Laser Flash Absorption Spectroscopy Study of Flavodoxin Reduction by Photosystem I in *Synechococcus* sp. PCC 7002[†]

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ABSTRACT: The photoreduction of flavodoxin by trimeric photosystem I, both from the cyanobacterium *Synechococcus* sp. PCC 7002, was investigated by flash absorption spectroscopy. After addition of flavodoxin in darkness, single flash experiments show that the transient signals change between individual flashes. This behavior is assigned to a progressive accumulation of flavodoxin semiquinone, which is relatively stable under most experimental conditions. Different conditions were devised in order to study the reduction of the oxidized and semiquinone forms of flavodoxin separately. Both processes were identified by their differential spectra measured between 460 and 630 nm. Detailed kinetic characteristics of flavodoxin reduction were obtained at pH 8.0 in the presence of salts. The kinetics of reduction of oxidized flavodoxin displays a single-exponential component. The rate of this component increases with the flavodoxin concentration up to an asymptotic value of about 600 s^{-1}. The semiquinone form of flavodoxin being protonated, this rate corresponds to a rate-limiting reaction which could be either an electron transfer reaction or a protonation reaction. In contrast, the reduction of flavodoxin semiquinone is biphasic. A fast first-order phase with $t_{1/2} \approx 10$ μs is interpreted as an electron transfer process within a preformed complex. A dissociation constant of 2.64 μM is calculated for this complex by assuming a simple binding equilibrium between photosystem I and flavodoxin semiquinone. The slower phase observed for semiquinone reduction is concentration dependent, and a second-order rate constant of 1.7×10^8 M^{-1} s^{-1} is calculated. For both one-electron reduction steps, different optimal salt concentrations are observed indicating slightly different interactions between photosystem I and flavodoxin in its oxidized and semiquinone states.

Photosystem I (PSI)[1] is a light-driven cytochrome c_6 (plastocyanin)−ferredoxin (flavodoxin) oxidoreductase. Together with photosystem II and the cytochrome b_6/f complex, this complex constitutes the membrane-bound part of the photosynthetic electron transport chain found in photosynthetic membranes of oxygen-evolving phototrophic organisms. Cyanobacterial PSI reaction centers contain at least eleven different polypeptidic subunits (Golbeck, 1994) and can be isolated in trimeric as well as in monomeric forms. A X-ray structure with a 6 Å resolution is available for PSI from the cyanobacterium *Synechococcus elongatus* (Krauss et al., 1993). The core of the complex is formed by the PsaA−PsaB heterodimer. Besides the light-harvesting pigments, this core contains all cofactors involved in electron transfer within PSI with the exception of the terminal acceptors F_A and F_B which are bound to the PsaC subunit resembling bacterial-type 2[4Fe−4S] ferredoxins (Zhao et al., 1992).

The soluble electron acceptor of PSI is a [2Fe−2S] soluble ferredoxin (Matsubara & Saeki, 1992) which is involved in many metabolic processes such as carbon, nitrogen, and sulfide assimilation (Knaff & Hirasawa, 1991). In most cyanobacteria, ferredoxin can be replaced under iron deprivation conditions by flavodoxin, an FMN containing protein (Rogers, 1987). Flavodoxins are widely distributed throughout the bacterial kingdom (Mayhew & Tollin, 1992) and, in addition, can also be found in green (Zumft & Spiller, 1971; Peleato et al., 1994) and red algae (Fitzgerald et al., 1978; Price et al., 1991). They are usually classified according to size in short- (16−18 kDa) and long-chain (18−20 kDa) types (Rogers, 1987; Mayhew & Tollin, 1992), the cyanobacterial and algal proteins belonging exclusively to the latter (Laudenbach et al., 1988; Wakabayashi et al., 1989; Fillat et al., 1991; Leonhardt & Straus, 1992). Flavodoxins are capable of performing two subsequent one electron transfer reactions thus shuttling the redox active FMN group between an oxidized form (Fld), a thermodynamically stabilized protonated semiquinone ($FldH^{\bullet}$) and a fully reduced hydroquinone form ($FldH^-$) (Mayhew & Ludwig, 1975). For flavodoxins found in photoautotrophic organisms, the corresponding redox potentials are in the −180/−240 mV region for the Fld/$FldH^{\bullet}$ couple (at pH 7) and in the −370/−470 mV region for the $FldH^{\bullet}$/$FldH^-$ couple (Sykes & Rogers, 1984). Though it has been suggested that flavodoxin may not be able to replace ferredoxin in all of its functions (Van der Plas et al., 1988), flavodoxin can react efficiently with PSI, ferredoxin−$NADP^+$ reductase and hydrogenase (Bothe, 1969; Mayhew & Ludwig, 1975; Fitzgerald et al., 1980; Fillat et al., 1988). The ability of flavodoxin to donate electrons to nitrogenase (Bothe & Neuer, 1988; Fillat et al., 1988) was

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[1] Abbreviations: PSI, photosystem I; FMN, flavin mononucleotide; Fld, oxidized form of flavodoxin; $FldH^{\bullet}$, semireduced form of flavodoxin; $FldH^-$, fully reduced form of flavodoxin; β-DM, *n*-dodecyl β-D-maltoside; DPIP, 2,6-dichlorophenolindophenol.

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recently questioned (Razquin et al., 1995). With regard to its function as a ferredoxin substitute, it is generally assumed, on the basis of their relative redox potentials, that flavodoxins shuttle between the semiquinone and the fully reduced forms under the physiological conditions of the cell, although this has not been thoroughly investigated (Rogers, 1987). The first wave of flavodoxin reduction (from Fld to $FldH^{\bullet}$) by PSI has been recently reported (Medina et al., 1992). In the present study, we report the observation of both PSI-mediated reduction steps of flavodoxin reduction by flash absorption spectroscopy. Both reaction partners were purified from the marine cyanobacterium *Synechococcus* sp. PCC 7002.

MATERIALS AND METHODS

Biological Materials. Flavodoxin from *Synechococcus* sp. PCC 7002 (Leonhardt & Straus, 1992) was obtained by overproduction in *Escherichia coli* using the pSE280 expression vector (Brosius, 1989). Flavodoxin was purified essentially following the procedure described by Fillat et al. (1991). Full reconstitution with FMN was achieved by incubation of the preparation with 5 mM FMN for 3−4 days at a flavodoxin concentration of 500 μM. Excess of cofactor was subsequently removed by gel filtration. A spectral ratio $A_{276}/A_{466} \approx 0.19$ was routinely obtained. PSI from *Synechococcus* sp. PCC 7002 was isolated essentially by the procedure described by Golbeck *et al*. (1988), except that Triton X-100 was substituted by 0.03% (w/v) β-dodecyl maltoside (β-DM) in the sucrose-gradient centrifugation step. The trimeric status of the preparation was confirmed by HPLC gel filtration.

Difference Spectra of Flavodoxin. The spectra of flavodoxin from *Synechococcus* sp. PCC 7002 were recorded from 350 to 700 nm in 1 × 1 cm cuvettes with flavodoxin concentrations of about 35 μM. The neutral semiquinone form of flavodoxin ($FldH^{\bullet}$) was obtained at pH 5.8 by adding 10 mM sodium dithionite and 0.5 μM benzyl viologen, and the fully reduced form ($FldH^{-}$) was obtained at pH 9.1 by adding 10 mM sodium dithionite and 0.5 μM methyl viologen. Contributions of dithionite and the viologens were subtracted from the spectra. The differential absorption coefficients were calculated assuming an absorption coefficient of 9500 M^{-1} cm^{-1} for oxidized flavodoxin at 466 nm (Hutber *et al.*, 1981). With this last assumption, a value of 3900 M^{-1} cm^{-1} was measured for the absorption coefficient of the semiquinone at 580 nm.

Flash Absorption Spectroscopy. Measurements were made at 296 K with a microsecond time resolution as described previously (Sétif & Bottin, 1994). Square cuvettes (1 × 1 cm) were used. The repetition rate of laser flash excitation used for averaging was 0.10−0.17 Hz. The measuring wavelength was selected with two interference filters placed before and after the cuvette. In order to minimize actinic effects of the measuring light during measurements in the visible region, a shutter was placed in front of the cuvette and opened 1 ms before flash excitation. Kinetic data were fitted to a multiexponential decay with a Marquardt least-squares algorithm. The PSI concentration of the samples was calculated from the photoinduced absorption changes at 820 nm assuming an absorption coefficient of 6500 M^{-1} cm^{-1} for $P700^{+}$ at this wavelength (Mathis & Sétif, 1981). The absorption coefficients measured in the visible region were also calculated by reference to the signals measured at 820 nm on the same sample.

For observing the second wave of flavodoxin reduction, it was necessary to preilluminate the sample containing PSI and flavodoxin, so that flavodoxin is in its semireduced state before recording the flash absorption signals. For small flavodoxin concentrations (≤ 1 μM), a series of preflashes was found to be sufficient for this purpose. However, for larger flavodoxin concentrations, a pretreatment with continuous illumination was used. As a control experiment, the absorption at 580 nm was measured in samples containing 20 μM flavodoxin within the flash absorption setup just after the illumination period, and it was found that the whole content of flavodoxin is reduced to semiquinone. After the illumination pretreatment, a few seconds of dark adaptation was found to be sufficient for reoxidation of fully reduced flavodoxin to the semiquinone form. This was checked by varying the duration of this dark adaptation period: the flash absorption signal was generally found to remain the same between 2−3 and 30 s after the end of illumination. It was also checked that the signal was not changed when using different repetition rates of flash excitation, thus ensuring that fully reduced flavodoxin is reoxidized (presumably by dissolved oxygen) to semiquinone between two consecutive flashes. The conditions used for measuring the spectra of the two waves of flavodoxin reduction by PSI are detailed in the Results section and in the legend of Figure 2.

The differential spectrum corresponding to the one-electron reduction of (F_A, F_B) was obtained as described in Sétif and Bottin (1995) following Ke (1973). The calculated spectra corresponding to electron transfer from $(F_A, F_B)^{-}$ to either oxidized or semireduced flavodoxin (as displayed in Figure 2) were obtained by adding the $[(F_A, F_B) - (F_A, F_B)^{-}]$ spectrum to either the ($FldH^{\bullet}$ − Fld) spectrum or the ($FldH^{-}$ − $FldH^{\bullet}$) spectrum.

RESULTS

Figure 1 shows the flash-induced absorption changes that are elicited at 580 nm in trimeric PSI reaction centers isolated from the cyanobacterium *Synechococcus* sp. PCC 7002 either in the absence of flavodoxin (traces 0) or after the addition of flavodoxin from the same organism. The wavelength of 580 nm is appropriate for observing absorption changes due to flavodoxin since it corresponds to the absorption maximum of the semiquinone form of the FMN cofactor. This semireduced form has been shown to be protonated at position N(5) (Müller et al., 1970; Entsch & Smillie, 1972; Mayhew & Ludwig, 1975) and will be thereafter designated as $FldH^{\bullet}$. In the absence of flavodoxin, light excitation eventually leads to charge separation between $P700^{+}$ and the terminal acceptor, either F_A^{-} or F_B^{-}, within a few hundreds of nanoseconds (Sétif & Brettel, 1993; Sétif & Bottin, 1994, 1995; Leibl et al., 1995). Formation of these two species corresponds to the initial absorption decrease which is observed for both traces 0. Some fast decaying signals ($t_{1/2} < 15$ μs) can also be observed on a faster time scale (not shown), which are due to some antenna triplet states. The slow decay observed on a 60 ms time scale (Figure 1) is mostly due to a recombination reaction between $P700^{+}$ and $(F_A, F_B)^{-}$. The two series of experiments shown in Figure 1 correspond to similar PSI concentrations (0.26 μM) but to different amounts of flavodoxin which were added in darkness (0.75 μM for part A and 5.62 μM for part B). The first few flashes following addition of flavodoxin were recorded separately and are shown with their corresponding flash numbers. An averaged signal is also shown

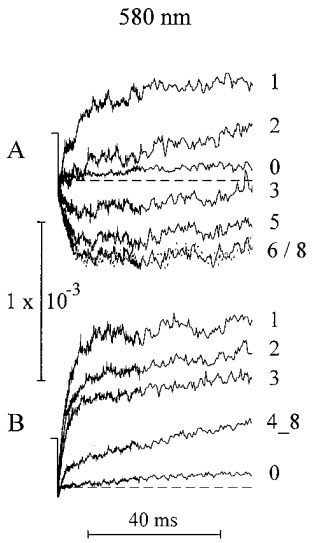

FIGURE 1: Flash-induced absorption changes measured with PSI trimers from *Synechococcus* sp. PCC 7002 at 580 nm in the presence or in the absence of flavodoxin from the same organism. Two series of experiments are shown with flavodoxin concentrations of 0.75 μM (part A) and 5.62 μM (part B). The PSI reaction centers (concentration of 0.26 μM) were suspended in 50 mM Tricine, pH 8.0, in the presence of 0.03% β-DM, 30 mM NaCl, 5 mM $MgCl_2$, 1 mM sodium ascorbate, and 20 μM DPIP. Traces 0 were recorded in the absence of flavodoxin. Flavodoxin was then added in darkness, and single-flash experiments were recorded (trace 1, first flash; trace 2, second flash, etc.). In part A, trace 6 is shown in dotted line in order to distinguish it from trace 8 which is very similar. In part B, trace 4−8 is the average of signals recorded from the fourth to the eighth flashes. During each series of experiments, the repetition rate of the laser flash excitation was 0.17 Hz.

corresponding to the fourth to eighth flashes in part B. It is immediately apparent that the signals change from one flash to the next, though the time interval between two consecutive flashes is as large as 6 s. When compared to the reference sample (traces 0), the first flash gives rise to a large absorption increase and the size of this absorption change is decreased for the second flash. For a small flavodoxin concentration (part A), the next flashes give rise to an absorption decrease by comparison to the reference sample. From the sixth flash, the signal becomes reproducible (maximum amount of absorption decrease) as it can be seen from the comparison between the sixth and eighth flashes (traces 6 and 8 in part A). When the flavodoxin concentration is much higher than the PSI concentration (part B), the absorption changes due to flavodoxin decrease in size with the flash number but stay always positive under the present experimental conditions. Control experiments were performed at 820 nm for recording the decay of $P700^+$ either in the absence or the presence of flavodoxin (not shown). These experiments show that flavodoxin addition inhibits the recombination reaction between $P700^+$ and $(F_A, F_B)^-$, as can be expected if flavodoxin is reduced from $(F_A, F_B)^-$.

The above experiments can be easily interpreted by assuming that the semiquinone form of flavodoxin ($FldH^\bullet$) is rather stable under our experimental conditions. The first flash thus corresponds to reduction of oxidized flavodoxin and formation of $FldH^\bullet$. This semiquinone form then accumulates along a series of successive flashes and competes with oxidized flavodoxin for electrons from PSI. When there is only a slight excess of flavodoxin over PSI (part A), it is possible to accumulate almost 100% of semiquinone within a few flashes. The semiquinone is then reduced by PSI which leads to an absorption decrease at 580 nm. It was also checked that under these conditions, fully reduced flavodoxin is completely reoxidized to the semiquinone form, presumably by dissolved oxygen, between two consecutive laser flashes (see Materials and Methods). When flavodoxin is in large excess over PSI (part B), complete accumulation of $FldH^\bullet$ during a flash series is not possible due to the competing process of semiquinone reoxidation, presumably by dissolved oxygen. Under such conditions, semireduced flavodoxin competes with oxidized flavodoxin for oxidizing $(F_A, F_B)^-$. This leads to some decrease in signal size, the reduction of oxidized flavodoxin remaining the dominant process. This interpretation can be further substantiated by a spectral analysis of the two processes of flavodoxin reduction, as described below.

For making this spectral characterization, two different types of experimental conditions were devised in order to promote one wave of flavodoxin reduction by PSI (reduction of either the oxidized form or the semireduced form) at the expense of the other. For studying the reduction of oxidized flavodoxin, the protein was added in complete darkness and a very small amount of PES (0.1 μM) was added to the sample. The redox potential of this redox mediator (+50 mV at pH 7) is such that it should be partially oxidized in the presence of ascorbate. Under these conditions, the signal was found to be reproducible and identical to the first flash signal (after addition of flavodoxin in darkness) when one flash is given to the sample every 10 min. This delay between two flashes appears to be necessary for complete reoxidation of semireduced flavodoxin. The reoxidation process is probably predominantly mediated by oxidized PES, as a larger than 30 min delay between two consecutive flashes is necessary in its absence. In order to investigate the effect of PES, control experiments were performed under identical conditions in the absence of flavodoxin. These experiments show that PES can accept electrons from the iron−sulfur centers of PSI (not shown). This process can be very efficient at high PES concentrations (not shown). However, under the conditions used for the spectral analysis described below (0.1 μM PES and 5.0 μM flavodoxin), this process is much slower ($t_{1/2}$ > 20 ms) than flavodoxin reduction which appears to be monophasic ($t_{1/2} \approx 2.3$ ms), so that little interference due to direct electron transfer from $(F_A, F_B)^-$ to PES is expected.

The upper spectrum of Figure 2 corresponding to the photoreduction of oxidized flavodoxin by PSI (closed circles) was obtained with a large excess of flavodoxin over PSI under the experimental conditions described above. The spectrum was obtained from the difference between the absorptions measured at 12 ms and 100 μs after the flash. 12 ms after flash excitation, flavodoxin reduction is fully completed, and 100 μs after the flash, this process has hardly began whereas the antenna triplet states have fully decayed. An absorption increase (e.g., at 580 nm) is plotted as a positive amplitude. The vertical scale is calibrated after measuring $P700^+$ at 820 nm on the same sample (see Materials and Methods). The spectrum corresponding to electron transfer from $(F_A, F_B)^-$ to oxidized flavodoxin was independently calculated from the ($FldH^\bullet$ - Fld) spectrum and the differential spectrum corresponding to the one-electron reduction of (F_A, F_B) (see Materials and Methods). It is shown as a dashed line after multiplication by a scaling factor of 0.7 which gives similar absorption coefficients for the two spectra at 580 nm.

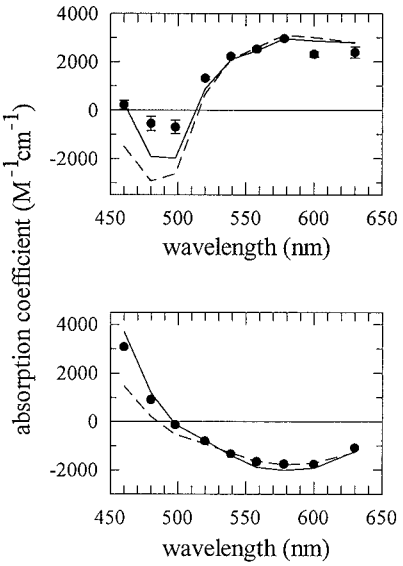

FIGURE 2: Spectra recorded between 460 and 630 nm corresponding to flavodoxin reduction. For an easier comparison of these spectra to the data of Figures 1 and 3, an absorption increase (decrease) is shown as a positive (negative) signal. Upper part: difference spectrum for the first wave of flavodoxin reduction (closed circles). Lower part: difference spectrum for the second wave of flavodoxin reduction (closed circles). The spectra are compared to the spectra calculated for these two waves (dashed lines) from the independent measurement of the differential spectra of (F_A, F_B) and both waves of flavodoxin reduction. The spectra expected for 100% oxidation of (F_A, F_B)$^-$ and 65% reduction of flavodoxin are also shown for the two waves of flavodoxin reduction (continuous lines). For both spectra, PSI reaction centers ($\approx$ 0.26 μM) were dissolved in 50 mM Tricine, pH 8.0, in the presence of 0.03% β-DM and 2 mM sodium ascorbate. Conditions for the first-wave spectrum:5.0 μM flavodoxin, 10 mM NaCl, 10 mM $MgCl_2$, 25 μM DPIP, and 0.1 μM PES; single-flash experiments were recorded, and the sample was kept in darkness for 10 min between two consecutive flashes. Conditions for the second-wave spectrum : 0.75 μM flavodoxin, 30 mM NaCl, 5 mM $MgCl_2$ and 5 μM DPIP; at each wavelength, seven preflashes were given to the sample and the average of 8 flashes was then recorded (repetition rate of flash excitation = 0.1 Hz).

As a prerequisite for measuring the second wave of flavodoxin reduction, flavodoxin must be completely reduced to its semiquinone form prior to the spectroscopic investigation. As suggested from the results shown in Figure 1A, this can be achieved by a series of preflashes in case that flavodoxin is present in only a small excess over PSI. After a series of seven preflashes, the observed signal is reproducible from flash to flash under these conditions and signal averaging can be performed. As discussed above, this behavior can be ascribed to complete accumulation of the flavodoxin semiquinone whereas fully reduced flavodoxin is reoxidized between two consecutive flashes. In line with this interpretation, it was also observed that the signal after seven preflashes is identical to the signal observed a few seconds after continuous illumination of the sample.

The lower spectrum of Figure 2 (closed circles) corresponds to the reduction of flavodoxin semiquinone by PSI. This spectrum was obtained with flavodoxin in small excess over PSI in the absence of PES after a series of seven preflashes. As described below, reduction of semireduced flavodoxin is a biphasic process. However, under the conditions used for measuring the spectrum, a slow phase is dominating the decay ($t_{1/2} \approx$ 2.4 ms). The spectrum was obtained from the difference between the absorptions measured at 12 ms after the flash in the sample containing flavodoxin and at 100 μs after the flash in a reference sample which was identical but without flavodoxin. As described in preceding papers studying the ferredoxin reduction process (Sétif & Bottin, 1994, 1995), this procedure is made necessary by the presence of antenna triplet signals. Vertical calibration is made by reference to the signal recorded at 820 nm. The spectrum corresponding to electron transfer from (F_A, F_B)$^-$ to semireduced flavodoxin was also independently calculated from the ($FldH^-$ $-FldH^\bullet$) spectrum and the (F_A, F_B) differential spectrum (dashed line). In that case, a scaling factor of 0.5 was used for convenient spectral comparison.

It appears immediately that the spectra recorded under these two different experimental conditions are in good qualitative agreement with the spectra expected for reduction of either oxidized or semireduced flavodoxin by the terminal acceptor of PSI, (F_A, F_B). Above 500 nm, the absorption changes due to either the formation or the disappearance of the flavodoxin semiquinone dominate the absorption changes, thus leading to approximately opposite spectra for the two different reduction steps. At 460 nm, the bleaching associated with the formation of flavodoxin semiquinone appears to be almost fully compensated by the absorption increase linked to (F_A, F_B)$^-$ reoxidation (upper spectrum). For the lower spectrum, this reoxidation makes the largest contribution to the 460 nm signal, as reduction of the flavodoxin semiquinone is accompanied by little absorption change at this wavelength. These results unambiguously support the above interpretation for the reduction of either oxidized or semireduced flavodoxin by PSI.

The quantitative discrepancies between the observed and calculated spectra can be tentatively rationalized as follows: as judged by the absorption coefficients at 580 nm, the observed signals are generally smaller than the ones expected for the reduction of both forms of flavodoxin. For the lower spectrum, this may be due to the slow kinetics of reduction of flavodoxin semiquinone ($t_{1/2} \approx$ 2.4 ms), as a competing process of reoxidation of fully reduced flavodoxin by dissolved oxygen is most probably occurring. For the upper spectrum, it is reasonable to assume that the semiquinone formed immediately after the flash may compete with oxidized flavodoxin for the remainder of the PSI reaction centers with a still reduced terminal acceptor. This would result in reduction of some oxidized flavodoxin to the fully reduced form. Despite these discrepancies in signal size observed in the visible region, the terminal acceptors of PSI are being fully reoxidized in the presence of flavodoxin as judged by comparison of the $P700^+$ rereduction kinetics at 820 nm in the presence and absence of flavodoxin. These experiments show that the recombination reaction between $P700^+$ and (F_A, F_B)$^-$ is almost completely inhibited under the conditions used for measuring both spectra (not shown; for the upper spectrum, the control experiment at 820 nm was performed in the absence of PES with a very low repetition rate).

When combining these observations, it appears that the difference spectrum for the reoxidation of (F_A, F_B)$^-$ contributes fully to the experimentally observed spectra while the difference spectra due to the different waves of flavodoxin reduction contribute only partially. This is shown by the continuous lines in Figure 2, which were calculated for both waves of flavodoxin reduction by assuming 100% of reoxidation of (F_A, F_B)$^-$ and only 65% of flavodoxin reduction. The agreement with the observed spectra is better in both cases than when assuming a scaling factor as

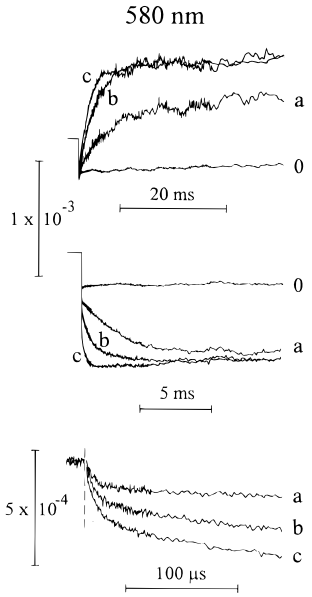

FIGURE 3: Flash-induced absorption changes measured at 580 nm showing the first wave (upper part) or the second wave (middle and lower parts) of flavodoxin reduction by photosystem I. PSI (0.26 μM) was suspended with the same reagents as described in Figure 1 (except for a higher DPIP concentration of 30 μM for traces c). Traces 0: control experiments in the absence of flavodoxin; traces a−c correspond to flavodoxin concentrations of 1.25, 5.62, and 20.5 μM, respectively. For the first wave of flavodoxin reduction (upper part), a new sample was prepared for each flavodoxin concentration and first-flash experiments were recorded after addition of flavodoxin in darkness (a and b, 1 experiment; c, average of four experiments). For recording the second wave of flavodoxin reduction (middle part), the flavodoxin semiquinone was accumulated by continuous illumination of the sample for 10 s (a and b) or 40 s (c). Single-flash experiments were recorded. The first four flashes exhibit similar kinetics and were averaged. The repetition rate of the laser flash excitation was 0.17 Hz. The lower part shows differences (from middle part) between each trace recorded in the presence of flavodoxin and the trace recorded in its absence after expansion on a faster time scale. The time of the flash is indicated by a vertical dashed line.

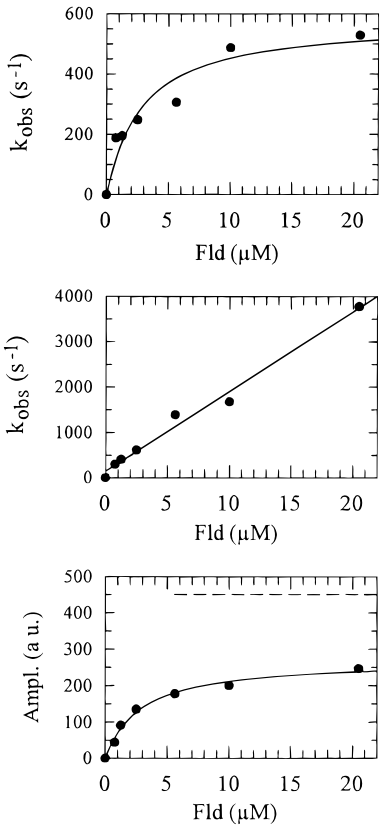

FIGURE 4: Dependence of the kinetic characteristics of the two waves of flavodoxin reduction upon flavodoxin concentration. Traces recorded under similar conditions to those described in Figure 3 were studied with a flavodoxin concentration ranging from 0.75 to 20.5 μM. Upper part: observed rate for the first wave of flavodoxin reduction (semiquinone formation). Middle part: observed rate of the slow phase for the second wave of flavodoxin reduction (semiquinone reduction). A second-order rate constant of 1.73×10^8 M^{-1} s^{-1} can be calculated from a linear fit of the data (continuous line). Lower part: amplitude of the fast phase ($t_{1/2} \approx 10$ μs) for the second wave of flavodoxin reduction. Two different models were assumed for fitting these data, which result in indistinguishable curves (continuous line; see text). The total amplitude due to both phases is shown as a dashed line.

described above (dashed lines). However, it can be noted that the above explanation is not fully satisfying for the upper spectrum (first wave) when one considers the different rates of the processes which would be involved in the two-step full reduction of oxidized flavodoxin.

The kinetics of flavodoxin reduction were further studied by variation of the flavodoxin concentration. The results are displayed in the upper parts of Figures 3 and 4 for absorption changes measured at 580 nm and corresponding to reduction of oxidized flavodoxin. Those for semiquinone reduction are shown in the middle and lower parts of both figures. Whereas traces 0 of Figure 3 are recorded in the absence of flavodoxin, traces a−c correspond to increasing concentrations of flavodoxin (1.25, 5.62, and 20.5 μM, respectively), all in excess to the concentration of PSI (0.26 μM). The upper part exhibiting first-flash experiments shows that the rate of reduction of oxidized flavodoxin increase with flavodoxin concentration. An increase in signal size is also observed between traces a and b (see above discussion of the spectral amplitude for a possible explanation). With the low signal to noise ratio of these experiments, these data could be satisfactorily fitted with one exponential component. The corresponding rate is plotted versus the flavodoxin concentration in the upper part of Figure 4. At high flavodoxin concentrations, the observed rate appears to reach an asymptotic value ($k_{obs} \approx 600$ s^{-1}) which indicates the occurrence of a rate-limiting step for the semiquinone formation. Similar observations were previously made by Medina et al. (1992) which were quantitatively interpreted within a two-step mechanism (complex formation followed by intracomplex electron transfer) by assuming that this asymptotic value corresponds to a limiting electron transfer rate constant.

As already discussed to measure the second wave of flavodoxin reduction it is necessary that flavodoxin is completely reduced to its semiquinone form before averaging the absorption transient signals. For this purpose, a series of preflashes is efficient when flavodoxin is in slight excess over PSI but this is not the case with a large excess of flavodoxin (see Figure 1). In the latter case, the sample containing flavodoxin was preilluminated for 30−60 s with white light for accumulating semireduced flavodoxin and signal averaging was then performed after a few seconds of dark adaptation (sufficient for reoxidation of fully reduced flavodoxin to the semiquinone form; see Materials and Methods). The middle part of Figure 3 shows that reduction of flavodoxin semiquinone is a biphasic process. A fast decay, which is only seen on a 15 ms time scale as an initial signal decrease compared to the control sample, is followed by a concentration dependent slower decay. At increasing

flavodoxin concentrations, the amplitude of this slow decay decreases as the amplitude of the fast component increases. With the present signal to noise ratio of our experiments, the slow decay can be satisfactorily fitted with one exponential component. The corresponding rate is plotted versus the flavodoxin concentration in the middle part of Figure 4. A linear dependence is observed up to the maximum flavodoxin concentration that was tested (20 μM). A second-order rate constant of 1.7×10^8 M^{-1} s^{-1} can be calculated from a linear fit of these data. It should be noted that this linear dependence strongly supports the assumption that flavodoxin is entirely in its semiquinone form prior to the flash, up to 20 μM of total flavodoxin.

Due to the presence of antenna triplet states decaying in the microsecond range [see Sétif and Bottin, (1994, 1995)], the fast decay is better defined after subtraction of the signal recorded in the absence of flavodoxin from the signal recorded in its presence. Such a procedure was used for the lower part of Figure 3 which exhibits such differences on a 200 μs time scale. This decay can be fitted with a single exponential component and its rate is not dependent upon the flavodoxin concentration (first-order process). Its halftime is about 10 μs, and it most probably corresponds to electron transfer within a complex between PSI and semireduced flavodoxin which is preformed before flash excitation. It should be also noted that, contrary to the observations made with ferredoxin (Sétif & Bottin, 1994, 1995), no submicrosecond phase of flavodoxin reduction is observed.

The amplitude of the fast phase is plotted versus the flavodoxin concentration in the lower part of Figure 4. At high flavodoxin concentrations, it appears to reach an asymptotic value which represents less than 60% of the total signal due to reduction of flavodoxin semiquinone (indicated as a dashed line). These data were fitted assuming a simple equilibrium (reaction 1) between PSI and flavodoxin

$$PSI^- + FldH^\bullet \overset{(1)}{\rightleftarrows} PSI^-\cdot FldH^\bullet \overset{(2)}{\rightarrow} PSI\cdot FldH^-$$

[PSI^- is an abbreviation for designating PSI with an electron on (F_A, F_B)].

For making this calculation, it is assumed that flavodoxin is entirely in its semireduced form before the flash, which is strongly supported by the linear dependence of the slower phase mentioned above. Both the dissociation constant and the maximum amplitude of the fast phase were used as free parameters for such a fit. The corresponding continuous curve of Figure 4 corresponds to a dissociation constant of 2.64 μM. However, within such a two-step model, the fast phase should represent 100% of the total signal amplitude at high flavodoxin concentrations. This is clearly not the case as the fast phase represents at most 60% of the total amplitude. Three different explanations can be proposed for the observed maximal amount of fast phase:

(1) The PSI preparation is partially damaged during solubilization or purification, with only 60% of reaction centers capable of binding flavodoxin semiquinone.

(2) Reaction 2 may not correspond to a quasi-irreversible reaction and should be replaced by an equilibrium with $[PSI\cdot FldH^-]/([PSI^-\cdot FldH^\bullet] + [PSI\cdot FldH^-]) \approx 60\%$. This could happen if the redox potentials of the partners of the electron transfer reaction are modified. For example, this can be due to a large negative shift of the $FldH^\bullet/FldH^-$ redox couple within the PSI−flavodoxin complex. We will not elaborate further in this direction in the absence of redox titration data. However, with this hypothesis, the above dissociation constant of 2.64 μM can still be directly interpreted as resulting from a simple equilibrium corresponding to complex formation between PSI and $FldH^\bullet$.

(3) A three-step model may be more appropriate to describe the present results

$$PSI^- + FldH^\bullet \overset{(3)}{\rightleftarrows} \underset{(A)}{PSI^-\cdots FldH^\bullet} \overset{(4)}{\rightleftarrows} \underset{(B)}{PSI^-\cdot FldH^\bullet} \rightarrow PSI\cdot FldH^-$$

with complex A being inactive for electron transfer to flavodoxin. Within such a model, the amplitude of the fast phase will depend evidently from the equilibria 3 and 4. The data were also fitted with this model by assuming two free parameters K_d ($= [PSI^-] \times [FldH^\bullet]/[PSI^-\cdots FldH^\bullet]$) and K ($= [PSI^-\cdot FldH^\bullet]/[PSI^-\cdots FldH^\bullet]$) and by fixing the signal corresponding to 100% of the total amplitude (see Figure 4: dashed line). The results of the fitting procedure are the following: $K_d = 6.5$ μM, and $K = 1.48$. The curve resulting from this fit is indistinguishable from the curve resulting from the previous fit.

Our present experimental data do not allow us to decide which of these three explanations is the correct one.

The salt dependences (at pH 8) of the rates of flavodoxin reduction are shown in Figure 5 (reduction of the oxidized form in the upper part and of the semireduced form in the lower part). For reduction of oxidized flavodoxin, optimal concentrations of NaCl and $MgCl_2$ are found around 40−80 and 15−20 mM, respectively. These optimal concentrations correspond to similar ionic strengths. However a specific role for Mg^{2+} cation, or probably for divalent cations in general, has to be invoked as the largest rate constants are observed only in the presence of $MgCl_2$ and the concentration dependence is apparently stronger than for NaCl. A similar specific role of Mg^{2+} cations has been found in several complexes of electron-transfer proteins and has been already reported for PSI and oxidized flavodoxin (Medina et al., 1992). Qualitatively similar salt dependences are observed for the slow phase of reduction of semireduced flavodoxin. However, in this latter case, the NaCl dependence is very weak whereas a clear optimum is seen for 5 mM $MgCl_2$. For the same process of reduction of semireduced flavodoxin, the amplitude of the fast phase is largest for NaCl and $MgCl_2$ concentrations of 40−60 and 2−5 mM, respectively. It is noteworthy that the $MgCl_2$ dependences are different for the first and second waves of flavodoxin reduction, optimal concentrations being respectively 15−20 and 2−5 mM.

The pH dependence (between 6.4 and 8.0) of the reduction of oxidized flavodoxin is plotted in the upper part of Figure 5, thus showing a rate increase at decreasing pH values. Studies below pH 6 or above pH 8 were less reliable as some significant decrease in signal amplitude was observed. Variations in amplitude were also found (with maximum amplitude at pH 7−8) for the second wave of flavodoxin reduction, thus impeding recording of reliable data. In addition, the stability of the semiquinone form was found to be very sensitive to pH, so that reliable averaging could not be performed under all conditions. This behavior is easily understandable, as the oxidation of the semiquinone is accompanied with deprotonation.

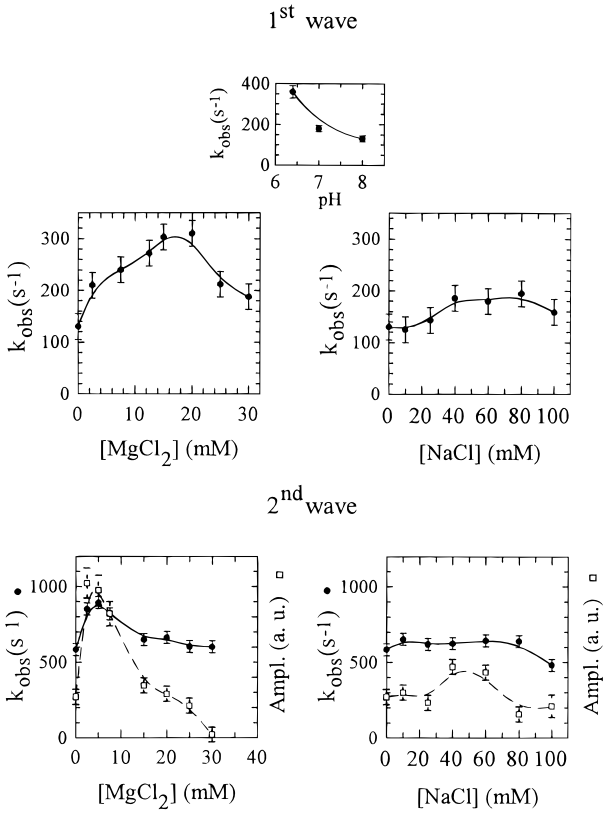

FIGURE 5: pH and salt dependences of the kinetic characteristics of flavodoxin photoreduction. pH dependence is only shown for the observed rate corresponding to the first wave of flavodoxin reduction (no salts). The salt dependences were measured at pH 8.0. Upper part: NaCl and $MgCl_2$ dependences for the rate observed for the first wave of flavodoxin reduction. Lower part: salt dependences for the rate of the slow phase (closed circles) and for the amplitude of the fast phase (open squares) for the second wave of flavodoxin reduction. Amplitudes are given in arbitrary units but can be compared directly between the two lowest figures (with NaCl or $MgCl_2$). Flavodoxin concentration, 2.5 μM; PSI concentration, 0.29 μM. Other experimental conditions are similar to those of Figure 3.

DISCUSSION

EPR detection of the semiquinone form of flavodoxin in cyanobacterial cells in darkness has been previously reported (Norris et al., 1972). This observation implies that the ambient redox potential of the cytoplasmic compartment is sufficiently low to maintain a high level of flavodoxin semiquinone. If this is the case, photoreduction of flavodoxin by PSI should involve primarily the reduction of the flavodoxin semiquinone and formation of the fully reduced form ($FldH^-$). Despite this, the only kinetic study of flavodoxin reduction by PSI was concerned with the photoreduction of oxidized flavodoxin (Medina et al., 1992). This study was performed with spinach PSI and flavodoxin from the cyanobacterium *Anabaena* sp. PCC 7119. Our study of electron transfer between PSI and flavodoxin, both from the cyanobacterium *Synechococcu*s sp. PCC 7002, basically agrees with the results of Medina and co-workers (1992) when considering reduction of oxidized flavodoxin. However the present study greatly extends these first results, as it is the first report concerning the reduction of semireduced flavodoxin by PSI. A spectral characterization of the two waves of flavodoxin reduction has been also performed. The observed spectra qualitatively agree with the spectra that can be calculated independently for electron transfer between $(F_A, F_B)^-$ and flavodoxin. It should be noted that it was possible to study the second wave of flavodoxin reduction because of the stability of the semiquinone form of flavodoxin under our experimental conditions. This apparent stability rendered signal averaging for studying the first wave of flavodoxin reduction very difficult, but it could be used to reduce flavodoxin to the semiquinone state by an illumination pretreatment. In this context, it should be noted that the overall effect of flavodoxin photoreduction by cyanobacterial PSI described in our study is very different from the one reported previously for PSI from spinach. Due to its apparent high stability the flavodoxin semiquinone is being accumulated within a series of consecutive flashes. This behavior, which we were able to reproduce using both reaction partners from *Synechocystis* sp. PCC 6803 (H. Bottin, P. Sétif, and U. Mühlenhoff, unpublished data), was not observed in the previous study from Medina et al. (1992). These last authors were apparently able to average signals due to the reduction of oxidized flavodoxin by spinach PSI without any signal loss. Currently, there is no explanation for these discrepancies since the experimental conditions in both studies were generally similar.

When the kinetics of both reduction processes are compared, it appears that first-order processes due to electron transfer within complexes preformed before the flash excitation are observed only for the second wave of flavodoxin reduction. This would imply that PSI has a larger affinity for the semiquinone than for oxidized flavodoxin. In addition, the difference in $MgCl_2$ dependence observed for the rates of the two waves of flavodoxin reduction suggest that the electrostatic interactions between PSI and flavodoxin may be slightly different for the two redox forms of flavodoxin. Since only minor conformational differences in the overall structure of oxidized and semireduced flavodoxin are observed (Watt et al., 1991), it appears likely that the higher affinity of the semiquinone is due to the more predominant changes in the protein environment of the FMN cofactor (see below). However these conclusions should be taken with caution owing to the poor signal to noise ratio of the experiments concerning the first wave of flavodoxin reduction. For this process, it cannot be excluded that electron transfer is biphasic and may thus include a fast phase of small amplitude that could correspond to a preformed complex between oxidized flavodoxin and PSI. At the moment, such uncertainties make impossible to determine precisely the affinity of oxidized flavodoxin for PSI.

Under identical salts and pH conditions, the limiting rate of reduction of oxidized flavodoxin is 2 orders of magnitude smaller than the reduction rate of semireduced flavodoxin within a preformed complex ($t_{1/2} \approx 1$ ms vs 10 μs). This appears quite surprising as the former reaction corresponds to a larger decrease in free energy, which is an essential factor in electron transfer processes (Marcus & Sutin, 1985). Two different types of explanations can be proposed for these observations:

(1) The structure of the PSI-flavodoxin complex is different whether flavodoxin is oxidized or semireduced. Whereas three different crystal structures of long-chain flavodoxins are available (Laudenbach et al., 1987; Fukuyama *et al*., 1992; Rao *et al*., 1992), comparison of X-ray structures between different redox states has been performed only in the case of short-chain flavodoxins (Smith et al., 1977; Watt et al., 1991). These two studies conclude that the first one-electron reduction of flavodoxin is accompanied by several changes at the FMN site whereas the structural

rearrangement between the semiquinone and hydroquinone states is small (Watt et al., 1991). Similar conclusions have also been reached by NMR studies of a short-chain flavodoxin (Peelen & Vervoort, 1994) and it seems likely that these results can be extended to the long-chain type. These data suggest that the PSI-flavodoxin interactions may indeed be different for the different redox forms of flavodoxin. If sufficiently large, these differences may be sufficient for modifying the distance or the electron transfer pathway between (F_A, F_B) and FMN, thus explaining the slower reduction of oxidized flavodoxin. Another possibility is that the activation energy for the first wave of flavodoxin reduction is much larger than for the second wave, in line with the larger rearrangement involved during the first wave.

(2) Electron transfer is not the rate-limiting step during reduction of oxidized flavodoxin. As the semiquinone form of flavodoxin is protonated and as only this protonated form is observable at 580 nm [Massey et al., 1969; see also Stankovich et al. 1978)], the slow reduction of the oxidized form can be explained by a rate-limiting protonation.

Although our results do not allow to decide which explanation is correct, it should be noted that such a rate-limiting protonation step has been recently put forward during the study of photoreduction of flavodoxin by 5-deazariboflavin (Navarro et al., 1994). The rate constant of this protonation step (450 s^{-1}) is similar to the limiting rate of reduction of oxidized flavodoxin by PSI (600 s^{-1}), which supports the second hypothesis for a rate-limiting protonation. In addition, rate-limiting protonation is in agreement with our experimental observation that the rate for photoreduction of oxidized flavodoxin increases at lower pH.

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