Laser Flash Photolysis Studies of the Reduction Kinetics of NADPH:Cytochrome P-450 Reductase†

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ABSTRACT: The reduction kinetics of NADPH:cytochrome P-450 reductase have been investigated by the laser flash photolysis technique, using the semiquinone of 5-deazariboflavin (5-dRfH•) as the reductant. Transients observed at 470 nm at neutral pH indicated that the oxidized reductase was reduced via second-order kinetics with a rate constant of 6.8×10^7 M^{-1} s^{-1}. The second-order rate constant corresponding to the formation of the protein-bound semiquinone (measured at 585 nm) was essentially the same as that obtained at 470 nm (7.1×10^7 M^{-1} s^{-1}). Subsequent to this rapid formation of protein-bound semiquinone, a partial exponential decay was observed at 585 nm. The rate of this decay remained invariant with protein concentration between pH 5.0 and 7.0, and a first-order rate constant of 70 s^{-1} was obtained for this process. This is assigned to intramolecular electron transfer from FADH• to FMN. Prior reduction of the enzyme to the one-electron level led to a decrease in both the second-order rate constant for reduction (2×10^7 M^{-1} s^{-1}) and the first-order intraflavin electron transfer rate constant (15 s^{-1}). The protein-bound FAD moiety of FMN-depleted reductase was reduced by 5-dRfH• with a second-order rate constant that was identical with that observed with the native enzyme (6.9×10^7 M^{-1} s^{-1}). However, with this species no significant decay of the FAD semiquinone was observed at 585 nm following its rapid formation, consistent with the above assignment of this kinetic process. Also consistent with this assignment is the fact that replacement of the FMN moiety by 7,8-Cl_2-FMN resulted in a ~2.5-fold increase in the observed intramolecular rate constant (170 s^{-1}). Replacement of EDTA as the electron donor (to the 5-dRf triplet) by oxalate led to no significant changes in the observed kinetic properties of the native reductase. In addition, no significant differences were observed between the reduction kinetics of the trypsin- and detergent-solubilized forms of the enzyme.

NADPH:cytochrome P-450 reductase is a component of the liver microsomal mixed-function oxidase system and participates in the transfer of two reducing equivalents in two separate electron transfer steps from NADPH to cytochrome P-450. It is also capable of transferring electrons to other heme-containing proteins such as cytochrome b_5 and heme oxygenase (Enoch & Strittmatter, 1979; Guengerich, 1978). It is an unusual flavoprotein in that it contains both FAD[1] and FMN moieties within the same polypeptide chain (Iyanagi & Mason, 1973) and is thus capable of direct electron transfer to cytochrome P-450 without the involvement of an intermediary iron–sulfur protein, as is the case for the cytochrome P-450 hydroxylase systems from *Pseudomonas putida* and adrenal mitochrondria (Katagiri et al., 1986; Omura et al., 1966). The enzyme has been successfully isolated in both the detergent- and trypsin-solubilized forms, although only the former is capable of direct electron transfer to cytochromes P-450 and b_5 (Gum & Strobel, 1979; French et al., 1980; Enoch & Strittmatter, 1979).

Studies with the native and the FMN-depleted forms of the reductase have established the FAD moiety as the site of electron entry into the enzyme, whereas the FMN is the electron-donating site to cytochromes P-450 and b_5, as well as to other artificial electron acceptors such as cytochrome *c* (Vermillion & Coon, 1976, 1978; Vermillion et al., 1981; Nisimoto & Shibata, 1982).

Reduction of the enzyme by dithionite leads initially to the formation of the neutral blue semiquinones of the respective flavins, followed by complete reduction of the enzyme to the fully reduced form (Vermillion & Coon, 1978). Subsequent reoxidation of the fully reduced species by molecular oxygen results in the formation of an air-stable semiquinone, which has been shown to be localized on the FMN moiety of the reductase (Otvos et al., 1982).

Potentiometric titrations have established the redox potentials of each flavin half reaction for the native reductase molecule; these are listed in Table I.

Table I: Redox Potentials of Flavin Half-Reactions for the Native Reductase Molecule[a]

half-reaction	$E_{m,7}$ (mV)
FMN/FMNH•	−110
FMNH•/$FMNH_2$	−270
FAD/FADH•	−290
FADH•/$FADH_2$	−365

[a] Values are from Iyanagi et al. (1974) and Vermillion and Coon (1978).

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[1] Abbreviations: 5-dRf, oxidized 5-deazariboflavin; 5-dRfH•, 5-deazariboflavin semiquinone; FAD, oxidized flavin adenine dinucleotide; FADH•, neutral FAD semiquinone; $FADH_2$, hydroquinone form of FAD; FMN, oxidized flavin mononucleotide; FMNH•, neutral FMN semiquinone; $FMNH_2$, hydroquinone form of FMN; 7,8-Cl_2-FMN, oxidized 7,8-dichloro-FMN; EDTA, ethylenediaminetetraacetate.

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Stopped-flow studies on the trypsin- and the detergent-solubilized enzyme in the presence of stoichiometric amounts of NADPH have shown that the two-electron reduction of the protein occurred simultaneously with the formation of protein-bound semiquinone. This observation suggested that the formation of the disemiquinoid species occurred at a rate that was at least comparable to, or even faster than, the rate of electron transfer from NADPH to the enzyme ($k > 28$ s^{-1}; Oprian & Coon, 1982). In the presence of greater than stoichiometric amounts of NADPH, decay of the protein-bound semiquinone occurred, consistent with formation of fully reduced flavin.

Although the enzyme has been the subject of numerous structural and kinetic investigations, the exact mechanisms by which the electrons are transferred from one flavin to the other remains obscure. In addition, the rate constants for these intramolecular electron transfer processes have yet to be determined. Our main aim in this study was to characterize the kinetic behavior of the system concomitant with and subsequent to one-electron reduction by laser flash generated free flavin semiquinones and thus to obtain rate constants for the various electron transfer reactions in the fully oxidized and partially reduced forms of the enzyme. We have previously used the laser flash photolysis technique to study intramolecular electron transfer in several protein redox systems with considerable success (Bhattacharyya et al., 1983, 1985; Cusanovich & Tollin, 1980; Ahmad et al., 1982; Tollin et al., 1982, 1986; Kipke et al., 1988). By using suitable electron donors (free flavin semiquinones), the reduction kinetics of the respective redox proteins can be optically monitored on varying time scales and at different wavelengths. An important advantage of this technique is the speed (<1 μs) with which the reductase can be reduced compared with stopped-flow spectrophotometry.

In the current study, the kinetic behavior of the native reductase molecule has been examined at several pH values. In addition, the kinetic properties of the FMN-depleted and 7,8-Cl_2-FMN-substituted reductases have also been investigated, and results obtained with the detergent- and the trypsin-solubilized forms of the enzyme have been compared. Due to the low redox potentials of the system under investigation, we have utilized the semiquinone of 5-deazariboflavin ($E_{m,7}$ = −650 mV; Blankenhorn, 1976) as the reductant in these reactions.

Materials and Methods

Liver microsomes were prepared from fresh male rabbit livers by the calcium precipitation method of Cinti et al. (1972). Detergent-solubilized NADPH:cytochrome P-450 reductase was prepared according to the method of Yasukochi and Masters (1976). Trypsin-solubilized P-450 reductase was isolated by the method of Iyanagi and Mason (1973) and further purified by 2′,5′-ADP–agarose affinity chromatography as described by Yasukochi and Masters (1976). FMN-depleted reductase was obtained by the KBr/charcoal dialysis treatment of Calhoun et al. (1987).

Reductase preparations were assayed at 30 °C for their ability to reduce cytochrome *c* in 0.33 M potassium phosphate buffer, pH 7.7, containing 1 mM EDTA. Flavin contents were determined by the fluorescence method of Faeder and Siegel (1973). These preparations typically contained 18–20 nmol of flavin/mg of protein. The ratio of FMN to FAD ranged from 0.8 to 0.85. FMN-depleted preparations of the reductase typically had FMN/FAD ratios of 0.05–0.07.

All chemicals were ACS reagent grade. 7,8-Cl_2-FMN was prepared as described by Shiga and Tollin (1976). 5-Deazariboflavin was a kind gift from Drs. W. McIntire and D. E. Edmondson.

All laser flash photolysis experiments were performed under anaerobic conditions and at ambient temperatures. The buffers used for the flash experiments were as follows: at pH 5.0, 10 mM sodium acetate, 0.5 mM EDTA, and 10% glycerol; at pH 6.0 and 7.0, 10 mM potassium phosphate, 0.5 mM EDTA, and 10% glycerol. A few experiments at pH 7.0 were performed in the presence of 10 mM sodium oxalate, instead of EDTA, as the electron donor to the 5-deazariboflavin excited triplet state. The concentration of 5-deazariboflavin in all flash experiments was ca. 130 μM.

All kinetic experiments were performed under pseudo-first-order conditions, in which the concentration of the oxidized protein was in large excess over the amount of 5-dRfH$^{\bullet}$ produced per flash [<1 μM; concentration based on an extinction coefficient of 3 cm^{-1} at 500 nm (Goldberg et al., 1981)]. This ensures that each reductase molecule reacts with a single 5-dRfH$^{\bullet}$ radical. In these experiments, protein reduction is always in competition with 5-dRfH$^{\bullet}$ disproportionation (Edmonson et al., 1972). Thus, depending upon the relative magnitudes of the second-order rate constants for these reactions (5-dRfH$^{\bullet}$ disproportionation rate constant is 4.3×10^9 M^{-1} s^{-1}) and the relative concentrations of the reactants, the kinetic traces corresponding to the reaction of protein with semiquinone obtained during a flash experiment will be influenced by the semiquinone disproportionation process. In the present case, as will be shown below, the protein reduction rate constants are comparatively small (<10^8 M^{-1} s^{-1}), and especially at the lower protein concentrations, it was necessary to deconvolute the kinetic transients in order to obtain a rate constant for protein reduction. This was done by taking advantage of the fact that semiquinone disproportionation contributes predominantly to the initial portion of the decay curve (due to its dependence on the square of the semiquinone concentration). Thus, an adequate representation of the kinetics of protein reduction could be obtained by hand-fitting a single exponential curve to the longer time portion of the kinetic trace. The validity of this procedure is attested to by the linear dependence of the first-order rate constants obtained in this way on the protein concentration (see below). For the systems dealt with in the present study, computer simulations indicate that this method of obtaining second-order rate constants for protein reduction tends to overestimate the actual value by an average of approximately 15% over the concentration range used (<15% at the higher end and >15% at the lower end of the concentration range of protein). Although, in principle, it is possible to use computer analysis to carry out this deconvolution, it has been our experience that this is less satisfactory than the above procedure. The number of laser flashes per experiment varied unless quantitation was required, in which case the same number of flashes were averaged per measurement. A description of the laser apparatus and the method of data collection and analysis has been published elsewhere (Przysiecki et al., 1985; Bhattacharyya et al., 1983). The experimental error in obtaining first-order rate constants was ≤10%.

Results

Kinetic Studies of Detergent-Solubilized Cytochrome P-450 Reductase. The complete absorbance spectra of the various oxidation states of cytochrome P-450 reductase are illustrated in the article by Oprian and Coon (1982). Only the features relevant to our experiments will be briefly outlined here. In the completely oxidized state, absorbance at 470 nm is maximum and diminishes roughly linearly with the addition of each

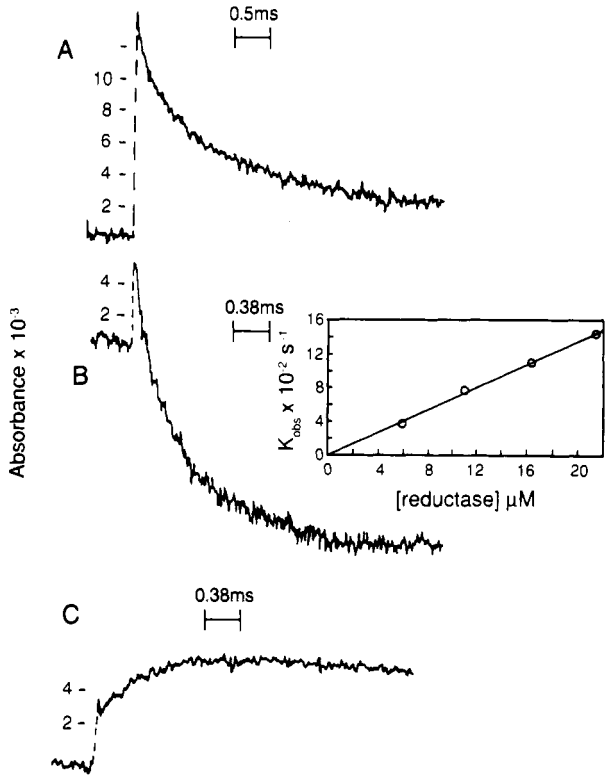

FIGURE 1: Reduction kinetics of oxidized detergent-solubilized NADPH:cytochrome P-450 reductase by 5-dRfH•. (A) Kinetic transient (obtained by summing absorbance from four flashes) obtained at 500 nm upon laser flash photolysis of a solution containing 130 μM 5-dRf. The buffer used was 10 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, and 10% glycerol. The rapid increase in absorbance followed by the slow decay correspond to the formation and subsequent disproportionation of 5-dRfH•. (B) Transient absorbance (sum of absorbance from three flashes) change obtained at 470 nm upon flash photolysis of 21.5 μM P-450 reductase in the presence of 5-dRf. The inset shows a second-order plot corresponding to the reaction between oxidized reductase and 5-dRfH• ($k = 6.8 \times 10^7$ M^{-1} s^{-1}). (C) Kinetic trace (sum of absorbance from four flashes) obtained at 585 nm upon flash photolysis of 21.5 μM reductase in the presence of 5-dRf. The exponential increase in absorbance represents formation of the neutral protein-bound semiquinone.

electron. The four-electron-reduced protein has approximately 10% of the absorbance at 470 nm compared to the completely oxidized reductase. One-electron reduction of either the holo or FMN-depleted reductase results in formation of the neutral blue semiquinone of the FMN or FAD moiety, respectively. The blue flavin semiquinone shows a characteristic broad absorbance at wavelengths above 500 nm and a loss of absorbance below 500 nm, with a maximum at 585 nm. Thus a difference spectrum between the one-electron-reduced form of the enzyme and the oxidized state should also reflect a broad positive absorbance above 500 nm and a more pronounced negative absorbance below 500 nm.

Reduction of Oxidized Cytochrome P-450 Reductase by 5-Deazariboflavin Semiquinone. Figure 1A shows a typical kinetic trace obtained at 500 nm upon flash photolysis of an anaerobic solution of 5-deazariboflavin plus EDTA in the absence of the reductase. The rapid increase in absorbance (<2 μs) followed by a second-order decay corresponds to the formation and disproportionation of 5dRfH•.

Figure 1B shows a typical kinetic trace obtained at 470 nm upon flash photolysis of 5-dRf in the presence of oxidized cytochrome P-450 reductase. In this case, the rapid increase in absorbance was followed by an exponential decay that eventually went below the preflash baseline. These changes are consistent with formation and subsequent reoxidation of 5-dRfH• by the flavoprotein leading to its reduction. The observed rate constant for protein reduction increased with increasing protein concentration (inset, Figure 1B), and a calculated second-order rate constant of 6.8×10^7 M^{-1} s^{-1} was obtained for this reaction.

When protein-bound flavin reduction was followed for 3–4 ms at 585 nm, an exponential increase in absorbance was observed, consistent with the formation of the protein-bound semiquinone (Figure 1C). Once again, the rate of formation of the protein-bound semiquinone increased with increasing protein concentration (not shown) and a calculated second-order rate constant of 7.1×10^7 M^{-1} s^{-1} was obtained, a value which, within experimental error, is the same as that obtained at 470 nm. Inclusion of NADP (1 mM) did not alter this rate constant (data not shown), indicating that the transfer of reducing equivalents from 5-dRfH• occurs at a site that is distinct from the NADP binding site on the enzyme.

Intramolecular Electron Transfer. The kinetic behavior of the protein-bound semiquinone was also monitored subsequent to its rapid formation. Figure 2A shows a typical transient obtained over an 80-ms interval at 585 nm following flash photolysis of the oxidized reductase in the presence of 5-dRf. The initial increase in absorbance (protein-bound semiquinone formation) was followed by a slow exponential decay that ended above the preflash baseline. The rate of this decay was invariant with protein concentration ($k_1 = 70$ s^{-1}; inset, Figure 2A), consistent with a first-order, i.e., intramolecular, process. Since the reported oxidized/one-electron redox potentials of the FAD and FMN moieties of the native reductase indicate that the predominant direction of electron transfer would be from FAD semiquinone (FADH•) to FMN, the first-order transient described above can tentatively be assigned to this intraflavin reaction (we will demonstrate below that, consistent with this assignment, both FMN removal and substitution by 7,8-dichloro-FMN significantly alter the kinetic properties of this transient signal).

When protein-bound flavin reduction was followed for 80 ms at 470 nm following the laser flash, an initial rapid bleaching was observed, followed by a minor slower exponential decrease in absorbance (Figure 2B). These changes are consistent with the rapid (second-order) reduction of the protein-bound flavin by 5-dRfH• and a subsequent slower reaction, which we have not investigated further.

Figure 2C shows the time-resolved flash-induced (reduced minus oxidized) difference spectra obtained between 470 and 610 nm, measured at $t = 4$ ms (when all the 5-deazariboflavin semiquinone had reacted with the oxidized reductase) and $t = 100$ ms following the laser flash (corresponding to the end point of the intraflavin reaction described above). Subsequent to $t = 100$ ms, no further transient absorbance changes were observed at time scales up to 5 s (data not shown). The solid line represents the enzyme-bound FAD semiquinone minus the spectrum of the oxidized enzyme, and the dashed line represents the FMN semiquinone minus the oxidized difference spectra obtained under steady-state conditions in a spectrophotometer. Although the overall shapes of the two flash-generated spectra resemble the steady-state semiquinone difference spectra, one evident discrepancy is an apparent shift in the isosbestic point in the enzyme-bound FAD spectrum. Although this may in part be due to the difference in experimental procedures, it is also possible that the transient FADH• spectrum is perturbed relative to the steady-state species. We will return to this again below.

Kinetic Studies of the FMN-Depleted Reductase. Kinetic studies were performed on FMN-depleted reductase that had

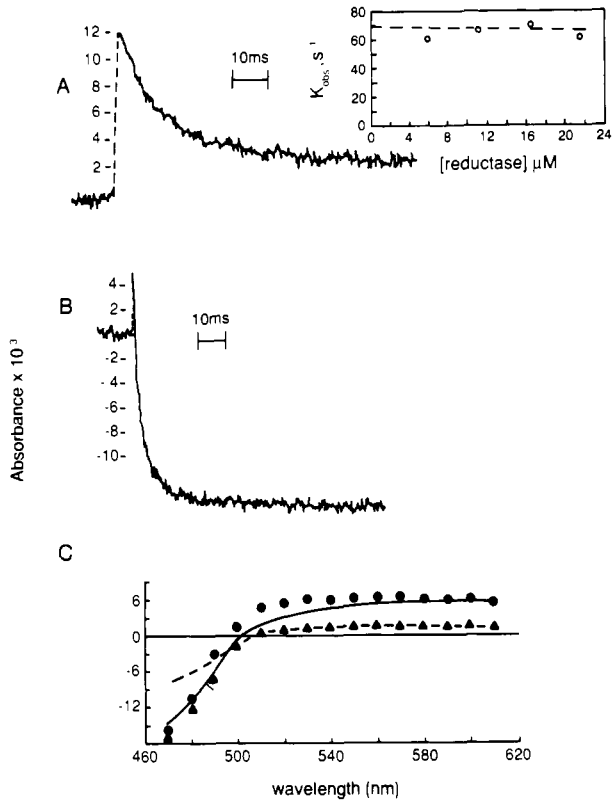

FIGURE 2: Kinetic behavior of the protein-bound semiquinone of detergent-solubilized cytochrome P-450 reductase. (A) Transient absorbance (sum of eight flashes) change at 585 nm. The protein concentration and buffer conditions were as in Figure 1. The inset shows the independence of the rate constant for semiquinone oxidation on the concentration of oxidized protein. (B) Kinetic trace (absorbance is the sum of four flashes) obtained at 470 nm showing the biphasic reduction of cytochrome P-450 reductase. Conditions as described in (A). (C) Flash-induced (reduced minus oxidized) time-resolved difference spectra associated with the reduction of cytochrome P-450 reductase. Laser flash data were obtained with a sample containing 21.5 μM P-450 reductase and 130 μM 5-dRf. Buffer conditions were as in Figure 1. The flash data represented by the closed circles correspond to the signal amplitude obtained at 4 ms following the laser flash (when all 5-dRfH• had reacted with the oxidized reductase), and the closed triangles represent the signal amplitude at 100 ms following the laser flash. The solid line represents the one-electron-reduced minus oxidized difference spectrum of protein-bound FAD obtained in a spectrophotometer by steady-state photoreduction of FMN-depleted reductase with the light/EDTA/5-dRf system. Spectral data were normalized to the flash data at 585 nm. The dashed line represents the one-electron-reduced minus oxidized spectrum of protein-bound FMN (air-stable semiquinone, obtained by partial reduction followed by air oxidation of native reductase) obtained in a spectrophotometer. Spectral data were normalized to the flash data at 585 nm.

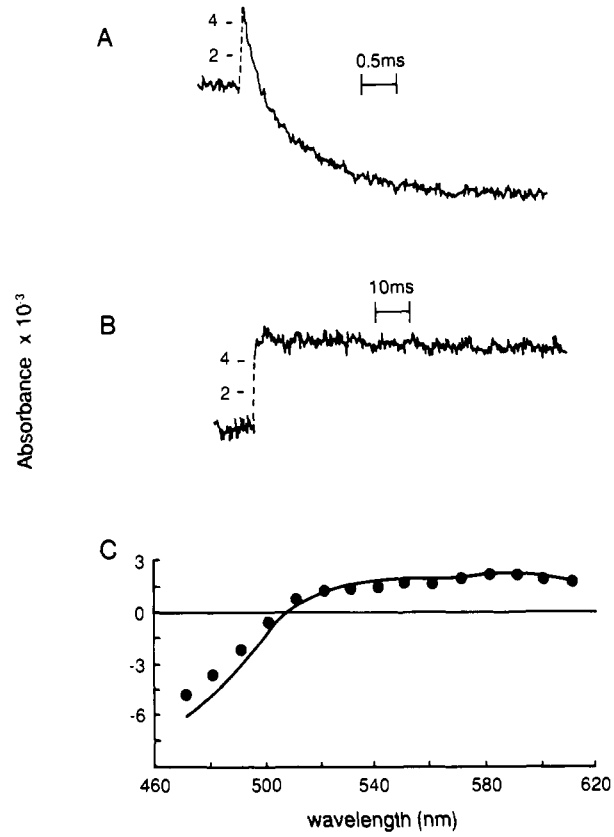

FIGURE 3: Reduction kinetics of FMN-depleted detergent-solubilized cytochrome P-450 reductase. (A) Transient absorbance (sum of three flashes) change obtained at 470 nm upon flash photolysis of 15.5 μM FMN-depleted reductase in the presence of 5-dRf. (B) Kinetic trace (absorbance is the sum of four flashes) obtained at 585 nm. Protein concentration was as described in (A). (C) Flash-induced (reduced minus oxidized) difference spectrum corresponding to the formation of the stable protein-bound semiquinone of FMN-depleted reductase. The experimental points represent the signal amplitude obtained at 100 ms following the laser flash. The solid line is a one-electron-reduced minus oxidized spectrum obtained by photoreduction of FMN-depleted enzyme in a spectrophotometer. Spectral data were normalized to the flash data at 585 nm.

approximately 5% residual cytochrome *c* reductase activity. Figure 3A shows reduction of the protein-bound FAD by 5-dRfH• at 470 nm. Once again, the observed rate of reduction of the FMN-depleted reductase increased with increasing protein concentration (data not shown), and a second-order rate constant of 6.9×10^7 M^{-1} s^{-1} was obtained for this reaction. This value is unchanged from that obtained with the native enzyme, in support of the contention that the FAD site is an entry point of electrons from 5-dRfH• (see below for further discussion).

In order to further determine whether the first-order decay of the protein-bound semiquinone observed with the native reductase was indeed due to reoxidation of the FAD radical by the oxidized FMN moiety, we examined the reduction kinetics of the protein-bound flavin semiquinone of the FMN-depleted reductase. Figure 3B shows a typical kinetic transient obtained at 585 nm on a 100-ms time scale following the laser flash. It is apparent that no significant decay of the semiquinone occurred. Figure 3C shows a flash-induced (reduced minus oxidized) difference spectrum obtained at 100 ms, corresponding to the reaction of 5-dRfH• with the FMN-depleted reductase. The solid line is a protein-bound FAD one-electron-reduced minus oxidized difference spectrum obtained in a spectrophotometer. The agreement between the flash and spectral data is reasonably good, although again there appear to be some differences in the shorter wavelength region. These results support the contention that the first-order decrease in the absorbance at 585 nm seen in the native reductase represents electron transfer from FADH• to FMN.

Electron Transfer Properties of the One-Electron-Reduced Cytochrome P-450 Reductase. The effect of prior one-electron reduction of the reductase on both the second-order rate of reaction with 5-dRfH• and the first-order intraflavin electron transfer reaction was examined. As in the case of the oxidized enzyme, an exponential bleach was observed upon laser photolysis at 470 nm, corresponding to further reduction of the partially reduced enzyme by 5-dRfH• (data not shown). However, the second-order rate constant corresponding to this electron transfer process was significantly smaller than that obtained for the fully oxidized protein ($k_2 = 2 \times 10^7$ M^{-1} s^{-1}).

Figure 4A shows the kinetic behavior of the protein-bound semiquinone at 585 nm upon flash photolysis of the one-

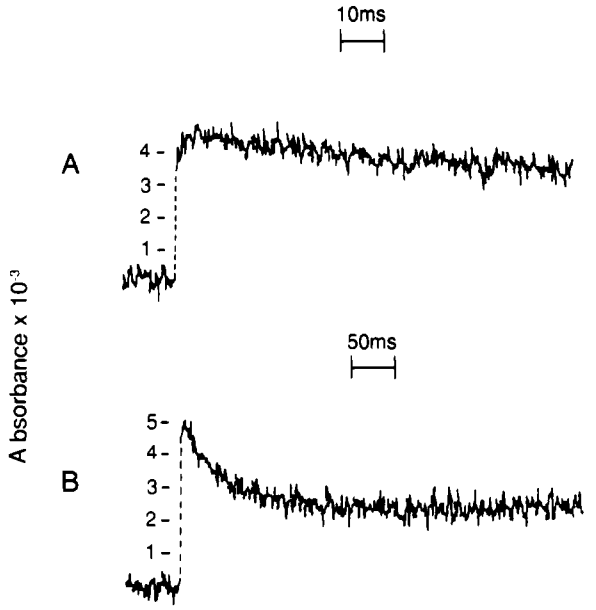

FIGURE 4: Kinetic behavior of one-electron-reduced detergent-solubilized P-450 reductase. (A) Absorbance change at 585 nm obtained upon flash photolysis of 21.5 μM one-electron-reduced reductase in the presence of 5-dRf, with buffer conditions as in Figure 1. Tracings in (A) and (B) were both obtained by summing absorbance from eight flashes. (B) Same as (A) except that the time scale was 500 ms.

electron-reduced reductase. It is apparent that the decay of the protein-bound radical was no longer complete on a 100-ms time scale (see Figure 2A for comparison). However, this decay was exponential over longer times (Figure 4B), and a first-order rate constant of 15 s^{-1} was obtained. In addition, the extent of decay was smaller than that obtained with the oxidized protein. A smaller decay magnitude would be expected on the basis of equilibration of electrons between the two flavin couples, since the one-electron-reduced enzyme ($FMNH^{\bullet}$) has a smaller redox potential difference between $FADH^{\bullet}$ and $FMNH^{\bullet}$ (ΔE = 20 mV) than that between $FADH^{\bullet}$ and FMN in the oxidized enzyme (ΔE = 180 mV).

The two intramolecular electron transfer reactions described above can be written as

$$FADH^{\bullet} + FMN \underset{k_b}{\overset{k_f}{\rightleftharpoons}} FAD + FMNH^{\bullet} \quad (1)$$

$$FADH^{\bullet} + FMNH^{\bullet} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} FAD + FMNH_2 \quad (2)$$

Since $k_{obs} = k_f + k_b$, $K_{eq} = k_f/k_b$, and $\Delta E = -0.06/(n \log K_{eq})$, both forward and reverse rate constants may be calculated from the observed first-order rate constants. Values of k_f = 69.9 s^{-1} and k_b = 0.1 s^{-1} were obtained for the oxidized reductase (reaction 1), and values of 10.3 and 4.8 s^{-1} were obtained for the forward and reverse rate constants for the one-electron-reduced reductase (reaction 2).

Effect of pH on the Reduction Kinetics of Cytochrome P-450 Reductase. Kinetic studies at pH 5.0 (data not shown) demonstrated that the oxidized reductase was reduced in a second-order manner with a rate constant that was approximately 3-fold larger than that observed at pH 7.0 ($k = 2 \times 10^8$ M^{-1} s^{-1} vs 6.9×10^7 M^{-1} s^{-1}).[2]

The kinetic behavior of the protein-bound semiquinone was followed at 585 nm. At pH 5.0, the first-order rate constant for semiquinone decay (not shown) was 62 s^{-1}, a value which is not very different from that observed at pH 7.0 (k_1 = 69 s^{-1}). Reduction of the flavoprotein to the one-electron level again resulted in a decrease in the rate constant of the first-order decay, giving a value (k_1 = 19 s^{-1}) similar to that obtained for the one-electron-reduced enzyme at pH 7.0. Furthermore the extent of decay at 585 nm remained unchanged between pH 5.0 and 7.0.

The invariance of the intraflavin electron transfer rate constant and the magnitude of the absorbance change with pH is consistent with the expected changes in the redox potentials of the protein-bound flavins. Since the redox potential of free flavins and most flavoproteins varies by ~60 mV/pH unit (Draper & Ingraham, 1968), decreasing the pH would be expected to result in a similar increase in the redox potentials of both flavins in P-450 reductase. Thus, the redox potential difference between the one-electron-reduced forms of FAD and FMN in the native reductase should remain unchanged, and this would be reflected in an unchanged intraflavin electron transfer rate constant and equilibrium constant.

Kinetic Studies of the 7,8-Cl_2-FMN-Substituted Cytochrome P-450 Reductase. Steady-state anaerobic photochemical reduction of reductase that had been depleted of FMN and reconstituted with 7,8-Cl_2-FMN indicated that the modified enzyme behaved in a manner similar to that reported for 7-Br-FMN-substituted reductase (Vermillion et al., 1981), in that complete reduction of the 7,8-Cl_2-FMN moiety was observed prior to the appearance of the semiquinone of FAD (data not shown). Thus, the two redox potentials of the bound 7,8-Cl_2-FMN also appear to be substantially closer together than those of FMN in the native enzyme. Figure 5A shows a typical kinetic transient obtained at pH 7.0 and 470 nm upon laser flash photolysis of the modified reductase in the presence of 5-dRf. The data demonstrated a more rapid second-order reduction of the enzyme ($k = 9 \times 10^7$ M^{-1} s^{-1}) by $5\text{-dRfH}^{\bullet}$. In addition, the kinetics of the protein-bound semiquinone were followed at 585 nm and are shown in Figure 5B, which shows the transient absorbance changes corresponding to the formation and subsequent decay of the protein-bound semiquinone ($FADH^{\bullet}$). The rate of this process again remained invariant with protein concentration, and a first-order rate constant of 170 s^{-1} was obtained, significantly larger than that observed with the native enzyme. Figure 5C shows a flash-induced time resolved (reduced minus oxidized) difference spectrum obtained at 2.5 ms (triangles, when all $5\text{-dRfH}^{\bullet}$ had reacted with the oxidized enzyme) and 50 ms (circles, corresponding to the end point of the intramolecular reaction described above) following the laser flash. This is similar to that obtained with the native enzyme.

The above results demonstrate that replacement of FMN by its dichloro analogue leads to a significant increase in the rate constant of the first-order decay of the $FADH^{\bullet}$ species. This result lends further support to the contention that the process observed at 585 nm corresponds to intraflavin electron transfer from $FADH^{\bullet}$ to FMN.

Reduction Kinetics of the Oxidized Cytochrome P-450 Reductase in the Absence of Glycerol Using Oxalate as the Electron Donor to 5-dRf. We have also examined the reduction kinetics of the detergent-solubilized reductase at neutral pH in the presence of sodium oxalate as the electron donor in place of EDTA. No significant differences were found in the transient kinetic behavior. This indicates the absence of any artifactual reactions due to the formation of donor photoproducts. Furthermore, the reduction kinetics of the oxidized reductase at pH 7.0 carried out in the absence

[2] Since the pH dependence of the one-electron redox potential of 5-dRf is not known, the effect of pH on the second-order rate constant for reduction of P-450 reductase cannot easily be predicted.

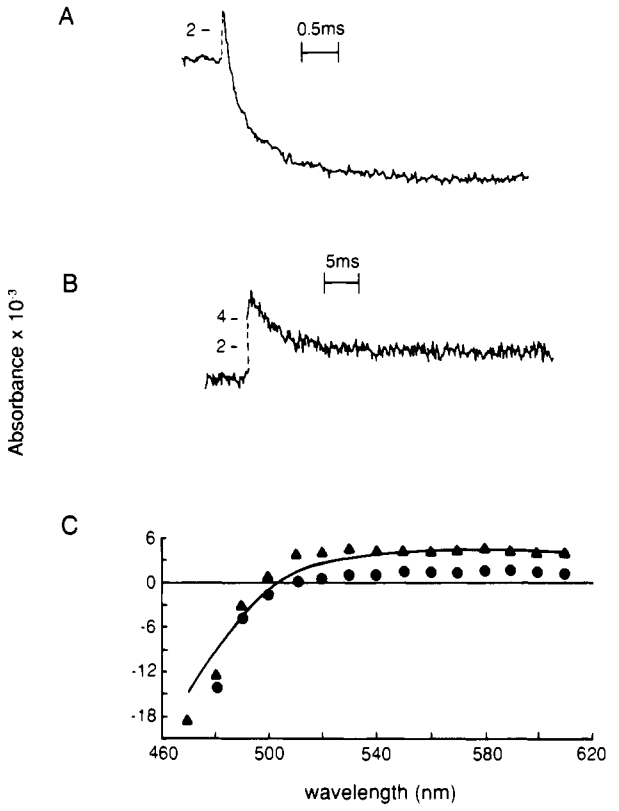

FIGURE 5: Reduction kinetics of 7,8-Cl_2-FMN-substituted detergent-solubilized cytochrome P-450 reductase. (A) Absorbance (sum of three flashes) changes over 5 ms at 470 nm. The protein concentration was 14.2 μM. Buffer conditions were as in Figure 1. (B) Absorbance (sum of six flashes) changes over 50 ms at 585 nm. Protein concentration and buffer conditions were as described in (A). (C) Time-resolved flash-induced (reduced minus oxidized) difference spectrum corresponding to the reduction of the substituted reductase. The experimental data points were obtained at 2.5 ms (solid triangles) and 50 ms (solid circles) following the laser flash, as described for Figure 2C. The line corresponds to the protein-bound one-electron-reduced minus oxidized FAD difference spectrum obtained with FMN-depleted reductase as described for Figure 2C. The spectral data were normalized to the flash data at 585 nm.

of glycerol were essentially the same as those obtained in its presence, once again indicating that the reactions observed were not due to artifacts created by any of the components of the buffer systems employed in these experiments.

Reduction Kinetics of the Trypsin-Solubilized Reductase. The reduction kinetics of the water-soluble trypsin-solubilized form of the reductase by 5-dRfH$^\bullet$ were compared to those obtained with the detergent-solubilized reductase at neutral pH and in the presence of EDTA. The second-order reduction of the enzyme by 5-dRfH$^\bullet$ (data not shown) occurred with a rate constant of 6.2×10^7 M^{-1} s^{-1}, a value not significantly different from that obtained with the detergent-solubilized form of the enzyme. Furthermore, no significant differences were detected in the kinetics of the protein-bound (FAD) semiquinone decay when compared to the detergent-solubilized form of the protein ($k = 66$ s^{-1}). These results are consistent with the results obtained from stopped-flow experiments, in which the reduction kinetics of both the trypsin- and detergent-solubilized forms of the enzyme with NADPH as the reductant were virtually identical. The results also indicate that the state of aggregation of the reductase (hexamer vs monomer) does not influence its reduction kinetics by either physiological or nonphysiological electron donors.

Discussion

The results presented above provide clear evidence for the rapid second-order reduction of P-450 reductase by 5-dRfH$^\bullet$, leading to the formation of a protein-bound semiquinone. Thermodynamic considerations predict that 5-dRfH$^\bullet$ should be capable of reacting with both the FAD and FMN centers in the native enzyme molecule. On the basis of the one-electron redox potential differences between 5-dRfH$^\bullet$ and FAD or FMN (260 or 540 mV), we would expect to see biphasic kinetics at 470 and 585 nm if both centers were reacting. However, the data clearly show monophasic behavior, indicating that 5-dRfH$^\bullet$ reacts either primarily with a single redox center (i.e., FAD) or with both centers at similar rates, thus making the two electron transfer processes unresolvable. We cannot at present distinguish between these two possibilities, although the former would seem more likely in view of the subsequent intramolecular reaction that we observe when FMN is present.

Sequence homology studies have led Porter and Casper (1987) to propose that the FAD- and FMN-binding domains of cytochrome P-450 reductase bear striking similarities to the FAD-binding domains of glutathione reductase and ferredoxin:NADP-reductase and to the FMN-binding domain of bacterial flavodoxin. The X-ray crystal structure of flavodoxin indicates that the dimethylbenzene portion of the flavin isoalloxazine ring is partially exposed to the solvent and hence is accessible to small external reductants (Mayhew & Ludwig, 1975). Flash photolysis studies on *Clostridium pasteurianum* flavodoxin ($E_{m,7}$; Fl_{ox}/FlH^- = −132mV) with 5-dRf as the reductant indicated that the oxidized FMN was rapidly reduced to the neutral semiquinone with a second-order rate constant of 3.7×10^8 M^{-1} s^{-1} (Simondsen & Tollin, 1983). If we accept the conclusion that the FAD is being preferentially reduced by 5-dRfH$^\bullet$, followed by internal electron transfer to the FMN, our results suggest that the flavin ring of the FMN moiety may be buried. Further work is required to resolve this question.

Reduction of the enzyme to the one-electron level led to a significant change in the second-order rate constant for reduction by 5-dRfH$^\bullet$ ($k_2 = 2 \times 10^7$ M^{-1} s^{-1} vs 6.8×10^7 M^{-1} s^{-1}). Since the FAD remains fully oxidized in this form of the reductase, one would not a priori expect a change in its rate of reduction. A possible explanation for this is that the accessibility of the FAD is altered by a conformational change occurring upon one-electron reduction. In support of this, fluorescence anisotropy measurements have shown that the mobility of the two flavins is considerably reduced upon one electron-reduction of the reductase (Blumberg et al., 1982). An alternative explanation is that the redox potential of the FAD moiety is altered due to reduction of the FMN moiety.

The intramolecular electron transfer process appears to be characterized by a decrease in absorbance of the protein-bound semiquinone subsequent to its rapid formation. Such a change is unexpected, since the extinction coefficients of the one-electron-reduced FAD and FMN moieties have been indirectly measured and are similar (Oprian & Coon, 1982; Iyanagi et al., 1974). If this were indeed the case, the absorbance changes corresponding to the reoxidation and the formation of the respective semiquinones would have canceled each other out. The data in Figure 2 indicate that the FAD semiquinone has the expected extinction coefficient ($\sim$3 mM^{-1} s^{-1}), while the FMN semiquinone (Figure 2C) has an extinction coefficient lower than expected (estimated to be $\leq$ 1 mM^{-1} cm^{-1} from the data of Figure 2), perhaps due to the experimental conditions or a protein environment different from that which exists in the equilibrium form of the one-electron-reduced enzyme (i.e., after the above-mentioned conformational

change). In fact, we cannot determine whether the kinetics we observe are due to the actual electron transfer event or to the presumed protein structural change. Another possibility, which can be ruled out, is that the transfer of reducing equivalents from FADH• to FMN leads initially to the formation of the anionic semiquinone, followed by protonation to the neutral form (Massey & Palmer, 1966). This is unlikely due to the absence of observed absorption changes subsequent to the initial (100 ms) decay of FADH• (up to 5 s, data not shown). We can also rule out the possibility of the formation of fully reduced protein-bound flavin (which would lead to a decrease in absorbance), since this would require formation of both FADH• and FMNH• by the reaction of two separate 5-dRfH• molecules with a single reductase molecule. This is not likely to occur under the current experimental conditions. The possibility of the same reaction occurring between two separate reductase molecules can also be ruled out since the kinetics of the observed absorbance decrease are protein concentration independent in both the detergent- and trypsin-solubilized forms of the enzyme.

The results obtained with the oxidized enzyme are consistent with the rate of intraflavin electron transfer being more rapid than the rate of enzyme reduction by NADPH (70 s^{-1} vs. 28 s^{-1}; Oprian & Coon, 1982). Such a rate constant for the intraflavin electron transfer process is not unreasonable considering that the two flavins are separated by a distance from 8 to 15 Å (Bonants et al., 1987).

In order to further characterize this system, kinetic studies should be performed on the FAD-depleted enzyme (Kurzban & Strobel, 1987), as well as other preparations of the reductase in which the FMN moiety is replaced by an analogue with modified redox and spectral properties, such as the 8-mercapto (see Vermillion et al., 1981) and 2-thio analogues of FMN. Such studies would permit the separate observation of the kinetic behavior of each protein-bound semiquinone species and thus further delineate the electron transfer properties of the reductase molecule.

Registry No. 5-dRfH•, 78548-68-2; FADH•, 73651-30-6; FMN, 146-17-8; NADPH–cytochrome P450 reductase, 9039-06-9.

References

Bhattacharyya, A. K., Tollin, G., Davis, M., & Edmondson, D. E. (1983) *Biochemistry 22*, 5270–5279.

Bhattacharyya, A. K., Tollin, G., McIntire, W., & Singer, T. P. (1985) *Biochem. J. 228*, 337–344.

Blankenhorn, G. (1976) *Eur. J. Biochem. 67*, 67–80.

Blumberg, W. E., Nisimoto, Y., & Maser, H. S. (1987) in *Oxygenases and Oxygen Metabolism*, pp 333–343, Academic Press, New York.

Bonants, P., Bastiaens, P., Visser, T., & Muller, F. (1987) in *Flavins and Flavoproteins* (Edmonson, D., & McCormick, D. B., Eds.) pp 357–360, Walter de Gruyter and Co., Berlin.

Calhoun, J. P., Ugent, S., & Masters, B. S. S. (1987) *Biochemistry 25*, 5344–5350.

Cinti, D. L., Moldeus, P., & Schenkman, J. B. (1972) *Biochem. Pharmacol. 21*, 3249–3256.

Cusanovich, M. A., & Tollin, G. (1980) *Biochemistry 19*, 3343–3347.

Draper, R. D., & Ingraham, L. L. (1968) *Arch. Biochem. Biophys. 125*, 802–808.

Edmondson, D. E., Barman, B., & Tollin, G. (1972) *Biochemistry 11*, 1133–1138.

Enoch, H. G., & Strittmatter, P. (1979) *J. Biol. Chem. 254*, 8976–8981.

Faeder, E. S., & Siegel, L. M. (1973) *Anal. Biochem. 53*, 332–336.

French, J. S., Black, S. D., Williams, C. H., Jr., & Coon, M. J. (1980) in *Microsomes, Drug Oxidations and Chemical Carcinogenesis* (Coon, M. J., Conney, A. H., Estabrook, R. W., Gelboin, H. V., Gillette, J. R., & O'Brien, J. P., Eds.) pp 387–390, Academic Press, New York.

Goldberg, M., Pecht, I., Keamer, H. E. A., Rainer, T., & Hemmerich, P. (1981) *Biochim. Biophys. Acta 673*, 570–593.

Guengerich, F. P. (1978) *Biochemistry 17*, 3633–3639.

Gum, J. R., & Strobel, H. W. (1979) *J. Biol. Chem. 254*, 4177–4185.

Iyanagi, T., & Mason, H. S. (1973) *Biochemistry 12*, 2297–2308.

Iyanagi, T., Makino, N., & Mason, H. S. (1974) *Biochemistry 13*, 1701–1710.

Katagiri, M., Ganguli, B. N., & Gunsalus, I. C. (1986) *J. Biol. Chem. 243*, 3343–3346.

Kipke, C. A., Cusanovich, M. A., Tollin, G., Sunde, R. A., & Enemark, J. H. (1988) *Biochemistry 27*, 2918–2926.

Mason, H. S. (1958) *Science (Washington, D.C.) 125*, 1185–1188.

Massey, V., & Palmer, G. (1966) *Biochemistry 5*, 3181–3189.

Masters, B. S. S., Kamin, H., Gibson, O. H., & Williams, C. H., Jr. (1965) *J. Biol. Chem. 240*, 921–931.

Mayhew, S. G., & Ludwig, M. L. (1975) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 12, pp 57–118, Academic Press, New York.

Mayhew, S. G., Foust, G. P., & Massey, V. (1969) *J. Biol. Chem. 244*, 803–810.

Nisimoto, Y., & Shibata, Y. (1982) *J. Biol. Chem. 257*, 12532–12539.

O'Donnel, M. E., & Williams, C. H., Jr. (1983) *J. Biol. Chem. 258*, 13795–13805.

Ohnishi, T., King, T. E., Salerno, J. C., Blum, H., Bowyer, J. R., & Maida, T. (1981) *J. Biol. Chem. 256*, 5577–5582.

Omura, T., Sanders, E., Estabrook, R. W., Cooper, D. Y., & Rosenthal, O. (1966) *Arch. Biochem. Biophys. 117*, 660–673.

Oprian, D. D., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 8935–8944.

Otvos, J. D., Krum, D. P., & Masters, B. S. S. (1986) *Biochemistry 25*, 7220–7228.

Porter, T. P., & Kasper, C. P. (1986) *Biochemistry 25*, 1682–1687.

Przysiecki, C. T., Bhattacharyya, A. K., Tollin, G., & Cusanovich, M. A. (1985) *J. Biol. Chem. 260*, 1452–1458.

Shiga, K., & Tollin, G. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) p 422, Elsevier Scientific Publishing Co., Amsterdam.

Simondsen, R. P., & Tollin, G. (1983) *Biochemistry 22*, 3008–3016.

Stankovich, M. A., Schopfer, L. M., & Massey, V. (1978) *J. Biol. Chem. 253*, 4971–4979.

Tollin, G., Meyer, T. E., & Cusanovich, M. A. (1980) *Biochemistry 21*, 3849–3856.

Vermillion, J. L., & Coon, M. J. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 674–678, Elsevier Scientific Publishing Co., Amsterdam.

Vermillion, J. L., & Coon, M. J. (1978) *J. Biol. Chem. 253*, 8812–8819.

Vermillion, J. L., Ballou, D. P., Massey, V., & Coon, M. J. (1981) *J. Biol. Chem. 256*, 266–277.