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Laser Flash Photolysis Studies of the Kinetics of Reduction of Ferredoxins and Ferredoxin–NADP$^+$ Reductases from *Anabaena* PCC 7119 and Spinach: Electrostatic Effects on Intracomplex Electron Transfer[1]

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The influence of electrostatic forces on the formation of, and electron transfer within, transient complexes between redox proteins was examined by comparing ionic strength effects on the kinetics of the electron transfer reaction between reduced ferredoxins (Fd) and oxidized ferredoxin–NADP$^+$ reductases (FNR) from *Anabaena* and from spinach, using laser flash photolysis techniques. With the *Anabaena* proteins, direct reduction by laser-generated flavin semiquinone of the FNR component was inhibited by complex formation at low ionic strength, whereas Fd reduction was not. The opposite results were obtained with the spinach system. These observations clearly indicate structural differences between the cyanobacterial and higher plant complexes. For the complex formed by the *Anabaena* proteins, the results indicate that electrostatic forces are not a major contributor to complex stability. However, the rate constant for intracomplex electron transfer had a biphasic dependence on ionic strength, suggesting that structural rearrangements within the transient complex facilitate electron transfer. In contrast to the *Anabaena* complex, electrostatic forces are important for the stabilization of the spinach Fd:FNR complex, and changes in ionic strength had little effect on the limiting rate constant for intracomplex electron transfer. This suggests that in this case the geometry of the initial collisional complex is optimal for reaction. These results provide a clear illustration of the differing roles that electrostatic interactions may play in controlling electron transfer between two redox proteins.

Ferredoxins (Fds)[4] are an important class of iron–sulfur proteins that function as electron transfer agents in biological processes such as photosynthesis, respiration, nitrogen fixation, and sulfur and carbon metabolism (1). The (2Fe–2S) chloroplast-type Fds (MW $\approx$ 10,000) are one-electron carriers with a $E_{m,7} \approx -400$ mV, whose active site consists of a dinuclear cluster represented as $Fe_2S_2(SR)_4$, where SR is a liganded protein cysteinyl sulfur (2). The X-ray structure of the Fd from the cyanobacterium *Spirulina platensis* has been determined (3). The iron–sulfur cluster is located near the surface of the molecule in a region that carries an overall negative charge and is highly conserved in other 2Fe–2S Fds (4).

A number of kinetic studies of the oxidation and reduction reactions of chloroplast-type Fds have been reported, using techniques such as stopped-flow (5–7), pulse radiolysis (8, 9), and electrochemical methods (10) and laser flash photolysis (11, 12). It has been concluded from this work that electrostatic forces play an important role in the electron transfer reactions of these proteins and that 1:1 complexes between Fd and ferredoxin–NADP$^+$ reductase (FNR) are involved in the reaction mechanism. The intracomplex electron transfer reaction between Fd_{red} and FNR_{ox} from spinach occurs within the dead time of the stopped-flow instrument at low ionic strengths (13–15). Utilizing the higher time resolution provided by laser flash photolysis, it has been possible to directly measure this rate constant for a transient complex formed between spinach Fd_{red} and FNR_{ox} at high ionic strengths (310 and 460 mM) (11).

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[4] Abbreviations used: Fd, Fd_{ox}, and Fd_{red}, ferredoxin and its oxidized and one-electron-reduced forms; FNR, FNR_{ox}, and FNR_{red}, ferredoxin–NADP$^+$ reductase and its oxidized and one-electron-reduced forms; dRf, dRfH$^\cdot$, and $dRfH_2$, the oxidized, one-electron-reduced, and fully reduced forms of 5-deazariboflavin; k_{obs}, observed rate constant.

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The goal of the present study was to obtain further insight into the structural factors which control the rates of electron transfer within physiologically relevant protein–protein complexes. Although no X-ray structure determinations are presently available for such complexes, computer graphics methods have been employed to simulate possible structures (16–18), with complementary electrostatic interactions between the proteins being utilized as one of the principal modeling criteria. However, in previous investigations of the complexes formed between cytochrome *c* and its physiological partners cytochrome *c* peroxidase (19) and cytochrome *c* oxidase (20), we have found that the electrostatically stabilized complex formed at low ionic strength is *not* optimized for electron transfer, thus calling into question the relevance of such computer models for understanding catalysis. The present study extends this line of investigation to the Fd and FNR isolated from the cyanobacterium *Anabaena* PCC 7119 and from spinach. As will be described below, laser flash photolysis experiments have shown that there are significant structural differences, different modes of stabilization, and different ionic strength effects on electron transfer kinetics, between the complexes formed by the spinach and cyanobacterial proteins, again emphasizing that complementary electrostatic interactions are not sufficient to account for all of the properties of redox protein electron transfer complexes.

MATERIALS AND METHODS

Anabaena PCC 7119 Fd was purified according to the method of Susor and Krogman (21), while FNR from the same source was purified using the method of Sancho *et al.* (22). Spinach Fd and FNR were purified according to the method of Zanetti and Curti (23). Extinction coefficients of 9.7×10^3 and 8.8×10^3 M^{-1} cm^{-1} at 420 and 465 nm, respectively, were used to determine the concentration of oxidized Fd. An extinction coefficient of 9700 M^{-1} cm^{-1} at 458 nm (22) was used to determine the concentration of *Anabaena* FNR, while an extinction coefficient of 10,300 M^{-1} cm^{-1} at the same wavelength was used for the spinach protein (24). Deazariboflavin was synthesized according to the procedure of Smit *et al.* (25). The buffer used throughout this study was 4.0 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA and approximately 100 μM 5-deazariboflavin (dRf). Sodium chloride was added to adjust the ionic strength.

All kinetic experiments were performed anaerobically at ambient temperature (23–25°C) in cuvettes which were deaerated by bubbling with water-saturated argon gas. Microliter volumes of a concentrated solution of protein were added via a syringe to 1–2 ml of buffer containing deazaflavin and EDTA subsequent to deoxygenation in the sealed cuvette. Traces of oxygen introduced during the addition of protein were removed by blowing argon gas across the surface of the solution. Laser photoexcitation of deazaflavin was carried out with a nitrogen laser-pumped dye solution (BBQ 2A386 dye from PRA, 396 nm maximum wavelength). A detailed description of the laser flash apparatus and the method of data collection has been published elsewhere (26, 27).

Laser flash photolysis rapidly generated 5-dRfH$^\bullet$ (in <1 μs) which, in the absence of protein, underwent disproportionation (28). In the presence of protein, electron transfer from dRfH$^\bullet$ to the oxidized protein occurred. The extent of the contribution of disproportionation to the observed transient decay kinetics in the presence of protein is determined by the magnitude of the second-order rate constant for protein reduction and the concentrations of the reacting species (i.e., protein vs dRfH$^\bullet$). On the basis of computer simulations, we estimate that the error due to disproportionation in the reported second-order rate constants for the reduction of *Anabaena* and spinach FNR, and for the reduction of spinach Fd at ionic strengths above 100 mM, is less than +5%, and for the reduction of *Anabaena* Fd is approximately +10–15%.

All kinetic experiments were performed under pseudo-first-order conditions, in which the concentration of protein acceptor (>4 μM) was in excess with respect to the amount of dRfH$^\bullet$ produced per flash (<0.6 μM). Kinetic traces were generally analyzed by fitting to an exponential curve, although some data were also analyzed using a computer fitting procedure (SIFIT, obtained from OLIS Co., Jefferson, GA), which gave equivalent results. The estimated error in the first-order rate constant determinations was $\leq \pm 10\%$, based upon standard deviations obtained from replicate measurements.

Nonlinear concentration dependencies of k_{obs} values for FNR reduction were obtained for experiments involving increasing amounts of FNR in the presence of excess Fd, implying a mechanism which involved intermediate complex formation (cf. Ref. (29)). The apparent dissociation constant for the complex between Fd_{red} and FNR_{ox} and the limiting first-order rate constant for intracomplex electron transfer were evaluated from these data by a nonlinear least squares computer-fitting procedure which has been described previously (17, 30).

RESULTS

In order to provide a basis for comparison of the Fd:FNR complexes of the *Anabaena* and spinach proteins, we determined the kinetics of reduction by dRfH$^\bullet$ of the individual proteins, both free and within preformed complexes, and the effects of ionic strength on the kinetics of electron transfer between Fd_{red} and FNR_{ox}.

Reduction of Anabaena Ferredoxin by dRfH$^\bullet$

The reduction of Fd by dRfH$^\bullet$ in the absence of FNR was monitored by the absorbance decrease at 465 nm, where the oxidized Fd has an absorption maximum. At 10 mM ionic strength, laser photolysis resulted in an initial increase in absorbance (Fig. 1) followed by an exponential

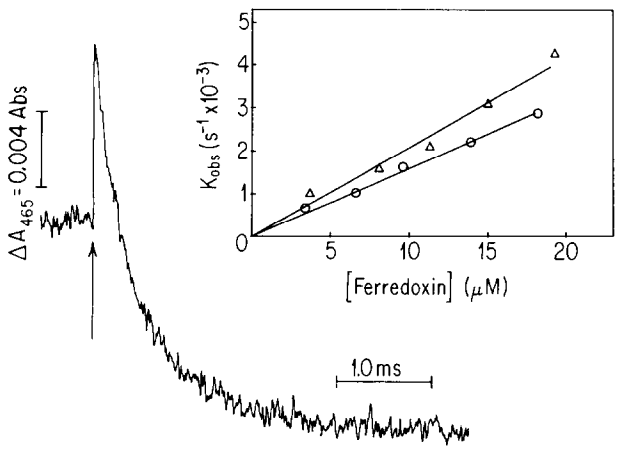

FIG. 1. Kinetic trace obtained for *Anabaena* PCC ferredoxin reduction by 5-dRfH$^\bullet$ monitored at 465 nm at I = 10 mM. Ferredoxin concentration was 10 μM. Arrow indicates time of laser flash. (Inset) Second-order plots of k_{obs} for ferredoxin reduction vs Fd_{ox} concentration at I = 10 mM (○) and I = 310 mM (△). The buffer conditions were: 4 mM potassium phosphate, pH 7.0, containing 0.5 mM EDTA and 100 μM dRf. An appropriate concentration of NaCl was included to adjust the ionic strength to 310 mM.

decay that eventually went below the preflash baseline (cf. Ref. (11)). These absorbance changes are consistent with the rapid formation of dRfH$^\cdot$ followed by its subsequent reoxidation by Fd_{ox}, leading to formation of Fd_{red}. From the slope of a plot of k_{obs} vs Fd_{ox} concentration, a second-order rate constant for Fd reduction of $1.6 \pm 0.15 \times 10^8$ M^{-1} s^{-1} was obtained (inset, Fig. 1). At high ionic strength (310 mM), the second-order rate constant was slightly increased ($2.1 \pm 0.3 \times 10^8$ M^{-1} s^{-1}). The ionic strength effect is similar to that observed previously with spinach Fd (11), although the increase in rate constant at high ionic strength was considerably larger (threefold) for the spinach protein. If such ionic strength effects are the result of alterations in redox potential and/or changes in protein structure induced by high salt concentrations, these are apparently smaller for the cyanobacterial than for the spinach Fd. The precise reasons for these differences require further study.

Reduction of Anabaena Ferredoxin-NADP$^+$ Reductase by dRfH$^\cdot$

Upon laser photolysis of oxidized *Anabaena* FNR in the presence of dRf, the absorbance monitored at 461 nm increased slightly and then decayed below the preflash baseline (data not shown). The initial absorbance increase is again due to the production of dRfH$^\cdot$, while the net bleaching of absorbance results from the one-electron reduction of the FAD cofactor via electron transfer from dRfH$^\cdot$. The corresponding formation of the FAD semiquinone was also observed as an increase in absorbance at 600 nm (data not shown; cf. Ref. (11) Fig. 1c for comparable results obtained with the spinach protein). Transients were well fit by a monoexponential curve and the observed pseudo-first-order rate constants obtained from the absorbance changes at these two wavelengths were identical, as expected. The k_{obs} values were linearly dependent on the concentration of FNR (data not shown; cf. Ref. (11), inset Fig. 1c for results obtained with the spinach protein), giving a second-order rate constant of $4.2 \pm 0.2 \times 10^8$ M^{-1} s^{-1} at an ionic strength of 10 mM. This value is similar to that reported previously for the reduction of the spinach FNR (6.3×10^8 M^{-1} s^{-1}; Ref. (11)). The second-order rate constant was relatively unaffected by changes in ionic strength ($k = 3.2 \times 10^8$ M^{-1} s^{-1} at 310 mM), consistent with results obtained with the spinach protein (11).

Reoxidation of Anabaena Ferredoxin by Anabaena Ferredoxin-NADP$^+$ Reductase

In the presence of FNR, the flash-induced reduction of Fd by dRfH$^\cdot$ was monitored at 507 nm, which is an isosbestic point for FNR reduction. With Fd alone, the absorbance at this wavelength rapidly decreased below the preflash baseline immediately following dRfH$^\cdot$ formation by the laser flash (data not shown; cf. Fig. 1 for comparable results). This decrease was well fit by a monoexponential decay which was dependent on the concentration of Fd_{ox}. As expected, the second-order rate constant calculated from these transients was identical to that obtained at 465 nm (see above). In the presence of substoichiometric amounts of *Anabaena* FNR, the absorbance decrease at 507 nm was followed by a reappearance of absorbance, shown at $I = 10$ mM in Fig. 2a. This increase corresponds to the reoxidation of Fd_{red} by FNR_{ox} (cf. Ref (11)). Consistent with this, an increase in absorbance which could also be fit by a single exponential was observed at 600 nm (Fig. 2b). Since absorbance changes at the longer wavelength are dominated by the formation of the FAD semiquinone of FNR, we interpret this increase as resulting from one-electron reduction of FNR_{ox} by Fd_{red}. As expected from this interpretation, the k_{obs} values obtained from transients collected at 600 nm were identical within experimental error to those calculated for the slow rise in absorbance at 507 nm.

Little or no direct reduction of the *Anabaena* FNR by dRfH$^\cdot$ was observed at $I = 10$ mM in the presence of equimolar or greater amounts of *Anabaena* Fd. This is apparent from Fig. 2b in which a single exponential fit to the increase in absorbance at 600 nm begins at a point which is close to the preflash origin. The small deviation (<1 ms) can be ascribed to absorbance changes associated with Fd reduction and reoxidation. Similar results were obtained at FNR concentrations of up to 30 μM (not shown). In view of the relative values of the reduction rate constants for the individual proteins (see above), this result suggests that the accessibility of the FAD cofactor of *Anabaena* FNR to dRfH$^\cdot$ is significantly decreased when *Anabaena* Fd is present at low ionic strength. This decreased accessibility presumably results from steric effects due to complex formation. Little or no decrease in Fd accessibility was noted, based on comparison of the rates of Fd reduction by dRfH$^\cdot$ obtained in the presence and absence of FNR (not shown). In contrast to the *Anabaena* complex, the accessibility of the FAD of spinach FNR has been found to be unaltered in the presence of spinach Fd at low ionic strengths, whereas the accessibility of the Fe/S center of spinach Fd was greatly diminished under these conditions (11). The apparent inaccessibility of *Anabaena* FNR to dRfH$^\cdot$ reduction in the presence of Fd was unaffected by increases in ionic strength up to 310 mM (data not shown). To illustrate this difference between the two complexes, a kinetic trace obtained with the spinach proteins at high ionic strength is also presented in Fig. 2c. Note that approximately equal amounts of both direct (initial rapid absorbance increase) and intracomplex reduction of FNR (subsequent slower absorbance increase) occurs with the spinach proteins, whereas this was not observed with the *Anabaena* proteins at either low or high ionic strengths (shown at low ionic strength in Fig. 2b). If the *Anabaena* complex was largely dissociated at high ionic strengths, one would have expected that reduction of FNR_{ox} would predominate at equimolar concentrations of the two proteins. The fact that this did

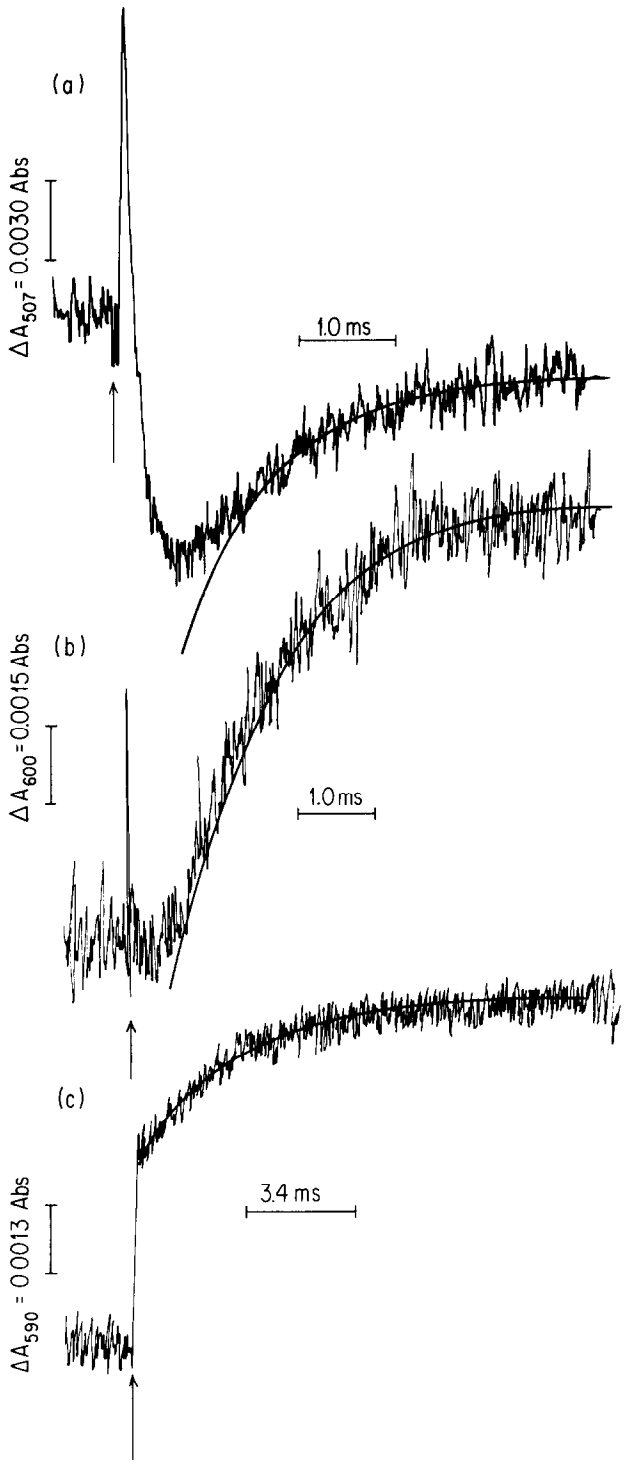

FIG. 2. Kinetic traces obtained during the reduction of mixtures of *Anabaena* Fd and FNR at 10 mM ionic strength. Arrows indicate time of laser flash. (a) Trace obtained at 507 nm, after the addition of 6 μM FNR to 35 μM Fd. Solid line corresponds to a monoexponential fit with a rate constant of 1200 s^{-1}. (b) Trace obtained at 600 nm under conditions identical to those of (a). Solid line corresponds to a monoexponential fit with a rate constant of 1200 s^{-1}. (c) Trace obtained at 590 nm showing FNR reduction during laser photolysis of 14 μM spinach Fd in the presence of 15.3 μM spinach FNR at 460 mM ionic strength. Solid line corresponds to a monoexponential fit with a rate constant of 340 s^{-1}. Each trace represents the sum of 10 transients.

not occur suggests that electrostatic forces do not contribute significantly to the stability of the *Anabaena* Fd_{ox}:FNR_{ox} complex (i.e., that the complex exists to approximately equal extents at both low and high ionic strengths). This result is again in contrast to those obtained with the spinach proteins (11), where electrostatic forces have been demonstrated to be a major contributing factor to complex stability (24, 31). We conclude from these results that the respective complexes formed between the oxidized proteins from *Anabaena* and those from spinach are structurally dissimilar and are stabilized differently, even though, individually, the corresponding proteins from these sources display similar properties (see below for further discussion).

During titration experiments in which aliquots of *Anabaena* FNR_{ox} were added to a solution containing an excess (ranging from 16 to 60 μM in different experiments) of *Anabaena* Fd_{ox}, the observed rate constants for electron transfer from Fd_{red} to FNR_{ox} obtained from transients collected at 600 nm displayed a hyperbolic dependence on FNR_{ox} concentration. This is illustrated in Fig. 3a for experiments performed at various ionic strengths. Such nonlinear behavior is generally consistent with a minimal kinetic mechanism of a second-order reaction, followed by a first-order reaction which becomes rate limiting at high concentrations (29), i.e.,

$$Fd_{red} + FNR_{ox} \overset{K_d}{\rightleftharpoons} (Fd_{red}{:}FNR_{ox}) \overset{k_{et}}{\rightarrow} Fd_{ox}{:}FNR_{red}.$$

This simple model, however, does not take into account a competing reaction involving the formation of the Fd_{ox}:FNR_{ox} complex. Formation of this complex would increase the apparent K_d value obtained from an analysis based upon the above equation by decreasing the free FNR_{ox} concentration. However, assuming that there is a single Fd binding site on FNR which leads to the formation of a productive complex (22), such competition would not affect the limiting rate constant (k_{et}) obtained by extrapolating to infinite FNR_{ox} concentrations.

The constants obtained from a computer fit of the data in Fig. 3a to the above scheme (cf. (17, 30) for details) are presented in Table I. An apparent K_d of 7.9 μM for the Fd_{red}:FNR_{ox} complex at I = 10 mM was obtained from the analysis. As discussed above, the inaccessibility of the FAD cofactor of *Anabaena* FNR toward direct reduction by dRfH• suggests that appreciable formation of the Fd_{ox}:FNR_{ox} complex occurs under these experimental conditions. Consistent with this conclusion, the k_{obs} values obtained during titration experiments were approximately independent of Fd_{ox} concentrations ranging from 16 to 60 μM. This suggests that formation of the Fd_{ox}:FNR_{ox} complex was at least 75% complete over this concentration range. An upper limit of 1 μM for the K_d of the fully oxidized *Anabaena* complex is predicted from these results. For comparison, a K_d of 0.05 μM is estimated for the fully oxidized complex formed between the spinach

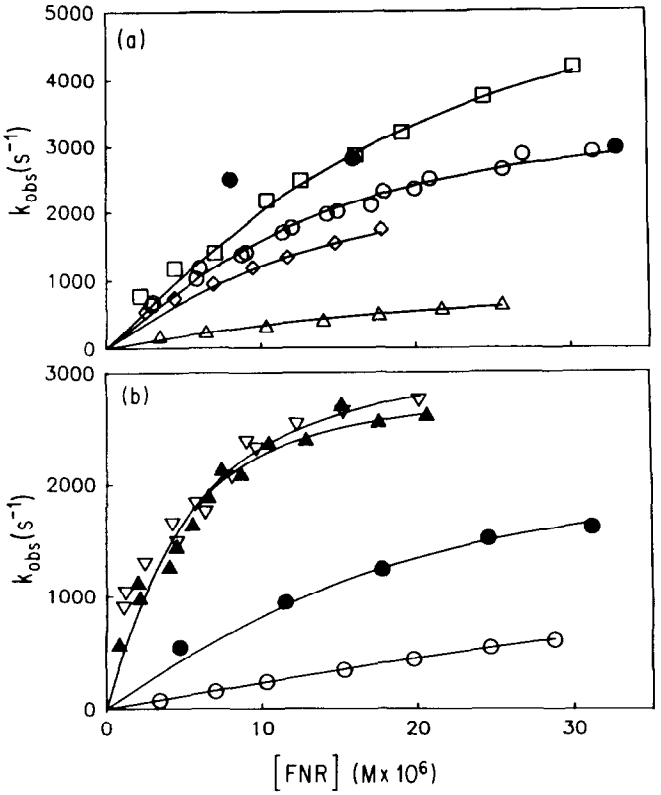

FIG. 3. (a) Concentration dependence of k_{obs} obtained for the reduction of *Anabaena* FNR_{ox} by Fd_{red} at (○) 10 mM (□) 110 mM, (◇) 210 mM, and (△) 310 mM ionic strengths. The closed circles represent the observed rate constants obtained for the electron transfer at 10 mM ionic strength with equimolar concentrations of Fd_{ox} and FNR_{ox}. Observed rate constants were obtained from transients collected at 600 nm. Fd_{ox} concentration was 40 μM at ionic strengths of 110, 210, and 310 mM. At 10 mM ionic strength, the Fd_{ox} concentration ranged from 15 to 60 μM. (b) Concentration dependence of k_{obs} obtained for the reduction of spinach FNR_{ox} by Fd_{red} at (▲) 110 mM, (▽) 210 mM, (●) 310 mM, and (○) 460 mM ionic strengths. Observed rate constants were obtained from transients measured at 600 nm. Fd_{ox} concentration was 20 μM at ionic strengths of 110 and 210 mM, and 15 μM at ionic strengths of 310 and 460 mM.

TABLE I

Kinetic Constants for Electron Transfer within the Fd_{red}:FNR_{ox} Complexes from *Anabaena* PCC 7119 and Spinach[a]

Ionic strength (mM)	K_d ($\times 10^{-6}$ M)	k_{et} (s^{-1})
Anabaena		
10	7.9	3830
110	12.1	6500
210	8	2800
310	10.5	1220
Spinach		
110	2.5	3000
210	3.0	3300
310	10.7	2490
460	40.7	2240

[a] Constants were calculated as described in Refs. (17, 30). For the *Anabaena* system, K_d values correspond to upper limits (see text).

proteins under comparable conditions of pH and ionic strength (cf. dashed line, Fig. 4). Therefore, the apparent K_d obtained from the kinetic analysis is most likely determined by the dissociation of the Fd_{ox}:FNR_{ox} complex. The apparent K_d was found to be insensitive to changes in ionic strength (Table I), which again indicates that electrostatic forces are not a dominant factor in the stabilization of the oxidized complex (see above).

The limiting first-order rate constant (k_{et}) for intracomplex electron transfer was estimated to be 3830 s^{-1} at 10 mM ionic strength (Table I). As expected, this rate constant was protein concentration independent, as shown by experiments in which increasing equimolar concentrations of the two *Anabaena* proteins at 10 mM ionic strength were subjected to laser flash photolysis (solid circles in Fig. 3a). In this latter experiment, direct reduction by dRfH$^\bullet$ of the preformed Fd_{ox}:FNR_{ox} complex predominates. This reaction could also account for the apparent nonzero intercept in the hyperbolic plots of k_{obs} vs FNR concentration (Fig. 3a). The fact that the k_{obs} values obtained with 1:1 mixtures of Fd_{ox} and FNR_{ox} are smaller than the extrapolated value of 3800 s^{-1} probably is a consequence of the presence of uncomplexed protein.

The importance of electrostatic forces in the formation of a productive electron-transfer complex can be assessed from the k_{et} values obtained at ionic strengths higher than 10 mM (Fig. 3a; Table I). This limiting rate constant was found to increase to 6500 s^{-1} at 110 mM, and then decrease as the ionic strength was progressively raised to 210 mM and then 310 mM (Table I). These results suggest that, while electrostatic forces facilitate electron transfer within the complex at moderate-to-high ionic strengths, at low ionic strengths these forces become inhibitory. This will be discussed further below.

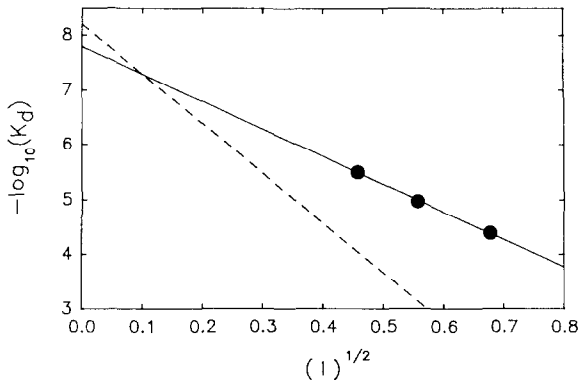

FIG. 4. Ionic strength dependence observed for the K_d of the spinach Fd_{red}:FNR_{ox} complex from 210 to 460 mM, obtained from kinetic analysis (closed circles; solid line). For comparison, the ionic strength dependence displayed by the fully oxidized complex, corrected to pH 7.0, is also included (broken line; data obtained from Ref. (31)).

Reoxidation of Spinach Ferredoxin by Spinach Ferredoxin-NADP$^+$ Reductase

The second-order rate constant obtained for the direct reduction of Fd by dRfH$^\cdot$ at 110 mM ionic strength was $5.6 \pm 0.68 \times 10^8$ M^{-1} s^{-1} (data not shown). This is close to that obtained earlier at 460 mM ionic strength (6.4×10^8 M^{-1} s^{-1}; Ref. (11)), indicating that a negligible change in the rate constant for the direct reduction of spinach Fd by dRfH$^\cdot$ occurs over this range of ionic strengths.

The kinetics of reduction by dRfH$^\cdot$ of the preformed complex between spinach Fd and FNR at low ionic strength are dominated by direct electron transfer to FNR (11). At high ionic strengths the electrostatic forces which stabilize the fully oxidized spinach complex are known to be weakened (24, 31) and a greater percentage of the component proteins are free in solution. Thus, under these conditions, the reduction of spinach Fd by dRfH$^\cdot$ can be observed and is followed by its slower reoxidation via electron transfer to FNR (monitored at 500 nm; cf. Ref. (11) Fig. 5a). The production of reduced FNR monitored at 600 nm occurs with the same rate constant as Fd reoxidation (cf. Ref. (11) Fig. 5b). In the present study, we have repeated these experiments at several ionic strengths. The observed rate constant obtained for the reduction of FNR (in the presence of excess Fd_{ox}) at ionic strengths higher than 100 mM displayed saturation kinetics when plotted vs FNR concentration (Fig. 3b). Such hyperbolic behavior is again consistent with the minimal kinetic model of a second-order reaction followed by a first-order reaction, as noted above. However, unlike the *Anabaena* proteins, the value of the apparent K_d for the spinach Fd_{red}:FNR_{ox} complex obtained from the kinetic data at high ionic strengths was not significantly affected by the formation of the fully oxidized complex. The constants obtained from the computer analysis of the data in Fig. 3b are included in Table I. The K_d value was found to increase substantially as the ionic strength was raised from 210 to 460 mM (Table I), which demonstrates the importance of electrostatic forces in the formation of the one-electron-reduced spinach Fd_{red}:FNR_{ox} complex, in contrast with the *Anabaena* case (see below for further discussion).

Comparison of the results obtained at 210 and 110 mM indicate that there was little change in apparent K_d. This effect was even more pronounced during experiments performed at 75 mM ionic strength (data not shown), where the observed rate constants were much smaller than those obtained at 110 mM, and no significant deviation from linearity of the k_{obs} vs concentration plot was obtained at FNR concentrations as high as 16 μM. Such results are unexpected for an electrostatically stabilized complex. We thus interpret this to be a consequence of increased competition from the formation of the Fd_{ox}:FNR_{ox} complex. Consistent with this interpretation, the reduction of FNR_{ox} by electron transfer from Fd_{red} at low ionic strengths has been found to be competitively inhibited by the presence of Fd_{ox} (13–15). Furthermore, this inhibition occurs on a comparable time scale to that of Fd_{ox}:FNR_{ox} complex formation (15).

The limiting rate constant for intramolecular electron transfer from Fd_{red} to FNR_{ox} (k_{et}) for the spinach proteins was not appreciably affected by changes in ionic strength (cf. Table I). This result suggests that electrostatic forces do not significantly influence the electron transfer reaction between Fd_{red} and FNR_{ox} once the collision complex is formed, again in contrast with the *Anabaena* results (see below for further discussion).

In the previous experiments of Bhattacharyya *et al.* (11), a decrease in ionic strength from 460 to 310 mM was reported to be accompanied by an appreciable increase in the limiting rate constant for intracomplex electron transfer. These earlier results were most likely due to a higher concentration of phosphate ions used to produce the high ionic strengths, in contrast to the present experiments. In agreement with this, when experiments were performed at 310 mM ionic strength using 100 mM phosphate buffer containing 134 mM NaCl, rather than 4 mM phosphate buffer containing 300 mM NaCl, results comparable to those reported previously were obtained. Thus, we attribute the apparent increase in the rate constant for intracomplex electron transfer reported in the earlier work to a specific effect of phosphate ions, which was counteracted by the increase in NaCl concentration as the ionic strength was raised from 310 to 460 mM (cf. Ref. (11)). The binding of various divalent anions to *c*-type cytochromes, and the resulting alterations in midpoint potential, has been noted previously (32). Thus, it is not unreasonable to suggest that a divalent anion such as phosphate may interact with proteins differently than monovalent anions such as chloride. Cytochrome *c* oxidase is another example of a protein which displays similar divalent ion effects (33, 34).

DISCUSSION

Our analysis of the kinetics of reduction of FNR by Fd has allowed an evaluation of the dissociation constant of the Fd_{red}:FNR_{ox} complex for the spinach proteins. It should be noted, however, that the K_d value for the one-electron reduced complex can, in principle, be quite different from that of the fully oxidized complex. Consequently, the ionic strength dependence displayed by the fully oxidized Fd:FNR complex from spinach (24, 31) may not accurately reflect the importance of electrostatic forces in the formation of the catalytically more relevant one-electron-reduced complex. Figure 4 illustrates the change in K_d as a function of ionic strength for both the fully oxidized (dashed line, derived from data in Ref. (31)) and the Fd_{red}:FNR_{ox} (solid line) complexes from spinach at pH 7.0. For reasons discussed above, the K_d for the Fd_{red}:FNR_{ox} complex obtained at 110 mM ionic strength was not included in this plot, and the value of K_d obtained at 460 mM ionic strength probably has a larger error as-

sociated with it because of the smaller amount of curvature in the data. Despite these limitations, the ionic strength dependence obtained for the Fd_{red}:FNR_{ox} complex is clearly not as pronounced as that observed with the fully oxidized complex. At ionic strengths greater than 10 mM, the K_d of the Fd_{red}:FNR_{ox} complex is smaller than that of the fully oxidized complex. Thus, for the spinach proteins at physiological ionic strengths (i.e., 120–150 mM), the binding of reduced ferredoxin to oxidized FNR is favored over the binding of oxidized ferredoxin. This is consistent with the direction of electron transfer in this system. However, this conclusion is in contrast with previous estimates, obtained from potentiometric titrations (31, 35), in which the K_d for the spinach Fd_{red}:FNR_{ox} complex was suggested to be appreciably larger than for the fully oxidized complex at 11 mM ionic strength. It must be noted, however, that the determination of a K_d for the spinach Fd_{red}:FNR_{ox} complex from potentiometric measurements is complicated by the presence of multiple equilibria and redox-active species. Additionally, potentiometric titrations of the Fd:FNR complex have yielded conflicting results (31, 35, 36).

The limiting rate constant for electron transfer within the *Anabaena* Fd_{red}:FNR_{ox} complex displayed an appreciable ionic strength dependence, increasing to a maximum value as the ionic strength was lowered to 110 mM, and then decreasing as the ionic strength was further lowered from 110 to 10 mM (Table I). Similar biphasic ionic strength behavior has been previously observed for intracomplex electron transfer between cytochrome *c* and cytochrome *c* peroxidase (19, 37), and cytochrome *c* and cytochrome *c* oxidase (20, 38). This has been interpreted in terms of flexibility within the initial collision complex which is required to maximize the rate constant for intracomplex electron transfer (19, 20). Thus, electrostatic forces probably assist in favorably orienting the two *Anabaena* proteins during the formation of the initial collision complex over the ionic strength range from 310 to 110 mM, without providing a major contribution to the stability of the complex. The decrease in rate constant below this ionic strength implies that an *optimal* orientation for electron transfer is only achieved by an additional rearrangement of the proteins within the collision complex. This further structural change is inhibited by the stronger electrostatic interactions which exist at 10 mM ionic strength, i.e., the proteins are "locked" into a less productive orientation. It is noteworthy in this context that covalent cross-linking of a 1:1 complex between *Anabaena* FNR and *Anabaena* flavodoxin leads to a decrease in the rate constant for intracomplex electron transfer from that obtained with the electrostatically stabilized complex at low ionic strength (39), and a similar effect has been obtained with the cytochrome *c*–cytochrome *c* peroxidase (40) and cytochrome *c*–plastocyanin (41) complexes. These may again be due to an inhibition of structural changes within the complexes which are required for optimal overlap between redox centers. Another possible contribution to the ionic strength effects is conformational changes within the FNR which influence its reactivity. Although we cannot completely rule this out, there is no corresponding ionic strength-dependent change in the second-order rate constant for the reduction of free FNR by dRfH$^\bullet$ (see above).

In the case of the spinach Fd:FNR complex, an increase from 110 to 460 mM had little or no effect on the limiting rate constant for electron transfer. This suggests that the relative orientation of the protein components and their respective cofactors must already be optimized with regard to electron transfer within the initial collision complex, i.e., nonproductive complexes are improbable. Therefore, electrostatic forces provide no additional benefit to electron transfer, beyond their contribution to the attractive forces that facilitate the formation of the initial complex.

The difference in the rate constant for intracomplex electron transfer obtained with the *Anabaena* proteins compared to that for the spinach proteins under physiological ionic strength conditions (6500 vs 3000 s^{-1}, respectively) is probably catalytically insignificant. The overall electron transfer process represented by the Fd-dependent reduction of $NADP^+$ is apparently rate-limited by some other step. The turnover number of the FNR isolated from the blue-green algae *Spirulina platensis* has been measured as 350 s^{-1} with respect to the two-electron reduction of $NADP^+$ (13, 14), and a similar value has been reported for spinach FNR (15). Consequently, the rate constants reported here for the intracomplex electron transfer step are much larger than those for catalytic turnover in the production of NADPH.

In summary, for both systems investigated here, it appears that electrostatic forces contribute to the formation of a protein complex that permits electron transfer. However, the role that these forces play clearly differs in the two cases. For the spinach proteins, electrostatic interactions are dominant in the formation and stabilization of an initial collision complex which is already optimized for intracomplex electron transfer. For the *Anabaena* proteins, electrostatic forces only lead to an approximately correct orientation during the formation of the initial collision complex, presumably by disfavoring nonproductive orientations (cf. Ref. (11)). An optimized orientation and a stabilized complex is then achieved by rearrangements favored by nonelectrostatic forces. Elucidation of the nature of these forces requires additional study. Furthermore, it is not possible at present to pinpoint the structural reasons for the differences in properties of these two Fd:FNR systems, and additional structural characterizations will be required in order to accomplish this. Clearly, however, the present results as well as our earlier studies (19, 20) demonstrate that modeling of electron transfer complex structures using computer graphics methods must take into consideration interactions other than charge pairing in order to accurately represent catalytic intermediates.

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