Anabaena *PCC 7119 FNR at pH 8.0.* Spectra of the reductive titration. Inset: Nernst plot of the data. The protein concentration was 29.15 μM in 50 mM Tris/HCl, pH 8.0 containing 15% (by vol.) glycerol. Also present were 100 μM PDQ^{2+} and 1 μM benzyl viologen

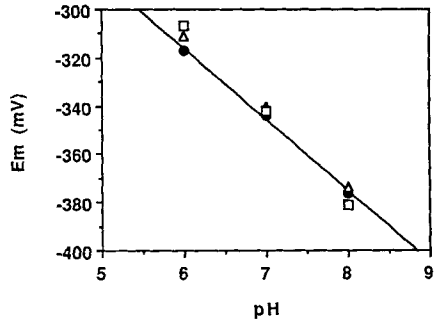

Fig. 5. *Effect of pH on the midpoint potential of* Anabaena *PCC 7119 FNR.* (●) FNR alone; (△) electrostatic and (□) covalent complexes of FNR and flavodoxin

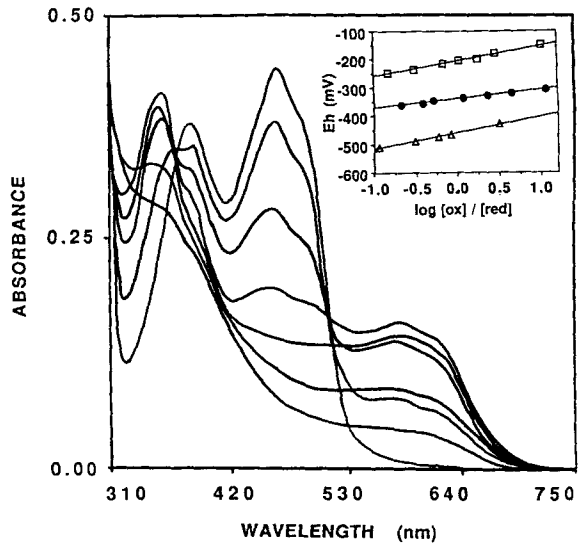

Fig. 6. *Potentiometric titration of* Anabaena *PCC 7119 FNR-flavodoxin electrostatic complex at pH 7.0.* The spectra were obtained during the reductive titration. Inset: Nernst plots of the data, (□) Fld_{ox}/Fld_{sq}; (●) FNR_{ox}/FNR_{red}; (△) Fld_{sq}/Fld_{red}. The protein concentration was 23.2 μM in 50 mM potassium phosphate, pH 7.0, containing 15% (by vol.) glycerol. Also present were 100 μM PDQ^{2+}, 1 μM methyl viologen, 1 μM benzyl viologen, 1 μM neutral red and 1 μM anthraquinone-2-sulfonate

of the two-electron reduction of the enzyme were determined (Fig. 4) and values for the difference in potential between the two one-electron steps were calculated from the concentration of semiquinone observed spectroscopically.

The Nernst plots of the redox data were consistent with a two-electron reduction; the slope of E_m versus pH was -0.03 V $\pm$ 0.004 V (Fig. 5), as described previously for FNR from spinach [27] and is consistent with the addition of two electrons and a single proton in the pH range studied. It was not possible to extend the measurements to lower pH values because the protein precipitated. The extent of semiquinone formation increased with decreasing pH, showing that the equilibrium $2\,FNR_{sq} \rightleftharpoons FNR_{ox} + FNR_{red}$ is shifted to the left at lower pH (sq, semiquinone; ox, oxidised; red, reduced). *Values* for the semiquinone-formation constant, K_{sq}, where K_{sq} = [(FNR semiquinone)^2/(oxidized FNR)(FNR hydroquinone)] were calculated at 50% reduction, using an assumed value of 5 $mM^{-1} \cdot cm^{-1}$ for the absorption coefficient of the semiquinone at 600 nm. The values calculated were 0.175, 0.079 and 0.022 for pH 6, 7 and 8, respectively. Values for E_2-E_1 can be calculated using the equation E_2-E_1 = 0.059 log K_{sq} [16], where E_2 is the potential for the oxidized FNR/FNR semiquinone and E_1 is the potential for the FNR semiquinone/FNR hydroquinone. The values calculated at pH 6, 7 and 8 are -0.045, -0.065 and -0.098 V, respectively.

Complexes of FNR and flavodoxin

Electrolytic reduction of the non-covalent complex of FNR and flavodoxin showed that, in keeping with the redox data for the separate proteins, flavodoxin is first reduced to the semiquinone, followed by the reduction of FNR to the hydroquinone with very little stabilization of its semiquinone, and finally reduction of the semiquinone of flavodoxin to the hydroquinone. These three steps in the reduction are sufficiently well resolved for redox potentials to be determined for each step from a single titration (Fig. 6, Table 1). Comparison of the potentials for the complex with the corresponding potentials of the separate proteins shows that, over the pH range studied (pH 6–8), the potentials of the two-electron reduction of FNR and the one-electron reduction of flavodoxin to the semiquinone have shifted slightly to less negative values, while the potential for the one-electron reduction of flavodoxin semiquinone to the hydroquinone has shifted by about 25 mV to more negative potentials. It seemed possible that small shifts of this kind might be due to an interaction with the positively charged dye PDQ^{2+}, which is present at a concentration of 100 μM. However, control experiments with only 1 μM PDQ^{2+} and also in the absence of glycerol, showed that, while the rate of electrolytic reduction of the complex was very much lower with the lower concentration of mediator, the three midpoint potentials were unaffected. The effects of pH on the potentials of the proteins in the complex are similar to the effects of pH on the separate proteins (Figs 2, 3 and 5).

The shifts observed in the redox potentials might be due to differences in the relative strengths of interaction of the three redox forms of flavodoxin with the two redox forms of

Table 1. *Midpoint potentials of* Anabaena *PCC 7119 FNR and flavodoxin alone and in electrostatic and covalent complexes at pH 6, 7 and 8*

Anabaena PC 7119 component	pH	E_m		
		$Fld_{ox/sq}$	$FNR_{ox/red}$	$Fld_{sq/red}$
		mV		
FNR	6		−317	
Flavodoxin		−164		−421
Electrostatic complex		−151	−311	−449
Covalent complex		−187	−307	−396
FNR	7		−344	
Flavodoxin		−212		−436
Electrostatic complex		−204	−340	−461
Covalent complex		−233	−342	−416
FNR	8		−376	
Flavodoxin		−261		−435
Electrostatic complex		−254	−374	−463
Covalent complex		−286	−381	−422

FNR, or to changes in the association of the FMN with apoflavodoxin and of FAD with apo-FNR, as a result of the protein-protein complex or to a combination of both effects. However, if it is assumed that the changes are due solely to a change in the relative strengths of interaction between the different redox forms of the two proteins, it is possible to estimate the size of the change. A similar kind of approach has previously been made for the spinach FNR-ferredoxin electrostatic complex [28]. Neglecting any possible contribution of the semiquinone of FNR, which is only formed in small amounts in the mixture, the different redox states of the complex can be represented as follows:

$$\begin{array}{ccc} Fld_{ox} + FNR_{ox} & \underset{K_{d1}}{\rightleftharpoons} & Fld_{ox} - FNR_{ox} \\ e^- \Downarrow & & \Downarrow e^- \\ Fld_{sq} + FNR_{ox} & \underset{K_{d2}}{\rightleftharpoons} & Fld_{sq} - FNR_{ox} \\ 2e^- \Downarrow & & \Downarrow 2e^- \\ Fld_{sq} + FNR_{red} & \underset{K_{d3}}{\rightleftharpoons} & Fld_{sq} - FNR_{red} \\ e^- \Downarrow & & \Downarrow e^- \\ Fld_{red} + FNR_{red} & \underset{K_{d4}}{\rightleftharpoons} & Fld_{red} - FNR_{red}, \end{array}$$

where the dissociation constants are defined by the following equations.

$$K_{d1} = [(Fld_{ox})(FNR_{ox})/(Fld_{ox} - FNR_{ox})];$$
$$K_{d2} = [(Fld_{sq})(FNR_{ox})/(Fld_{sq} - FNR_{ox})];$$
$$K_{d3} = [(Fld_{sq})(FNR_{red})/(Fld_{sq} - FNR_{red})];$$
$$K_{d4} = [(Fld_{red})(FNR_{red})/(Fld_{red} - FNR_{red})].$$

Dissociation constants for the various complexes can be derived from the measured redox potentials as follows

$$E_{m,ec}\ (Fld_{ox/sq}) = E_m\ (Fld_{ox/sq} + 0.059 \log (K_{d1}/K_{d2});$$
$$E_{m,ec}\ (FNR_{ox/red}) = E_m\ (FNR_{ox/red}) + (0.059/2) \log (K_{d2}/K_{d3});$$
$$E_{m,ec}\ (Fld_{sq/red}) = E_m\ (Fld_{sq/red}) + 0.059 \log (K_{d3}/K_{d4}).$$

E_m is the midpoint potential of the free protein and $E_{m,ec}$ is the midpoint potential of the protein in the electrostatic complex.

These equations allow values for K_{d2}, K_{d3} and K_{d4} to be calculated using the known value for K_{d1} (6.4 μM) [25] and the values measured for E_m and $E_{m,c}$ for the free proteins and their electrostatic complexes. The values calculated for K_{d2}, K_{d3} and K_{d4} are 4.7, 3.2 and 9 μM, respectively.

If this interpretation of the data is correct, oxidized FNR interacts more strongly with the semiquinone of flavodoxin than with oxidized flavodoxin and reduced FNR interacts even more strongly than oxidized FNR with the flavodoxin semiquinone. The weakest interaction is between reduced FNR and fully reduced flavodoxin. The interactions between oxidized FNR and fully reduced flavodoxin and between reduced FNR and oxidized flavodoxin, cannot be determined from these measurements, yet both are important in catalysis as they represent states prior to electron transfer. However, it is known that there is very little change in the structure of the protein when flavodoxin from *C. beijerinckii* is reduced from the semiquinone to the hydroquinone [29] and it is therefore likely that, with the possible exception of a new interaction due to the negative charge at N1 of the FMN in flavodoxin hydroquinone, the interactions between the hydroquinone and oxidized FNR are similar to those of the semiquinone with oxidized FNR.

Redox titrations of the covalent complexes were similar to those of the corresponding non-covalent complexes (Fig. 7); the complex of FNR and flavodoxin was reduced successively to flavodoxin semiquinone, reduced FNR and flavodoxin hydroquinone. The data from titrations of this complex at pH values of 6, 7 and 8 show that the potentials are again shifted relative to those of the separate proteins, but that, in general, the shifts are different from those in the non-covalent complex (Table 1). The small shift for FNR is to a less negative potential at pH 6 and pH 7, as was observed with the electrostatic complex, but at pH 8 the shift is in the opposite direction. The potential for the oxidized flavodoxin/flavodoxin semiquinone couple is shifted by 21 – 25 mV to more negative values at all pH values, while the semiquinone/hydroquinone couple is shifted to less negative values; in this case, the shift decreases with pH from 25 mV at pH 6 to 13 mV at pH 8.

Since the proteins are linked covalently, these shifts cannot be attributed to changes in the strength of interaction between the two proteins as their redox states change. The shifts probably reflect changes in the dissociation constants for the dissociation of FMN and FAD from the complex. It is possible to use the data for the covalent complex to make an additional estimate of the K_d values for the protein-protein interaction in the electrostatic complex. It is necessary to assume that the conformation of the two oxidized proteins is the same in both the covalent and electrostatic complexes. The covalent complex then becomes a particular case in which the changes in potentials are a result of only the changes in the interaction of the flavin cofactors with the apoproteins. By comparing the data for the electrostatic complex with those for the covalent complex rather than with those of the free proteins, the contributions due to changes in the binding of flavins to the apoproteins are eliminated from the calculations, since they are already included in the potentials of the covalent complex.

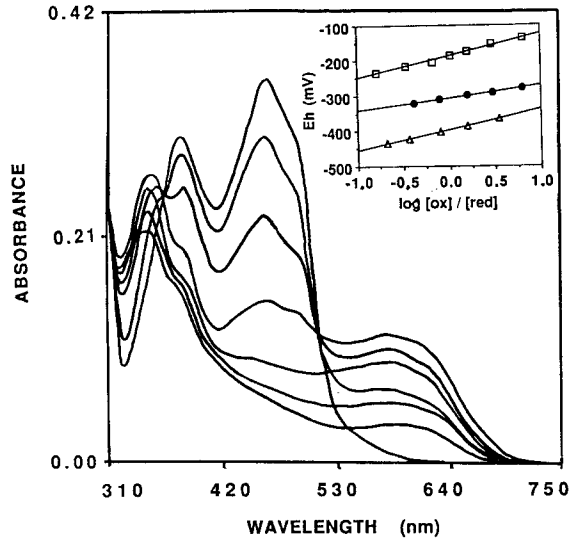

Fig. 7. *Potentiometric titration of* Anabaena *PCC 7119 FNR-flavodoxin covalent complex at pH 6.0.* The spectra were obtained during the reductive titration. Inset: Nernst plots of the data, (□) Fld_{ox}/Fld_{sq}; (●) FNR_{ox}/FNR_{red}; (△) Fld_{sq}/Fld_{red}. The protein concentration was 19.15 μM in 50 mM potassium phosphate, pH 6.0, containing 15% (by vol.) glycerol. Also present were 100 μM PDQ^{2+}, 1 μM methyl viologen, 1 μM benzyl viologen, 1 μM neutral red, 1 μM anthraquinone-2-sulfonate and 1 μM anthraquinone-2,6-disulfonate

The dissociation constants can then be calculated from the following equations:

$$E_{m,cc}\ (Fld_{ox/sq}) = E_{m,cc}\ (Fld_{ox/sq}) + 0.059 \log (K_{d1}/K_{d2});$$

$$E_{m,ec}\ (FNR_{ox/red}) = E_{m,cc}\ (FNR_{ox/red}) + (0.059/2) \log (K_{d2}/K_{d3});$$

$$E_{m,ec}\ (Fld_{sq/red}) = E_{m,cc}\ (Fld_{sq/red}) + 0.059 \log (K_{d3}/K_{d4}).$$

$E_{m,cc}$ is the midpoint potential of the protein in the covalent complex and $E_{m,ec}$ is the midpoint potential of the protein in the electrostatic complex.

The dissociation constants calculated in this manner are K_{d2} = 2.1 μM, K_{d3} = 1.8 μM and K_{d4} = 10.2 μM. All of them are in the same order of magnitude as those calculated above by comparing data for the electrostatic complex with those for the free proteins and again indicate that complexes formed when the proteins are in different oxidation-reduction states are stronger than those formed when the proteins are both fully oxidized or both fully reduced.

The difference in redox potentials between the covalent and electrostatic complexes of *Anabaena* PCC 7119 FNR and flavodoxin are probably due to the fact that the association state of the two proteins cannot change in the covalent complex. The orientation of the redox cofactors in the covalent complex may therefore be suboptimal, and any structural reorganization that is required for electron transfer may be impeded. The rate of electron transfer between the two proteins is lower in the covalent complex than in the electrostatic complex [10] and the $NADP^+$ photoreductase activity is also lower for the covalent complex [9].

Redox potentials of the complexes of FNR from A. variabilis *and flavodoxin from* A. vinelandii

Similar measurements to those described above were made with the electrostatic and covalent complexes of FNR and flavodoxin from different organisms. FNR from *A. variabilis* and flavodoxin from *A. vinelandii* are known to form a strong electrostatic complex (K_{d1} = 7 μM) [30]. The values for the redox potential determined for this complex and for the covalent complex of the two proteins were compared with published values for the redox potentials of the free flavodoxin [31] and with a measured value for free FNR (Table 2). The changes on complex formation are qualitatively similar to those described above for the complexes of the two proteins from the PCC 7119 strain of *Anabaena*. When the values for the electrostatic and covalent complexes were compared using similar calculations to those made above and using a value of K_{d1} = 7 μM, values for K_{d2}, K_{d3} and K_{d4} of 0.4, 0.2 and 4.0 μM, respectively, were obtained. The difference of one order of magnitude between either K_{d2} or K_{d3} and either K_{d1} or K_{d4}, again shows that oxidized FNR interacts more strongly with the semiquinone of flavodoxin than with oxidized flavodoxin, while reduced FNR interacts more strongly than oxidized FNR with the flavodoxin semiquinone. The weakest interactions are when the two proteins are fully oxidized or fully reduced.

Table 2. *Midpoint potentials of* A. variabilis *FNR and* A. vinelandii *flavodoxin alone and in electrostatic and covalent complexes at pH 8*
See [31] for flavodoxin

A. vinelandii	E_m		
	$Fld_{ox/sq}$	$FNR_{ox/red}$	$Fld_{sq/red}$
	mV		
FNR		−377	
Flavodoxin	−200		−500
Electrostatic complex	−200	−367	−508
Covalent complex	−274	−377	−427

Changes in redox potentials have been described for the electrostatic complex between FNR and ferredoxin from spinach [28, 32]. The midpoint potential of FNR in the complex becomes some 20 mV more positive and the midpoint potential of ferredoxin becomes more negative by either 20 mV [32] or 90 mV [28]. The shifts found in the present work for FNR in the FNR-flavodoxin electrostatic complex were smaller but also positive. The potential for the semiquinone/hydroquinone couple of flavodoxin becomes about 25 mV more negative and, therefore, it behaves in a similar way to ferredoxin, as might be expected from their similar physiological roles. A further physiological consequence of these results can be considered; since complex formation causes the E_1 of flavodoxin to become more negative and therefore makes flavodoxin a stronger reductant, the association of FNR and flavodoxin facilitates the reduction of FNR from a thermodynamic point of view.

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REFERENCES

1. Zanetti, G. & Curti, B. (1980) *Methods Enzymol. 69*, 250–253.
2. Rogers, L. J. (1987) in *The cyanobacteria* (Fay, P. & van Baalen, C., eds) pp. 35–67, Elsevier Science Publication, Amsterdam.

3. Mayhew, S. G. & Ludwig, M. L. (1975) in *The enzymes*, 3rd edn (Boyer, P. D., ed.) pp. 57–118, Academic Press, London.
4. Nelson, N. & Neumann, J. (1968) *Biochim. Biophys. Res. Commun. 30*, 142–147.
5. Shin, M. & San Pietro, A. (1968) *Biochim. Biophys. Res. Commun. 33*, 38–42.
6. Foust, G. P., Mayhew, S. G. & Massey, V. (1969) *J. Biol. Chem. 244*, 964–970.
7. Zanetti, G., Aliverti, A. & Curti, B. (1984) *J. Biol. Chem. 259*, 5175–5178.
8. Pueyo, J. J., Edmondson, D. E. & Gómez-Moreno, C. (1989) *Eur. J. Biochem. 183*, 539–544.
9. Pueyo, J. J. & Gómez-Moreno, C. (1991) *Biochim. Biophys. Acta*, in the press.
10. Walker, M. C., Pueyo, J. J., Gómez-Moreno, C. & Tollin, G. (1990) *Arch. Biochem. Biophys. 281*, 76–83.
11. Sancho, J., Peleato, M. L., Gómez-Moreno, C. & Edmondson, D. E. (1988) *Arch. Biochem. Biophys. 260*, 200–207.
12. Fillat, M. F., Sandmann, G. & Gómez-Moreno, C. (1988) *Arch. Microbiol. 150*, 160–164.
13. Edmondson, D. E. & Tollin, G. (1980) *Methods Enzymol. 69*, 392–405.
14. Stankovich, M. T. (1980) *Anal. Biochem. 109*, 295–308.
15. Edmondson, D. E. & Tollin, G. (1971) *Biochemistry 10*, 124–132.
16. Clark, W. M. (1960) *Oxidation-reduction potentials of organic systems*, the Williams & Wilkins Company, Baltimore.
17. Mayhew, S. G., Foust, G. P. & Massey, V. (1969) *J. Biol. Chem. 244*, 803–810.
18. Mayhew, S. G. (1971) *Biochim. Biophys. Acta 235*, 276–288.
19. Entsch, B. & Smillie, R. M. (1972) *Arch. Biochem. Biophys. 151*, 378–386.
20. Draper, R. D. & Ingraham, L. L. (1968) *Arch. Biochem. Biophys. 125*, 802–808.
21. Franken, H. D., Rüterjans, H. & Müller, F. (1984) *Eur. J. Biochem. 138*, 481–489.
22. Vervoort, J., Müller, F., Mayhew, S. G., van der Berg, W. A. M., Moonen, C. J. W. & Bacher, A. (1986) *Biochemistry 25*, 6789–6799.
23. Stockman, B. J., Westler, W. M., Mookerry, E. S. & Mahley, J. L. (1988) *Biochemistry 27*, 136–142.
24. Ludwig, M. L., Schopfer, L. M., Metzger, A. L., Pattridge, K. A. & Massey, V. (1990) *Biochemistry 29*, 10364–10375.
25. Fillat, M. F., Edmondson, D. E. & Gómez-Moreno, C. (1990) *Biochim. Biophys. Acta 1040*, 301–307.
26. Paulsen, K. M., Stankovich, M. T., Stockman, B. J. & Markley, J. L. (1990) *Arch. Biochem. Biophys. 280*, 68–73.
27. Keirns, J. J. & Wang, J. H. (1982) *J. Biol. Chem. 247*, 7374–7382.
28. Batie, C. J. & Kamin, H. (1981) *J. Biol. Chem. 256*, 7756–7763.
29. Ludwig, M. L., Burnett, R. M., Darling, G. D., Jordan, S. R., Kendall, D. S. & Smith, W. W. (1976) in *Flavins and flavoproteins* (Singer, T. P., ed.) pp. 393–404, Elsevier, Amsterdam.
30. Gómez-Moreno, C., Sancho, J., Fillat, M. F., Pueyo, J. J. & Edmondson, D. E. (1987) in *Flavins and flavoproteins* (Edmondson, D. E. & McCormick, D. B., eds) pp. 335–359, W. de Gruyter & Co., Berlin.
31. Watt, G. D. (1979) *Anal. Biochem. 99*, 399–407.
32. Smith, M., Smith, W. H. & Knaff, D. B. (1981) *Biochim. Biophys. Acta 635*, 405–411.