Photochemistry and Photobiology, Vol. 60, No. 3, pp. 231–236, 1994
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0031-8655/93 $05.00+0.00
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# LASER FLASH-INDUCED PHOTOREDUCTION OF PHOTOSYNTHETIC FERREDOXINS AND FLAVODOXIN BY 5-DEAZARIBOFLAVIN AND BY A VIOLOGEN ANALOGUE

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(*Received* 10 *February* 1994; *accepted* 22 *April* 1994)

**Abstract**—Laser flash photolysis has been used to compare the kinetics of reduction of ferredoxin isoforms from the green alga *Monoraphidium braunii*, and the ferredoxin and flavodoxin from the cyanobacterium *Anabaena* PCC 7119, by 5-deazariboflavin semiquinone (dRfH·) and the viologen analogue 1,1′-propylene-2,2′-bipyridyl (PDQ·+). Similar ionic strength-independent second-order rate constants ($1.4 \times 10^8 M^{-1} s^{-1}$) were obtained for the reduction of both algal ferredoxin isoforms by dRfH·. For the reduction of oxidized flavodoxin by dRfH·, a more complex behavior was observed, with a second-order rate constant for dRfH· decay of $1.8 \times 10^8 M^{-1} s^{-1}$, and a first-order (*i.e.* protein concentration independent) rate constant of 450 $s^{-1}$, that probably corresponds to the protonation of the FMN semiquinone cofactor, which occurs subsequent to electron transfer. A value of $5 \times 10^7 M^{-1} s^{-1}$ was obtained for the second-order rate constant of flavodoxin semiquinone reduction by dRfH·. The reduction of ferredoxins and flavodoxin semiquinone by PDQ·+ showed nonlinear protein concentration dependencies, consistent with a minimal two-step mechanism involving complex formation followed by intracomplex electron transfer. A negative ionic strength effect on the kinetic constants was obtained, indicating the existence of attractive electrostatic interactions during electron transfer. With all the ferredoxins the $k_\infty$ values (rate constants extrapolated to infinite ionic strength) for the second-order step of the reduction process (complex formation) are smaller than previously reported for spinach ferredoxin, although *Anabaena* ferredoxin is somewhat more reactive than are the algal ferredoxins with the viologen. In contrast, the $k_\infty$ values for the first-order component of ferredoxin reduction (intracomplex electron transfer) for the algal ferredoxins are comparable to that for spinach ferredoxin, whereas for this reaction the ferredoxin from *Anabaena* has a smaller intrinsic reactivity. As compared with the ferredoxins, *Anabaena* flavodoxin has significantly smaller $k_\infty$ values for its interaction with the viologen analogue, both for complex formation and for electron transfer. In all cases the existence of nonproductive electrostatic interactions between the viologen analogue and the proteins is suggested by the data.

## INTRODUCTION

The [2Fe–2S] chloroplast-type ferredoxins (Fd)† (molecular weight 10 kDa) are one-electron carriers ($E_{m,7} \simeq -400$ mV) that function as electron transfer agents in photosynthesis.[1] In these proteins, the iron–sulfur cluster is located near the surface of the molecule, in a region that carries an overall negative charge, which is highly conserved.[2]

The occurrence of two different molecular species of Fd (Fd I and Fd II) in cyanobacteria, algae and higher plants is now well established.[3–5] They can be distinguished by their different amino acid sequences and isoelectric points. Although several attempts have been made to assign different functions to both isoproteins, until now no conclusive evidence has been obtained in this respect.[6]

Most cyanobacteria and some algae synthesize the flavoprotein flavodoxin (Fld) instead of Fd when grown under iron-deficient conditions. In photosynthetic organisms, flavodoxin is an acidic low molecular weight (20–23 kDa) flavin mononucleotide (FMN)-containing protein ($E_{m,7} = -420$ mV).[7] It has been proposed that Fld can substitute for Fd, under low iron growth conditions, in all reactions in which the iron-containing protein participates.[3,5] It has been further suggested that in such reactions Fld acts as a one-electron carrier by cycling between the semiquinone and hydroquinone forms.[7] Crystal structures of several Fld have been determined, that of the protein from *Anabaena* PCC 7120 being the most recent achievement.[8] In all the structures, the FMN cofactor is located on one side of the molecule with the ribityl phosphate side chain extending toward the center of the protein and solvent access to the flavin being restricted to the dimethylbenzene region of the isoalloxazine ring. The charge distribution in Fld is asymmetric, with most of the negative charge clustered on the surface of the protein, which contains the exposed flavin edge.[8]

Several kinetic studies of the oxidation and reduction reactions of both chloroplast-type Fd and Fld using different

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†*Abbreviations:* dRf and dRfH·, 5-deazariboflavin and its one-electron reduced form; Fd, $Fd_{ox}$ and $Fd_{red}$, ferredoxin and its oxidized and one-electron reduced forms; Fld, $Fld_{ox}$, $Fld_{sq}$ and $Fld_{hq}$, flavodoxin and its oxidized, one-electron and two-electron reduced forms; FMN, flavin mononucleotide; $FMN_{sq}$ and $FMN_{hq}$, FMN cofactor of flavodoxin in its one-electron and two-electron reduced forms; $k_{obs}$, observed rate constant; $PDQ^{2+}$ and PDQ·+, 1,1′-propylene-2,2′-bipyridylium (propylene diquat) and its one-electron reduced radical form.

techniques, including laser flash photolysis, have been reported.[9–14] From these investigations it has been concluded that electrostatic forces play an important role in the electron transfer reactions of these proteins.

The specific aim of the present study was to continue our comparisons of the electron transfer reactivities of pairs of interchangeable redox proteins, as we have done in our previous studies with plastocyanin/cytochrome $c_6$[15,16] and Fd/Fld.[17] In this work we compare the reactivities of the two Fd isoforms of the green alga *Monoraphidium braunii*, as well as of Fd and Fld from the cyanobacterium *Anabaena* PCC 7119, toward the electrically neutral one-electron reduced 5-deazariboflavin radical (dRfH·) and a positively charged viologen analogue, as previously described for spinach Fd.[12] By using both molecules as probes, the electrostatic and steric factors controlling the electron transfer process in those proteins can be investigated. Our results indicate that, although the kinetic properties of the Fd and one-electron reduced Fld ($Fld_{sq}$) are similar to one another toward reduction by dRfH·, oxidized Fld ($Fld_{ox}$) reduction by dRfH· is more complex. Moreover, significant kinetic differences do exist in the interaction of $Fld_{sq}$ with the viologen analogue as compared with Fd.

## MATERIALS AND METHODS

Purification of *M. braunii* Fd I and II was based on the protocol described by Campos *et al.*[18] for cytochrome $c_6$. After the adsorption of the proteins to a DEAE-cellulose column, the column was washed extensively with 0.2 *M* NaCl. Elution of Fd was carried out with a 0.2–0.6 *M* NaCl gradient. Two Fd bands were removed from the column, corresponding to Fd II and Fd I, respectively. Each fraction was pooled, dialyzed and separately chromatofocused in the pH range 4–7. Fractions containing each Fd type were pooled, dialyzed, concentrated on an Amicon pressure dialysis cell and stored at −80°C. For the purest Fd fractions, ratios $A_{420}/A_{270}$ of 0.52 (Fd II) and 0.59 (Fd I) were obtained. Molecular weight and isoelectric point determinations of Fd were carried out as described.[19,20]

*Anabaena* PCC 7119 Fd and Fld were purified according to Pueyo and Gómez-Moreno.[21] An extinction coefficient of $8.8 \times 10^3\ M^{-1}\ cm^{-1}$ at 465 nm was used to determine the concentrations of oxidized Fd. An extinction coefficient of $9.4 \times 10^3\ M^{-1}\ cm^{-1}$ at 465 nm was used in the case of *Anabaena* Fld.[22] The diquat analogue, 1,1′-propylene-2,2′-bipyridylium ($PDQ^{2+}$), was synthesized according to Homer and Tomlinson.[23] 5-Deazariboflavin (dRf) was synthesized using the procedure described by Smit *et al.*[24]

The standard reaction mixture contained, in a final volume of 1.5 mL, 5 m*M* potassium phosphate, pH 7.0, 2 m*M* EDTA, 100 $\mu M$ 5-dRf and the concentration of protein indicated in the figure legends. When noted, $PDQ^{2+}$ (1 m*M*) and NaCl at varying concentrations were added to the reaction mixture.

Laser flash photolysis experiments were performed anaerobically at room temperature as previously described.[12,13] In the absence of protein, the laser flash generated 5-dRfH·, which either disproportionated or, in the presence of $PDQ^{2+}$, rapidly (<1 μs) underwent electron transfer to form $PDQ^{\cdot +}$. In the presence of oxidized protein (or $Fld_{sq}$), electron transfer from dRfH· (in the absence of $PDQ^{2+}$) or from $PDQ^{\cdot +}$ to the protein takes place. The neutral semiquinone form of the Fld was produced by visible light anaerobic photoreduction in the same sample previous to laser flash[25] and was followed spectrophotometrically until all of the $Fld_{ox}$ originally present in the sample was in the semiquinone form. Unless quantitation was required, the number of flashes averaged per kinetic trace varied between 2 and 5. Kinetic traces were adjusted to an exponential curve using a computer-fitting procedure (SIFIT, from OLIS Co.). The estimated error in the rate constants determination was ≤10%.

Nonlinear concentration dependencies for the observed rate constant ($k_{obs}$) values were obtained for the reduction of proteins by $PDQ^{\cdot +}$, implying a mechanism that involved intermediate complex formation preceding electron transfer.[25,26] The second-order rate constants ($k_{12}$) and the limiting first-order ($k_{23}$) rate constants for protein reduction were evaluated from these data by a nonlinear least-squares computer-fitting procedure as described previously.[25] The estimated error in these values is ≤15%. The ionic strength dependencies of the rate constants were fitted with a theoretical model for electrostatic interactions developed by Watkins[27] and previously described[28,29] to obtain an electrostatically corrected rate constant ($k_\infty$) as well as the electrostatic interaction energy ($V_{ii}$) existing between the reactants during the electron transfer reaction. A detailed description of the laser flash apparatus and the method of data collection have been previously published.[30]

## RESULTS

### *Reduction of Fd and Fld by dRfH·*

Reduction of *M. braunii* Fd by dRfH· was monitored by the absorbance decrease at 465 nm, which corresponds to the rapid formation of dRfH· followed by its subsequent reoxidation by $Fd_{ox}$, leading to formation of $Fd_{red}$.[12,14] From the slopes of plots of $k_{obs}$ *vs* $Fd_{ox}$ concentration, a similar second-order rate constant of $1.4 \times 10^8\ M^{-1}\ s^{-1}$ for the reduction of both algal Fd was obtained (data not shown). On the other hand, reduction of *Anabaena* $Fld_{ox}$ by the dRf radical was characterized by complex kinetics at 465 nm, where both $Fld_{ox}$ and dRfH· disappearance are measured. Biphasic absorbance decays were observed, with an initial fast phase followed by a slower further decay to below the preflash baseline (Fig. 1). Consequently, dRfH· decay and Fld reduction were followed separately by measuring absorbance changes at 520 nm (an isosbestic point for Fld at which dRfH· absorbs) and at 600 nm (where it is possible to observe $Fld_{sq}$ formation without appreciable interference from dRfH·).[13] In the presence of $Fld_{ox}$ at low ionic strength (Fig. 1), dRf-sensitized laser photolysis resulted in an initial increase of absorbance at 520 nm followed by an exponential decay to the preflash baseline. These absorbance changes are consistent with the rapid formation of dRfH·, which has positive absorbance in the 450–550 nm region[31] (see below), followed by its subsequent reoxidation by $Fld_{ox}$. As dRfH· disproportionates in the absence of protein to yield the fully oxidized and reduced species, the second-order rate constant for dRfH· oxidation in the presence of $Fld_{ox}$ was obtained from Stern–Volmer plots of dRf radical decay at increasing concentrations of Fld (data not shown). A second-order rate constant of $1.8 \times 10^8\ M^{-1}\ s^{-1}$ was calculated from these data; the $Fld_{ox}$ concentration dependence of the $k_{obs}$ values for dRfH· decay is shown as a Stern–Volmer plot in Fig. 1 (inset).

In the presence of $Fld_{ox}$, laser photolysis resulted in an increase of absorbance at 600 nm (Fig. 1), consistent with the formation of the semiquinone species of Fld as a result of the one-electron reduction of the FMN cofactor in the protein.[13] This process was appreciably slower than the previously mentioned dRfH· decay and was similar to the slow phase of the decay observed at 465 nm. Furthermore, the $k_{obs}$ values were not dependent on $Fld_{ox}$ concentration (inset, Fig. 1), nor on the ionic strength of the buffer (data not shown), but were pH dependent, with $k_{obs}$ values of 1200, 650 and 450 $s^{-1}$ at pH 5, 6 and 7, respectively (see Discussion). We conclude that the biphasic kinetics obtained at 465 nm are the result of the superposition of two different pro-

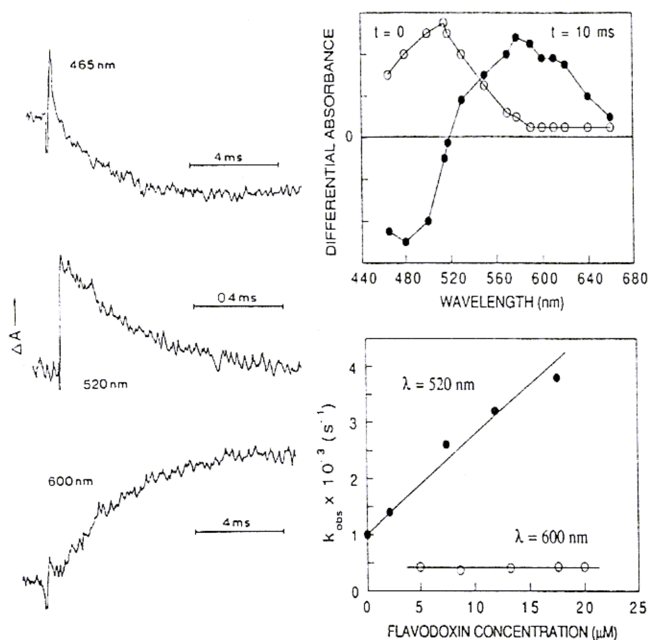



Figure 1. Kinetic traces for the reduction of *Anabaena* Fld by dRfH· at different wavelengths. The Fld concentration was 12 $\mu M$. (Inset) upper, difference spectra of solutions containing dRf/EDTA and Fld at t = 0 (immediately after laser flash) and t = 10 ms; Fld concentration was 20 $\mu M$. (Inset) lower, protein concentration dependence of $k_{obs}$ for dRfH· decay measured at 520 nm and for $Fld_{sq}$ formation measured at 600 nm. The buffer conditions were: 10 m$M$ potassium phosphate, pH 7.0, containing 2 m$M$ EDTA, 100 $\mu M$ dRf and different concentrations of Fld.

cesses: the initial fast dRfH· decay and the slower subsequent formation of the blue $FMN_{sq}$ cofactor of Fld (see below). From the plot of $k_{obs}$ *vs* $Fld_{ox}$ concentration, a first-order rate constant of 450 $s^{-1}$ was determined for this latter process. Figure 1 (inset) also shows time-resolved difference spectra of dRf/Fld solutions at t = 0 (just after the laser flash) and t = 10 ms (when the kinetic traces are stabilized). From these spectra it is clear that the initial dRfH· formation, measured in the 450–550 nm region,[31] is followed by a slower appearance of the $FMN_{sq}$ cofactor of Fld, the latter showing the expected loss of absorbance at around 480 nm, an isosbestic point around 520 nm and an absorbance maximum around 580 nm.[13]

The $Fld_{sq}$ reduction by 5-dRfH· was monitored as an absorbance decrease at 600 nm, a wavelength at which 5-dRfH· has negligible absorption. Again, the decay of the dRf radical was monitored at 520 nm, where its absorbance predominates over the changes due to $Fld_{sq}$ reduction. In contrast to what is observed for $Fld_{ox}$, at both wavelengths we observe equivalent monoexponential kinetics that depend linearly on $Fld_{sq}$ concentration (data not shown). We attribute this to the one-electron reduction of the $FMN_{sq}$ by 5-dRfH·, with a second-order rate constant of 5 × $10^7$ $M^{-1}$ $s^{-1}$ for this process.

*Reduction of ferredoxins by PDQ·⁺*

In the presence of an excess of $PDQ^{2+}$, all the dRfH· generated by the laser flash reacts with the viologen analogue resulting in the formation of $PDQ^{\cdot +}$. The Fd reduction by

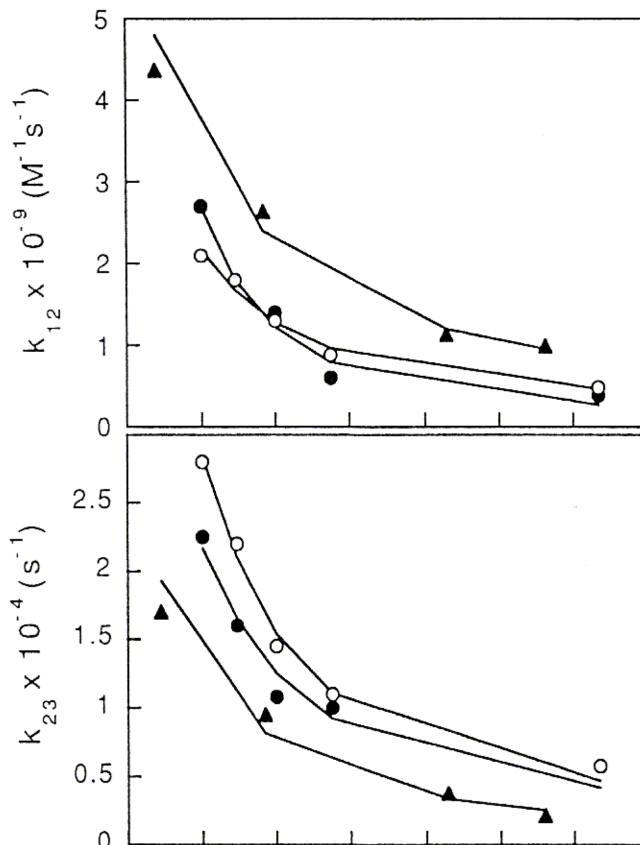
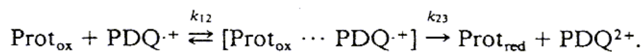



Figure 2. Effect of ionic strength on the (upper) second- ($k_{12}$) and (lower) first-order ($k_{23}$) rate constants for the electron transfer reaction from $PDQ^{\cdot +}$ to Fd. (●) *Monoraphidium braunii* Fd I; (○) Fd II; (▲) *Anabaena* Fd.

this latter radical could be followed by the absorbance decrease at 465 nm, where the contribution of $PDQ^{\cdot +}$ is relatively small.[12] A nonlinear $Fd_{ox}$ concentration dependence of $k_{obs}$ for the reduction of Fd was observed with the three proteins; in all cases, the observed rate constants decreased as the ionic strength increased (data not shown). These results are closely analogous to those previously observed with the spinach protein,[12] which have been interpreted in terms of the following minimal two-step mechanism:



$$Prot_{ox} + PDQ^{\cdot +} \overset{k_{12}}{\rightleftarrows} [Prot_{ox} \cdots PDQ^{\cdot +}] \overset{k_{23}}{\longrightarrow} Prot_{red} + PDQ^{2+}.$$

By fitting the data to this model it is possible to obtain individual values for the second-order rate constant of complex formation $k_{12}$ (a minimal value is obtained by this procedure), and the limiting first-order rate constant for intracomplex electron transfer $k_{23}$.[25] The ionic strength dependencies of the $k_{12}$ and $k_{23}$ values obtained from this analysis are plotted in Fig. 2. Using the electrostatic model of Watkins and coworkers,[27–29] it is possible to fit the ionic strength dependence data of Fig 2 (theoretical fits are shown as solid lines). Extrapolation of these theoretical fits to infinite ionic strength leads to values for $k_\infty$ (Table 1), which represent the intrinsic rate constants under conditions of negligible electrostatic effects.[28] The data analysis procedure also allows us to obtain an estimate for the electrostatic interaction en-

Table 1. Electrostatic analysis of the ionic strength dependence of the reduction of ferredoxins and flavodoxins by PDQ·+*

| Protein | $k_{12}$ ($M^{-1}$ $s^{-1}$) | $V_{ii}$ (kcal $mol^{-1}$) | $k_{23}$ ($s^{-1}$) | $V_{ii}$ (kcal $mol^{-1}$) |
|---|---|---|---|---|
| *Monoraphidium* Fd I | $1.3 \times 10^8$ | −5.9 | 2650 | −4.1 |
| *Monoraphidium* Fd II | $2.9 \times 10^8$ | −3.9 | 2800 | −4.5 |
| *Anabaena* Fd | $5.0 \times 10^8$ | −3.4 | 1100 | −4.3 |
| *Anabaena* $Fld_{sq}$ | $2.8 \times 10^7$ | −6.9 | 490 | −7.3 |
| Spinach Fd† | $1.3 \times 10^9$ | −2.5 | 2300 | −4.8 |

*In analyzing the rate constants *vs* ionic strength data, the following parameters were used: *n* (the radius of the active site) = 5.5 Å, $r_{12}$ (the distance between reactants in the intermediate complex) = 3.5 Å, $D_\epsilon$ (the dielectric constant within the interaction domain) = 50. See Tollin *et al.*[28] for further details.
†Data from Navarro *et al.*[12]

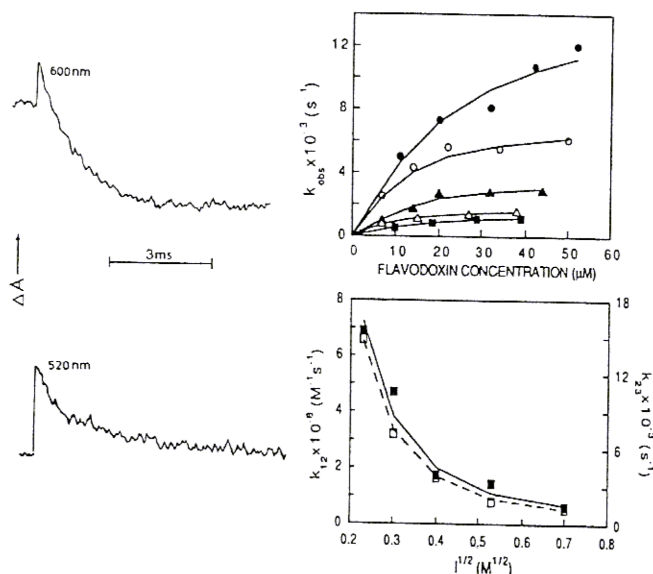



Figure 3. Kinetic traces for the reduction of *Anabaena* $Fld_{sq}$ by PDQ·+ at 520 and 600 nm. (Inset) upper, dependence of $k_{obs}$ for *Anabaena* $Fld_{sq}$ reduction upon protein concentration at different ionic strengths. Ionic strength values were (●) 53 m*M*; (○) 91 m*M*; (▲) 161 m*M*; (△) 281 m*M*; (■) 491 m*M*. Lower, effect of ionic strength on the (■) second- ($k_{12}$) and (□) first-order ($k_{23}$) rate constants for the electron transfer reaction.

ergy between the reactants during electron transfer, $V_{ii}$, which provides information on the strength of the interaction (Table 1).[28]

### *Anabaena* flavodoxin semiquinone reduction by PDQ·+

Flavodoxin semiquinone reduction by the viologen radical was followed both at 520 nm (where absorbance due to the viologen analogue predominates) and at 600 nm (where disappearance of $Fld_{sq}$ can be monitored). At 600 nm, an initial rapid increase and subsequent monoexponential bleaching of absorbance was obtained (Fig. 3), the latter being interpreted as a consequence of the one-electron reduction of the $FMN_{sq}$ cofactor of Fld by PDQ·+. The kinetics of the absorbance decrease for the decay of PDQ·+ at 520 nm were equivalent to those observed at 600 nm (Fig. 3), indicating that both correspond to the same process, *i.e.* the direct reduction of the $FMN_{sq}$ cofactor by PDQ·+. As can be seen in Fig. 3, the observed rate constants for $Fld_{sq}$ reduction (measured at 600 nm) showed a nonlinear protein concentration dependence and a decrease with increasing ionic strength. This again indicates the occurrence of a (minimal) two-step mechanism involving electrostatic-stabilized complex formation followed by intracomplex electron transfer. Theoretical fits obtained according to this mechanism are shown as solid lines in Fig. 3.

The ionic strength dependence of the $k_{12}$ and $k_{23}$ values obtained from the theoretical analysis are also plotted in Fig. 3. Extrapolation of these theoretical fits leads to the estimation of the respective rate constants at infinite ionic strength and the calculation of values for $V_{ii}$ (Table 1).

## DISCUSSION

Two isoforms of Fd (Fd I and Fd II) can be obtained from the green alga *M. braunii* by a purification procedure based upon their differential affinity to anionic exchange columns. Although the Fd have similar molecular weights of 12.3 (Fd I) and 11.6 kDa (Fd II), they have different pI values of 3.6 and 4.5, for Fd I and II, respectively. The second-order rate constant ($1.4 \times 10^8$ $M^{-1}$ $s^{-1}$) obtained for the reduction of both Fd by dRfH· at low ionic strength compares well with those previously reported for spinach ($2.0 \times 10^8$ $M^{-1}$ $s^{-1}$) and *Anabaena* ($1.6 \times 10^8$ $M^{-1}$ $s^{-1}$) Fd.[14,32] This is consistent with their similarities in structure and redox potential.

In a previous less extensive study of the reduction of *Anabaena* $Fld_{ox}$ by dRfH·, a second-order rate constant of $1.6 \times 10^8$ $M^{-1}$ $s^{-1}$ was reported, with only simple monoexponential kinetics being noted.[13] This contrasts with the results obtained in the present work, where a more complex kinetic behavior was observed. The second-order rate constant determined here for the disappearance of dRfH· (presumably due to electron transfer to $Fld_{ox}$) is $1.8 \times 10^8$ $M^{-1}$ $s^{-1}$, which is closely similar to that previously reported[13] and comparable to the values determined for Fd reduction by dRfH·. However, a protein concentration-independent first-order rate constant of 450 $s^{-1}$ for the formation of the $FMN_{sq}$ cofactor (as clearly deduced from the final difference spectrum after the laser flash) was also determined. These results strongly indicate the existence of two different processes for $Fld_{ox}$ reduction by dRfH·: (1) a rapid second-order redox reaction of the dRfH· with the protein; and (2) a slow protein concentration-independent generation of the blue $FMN_{sq}$ form. This latter process was not previously observed because $Fld_{ox}$ reduction was mainly followed at 465 nm.

As noted above, these slow kinetics at 600 nm, which were not observed for the reduction of $Fld_{sq}$ by 5-dRfH·, were shown to be pH dependent, with first-order rate constant values of 1200, 650 and 450 $s^{-1}$ at pH 5, 6 and 7, respectively. We thus attribute this process to be the result of a fast initial formation of the red $Fld_{sq}$ anion by reduction with 5-dRfH· (with no absorbance increase at 600 nm) and its subsequent slower protonation to the blue neutral semiquinone form. Anderson *et al.*[33] have reported rate constants of $2.6 \times 10^5$ $s^{-1}$ and $1.1 \times 10^5$ $s^{-1}$ for the protonation of the bacterial

*Megasphaera eldsenii* flavodoxin at pH 6.1 and 9.15, respectively, in anaerobic solutions under pulse radiolysis. In this work we have not carried out a more extensive pH study, inasmuch as the pH-dependent redox potential changes of Fld make it difficult to determine accurately the $pK_a$ value for the protonation process. Slow kinetics similar to those obtained here with *Anabaena* Fld were observed for the reduction of *Synechocystis* 6803 $Fld_{ox}$ by $dRfH^{\cdot}$ (data not shown), which could indicate that the protonation kinetics are of similar order of magnitude in cyanobacterial Fld.

The observed second-order rate constant for the reduction of $Fld_{sq}$ by 5-$dRfH^{\cdot}$ is about three times smaller than that for $Fld_{ox}$ reduction. This is in agreement with the differences in redox potential between the $Fld_{ox}/Fld_{sq}$ ($E_{m,7} = -212$ mV) and $Fld_{sq}/Fld_{hq}$ ($E_{m,7} = -436$ mV) couples.[34] Kinetic interferences at 600 nm arising from the $Fld_{sq}$ protonation process made it impossible to follow *Anabaena* $Fld_{ox}$ reduction by $PDQ^{\cdot+}$ at this wavelength, although $Fld_{sq}$ reduction by the viologen analogue was easily measured.

The negative ionic strength dependencies and nonlinear protein concentration effects on the kinetic constants for reduction of all of the Fd and Fld by $PDQ^{\cdot+}$ demonstrate a plus–minus attractive electrostatic interaction between the electron donor and the proteins, leading to the formation of an electrostatically stabilized transient complex. Similar behavior has been previously reported for spinach Fd reduction by this viologen analogue,[12] suggesting that the functional electron transfer site in all Fd and $Fld_{sq}$ carries an appreciable negative charge. In this respect, it is well known that Fd and Fld form electrostatically stabilized complexes with both photosystem I[17,35] and ferredoxin-$NADP^+$ reductase.[13,14] The differences in $k_\infty$ values for the second-order complex formation reaction for the four proteins investigated here, as well as for spinach Fd, indicate a more effective association process between spinach Fd and $PDQ^{\cdot+}$ as compared with the algal and cyanobacterial Fd and Fld, although the *Anabaena* Fd is more efficient in this regard than are its own Fld and *Monoraphidium* Fd. It is also worth noting that *Anabaena* $Fld_{sq}$ is appreciably less effective in complex formation with $PDQ^{\cdot+}$ than the other proteins. The differences in $k_\infty$ values for the first-order component of protein reduction indicate a lower intrinsic reactivity of both *Anabaena* proteins toward the electron donor, which is again especially clear in the case of Fld. We presume that structural factors that lead to steric effects and the existence of possible nonoptimal interaction sites for the electron transfer are responsible for these reactivity differences (see below).

The higher $V_{ii}$ values ($-5.9$ and $-6.9$ kcal $mol^{-1}$) obtained for the ionic strength dependencies of $k_{12}$ in *Monoraphidium* Fd I and *Anabaena* Fld as compared with the other Fd suggest that a larger negative electrostatic charge exists close to the iron–sulfur cluster and the flavin cofactor in these proteins. In contrast, spinach Fd yields the lowest value for $V_{ii}$ ($-2.5$ kcal $mol^{-1}$)[12] and thus probably has the smallest negative charge close to the redox center. The values for $V_{ii}$ calculated from the ionic strength dependence of $k_{23}$ are similar in all of the Fd studied (*ca* $-4.5$ kcal $mol^{-1}$) but smaller than the value obtained for *Anabaena* Fld ($-7.3$ kcal $mol^{-1}$). This suggests that the electrostatic potential distribution in regions away from the iron–sulfur cluster and the number of possible nonoptimal sites are similar in all these Fd.[12,32] In the case of *Anabaena* $Fld_{sq}$, with a low $k_\infty$ value and higher $V_{ii}$ for the first-order electron transfer, it is possible that the viologen analogue could interact with $Fld_{sq}$ at a site relatively distant from the exposed FMN edge. Consistent with this, the electrostatic map of *Anabaena* 7120 Fld deduced from its crystal structure shows the existence of regions of negative charge located away from the FMN exposed edge (data not shown).

In summary, the above results allow us to conclude that the electron transfer properties of the different Fd and Fld with respect to small nonphysiological reductants are qualitatively quite similar, which in the case of the *Anabaena* proteins is in good agreement with their equivalent physiological role. Quantitative differences do exist, which most probably reflect structural and steric differences between the redox sites. In addition, *Anabaena* Fld shows a complex mechanism of electron transfer from $dRfH^{\cdot}$ probably due to further protonation of the FMN cofactor after an initial tron transfer. For the reduction of this latter protein by $PDQ^{\cdot+}$, differences both in complex formation and electron transfer reflect a smaller intrinsic reactivity of this flavoprotein as compared with Fd.

*Acknowledgements*—This work was supported by grants from the Dirección General de Universidades e Investigación (SEUI PB90-0099 to M.A.R.), Junta de Andalucía (PAI3182 to M.A.R.), Comisión Asesora de Investigación Científica y Técnica (BIO91-1124 to C.G.M.), National Institutes of Health (DK15057 to G.T.) and NATO (joint project No. CRG.900065). We thank Dr. H. Bottin for the gift of *Synechocystis* Fld.

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