Comparison of the Kinetics of Reduction and Intramolecular Electron Transfer in Electrostatic and Covalent Complexes of Ferredoxin–NADP⁺ Reductase and Flavodoxin from *Anabaena* PCC 7119¹

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The kinetics of reduction and intracomplex electron transfer in electrostatically stabilized and covalently crosslinked complexes between ferredoxin–NADP⁺ reductase (FNR) and flavodoxin (Fld) from the cyanobacterium *Anabaena* PCC 7119 were compared using laser flash photolysis. The second-order rate constant for reduction by 5-deazariboflavin semiquinone (dRfH[•]) of FNR within the electrostatically stabilized complex at 10 mM ionic strength ($4.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) was identical to that for free FNR. This suggests that the FAD cofactor of FNR is not sterically hindered upon complex formation. A lower limit of approximately 7000 s^{-1} was estimated for the first-order rate constant for intracomplex electron transfer from FNR_{red} to Fld_{ox} under these conditions. In contrast, for the covalently crosslinked complex, a smaller second-order rate constant ($2.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$) was obtained for the reduction of FNR by dRfH[•] within the complex, suggesting that some steric hindrance of the FAD cofactor of FNR occurs due to crosslinking. A limiting rate constant of 1000 s^{-1} for the intracomplex electron transfer reaction was obtained for the covalent complex, which was unaffected by changes in ionic strength. The substantially diminished limiting rate constant, relative to that of the electrostatic complex, may reflect either a suboptimal orientation of the redox cofactors within the covalent complex or a required structural reorganization preceding electron transfer which is not allowed once the proteins have been covalently linked. Thus, although the covalent complex is biochemically competent, it is not a quantitatively precise model for the catalytically relevant intermediate along the reaction pathway. © 1990 Academic Press, Inc.

Flavoproteins participate in a large number of oxidation/reduction reactions that function either in energy transduction or in the biosynthesis and degradation of metabolic intermediates (cf. (1)). Due to their unique ability to transfer either one or two electrons, they are utilized for electron transfer between pyridine nucleotides and the metal-containing heme or iron–sulfur proteins. They also participate in the transfer of electrons to (or from) other flavin-containing proteins, which raises interesting questions relating to the thermodynamic and structural requirements for efficient flavin-to-flavin electron transfer. Among these requirements are: (i) the individual proteins must be able to form a productive complex; (ii) the redox potentials of both flavin cofactors must have the appropriate values for the electron transfer reaction to proceed in the required direction; (iii) the flavin cofactors should lie sufficiently close within the complex, and with the appropriate geometry, to facilitate the rapid transfer of electrons.

Ferredoxin–NADP⁺ reductase (FNR)³ is an FAD containing flavoprotein that participates in the reductive

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³ Abbreviations used: FNR, FNR_{ox}, and FNR_{sq}, ferredoxin–NADP⁺ reductase and its oxidized and one-electron reduced semiquinone forms; Fld, Fld_{ox}, and Fld_{sq}, flavodoxin and its oxidized and one-electron reduced semiquinone forms; Fd and Fd_{red}, ferredoxin and its one-electron reduced form; EDTA, ethylenediaminetetraacetic acid disodium salt; dRf and dRfH[•], 5-deazariboflavin and its one-electron reduced semiquinone form; k_{obs} , observed pseudo-first-order rate constant; SHE, standard hydrogen electrode.

side of photosynthesis, transferring electrons from reduced ferredoxin (Fd) to NADP⁺ (2, 3). It is an essential protein that mediates the flow of electrons generated in the light reactions of photosynthesis which constitute a source of energy for most organisms. The protein isolated from the filamentous cyanobacterium *Anabaena variabilis* has a molecular weight of 36,000 Da and contains one noncovalently bound FAD cofactor. A redox potential of -320 mV has been determined for the FAD oxidized/hydroquinone couple by potentiometric titration and by equilibration with NADPH/NADP⁺ (4). This value is 30–50 mV more positive than that reported for spinach FNR (5–7).

Flavodoxin (Fld) is a low-molecular-weight FMN containing protein that is widely present in microorganisms, either constitutively or when they are subjected to iron deprivation. It has been proposed that Fld can substitute for Fd in all reactions in which the iron-containing protein participates (8). This includes photoreduction of NADP⁺ by illuminated chloroplasts, or the NADPH-cytochrome *c* reductase activity, both of which are dependent on FNR. In these reactions electrons must flow either from reduced Fld to FNR, as is the case for the reduction of NADP⁺, or from reduced FNR to Fld, as occurs in the NADPH-FNR-Fld-cyt *c* electron transport chain. Both reactions proceed via the formation of a stable electrostatic complex that involves negative charges on Fld and positive charges on FNR (9). The intermediate complex thus allows electrons to flow in either direction, depending on the thermodynamic characteristics of the other components present. As a consequence of these properties, FNR and Fld represent a convenient physiological system with which the structural requirements for efficient electron transfer between flavin cofactors can be studied.

The strain of *A. variabilis* (ATCC 29211 or CCAP 1403.4b) from which the FNR has been previously isolated is unable to synthesize Fld even under conditions of strict iron limitation (10). The strain PCC 7119 of *Anabaena* (ATCC 29151), on the other hand, induces the synthesis of large amounts of Fld when cells are deprived of iron in the culture medium. This Fld has been isolated and its molecular and physicochemical properties have been characterized (11); these are consistent with those described for other large Flds (8). The corresponding FNR from this strain of *Anabaena* has also been isolated, and all of the molecular properties studied (molecular weight, amino acid composition, N-terminal amino acid sequence, etc.), as well as the kinetic behavior, show similarities to those of the FNR obtained from *A. variabilis* (4, 11). Therefore, the PCC 7119 strain serves as an appropriate system to investigate Fld-FNR interactions.

Chemical crosslinking of the amino and carboxyl residues that are involved in the formation of 1:1 protein complexes has been extensively used with a variety of redox proteins as a device for allowing more convenient

study of what would otherwise be a transient kinetic intermediate (12–18). Thus, for example, by the appropriate analytical procedures, one could identify the amino acid residues involved in complex formation. Cross-linked complexes may also prove useful in the growth of crystals which, upon X-ray analysis, could provide important information on the structural requirements at the interface that promote efficient electron transfer between the components. Further, such complexes would in principle allow the study of redox processes over a wide range of salt concentrations in order to determine the effect of the ionic strength on the electron transfer process independent of any effect on complex formation. It is therefore important in this context to ascertain whether the covalently crosslinked complex is an appropriate model for the electrostatic complex. In at least two cases, cytochrome *c*-plastocyanin (17) and cytochrome *c*-cytochrome *c* peroxidase (19), significant differences in electron transfer processes have previously been found between covalent and electrostatic complexes of redox proteins.

Covalently crosslinked complexes between *Anabaena* FNR and *Azotobacter* Fld and between *Anabaena* FNR and *Anabaena* Fld have been described (20, 21). In the case of the *Azotobacter* Fld complex, it was demonstrated that the NADPH binding site of FNR was not rendered inaccessible by the crosslinking since electron transfer from NADPH to Fld was observed. It has also been shown that the redox potential of the semiquinone/hydroquinone couple of covalently bound Fld is approximately 100 mV more positive than that of the free protein at the same pH. This shift in the redox potential upon complex formation would suggest that the complex behaves as a catalytic intermediate that facilitates the transfer of electrons from FNR to Fld. In the case of the *Anabaena* Fld covalent complex, activity was obtained in the chloroplast-dependent photoreduction of NADP⁺ (21), and thus the complex is clearly biochemically competent. These results indicate that the crosslinked FNR:Fld complexes represent appropriate models for the determination of the factors which influence the kinetic parameters involved in electron transfer between flavin cofactors in these proteins.

In the present study, we have used laser flash photolysis to investigate the kinetics of reduction and intramolecular electron transfer between protein components in the covalently crosslinked and electrostatically stabilized complexes between *Anabaena* PCC 7119 FNR and Fld. This technique is appropriate for these studies since it involves the generation of a strong reductant *in situ* which allows the measurement of electron transfer reactions that are too fast to be determined by rapid-mixing methods (19, 22, 23). As will be demonstrated below, we have observed significant quantitative differences in the kinetic properties of these two types of complexes which indicate that the covalent complex is not an entirely

suitable model for the catalytic complex occurring along the reaction pathway. We have also been able to obtain an estimate of the intracomplex electron transfer rate constant between FNR semiquinone and oxidized Fld within the electrostatically stabilized complex formed at low ionic strength.

MATERIALS AND METHODS

Fld and FNR were isolated from *Anabaena* PCC 7119 as described by Sancho *et al.* (4) and Fillat *et al.* (11) and were dialyzed against phosphate buffer. The covalently crosslinked FNR:Fld complex was prepared as described by Pueyo and Gómez-Moreno (21). 5-Deazariboflavin (dRf) was synthesized as described by Smit *et al.* (24). During the reduction of the individual proteins, the FNR concentration was determined from its absorbance at 459 nm, using an extinction coefficient of $9700 \text{ M}^{-1} \text{ cm}^{-1}$ (4), while the Fld concentration was determined from its absorbance at 465 nm, using an extinction coefficient of $9400 \text{ M}^{-1} \text{ cm}^{-1}$ (11). The concentration of the covalent complex was determined from its absorbance at 461 nm, using an extinction coefficient of $21,400 \text{ M}^{-1} \text{ cm}^{-1}$ (21). All flash photolysis experiments were performed in 4 mM phosphate buffer containing 0.5 mM EDTA, pH 7.0 ($I = 10 \text{ mM}$). Ionic strengths greater than 10 mM were achieved by the addition of NaCl to this buffer.

Steady-state photoreductive titrations were done in the presence of $5 \mu\text{M}$ dRf and 1.3 mM EDTA using 3-ml spectrophotometer cells equipped with a tapered neck and sealed with a rubber septum. Buffer solutions were made anaerobic by bubbling with argon for at least 1 h before the addition of aliquots of a concentrated enzyme stock. When necessary, trace amounts of oxygen introduced into the sample by this addition were removed by passing argon over the surface of the solution. Visible spectra were recorded on an On-Line Instrument Systems modification of a Cary 15 spectrophotometer.

Laser flash photolysis experiments were performed anaerobically at room temperature in the presence of approximately $90 \mu\text{M}$ dRf. Photoexcitation of dRf was accomplished using a Photochemical Research Associates Model LN100 dye-pumped nitrogen laser equipped with BBQ dye (PRA 2A386; excitation wavelength, 395 nm; pulse duration, approx 1 ns). The optical system used to monitor the reaction has been previously described (25). Signals were acquired using a Nicolet Model 1170 signal averager and the output directed to a strip-chart recorder and an XT-type computer.

Laser flash photolysis generated 5-dRfH[•] which either disproportionated (26) or, in the presence of protein, rapidly underwent electron transfer to produce reduced protein (cf. (22)). All kinetic experiments were performed under pseudo-first-order conditions, in which the concentration of protein acceptor ($>2 \mu\text{M}$) was in large excess over the amount of dRfH[•] produced per flash ($<0.7 \mu\text{M}$). In experiments such as these protein reduction is always in competition with dRfH[•] disproportionation. The extent of the contribution of the disproportionation reaction to the observed transient decay kinetics 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[•]). In the present experiments, this complication was insignificant, inasmuch as we were able to fit the observed transients well with a single exponential whose magnitude was protein concentration dependent. Unless quantitation was required (e.g., for bleaching measurements) the number of flashes averaged per kinetic trace varied. Kinetic traces were generally analyzed by fitting the data to an exponential curve, ignoring the initial transient due to dRf reduction. Kinetic 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 rate constant determinations was $\leq \pm 10\%$, based upon standard deviations from replicate measurements.

FIG. 1. Spectral changes observed during steady-state phototitrations. (a) *A. PCC 7119* Fld in 0.1 M phosphate buffer, pH 7.4, containing $5 \mu\text{M}$ dRf and 1.3 mM EDTA. (b) *A. PCC 7119* FNR. Experimental conditions were identical to those in a. (c) A 1:1 molar ratio of *A. PCC 7119* FNR and Fld. The titration was performed at 10 mM ionic strength, using a concentration of $26 \mu\text{M}$ for each component. (d) Covalently crosslinked complex between *A. PCC 7119* FNR and Fld. Experimental conditions were identical to those in c.

RESULTS AND DISCUSSION

Photoreductive Titrations of Free and Complexed Proteins

As illustrated in Fig. 1a, steady-state photoreduction of free *Anabaena* Fld by dRfH[•] was accompanied by the bleach of absorbances centered at 465 and 377 nm, and an increase in absorbance at 578 nm, with a shoulder at 608 nm. Isosbestic points were observed at 516 and 361 nm. These results are interpreted as due to the accumulation of the one-electron reduced semiquinone form of the FMN cofactor (8). The large amount of semiquinone observed with *Anabaena* Fld is a consequence of the higher (more positive) midpoint potential for the oxidized/semiquinone couple (-195 mV vs SHE) as compared to that of the semiquinone/hydroquinone couple (-390 mV vs SHE (27)), and reflects the general behavior of all known Flds (28).

The absorbance changes associated with the steady-state phototitration of free *Anabaena* FNR are presented in Fig. 1b. Reduction of the FAD cofactor resulted in the bleaching of absorbances centered at 458 and 390 nm. A broad band of increased absorbance centered around 600 nm developed during the initial stages of reduction, with isosbestic points at 357 and 507 nm. This is interpreted as being due to the formation of a small amount of the one-electron reduced FAD (FADH[•]). Previous potentiometric measurements performed using the FNR obtained from *A. variabilis* (strain 1403.4b)

yielded midpoint potentials of -370 and -270 mV (vs SHE) for the first and second one-electron reductions of the FAD cofactor (4), indicating that the hydroquinone form is the more thermodynamically stable reduced species. This example of protein-mediated destabilization of the flavin semiquinone, relative to the hydroquinone, is characteristic of pyrimidine-dependent electron transferring flavoproteins (28). The small amount of semiquinone generated during the titration, relative to that observed with Fld, is consistent with this behavior.

Steady-state phototitration of the 1:1 electrostatic complex formed by Fld and FNR at 10 mM ionic strength demonstrated that the absorbance changes above 500 nm which accompanied limited reduction were similar to those observed during the reduction of Fld alone. As illustrated in Fig. 1c, an isosbestic point at 516 nm, the general shape of the spectral profile obtained at longer wavelengths, and the size of the absorbance change indicated the formation of the FMNH[•] of Fld. Preferential accumulation of FMNH[•] during limited reduction is consistent with the more positive redox potential for the oxidized/semiquinone couple of the FMN of Fld, relative to that for the FAD of FNR, as discussed above (-195 mV vs -270 mV, respectively). As more reducing equivalents were introduced during the phototitration the isosbestic point shifted to shorter wavelengths, consistent with the one-electron reduction of FNR (data not shown). Phototitration of the covalent FNR:Fld complex displayed spectral behavior similar to that observed during the reduction of the electrostatic complex (Fig. 1d), and were interpreted in a similar manner. Differences apparent between Figs. 1c and 1d, especially below the isosbestic point, may reflect differences in the spectral perturbations resulting from protein-protein interactions occurring in the respective complexes.

Laser Flash-Induced Reduction of the Individual Proteins

When a solution of dRf is illuminated by a brief laser flash, in the presence of EDTA as a sacrificial electron donor, the rapid formation of a species that absorbs at 500 nm is observed (Fig. 2a). This corresponds to the formation of the one-electron reduced dRfH[•] by EDTA-mediated reduction of the flavin triplet state. This semiquinone species absorbs in the 450–550 nm region which is similar to other neutral flavin semiquinones (26). The semiquinone is very unstable in solution and, in the absence of protein, disproportionates to the fully oxidized and fully reduced species of dRf, neither of which appreciably absorbs light at 500 nm. In the presence of Fld the absorbance measured at 465 nm rapidly decayed below the preflash baseline (Fig. 2b). At this wavelength the absorbance contribution from dRfH[•] is much smaller, and thus the initial increase and the subsequent net

FIG. 2. (a) Formation and decay of dRfH[•] produced during the laser pulse, as monitored at 500 nm. The solution contained 72 μ M dRf in 4 mM phosphate buffer containing 0.5 mM EDTA, pH 7.0 ($I = 10$ mM). (b) Bleaching of absorbance following the laser flash as observed at 465 nm in the presence of 5.4 μ M A. PCC Fld. The buffer conditions were identical to those in a. (c) Increase in absorbance observed at 580 nm. The conditions were identical to those in b. The small positive and negative absorbance changes immediately following the laser flash are due to the production and subsequent decay of dRfH[•]. The contribution from dRfH[•] became less apparent at higher protein concentrations. Each trace represents the sum of six transients. Solid lines are single exponential curves drawn to best fit the data.

bleaching of absorbance is interpreted as a result of the one-electron reduction of the FMN cofactor of Fld by dRf semiquinone formed during the flash. The corresponding kinetics for the formation of the FMN semiquinone was followed at 580 nm (Fig. 2c). At this wavelength the dRfH[•] species does not absorb strongly and a net increase in absorbance due to the formation of FMNH[•] is observed. The pseudo-first-order rate constants obtained from the absorbance changes at 465 and 580 nm were identical, as expected, since they both correspond to the same process, i.e., the one-electron reduction of the FMN cofactor. Transients obtained at these wavelengths were dependent on the concentration of Fld (Fig. 3a, closed circles), giving a second-order rate constant of $1.6 \pm 0.1 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ at an ionic strength of 10 mM. For comparison, the second-order rate constant for the reduction of *C. pasteurianum* Fld by dRfH[•] has been reported to be $0.69 \pm 0.05 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ (23) under identi-

FIG. 3. (a) Concentration dependencies for the laser flash-induced reduction of *A. PCC* 7119 Fld (●) and FNR (○). The buffer conditions were identical to those in Fig. 2a. (b) Concentration dependence of transients obtained at 461 (○) and 600 nm (●) during the reduction of the electrostatically stabilized 1:1 complex between Fld and FNR. The buffer conditions were identical to those in Fig. 2a.

cal conditions of pH and ionic strength. Thus, these two Flds are not too different in their reactivities toward 5-dRf semiquinone, which suggests a similar (but probably not identical) steric environment of the FMN cofactor (cf. (22)).

The kinetic constant for the reduction of *A. PCC* FNR by dRfH[•] will be reported elsewhere (29). Briefly, kinetic data were obtained from the bleaching of absorbance at 461 nm and the absorbance increase at 600 nm (not shown). These wavelengths correspond to the absorbance maxima of the oxidized and one-electron reduced forms, respectively, of the FAD cofactor of FNR. The observed rate constants obtained from transients collected at these wavelengths were found to be protein concentration dependent (cf. Fig. 3b, open circles), yielding a second-order rate constant of $4.2 \pm 0.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$. This value is approximately twice that for the reduction of *Anabaena* Fld and is similar to that reported for the reduction by dRfH[•] of the FNR isolated from spinach ($6.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$; (30)), again suggesting structural homology.

Laser Flash-Induced Reduction of the Electrostatic Complex

The kinetics of reduction of the FNR:Fld electrostatic complex formed at 10 mM ionic strength was monitored

at 461 nm, since this corresponds to the wavelength maximum observed with an equimolar mixture of FNR and Fld. At this wavelength, the absorbance contains approximately equal contributions from the two oxidized protein components. The laser flash was followed by a monoexponential decay of absorbance below the pre-flash baseline, similar to that obtained with the individual proteins (data not shown). The concentration dependence of the observed rate constant obtained from transients collected at 461 nm was determined by the addition of equimolar concentrations of Fld and FNR (Fig. 3b, open circles). A second-order rate constant of $4.0 \pm 0.6 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ was obtained, which is within the same experimental error as that obtained for the reduction of free FNR by dRf semiquinone, and significantly faster than that for Fld alone.

Formation of the one-electron reduced FNR:Fld complex was monitored at 600 nm. As with the individual proteins, the laser flash was immediately followed by an increase in absorbance due to the production of flavin cofactor semiquinone (data not shown). The observed rate constants for reduction obtained at this wavelength were appreciably different from those obtained from the measurements performed at 461 nm (cf. Fig. 3b, closed circles). This was particularly evident at the highest enzyme concentration, where the observed rate constant obtained at 600 nm was approximately half of that obtained at 461 nm. This strongly suggests that different kinetic processes were being monitored at these two wavelengths under these conditions. The protein concentration dependence of the 600-nm transient was significantly nonlinear, which is consistent with a minimal kinetic mechanism of a second-order reaction, followed by a first-order reaction which becomes rate-limiting at high concentrations (31, 32). The simplest interpretation of this rate-limiting first-order process is intracomplex electron transfer from FNR semiquinone, formed via a second-order reaction with dRfH[•], to oxidized Fld, as follows:

As will be shown below, differences between data obtained at 461 and 600 nm were even more striking for the covalent complex, which provides further support for this interpretation.

A lower limit of 7000 s^{-1} is estimated from Fig. 3b for the rate constant corresponding to this intracomplex electron transfer (reaction [b]). It is worth noting that a value of 3800 s^{-1} has been observed under comparable conditions for the intracomplex electron transfer rate constant from reduced Fd to oxidized FNR from *Anabaena* PCC 7119 (29). In both cases, this is considerably faster than enzyme turnover.

The value of the second-order rate constant obtained from the transients at 461 nm is consistent with reaction [a], i.e., reduction occurring by electron transfer from dRfH[•] to the FNR component within the complex. The close agreement between this value and that for the reduction of free FNR suggests that little or no steric hindrance of the FAD of FNR occurs upon complex formation. Preferential reduction of a single component within a preformed FNR complex has been previously observed. In the case of the electrostatic complex formed at low ionic strengths between the Fd and FNR isolated from spinach, the kinetics were dominated by the direct reduction of FNR by dRf semiquinone, with a second-order rate constant identical to that obtained with FNR alone (30). It was further demonstrated, by examining the kinetics of reduction of the 1:1 complex formed between fully reduced spinach FNR and oxidized spinach Fd, that the Fe/S center of Fd was made approximately 20 times less accessible to dRfH[•] as a consequence of complex formation. An analogous experiment utilizing oxidized Fld and reduced FNR isolated from *Anabaena* PCC 7119 was not possible, since steady-state phototitrations of the 1:1 complex between FNR and Fld demonstrated that reducing equivalents equilibrated to the Fld (see above). Thus, we could not directly determine the extent of steric hindrance of the FMN of Fld as a result of complex formation. A possible way to overcome this problem would be to follow the reduction of Fld in a complex formed with apo-FNR, i.e., the protein in which the FAD cofactor has been removed. This requires, however, that apo-FNR binds to Fld with a similar affinity as does the holo form. Although we have not determined this, we have data which indicate that the complex formed between apo-Fld and FNR is 10-fold weaker than that formed between the holo-proteins (Sancho, J. and Gómez-Moreno, C., unpublished results). This requires further study.

Due to the spectral similarities between Fld and FNR it was not possible to follow the reduction of Fld independently of the reduction of FNR. Although isosbestic points obtained from the phototitrations of free Fld and FNR were separated by approximately 10 nm, we found that this difference could not be exploited to resolve the two reduction processes in the complex. This is perhaps a result of spectral changes which occur upon complex formation (9, 21). Such changes in absorbance have been used to calculate the extent of complex formation and to determine the binding constants for the FNR:Fld electrostatic complex (9), as well as for the spinach Fd:FNR complex (33).

Transfer of an electron within the complex via reaction [b] would not contribute any additional change in absorbance at 461 nm, since the extinction coefficients for the oxidized Fld and oxidized FNR are similar at this wavelength, and thus, this process would be undetectable. The kinetic transients obtained at 600 nm are in

FIG. 4. Concentration dependence of the reduction of the covalently crosslinked FNR:Fld complex (a) from transients obtained at 461 nm, and (b) from transients obtained at 600 nm. The buffer conditions were identical to those in Fig. 2a.

fact probably composed of contributions from both the direct reduction of FNR within the complex and intra-complex electron transfer to Fld. The rate constants for these two processes are too similar to one another, however, to result in biphasic kinetic traces at this wavelength. As will be demonstrated below, this is not the case for the covalent complex.

The laser flash-induced reduction of mixtures of FNR and Fld was also examined at 310 mM ionic strength. Under these conditions the electrostatic forces which stabilize the complex were weakened and a larger proportion of the component proteins existed free in solution. The transients obtained at both 461 and 600 nm were clearly multiexponential (data not shown) and could not be readily analyzed. This was interpreted to be a consequence of the overlapping reductions by dRfH[•] of free FNR, free Fld, and also intramolecular electron transfer within the complex, which could not be easily resolved from one another.

Laser Flash-Induced Reduction of the Crosslinked Complex

Reduction of the covalent complex obtained from the chemical crosslinking of *A. PCC* 7119 Fld and FNR was monitored in a manner identical to that described for the electrostatic complex. The absorbance changes observed at 461 nm were again monoexponential and linearly dependent on concentration (Fig. 4a), giving a second-or-

der rate constant of $2.1 \pm 0.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ for the reduction of the complex by dRfH'. Although this value is similar to that obtained for the reduction of Fld alone this does not necessarily indicate that reduction of the covalent complex proceeded via direct reduction of Fld, which would be in contrast to what was observed with the electrostatic complex (see below). Like the electrostatic complex, the rate constants and the pattern of concentration dependence obtained from transients observed at 600 nm differed from that obtained at 461 nm. The observed rate constants determined at the longer wavelength clearly approached a concentration-independent limiting value, and at $30 \mu\text{M}$ protein the k_{obs} was approximately six times smaller than that obtained at 461 nm at the same concentration (Fig. 4b). This strongly demonstrates again that different kinetic processes were being observed at these two wavelengths, and that a rate-limiting first-order step was involved in the mechanism. As noted above, phototitrations showed that a single electron introduced into the covalent complex equilibrated to the Fld, as was the case with the electrostatic complex. Thus, the first-order rate-limiting process observed at 600 nm in the flash experiments can again be interpreted in terms of the reduction of Fld by the concentration-independent intracomplex transfer of an electron from reduced FNR.

The first-order rate constant obtained for the limiting kinetic process was 1000 s^{-1} , and was found to be unaffected by changes in ionic strength (data not shown), as would be expected for a covalently crosslinked complex. It is important to note that this value is almost an order of magnitude smaller than that estimated for the electrostatic complex. This diminished rate constant for intracomplex electron transfer observed with the covalent complex correlates with a decreased steady-state rate constant obtained with the NADP⁺ reductase assay (21).

It is not possible to ascertain whether the transient absorbance changes observed with the covalent complex at 461 nm contained contributions from the direct reduction of Fld as well as of FNR. Such a possibility, as well as steric hindrance of the FNR by the covalent crosslinking, could account for the smaller second-order rate constant for direct reduction of the complex by dRfH', relative to that obtained with the electrostatic complex. Direct reduction of Fld by dRfH', in addition to the intracomplex electron transfer process, would be expected to result in a biphasic increase in absorbance at 600 nm under conditions in which the two rate constants were sufficiently different. Indeed, two phases of reduction could be detected at high protein concentrations, where the fast and slow phases were separated by a factor of 4 or greater. Figure 5 illustrates the quality of fit and the residuals for monophasic (Fig. 5a) and biphasic (Fig. 5b) exponential computer fits to a transient obtained at 600 nm with the covalently crosslinked com-

FIG. 5. (a) Monoexponential and (b) biexponential computer fits illustrating the biphasic nature of transients obtained at 600 nm during the reduction of $26 \mu\text{M}$ covalent complex at $I = 10 \text{ mM}$. Solid curves in the lower panel correspond to the theoretical fits. The observed rate constants obtained from the biphasic computer fit are 4200 and 830 s^{-1} for the fast and slow phases, respectively. These values are consistent with the data in Figs. 4a and 4b, considering the difficulties in analyzing biphasic curves in which the respective rate constants are separated by a factor of 5 or less. The absorbance scales are in arbitrary units.

plex at a concentration of $26 \mu\text{M}$. It is clear that the biexponential fit is superior. Note that the rate constants obtained for the two phases of reduction are separated by a factor of 5. The second-order rate constant obtained from the concentration-dependent fast phase of reduction was similar to that obtained from the monoexponential transients observed at 461 nm. Thus, it is indeed possible that the transients observed at 461 nm during the reduction of the covalent complex in part result from a contribution from the direct reduction of Fld by dRf semiquinone, in addition to reaction with a sterically hindered FNR component.

Previous reports have also demonstrated that rates of intramolecular electron transfer within 1:1 protein complexes can be diminished by covalent crosslinking (17,

19). This may be a consequence of constraining the two proteins to an unfavorable orientation, or of preventing a required structural change within the initial collision complex prior to electron transfer. Another study has indicated that reorientation of the components within the preformed A. PCC Fd:FNR complex facilitates the efficient transfer of an electron from Fd_{red} to FNR_{ox} (29). A similar reorientation of the components of the FNR:Fld collisional complex may also be required in order to optimize the geometries of the respective cofactors toward efficient electron transfer. Such a reorientation step might not be permitted by the covalent crosslinks, resulting in a decreased rate constant for the intramolecular electron transfer. This requires further study. However, it is clear from the present results that the covalent complex can only be considered as an approximation to the kinetically optimized transient electron transfer complex.

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