Preparation and Characterization of a 5′-DeazaFAD T491V NADPH−Cytochrome P450 Reductase†

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ABSTRACT: NADPH−cytochrome P450 reductase is a flavoprotein which contains both an FAD and FMN cofactor. Since the distribution of electrons is governed solely by the redox potentials of the cofactors, there are nine different ways the electrons can be distributed and hence nine possible unique forms of the protein. More than one species of reductase will exist at a given level of oxidation except when the protein is either totally reduced or oxidized. In an attempt to unambiguously characterize the redox properties of the physiologically relevant $FMNH_2$ form of the reductase, the T491V mutant of NADPH−cytochrome P450 reductase has been reconstituted with 5′-deazaFAD which binds to the FAD-binding site of the reductase with a K_d of 94 nM. The 5′-deazaFAD cofactor does not undergo oxidation or reduction under our experimental conditions. The molar ratio of FMN to 5′-deazaFAD in the reconstituted reductase was 1.1. Residual FAD accounted for less than 5% of the total flavins. Addition of 2 electron equivalents to the 5′-deazaFAD T491V reductase from dithionite generated a stoichiometric amount of the FMN hydroquinone form of the protein. The 5′-deazaFAD moiety remained oxidized under these conditions due to its low redox potential (−650 mV). The 2-electron-reduced 5′-deazaFAD reductase was capable of transferring only a single electron from its FMN domain to its redox partners, ferric cytochrome *c* and cytochrome b_5. Reduction of the cytochromes and oxidation of the reductase occurred simultaneously. The $FMNH_2$ in the 5′-deazaFAD reductase autoxidizes with a first-order rate constant of 0.007 s^{-1}. Availability of a stable NADPH−cytochrome P450 reductase capable of donating only a single electron to its redox partners provides a unique tool for investigating the electron-transfer properties of an intact reductase molecule.

NADPH−cytochrome P450 reductase from the rat is a 78225 kDa flavoprotein anchored to the endoplasmic reticulum via a transmembrane α helix at its amino terminus. It contains two flavin cofactors, FAD[1] and FMN, which sequentially transfer two reducing equivalents from NADPH to cytochrome P450 (*1*−*4*). The sequence of electron transfer is NADPH → FAD → FMN → cytochrome P450 (*5*, *6*). Additional electron acceptors of the reductase are heme oxygenase (*7*), cytochrome b_5 (*8*), fatty acid elongase (*9*), and cytochrome *c* (*10*). Ferricyanide and 2,3-dichloroindophenol are small molecule acceptors (*11*, *12*). There are four other FAD- and FMN-containing reductases which share 30−40% identity with cytochrome P450 reductase; they include bacterial sulfite reductase (*13*), nitric oxide synthase reductase (*14*), methionine synthase reductase (*15*), and novel reductase (*16*). Sequence analysis has shown that the amino-terminal FMN-binding domain is homologous to bacterial flavodoxins, and the carboxyl-terminal FAD-binding domain resembles the ferredoxin−$NADP^+$ reductase (*1*). The crystal structure of rat cytochrome P450 reductase has confirmed the structural homology with flavodoxins and the ferredoxin−$NADP^+$ reductase (*17*). An interflavin connecting region with no sequence similarity to any known protein joins the flavin domains and has been postulated to orient the two flavin cofactors for optimal electron transfer. In the crystal structure, the isoalloxazine rings are aligned with the dimethylbenzene rings facing each other with 3.85 Å between their C8 methyl groups. The flavin cofactors are, thus, appropriately positioned for rapid intramolecular electron transfer. Molecular orbital calculations using optimized geometries reveal an increase in partial charges at the C8 position upon reduction, consistent with a role for the methyl groups in electron transfer (*18*).

The distribution of electrons in the reductase is governed solely by the redox potentials of the flavin (*19*). The redox potentials of the flavins in the rabbit cyt P450 reductase are (*5*, *20*)

$$\mathrm{FMN} + e \rightarrow \mathrm{FMNH}\cdot \qquad E_1 = -110\ \mathrm{mV}$$

$$\mathrm{FMNH}\cdot + e \rightarrow \mathrm{FMNH_2} \qquad E_2 = -270\ \mathrm{mV}$$

$$\mathrm{FAD} + e \rightarrow \mathrm{FADH}\cdot \qquad E_3 = -290\ \mathrm{mV}$$

$$\mathrm{FADH}\cdot + e \rightarrow \mathrm{FADH_2} \qquad E_4 = -365\ \mathrm{mV}$$

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[1] Abbreviations: ATP, adenosine triphosphate; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; 5′-deazaFAD, 5′-deazariboflavin adenine dinucleotide; cyt P450 reductase, NADPH−cytochrome P450 reductase; cyt P450, cytochrome P450; cyt b_5, cytochrome b_5; cyt *c*, cytochrome *c*.

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Intraflavin electron transfer occurs with a rate constant of 11−70 s^{-1} (*21*, *22*). Each flavin can exist in three redox states (oxidized, 1-electron reduced, and 2-electron reduced). Therefore, nine unique species of reductase are possible. At any given level of reduction except completely oxidized or reduced, a mixture of reductase species will be present. For instance, in a 3-electron-reduced reductase preparation a reductase molecule may contain either 2, 3, or 4 electrons. To further complicate matters, it is also possible for the 3 electrons in a reductase molecule to be distributed differently between the flavin cofactors. This complexity makes it virtually impossible to deconvolute the spectral changes and assign them to a specific electron-transfer process (*19*). For example, the first electron will largely reduce FMN to the semiquinone ($E_1 = -110$ mV), but addition of the second electron will lead to the formation of four different species of the reductase which readily interconvert.

To investigate the function of the individual FAD and FMN cofactors, two different approaches have been employed. In one method, either the FAD or FMN was selectively removed to generate either the FAD- or FMN-depleted reductase (*5*, *6*, *23*, *24*). The FMN cofactor with a K_d of ∼10 nM is more readily removed (*5*, *6*). In contrast, the FAD cofactor is bound tightly to the reductase with a K_d of <1 nM and is difficult to remove. In the second method, the separate soluble FMN- and FAD-binding domains have been cloned, expressed, and characterized (*25*−*27*). These studies have provided important information about the function and chemistry of the individual flavin cofactors in the reductase. Furthermore, they have also demonstrated that although FMN and FAD bind to their respective domains in the absence of the second domain, alterations in one domain do have an impact on the structural integrity and activity of the remaining domain (*6*, *23*, *24*, *28*).

To be able to investigate the ability of the FMN domain of the reductase to donate a single electron to a protein acceptor, it was necessary to both simplify the complex spectral changes occurring in the native reductase during electron transfer and to conduct experiments with a reductase molecule that had normal activity with its redox partners. The T491V mutant of cyt P450 reductase was utilized for this purpose. This mutant reductase was selected for our studies because it binds FAD 100-fold less tightly than the native reductase but otherwise functions normally (*28*). The side chain of T491 forms a hydrogen bond with the phosphate group of FAD which is lacking when valine replaces threonine in the mutant protein. The diminished affinity of the T491V reductase for FAD facilitates the replacement of FAD with 5′-deazaFAD, a low-potential flavin analogue, which is not reduced either by NADPH or by dithionite under our experimental conditions. Characterization of the 5′-deazaFAD T491V reductase, from hereon referred to as 5′-deazaFAD reductase, revealed that the FMN domain was capable of donating a single electron to two different heme proteins, cytochrome b_5 and cytochrome *c*, at rates similar to those of the native reductase. Availability of this spectroscopically and functionally characterized reductase will make possible further studies with its physiological redox partners.

MATERIALS AND METHODS

Materials. All chemicals used are of ACS grade unless otherwise specified. FMN, FAD, sodium dithionite, and horse heart cyt *c* were purchased from Sigma-Aldrich (St. Louis, MO). ATP was purchased from Boehringer Mannheim (Indianapolis, IN), and dilauroylphosphatidylcholine (DLPC) was from Doosan Serdary Research Laboratory (Toronto, Canada). Horse heart cyt *c* was further purified on a 10 × 64 mm ion-exchange column (Bio-Scale S5, Bio-Rad, Berkeley, CA) using a Waters 650E protein purification system. It is of note that impurities existing in commercial glycerol, even in spectrophotometric grade, are capable of reducing cyt *c* as well as cyt b_5 under anaerobic conditions. Therefore, the glycerol used for all of our stopped-flow studies was distilled twice under vacuum to remove the impurities. 5′-Deazariboflavin was a gift from Dr. Vincent Massey (University of Michigan, Ann Arbor, MI), Dr. William McIntire (University of California, San Francisco, CA), and Dr. Marilyn Jorns (Hahnemann School of Medicine, Philadelphia, PA).

Synthesis and Purification of 5′-DeazaFAD. 5′-DeazaFAD was synthesized from 5′-deazariboflavin in the presence of ATP and FAD synthetase as previously described (*29*). Free FAD was removed from FAD synthetase (a gift from Dr. William McIntire, University of California, San Francisco, CA) by washing the FAD synthetase with buffer A (10 mM Tris-HCl, 200 mM NaCl, 3 mM ATP, pH 7.4) at 10 °C until the absorbance of the filtrate at 450 nm decreased to <0.005 au. In a typical reaction, 1 mL of *N*,*N*-dimethylformamide containing 5 mg of 5′-deazariboflavin was added to 250 mL of buffer B (10 mM potassium phosphate, 8 mM $MgCl_2$, 2 mM ATP, pH 6.7). The reaction was initiated by addition of 7 mg of FAD synthetase. The reaction mixture was incubated at 37 °C with shaking at 110 rpm. After 3 h of incubation, another 7 mg of FAD synthetase was added to the reaction mixture. Aliquots of the reaction mixture were withdrawn at various times and examined by thin-layer chromatography (TLC) to determine whether the reaction was complete. TLC was performed on a silica gel coated polyester film (Whatman Ltd., Hillsboro, OR). The mobile phase consisted of butanol, acetic acid, and water at a ratio of 12:3:5. Under these conditions, the rate of migration is 5′-deazariboflavin > 5′-deazaFMN > 5′-deazaFAD. The flavins were detected by their fluorescence under UV light.

Upon completion of the reaction, the reaction mixture was centrifuged at 12000 × *g* for 15 min to remove particulate matter. The supernatant containing the 5′-deazaFAD was loaded onto a Sep-Pak C18 cartridge (Waters Corp.) that had previously been washed with 3−5 volumes of 100% acetonitrile and subsequently equilibrated with distilled water. The cartridge with the adsorbed 5′-deazaFAD was then washed with 3 volumes of distilled water, and the 5′-deazaFAD was eluted with a solution of 10% acetonitrile: 90% H_2O. Pure 5′-deazaFAD was obtained by evaporating the solvent.

Expression and Purification of Cyt b_5 and T491V Cyt P450 Reductase from Escherichia coli. The detergent-solubilized form of rabbit cyt b_5 was expressed and purified as previously described (*30*). Briefly, the expressed apo-cyt b_5 was extracted from the lysed *E. coli* and subsequently reconstituted with exogenous heme. The first step in the purification

procedure was chromatography on a DEAE-Sepharose anion-exchange column. This was followed by size-exclusion chromatography on a Superdex 75 column. The purified cyt b_5 has a specific content of 50 nmol of heme/mg of protein. The concentration of cyt b_5 was determined spectrophotometrically using an extinction coefficient of $\Delta\epsilon_{409-424nm}$ of 185 mM^{-1} cm^{-1} between reduced and oxidized cyt b_5.

Rat wild-type and T491V cyt P450 reductase were expressed in *E. coli* and purified as previously described (*31*). Briefly, the bacterial membrane fraction was isolated and solubilized with 0.1% Triton X-100. The solubilized reductase was purified on a 2′,5′-ADP Sepharose affinity column. Residual detergent was removed from the cyt P450 reductase on a hydroxyapatite column. The purified protein was suspended in buffer C (0.1 M potassium phosphate, 15% glycerol, pH 7.4) and stored at −80 °C. The concentration of FAD- and FMN-containing holo-cyt P450 reductase was determined from its flavin content using an extinction coefficient of 21.4 $mM^{-1}cm^{-1}$ at 454 nm (*19*). Protein concentration was determined using a kit purchased from Pierce (no. 23240). Bovine serum albumin was used as a standard.

Preparation of 5′-DeazaFAD T491V Cyt P450 Reductase. Substitution of 5′-deazaFAD for FAD in T491V reductase was achieved by incubating the T491V reductase in the presence of excess 5′-deazaFAD at 20 ± 2 °C. In a typical exchange reaction, the T491V reductase was suspended at ∼1 μM concentration in buffer C that contained a 3-fold molar excess of 5′-deazaFAD. FMN (∼5 μM) was also included in the suspension to prevent dissociation of FMN from the reductase. The solution was incubated at 20 °C for 30 min to allow flavin exchange to occur. Free 5′-deazaFAD and FMN were removed from the reductase by centrifuging the mixture at 6000 rpm for 30 min in an Apollo concentrator (Orbital Biosciences). The reductase was then resuspended in buffer C. The exchange process was repeated until greater than 95% of the FAD molecules in the reductase had been replaced by 5′-deazaFAD. Typically, this occurred after three exchange reactions but occasionally required four.

The extent of FAD to 5′-deazaFAD exchange was evaluated by measuring the ability of the reductase to reduce cyt *c* in the presence of NADPH after each cycle of exchange. Only those reductase molecules that contain both FAD and FMN will be able to accept electrons from NADPH and reduce cyt *c*. The assay was performed as previously described in 0.27 M potassium phosphate buffer (pH 7.7) at 30 °C unless otherwise specified (*28*). The formation of ferrous cyt *c* was monitored at 550 nm on a Cary 300 spectrophotometer. An extinction coefficient of 21.1 mM^{-1} cm^{-1} was used to determine the amount of cyt *c* reduced. The specific activity of the reductase was determined from the initial rate of the reaction and expressed as micromoles of cyt *c* reduced per minute per milligram of reductase.

Determination of the Flavin Content of 5′-DeazaFAD T491V Cyt P450 Reductase by HPLC. The amount of FAD, FMN, and 5′-deazaFAD in the reductase samples was measured using a modification of a previously described HPLC method (*32*, *33*). The HPLC assay was performed on a Waters HPLC system consisting of two Waters 510 pumps, a system interface module, a Waters 590 UV−visible detector, and a PC workstation. Separation of the three flavins was achieved on a reverse-phase C18 column (4.6 × 250 mm, Lichrospher 5RP18, Varian Inc.) with a mobile phase of 20% methanol/80% potassium phosphate buffer (5 mM, pH 6.0). A 10 × 3 mm guard column (ChromGuard, Varian Inc.) was placed in front of the C18 column to remove small particles. Elution of the flavins was monitored at both 450 and 268 nm. The concentration of the standard solutions of FAD, FMN, and 5′-deazaFAD was determined using extinction coefficients of $\epsilon_{450nm} = 11.3$ mM^{-1} cm^{-1} (*34*), $\epsilon_{450nm} = 12.2$ mM^{-1} cm^{-1} (*35*), and $\epsilon_{399nm} = 11.5$ mM^{-1} cm^{-1} (*36*), respectively. A standard curve for quantification of each of the three flavins was obtained by integrating the area under the 268 nm absorbance peak of the flavin and plotting this area versus the amount of flavin loaded onto the column. The flavin calibration curve and reductase samples were prepared and analyzed on the same day. Typically, aliquots of 50−100 μL of reductase (5−10 μM) were sealed in a microtube and heated at 95 °C for 5 min in the dark to release flavins from the reductase. The heated samples were then placed on ice for 3−5 min and subsequently centrifuged for 10 min to remove the denatured protein. A 10−20 μL aliquot of the supernatant was loaded onto the HPLC column and eluted with 20% methanol/80% potassium phosphate buffer (5 mM, pH 6.0) at a flow rate of 0.8 mL/min.

Reduction of the Cyt P450 Reductase Preparations with a Stoichiometric Amount of Dithionite. The redox properties of 5′-deazaFAD reductase were examined by reducing the reductase with sodium dithionite under anaerobic conditions. Oxygen was removed from buffer solutions, glassware, and reductase samples by incubating them overnight in an anaerobic glovebox (Belle Technology, Portesham, Dorset, England). After an overnight incubation in the glovebox, the oxygen concentration in the buffer was ∼0.1 μM. The reductase sample was maintained at 4 °C overnight in the glovebox to prevent inactivation. Typically, 1.5 mL of a deoxygenated solution of reductase (5−10 μM) in buffer C was reduced in a tonometer with a standardized solution of dithionite at 25 °C. Methyl viologen, which functions as an electron mediator, was present at a final concentration of 0.1 μM. Dithionite was added in 2 μL increments using a Hamilton syringe. The reduction of the reductase by an aliquot of dithionite was considered complete when the absorbance change at 450 nm stabilized, typically, after 10−15 min. The dithionite solution was standardized by determining how much dithionite was required to reduce a known amount of cyt b_5.

Kinetics of Electron Transfer from the Wild-Type, T491V, and 5′-Deaza T491V Reductase to Oxygen, Ferric Cyt c, and Ferric Cyt b_5. The kinetics of electron transfer from the various reductase preparations to an protein electron acceptor were studied under anaerobic conditions using a SF61DX2 stopped-flow spectrophotometer (Hi-Tech) housed in an anaerobic glovebox. To characterize the autoxidation of the different reductases, the reduced protein was loaded into one of the driving syringes while buffer C which had been equilibrated with room air was loaded into the second driving syringe. The concentration of oxygen at 15 °C in buffer C is ≅320 μM (*37*). The final concentration of the reductase in the observation cell was 2.5−5 μM. The contents of the two syringes were rapidly mixed, and the absorbance changes occurring during the autoxidation of the reductase were recorded in the single wavelength mode at 450 and 585 nm.

Electron transfer from the mutant and wild-type reductases to ferric cyt *c* was characterized under pseudo-first-order conditions. The final concentration of the 4-electron-reduced wild-type and T491V reductase in the observation cuvette was ~2.5 μM whereas the 2-electron-reduced 5′-deazaFAD reductase was present at ~5 μM. The four final concentrations of ferric cyt *c* were 7.5, 15, 30, and 50 μM, respectively. Reduction of cyt *c* was followed at 550 nm ($\Delta\epsilon = 21.1$ mM^{-1} cm^{-1}) (*38*). At this wavelength the reductase also exhibits a small increase in absorbance due to formation of the semiquinone ($\Delta\epsilon_{550} = 2.5$ mM^{-1} cm^{-1}). Oxidation of the reductase was monitored at 431 nm, which is an isosbestic point for cyt *c*. Kinetic traces at 550 and 431 nm were fitted with two exponential functions, and two apparent rate constants (k_1^{app}, k_2^{app}) were obtained. A plot of k^{app} vs concentration of cyt *c* yields the second-order rate constant.

Electron transfer from 5′-deazaFAD reductase to ferric cyt b_5 under anaerobic conditions was also characterized using stopped-flow spectrophotometry. Reduction of cyt b_5 was monitored at 420 nm ($\Delta\epsilon = 80$ mM^{-1} cm^{-1}) where the spectral changes from 5′-deazaFAD reductase are negligible ($\Delta\epsilon = 0.6$ mM^{-1} cm^{-1}) (see Figure 3). Oxidation of the reductase was monitored at 567 nm ($\Delta\epsilon = 2.5$ mM^{-1} cm^{-1}), which is an isosbestic point for cyt b_5 (*39*). Rate constants and amplitudes were obtained by fitting the kinetic traces with multiple exponential functions using KinetAsyst 2 software (Hi-Tech).

RESULTS AND DISCUSSION

Determination of the Dissociation Constant, K_d, between 5′-DeazaFAD and T491V Cyt P450 Reductase. Preliminary experiments indicated that the FAD-depleted T491V reductase was unstable and became inactive shortly after FAD was removed. Other investigators have made similar observations and noted that although the individual domains are competent in binding their respective FMN and FAD cofactors, alterations in one domain do impact the integrity of the second domain (*6*, *24*, *28*). It was, therefore, necessary to reconstitute the protein with a flavin analogue that would maintain the structural integrity of the reductase, yet be redox inactive under our experimental conditions. 5′-DeazaFAD was selected because it is structurally similar to FAD, differing only at position 5 where the nitrogen of FAD has been replaced by a carbon which is inert in redox reactions compared to nitrogen. It is known that the N5 nitrogen of FAD receives the hydride ion from NADPH. As a result of this substitution, 5′-deazaFAD is 3−4 orders of magnitude less reactive with NADPH. In addition, the 5′-deazaFAD semiquinone is also destabilized both in the protein and free in solution where the potential between the oxidized and semiquinone flavin is −650 mV. Thus, 5′-deazaFAD should maintain the structural integrity of the FAD domain but not undergo electron-transfer reactions under our experimental conditions.

To minimize the amount of the scarce 5′-deazaFAD required for preparation of 5′-deazaFAD reductase, the K_d between 5′-deazaFAD and T491V reductase was determined. This information was then used to devise an efficient method of exchanging FAD for 5′-deazaFAD. The K_d between 5′-deazaFAD and T491V reductase was determined by monitoring the activity of T491V reductase in the presence of various molar ratios of free 5′-deazaFAD and FAD. When 5′-deazaFAD displaces FAD and binds to the T491V reductase, the reductase is unable to accept a hydride ion from NADPH. It will, therefore, not be able to reduce cyt *c* which receives an electron from the $FMNH_2$ of the reductase. Table 1 shows that the activity of the T491V reductase progressively decreases when it is incubated in the presence of 10 μM FAD and increasing concentrations (0, 10, and 20 μM) of 5′-deazaFAD. This decrease in activity is attributed to the substitution of 5′-deazaFAD for FAD.

Table 1: Cyt *c* Reductase Activity of T491V Reductase in the Presence of Excess Free FAD and 5′-DeazaFAD

FAD:5′-deazaFAD	ν (nmol of cyt *c* reduced/min)[a]	K_d^R [b]
1:0	35.9	
1:1	15.7	0.78
1:2	10.5	0.78

[a] The assay was performed as described in Materials and Methods with the following modifications. The reaction was performed at 30 °C in 0.3 M potassium phosphate buffer (pH 7.7), 10 μM FAD, and either 0, 10, or 20 μM 5′-deazaFAD. The reaction was initiated by addition of NADPH to a final concentration of 0.3 mM. [b] Relative dissociation constant (see text for details).

At equilibrium, the following two equations define the dissociation constant between the reductase and each of the flavins:

$$K_d^{5'-deaza} = \frac{[PR][5'\text{-deazaFAD}]}{[5'\text{-deazaFAD}-PR]} \quad (1)$$

$$K_d^{FAD} = \frac{[PR][FAD]}{[FAD-PR]} \quad (2)$$

where PR is the free reductase without FAD or 5′-deazaFAD, 5′-deazaFAD−PR and FAD−PR are the complexes between reductase and either 5′-deazaFAD or FAD, respectively, and 5′-deazaFAD and FAD are the unbound free flavins. The relative dissociation constant K_d^R is given by

$$K_d^R = \frac{K_d^{5'-deazaFAD}}{K_d^{FAD}} = \frac{[FAD-PR][5'\text{-deazaFAD}]}{[FAD][5'\text{-deazaFAD}-PR]} \quad (3)$$

In the presence of both FAD and 5′-deazaFAD the ratio of [FAD-PR]/[5′-deazaFAD-PR] is proportional to $\nu/(\nu_0 - \nu)$, where ν_0 is the activity of the FAD-containing T491V reductase in the absence of 5′-deazaFAD and ν is the activity of the T491V reductase in the presence of both FAD and 5′-deazaFAD. The relative dissociation constant can be further simplified to eq 4 by substituting ν and $\nu_0 - \nu$ into eq 3:

$$K_d^R = \frac{[5'\text{-deazaFAD}]\nu}{[FAD](\nu_0 - \nu)} \quad (4)$$

The activity of the T491V reductase was measured in the presence of varying concentrations of FAD and 5′-deazaFAD. Since the added free FAD and 5′-deazaFAD were present at ~1000-fold excess relative to the reductase, the concentration of the free flavins was considered to be the added concentration. By substituting the values for the activity of the reductase in the presence of varying ratios of

Table 2: Flavin Content of Wild-Type, T491V, and 5′-DeazaFAD T491V Cyt P450 Reductase[a]

protein sample	FAD (mol/mol of protein)	FMN (mol/mol of protein)	5′-deazaFAD (mol/mol of protein)	FAD:FMN:5′-deazaFAD
wild type	0.87 ± 0.09	0.88 ± 0.11	0	1:1:0
T491V	0.71 ± 0.06	0.86 ± 0.05	0	1:1.2:0
5′-deazaFAD reductase	0.005 ± 0.003	0.60 ± 0.08	0.54 ± 0.02	0.001:1.1:1

[a] The flavin content was determined as described in Materials and Methods. The results are an average of measurements from two different preparations.

high concentrations of the added flavins into eq 4, a relative dissociation of 0.78 was calculated (Table 1). The K_m of FAD for the T491V reductase is 0.12 μM, and it has been assumed in these experiments that the K_m and K_d are similar (*28*). Substituting these values into eq 3 indicates that the K_d between 5′-deazaFAD and T491V reductase is 94 nM at 30 °C. The relative K_d^R for 5′-deazaFAD and FAD is 0.78, which indicates that the two flavins bind with similar affinity to the FAD domain of T491V reductase. The almost identical affinity of FAD and 5-deazaFAD for the reductase provided the theoretical basis for preparing the 5′-deazaFAD reductase.

Preparation of 5′-DeazaFAD T491V Reductase. The 5′-deazaFAD reductase was prepared by incubating the T491V reductase with a 3-fold molar excess of 5′-deazaFAD. At equilibrium, the reductase should contain 25% FAD and 75% 5′-deazaFAD. Following incubation of the reductase with the 5′-deazaFAD, the unbound FAD and 5′-deazaFAD were removed from the reductase. The reductase now containing a mixture of 25% FAD and 75% 5′-deazaFAD was incubated a second time with 5′-deazaFAD to continue replacing FAD with 5′-deazaFAD. Repetition of this process three to four times resulted in a reductase molecule in which the 5′-deazaFAD replaced the FAD by >95% (see later section). This 5′-deazaFAD T491V reductase was shown to be active in reducing cyt *c* by subsequently replacing the 5′-deazaFAD with FAD and measuring the ability of the "back-exchanged" reductase to reduce cyt *c* in the presence of NADPH. Approximately 64−75% of the original T491V reductase activity could be recovered by replacing the 5′-deazaFAD with FAD. Treatment of the FAD-containing T491V reductase in a parallel manner resulted in a 16% loss in activity. The temperature at which the flavins are exchanged is critical. At 30−35 °C, the flavin exchange occurred rapidly but the reductase was inactivated. However, between 4 and 10 °C, the reductase was stable but the substitution of one flavin for another was too slow. As a compromise, the flavin exchange was conducted at 20−22 °C for 30 min.

Determination of the Flavin Content of 5′-DeazaFAD T491V Cyt P450 Reductase. An unambiguous interpretation of experiments with the 5′-deazaFAD reductase requires that the FAD in the reductase is replaced by 5′-deazaFAD. The flavin content of the 5′-deazaFAD T491V reductase was determined using an HPLC assay capable of resolving the three possible flavins, i.e., FMN, 5′-deazaFAD, and FAD. The UV−visible spectra of the free oxidized FAD, FMN, and 5′-deazaFAD are shown in Figure 1. Free FAD and FMN have absorption maxima at 440 and 369 nm in the visible spectrum and at 263 nm in the UV. The absorption maxima of free 5′-deazaFAD are blue shifted by approximately 40 nm to 400 and 336 nm in the visible region while the absorption maximum in the UV spectrum is blue shifted to 255 nm. The spectrum of 5-deazaFAD in Figure 1 is identical

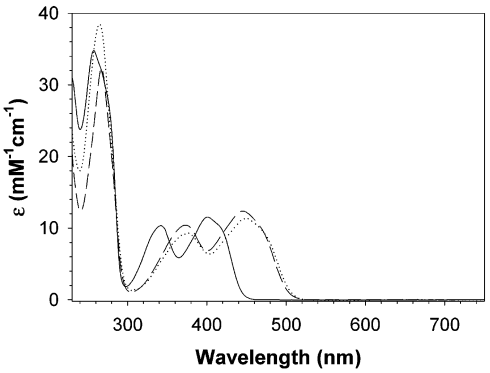

FIGURE 1: UV−visible spectra of oxidized FAD, FMN, and 5′-deazaFAD. The spectra were recorded in 0.1 M potassium phosphate buffer (pH 7.0): (•••), FAD; (---), FMN; (—), 5′-deazaFAD.

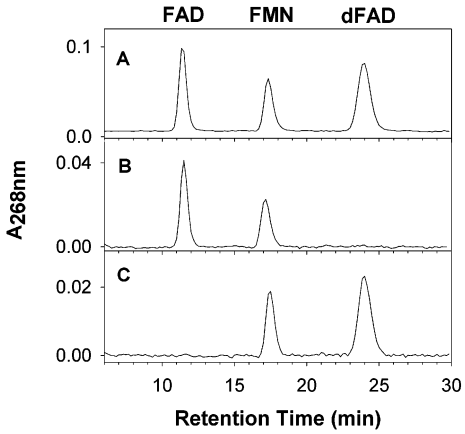

FIGURE 2: HPLC chromatograms of FAD, FMN, and 5′-deazaFAD. (A) Standard flavin solution that contained 150 pmol of each of the three flavins, FAD, FMN, and 5′-deazaFAD. (B) Flavins from 66 pmol of wild-type reductase. (C) Flavins from 48 pmol of 5′-deazaFAD reductase. The flavins were chromatographed on a C18 Lichrospher column and eluted and detected as described in Materials and Methods.

to that reported by Spencer and Walsh (*36*). Reductase-bound flavins are expected to have spectra similar to those of free flavins. A calibration curve which was linear when 0−200 pmol of an individual flavin was injected onto the column was constructed. In the wild-type reductase, the molar ratio of FMN:FAD:reductase was 0.87:0.88:1 (Table 2). As expected, no 5′-deazaFAD was detected in the native reductase (Figure 2B). In the 5′-deazaFAD reductase the average molar ratio of FMN:5′-deazaFAD:T491V reductase was 0.6:0.54:1 (Table 2). The amount of FAD present in the 5′-deazaFAD reductase was at the limit of detection for the assay and accounted for less than 5% of the amount of 5′-deazaFAD in the sample. These results unequivocally demonstrate that the 5′-deazaFAD reductase preparations contain an approximately equal amount of FMN and 5′-

Table 3: Catalytic Activity of 5′-DeazaFAD T491V, T491V, and Wild-Type Cyt P450 Reductase with Cyt *c* in the Presence of Added FAD and FMN[a]

reductase	[FAD] (μM)	[FMN] (μM)	μmol of cyt *c* reduced min^{-1} (mg of protein)$^{-1}$
5′-deazaFAD			0.022 ± 0.010
T491V	0.1		13.7 ± 1.5
	1.0		20.4 ± 2.0
	5.0		22.03 ± 1.8
	10.0		23.63 ± 2.2
	20.0		23.31 ± 1.9
	20.0	20.0	22.35 ± 1.8
T491V			17.5 ± 2.1
	0.1		21.0 ± 1.8
	1.0		29.0 ± 2.5
	5.0		34.1 ± 3.1
	10.0		37.1 ± 4.0
	20.0		36.1 ± 2.8
	20.0	20.0	36.1 ± 3.0
wild type			30.8 ± 4.0
	0.1		30.8 ± 3.5
	10.0		31.3 ± 2.8
	20.0	20.0	31.0 ± 2.9

[a] Cyt *c* reductase activity was measured at 20 °C in 0.27 M potassium phosphate buffer, pH 7.7, containing 65 μM NADPH and 50 μM cyt *c*. The reductase (final concentration 10 nM) was added to start the reaction.

deazaFAD with a negligible amount of residual FAD (Table 2).

Effect of Supplemental FAD and FMN on the Activity of T491V and 5′-DeazaFAD T491V Cytochrome P450 Reductase. To ensure that all available flavin binding sites on the reductase were occupied, the activity of the FAD-containing wild-type and 5′deazaFAD T491V reductase preparations was measured in the presence of increasing concentrations of FAD and FMN. As expected, addition of FAD to the 5′-deazaFAD T491V reductase stimulated its activity by ≅1000-fold; in contrast, addition of a 2000-fold excess of FMN to the maximally FAD stimulated T491V reductase did not result in any further increase in activity (Table 3). This observation indicates that the low specific flavin content of our reductase preparation cannot be attributed to unoccupied but otherwise competent FMN binding sites. If the low specific flavin content of our reductase were due to a random distribution of FMN and FAD in different reductase molecules, addition of an excess of FMN to a T491V reductase preparation saturated with FAD would double the activity. Clearly, this did not occur. It has been our experience that for unknown reasons the specific flavin content of the reductase depends on the particular enzyme preparation. Either the preparation has impurities or inactive reductase incapable of binding exogenous flavins is present or both. Since there is only a single band on the SDS−PAGE, it is likely that the flavin content of the 5′-deazaFAD T491V reductase used in the experiments reported here is low because of inactive enzyme which, in turn, is secondary to an extensive enzyme preparation procedure and the instability of the reductase in the absence of bound FAD. Despite the variable specific content of the enzyme preparations, the enzymatic activity correlates with the amount of FMN in the reductase preparation and cannot be significantly increased with additional FMN. As will be shown later the non-flavin-containing protein does not influence the kinetics of electron transfer.

Table 3 also illustrates the effect of addition of FAD and FMN to T491V reductase. As predicted from the K_d (0.12 μM) of FAD for the T491V reductase and the final concentration of reductase (10 nM) in the cyt *c* assay, a majority of the FAD will dissociate from the reductase when it is diluted into the cyt *c* assay reaction mixture. Thus FAD in the reaction mixture enhances the activity of the T491V reductase by ensuring the enzyme binds its full complement of the cofactor (*28*). FMN did not stimulate the activity of the FAD-saturated T491V reductase, indicating that all competent FMN binding sites were occupied. The activity of the wild-type reductase which had a specific flavin content similar to the T491V enzyme was not stimulated by addition of either flavin (Table 3). Using Dyna Fit software, the K_d of FAD for the T491V and 5′-deazaFAD T491V preparations was determined to be 0.084 ± 0.012 μM and 0.08 ± 0.01 μM, respectively, at 15 °C (*40*).

Reduction of 5′-DeazaFAD T491V Cyt P450 Reductase with a Stoichiometric Amount of Dithionite. The spectra of the oxidized and 1-electron- and 2-electron-reduced 5′-deazaFAD reductase are presented in Figure 3A. The reductase was reduced anaerobically with a standardized solution of dithionite. The spectrum of 5′-deazaFAD reductase shows an absorption maximum at 400 nm with a shoulder at 450 nm. The absorption maximum at 400 nm is a result of absorption by both 5′-deazaFAD (maximum at 400 nm) and FMN while the shoulder at 450 nm is due almost exclusively to FMN (see Figure 1). In Figure 3B, the amount of dithionite added has been corrected to account for reduction of the trace amount of oxygen present at the beginning of the titration. The data are also corrected for dilution by the added dithionite. The reduction of the reductase was followed at 585 nm to monitor the FMNH· blue semiquinone and at 450 nm to monitor the overall reduction of FMN.

As expected, the concentration of FMNH· was maximum when 1 electron equivalent had been added to the reductase (*41*). In addition, two isosbestic points were observed between the spectra of the oxidized flavin and the semiquinone at 503 and 362 nm (Figure 3A). The difference spectrum between the oxidized and semiquinone form of the reductase is illustrated in Figure 3C (—). It clearly illustrates the isosbestic points between the two spectra and the extinction coefficients for the decrease in absorbance at 450 nm and the increase between 530 and 670 nm. The spectral changes occurring during 1-electron reduction are very similar to those observed for wild-type reductase during 1-electron reduction (*12*).

However, addition of a second electron equivalent to the FMNH· semiquinone completely bleached the semiquinone absorbance between 530 and 670 nm and continued to decrease the absorbance at 450 nm, which is a measure of flavin oxidation state. An isosbestic point at 400 nm was observed when the semiquinone was reduced to the hydroquinone (Figure 3A,C). The occurrence of an isosbestic point during reduction of the semiquinone to the hydroquinone provides additional evidence that the 5′-deazaFAD is not reduced under our experimental conditions. The spectral changes which are observed, i.e., the difference spectrum between the hydroquinone and semiquinone, are shown in Figure 3C (···) along with their associated extinction coefficients. These spectral changes contrast with those

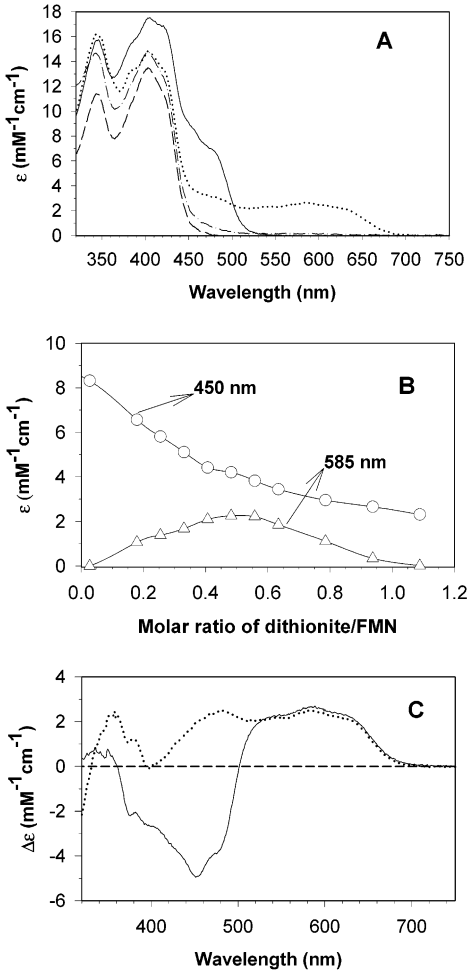

FIGURE 3: UV−visible spectra of oxidized and 1- and 2-electron-reduced 5′-deazaFAD T491V cyt P450 reductase. The reductase was reduced by sodium dithionite in a tonometer that contained 1.5 mL of 7.3 μM 5′-deazaFAD T491V reductase and 0.1 μM methyl viologen which was included as the electron mediator as described in Materials and Methods. (A) UV−visible spectra of oxidized (—), 1-electron-reduced (···), and 2-electron-reduced 5′-deazaFAD reductase (— · —) and the difference spectrum between the 2-electron-reduced 5′-deazaFAD reductase and free $FMNH_2$ (---). (B) Absorbance changes at 585 nm (triangle) and 450 nm (open circle) during the course of titration. (C) Difference spectra of 5′-deazaFAD reductase: (—) difference spectrum of the 1-electron-reduced minus oxidized reductase; (···) difference spectrum between the 1-electron-reduced minus the 2-electron-reduced reductase.

observed in the wild-type FAD-containing reductase where addition of the second electron generates approximately equal amounts of both $FMNH_2$ and FADH· semiquinone, which continues to absorb at 585 nm. The absorbance at 585 nm does not decrease until the total amount of the FADH· and FMNH· semiquinones begins to decrease as the fully reduced $FMNH_2$ is formed after approximately 2 reducing equivalents has been added (*12*). That the 5′-deazaFAD is not being reduced by addition of the second electron can also be confirmed by noting that the absorbance at 396 nm (maximum of oxidized 5′-deazaFAD) does not decrease during the second electron addition. When the expected spectrum of free $FMNH_2$ is subtracted from the spectrum of the 2-electron-reduced 5′-deazaFAD reductase, a spectrum not significantly different from that of free 5′-deazaFAD is observed (Figures 1 and 3A). This further supports the

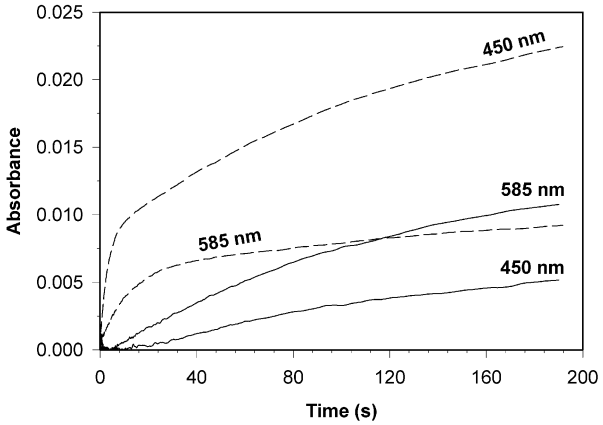

FIGURE 4: Kinetics of the autoxidation of the 2-electron-reduced 5′-deazaFAD T491V cyt P450 reductase and the 4-electron-reduced T491V cyt P450 reductase. The reductase samples were stoichiometrically reduced by sodium dithionite in the presence of 0.1 μM methyl viologen in 0.1 M potassium phosphate/15% glycerol buffer (pH 7.4) and then rapidly mixed with air-saturated phosphate/glycerol buffer in the stopped-flow spectrophotometer. The final concentration of 5′-deazaFAD T491V reductase (—) and T491V reductase (---) in the observation cell was 4.6 and 4.2 μM, respectively. The final concentration of oxygen was 0.16 mM.

conclusion that the 5′-deazaFAD has remained oxidized while the FMN has been completely reduced.

Autoxidation of 2-Electron-Reduced 5′-DeazaFAD T491V Cyt P450 Reductase. To characterize the kinetics of reduction of the redox partners of cyt P450 reductase under aerobic conditions, the kinetics of the spontaneous reaction of the reductase with oxygen, i.e., autoxidation, were delineated. Hydroquinones and semiquinones in many flavoproteins including cyt P450 reductase react with molecular oxygen and superoxide to produce superoxide and hydrogen peroxide, respectively (*42*). Autoxidation of the $FMNH_2$ of cyt P450 reductase produces an air-stable semiquinone and superoxide while the reaction of $FADH_2$ with oxygen results in a completely oxidized flavin (*12*, *20*, *43*). It is unknown whether superoxide and/or hydrogen peroxide are (is) the product(s) of oxidation of the $FADH_2$ hydroquinone. The kinetics of the autoxidation of the 4-electron-reduced T491V reductase and the 2-electron-reduced 5′-deazaFAD reductase at 15 °C are compared in Figure 4. The kinetics of autoxidation of the 4-electron-reduced T491V reductase were followed at both 450 and 585 nm. The kinetics are triphasic at both wavelengths (Figure 4 and Table 4). An interesting but unexplained feature of the autoxidation of the 4-electron-reduced T491V reductase is the existence of a single minor (amplitude 5−8%) fast phase with a rate constant of ∼15−24 s^{-1} followed by two slow phases. The two slow phases, designated phase 2 and phase 3, exhibited rate constants of 0.33 s^{-1} and 0.006 s^{-1}, respectively, with amplitudes of 27% and 65% when observed at 450 nm. Monitoring semiquinone formation at 585 nm yielded rate constants of 0.1 s^{-1} and 0.0056 s^{-1} with amplitudes of 44% and 45%, respectively, for the two slower phases (Table 4). Experiments described below indicate that the phase with the rate constant of 0.006 s^{-1} can be attributed to oxidation of the FMN hydroquinone to the semiquinone.

The autoxidation of the 5′-deazaFAD reductase was characterized under the same experimental conditions. In this case, only $FMNH_2$ will react with oxygen. As shown in

Table 4: Summary of Rate Constants and Phase Amplitudes for Oxidation of Reduced T491V and 5′-DeazaFAD Reductase by Cyt b_5 and Oxygen[a]

syringe 1	syringe 2	λ (nm) obsd	species obsd	phase 1 amp (%)	phase 1 $k_1 \pm$ SD (s^{-1})	phase 2 amp (%)	phase 2 $k_2 \pm$ SD (s^{-1})	phase 3 amp (%)	phase 3 $k_3 \pm$ SD (s^{-1})
4e-reduced T491V	O_2	450	reductase	8 ± 1	15 ± 3	27 ± 2	0.33 ± 0.033	65 ± 3	0.0065 ± 0.0003
	O_2	585	reductase	10 ± 5	24 ± 6	44 ± 2	0.1 ± 0.003	46 ± 3	0.0056 ± 0.0002
2e-reduced 5′-deazaFAD T491V	O_2	450	reductase	100 ± 11	0.007 ± 0.001				
		585	reductase	100 ± 8	0.007 ± 0.0004				
2e-reduced 5′-deazaFAD T491V	cyt b_5^{3+}	420	cyt b_5	98 ± 10	0.0015 ± 0.0002				
		567	reductase	100 ± 12	0.0017 ± 0.0004				

[a] The reactions were conducted as described in Materials and Methods.

Figure 4 (—) and Table 4, the kinetics of the autoxidation of 5′-deazaFAD reductase at 585 and 450 nm are monophasic with an apparent rate constant of 0.007 s^{-1} after a short lag phase. This rate of autoxidation corresponds to the slow phase of 0.006 s^{-1} observed in the autoxidation of 4-electron-reduced T491V reductase and represents the oxidation of the $FMNH_2$ to FMNH•. Therefore, the intermediate phase with a rate constant of 0.1−0.33 s^{-1} observed in 4-electron-reduced T491V reductase represents autoxidation of $FADH_2$. It is clear from this study that $FADH_2$ autooxidizes more rapidly than the $FMNH_2$, consistent with the fact that $FADH_2$ has a lower redox potential and FADH• is unstable (*5*). The lack of a fast and intermediate phase during oxidation of 5′-deazaFAD reductase is further evidence that significant amounts of FAD are not present in this reductase preparation. The monophasic kinetics of autoxidation also suggest that the low flavin content of our 5′-deazaFAD reductase is not influencing our kinetic results.

Anaerobic Reduction of Ferric Cyt c and Cyt b_5 by the 2-Electron-Reduced 5′-DeazaFAD T491V Cyt P450 Reductase. The activity of 5′-deazaFAD reductase was evaluated by measuring its ability to transfer electrons to its redox partners, ferric cyt *c* and cyt b_5, under anaerobic conditions. The kinetic traces for these redox reactions are presented in Figure 5. Rapid mixing of the 2-electron-reduced 5′-deazaFAD reductase with ferric cyt *c* results in rapid absorbance changes at 550 and 431 nm (Figure 5A). The absorbance increase at 431 nm reflects the formation of the FMN semiquinone of 5′-deazaFAD reductase since 431 nm is an isosbestic point for cyt *c* (*38*). At 550 nm 90% of the absorbance increase reflects reduction of cyt *c*, and 10% reports on the oxidation of 5′-deazaFAD reductase. Nonetheless, the absorbance changes at 550 and 431 nm occur simultaneously, indicating that there are no long-lived intermediates in the electron-transfer reaction. The kinetic traces at 550 and 431 nm were fit with two exponentials. It is of note that k_1^{app} and k_2^{app} are linearly dependent on ferric cyt *c* concentration in the experimental range (7.5−50 μM). The concentration dependence of phase 1 and phase 2 allows us to determine the second-order rate constants. As illustrated in Table 5, the second-order rate constants for reduction of cyt *c* by 5′-deazaFAD reductase are 1.7×10^5 and 5.6×10^4 M^{-1} s^{-1}. Since the electron distribution in wild-type and T491V reductase is different from that of 5′-deazaFAD reductase, no control can be identical in all of its redox properties to 5′-deazaFAD reductase. The 4-electron-reduced wild-type and T491V reductases were selected as controls since they are single species with a known electron distribution. Under the identical experimental conditions used for 5′-deazaFAD reductase, wild-type and T491V reductase exhibit kinetic profiles similar to those of 5′-deazaFAD reductase even though these two reductases contain the FAD cofactor. The nearly identical rate constants for reduction of cyt *c* by wild-type, T491V, and 5′-deazaFAD reductase demonstrate that the FMN domain of the 5′-deazaFAD reductase is intact and delivers electrons to cyt *c* in the same fashion as the wild-type and T491V reductase. Therefore, mutation of threonine 491 to valine and replacement of FAD with 5′-deazaFAD do not alter the mechanism of electron

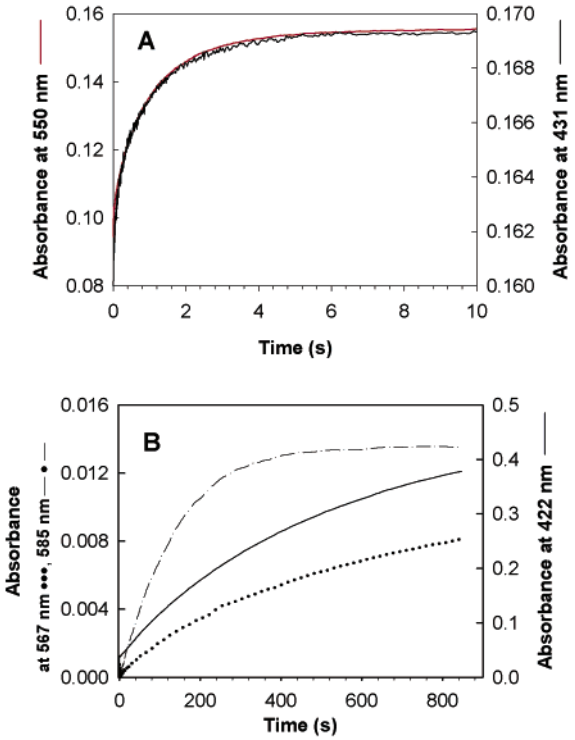

FIGURE 5: Kinetics of electron transfer from 2-electron-reduced 5′-deazaFAD T491V cyt P450 reductase to cyt *c*, cyt b_5, and oxygen. (A) Electron transfer to cyt *c*. Reductase samples were stoichiometrically reduced by dithionite in the presence of 0.1 μM methyl viologen in 0.1 M potassium phosphate/15% glycerol buffer (pH 7.4) and then mixed with four concentrations of ferric cyt *c* under anaerobic conditions as described in Materials and Methods. The final concentration of 5′-deazaFAD reductase is 4.3 μM in the observation cell. Key: absorbance change at 550 nm (red plot) and 431 nm (black plot). (B) Electron transfer to cyt b_5 and oxygen. Reductase samples were prepared as described in (A). The final concentration of 5′-deazaFAD reductase and cyt b_5 in the observation cell was 5 μM. Key: absorbance change at 422 nm (—) and 567 nm (•••) during cyt b_5 reduction and at 585 nm (— • —) during 5′-deazaFAD reductase autoxidation.

Table 5: Rate Constants for Reduction of Cyt *c* by Various Preparations of Cyt P450 Reductase

	second-order rate constant ($M^{-1} s^{-1}$)	
reductase	phase 1	phase 2
wild type	$(2.1 \pm 0.28) \times 10^5$	$(4.2 \pm 0.48) \times 10^4$
T491V	$(1.4 \pm 0.14) \times 10^5$	$(3.0 \pm 0.98) \times 10^4$
5′-deazaFAD T491V	$(1.7 \pm 0.28) \times 10^5$	$(5.6 \pm 0.77) \times 10^4$

transfer by T491V and 5′-deazaFAD reductase since both proteins behave exactly like wild-type reductase in this regard.

Although it is unclear what each phase represents, previous investigators have demonstrated that the kinetic mechanism of the reduction of cyt *c* by native cyt P450 reductase in the ionic strength range of 200−750 mM is random sequential with formation of a ternary complex consisting of two molecules of ferric cyt *c* and a single molecule of reductase (*44*). Each of the cyt *c* molecules is presumably reduced at a different rate. Thus the different phases may represent electron transfer to cyt *c* from two different binding sites on the reductase (*28*). In addition, the crystal structure of the reductase suggests that, in order for the FMN domain to both receive electrons from FAD and subsequently transfer them to a second protein, an intramolecular conformational change must occur. In view of the complex, kinetic mechanism of the reduction of cyt *c* and the requirement for intrareductase conformational changes for electron transfer, the multiphasic kinetics of the reduction of cyt *c* are not unexpected (*17*, *22*, *44*).

The ability of 5′-deazaFAD reductase to interact with membrane proteins was evaluated by measuring the rate of electron transfer from 2-electron-reduced 5′-deazaFAD reductase to the amphipathic form of ferric cyt b_5 following rapid mixing of the two proteins under anaerobic conditions. The reduction of ferric cyt b_5 was followed at 422 nm, and the 1-electron oxidation of the FMN hydroquinone to the blue semiquinone of the 5′-deazaFAD reductase was followed at 567 nm, which is an isosbestic point for cyt b_5 (*39*). As observed during electron transfer between cyt *c* and 5′-deazaFAD reductase, the 1-electron oxidation of the $FMNH_2$ of 5′-deazaFAD reductase and reduction of ferric cyt b_5 proceed simultaneously without observable intermediates (Figure 5B). Fitting the monophasic kinetic traces at 567 and 420 nm yields a single rate constant of 0.0015 s^{-1} for the reductase and 0.0017 s^{-1} for cyt b_5, respectively. A similar rate constant was observed (0.003 s^{-1}) for electron transfer between wild-type cyt P450 reductase and the detergent-soluble form of cyt b_5 (*45*). In contrast, the rate of reduction of ferric cyt b_5 by cyt P450 reductase is more rapid (k_1 of 4.1 s^{-1} and k_2 of 0.8 s^{-1}) when the two proteins are premixed and the reaction is initiated with NADPH (*45*). Therefore, the slow rate of cyt b_5 reduction under our experimental conditions can be attributed to formation of an interprotein complex which is capable of electron transfer.

The surface of the reductase surrounding the FMN is negatively charged (*17*). The heme on cyt b_5 is also surrounded with anionic amino acids (*46*) whereas the heme in cyt *c* is encircled by positively charged amino acids (*38*). It has, therefore, been concluded that a different ensemble of residues on the surface of the reductase mediates binding and electron transfer to cyt *c* and cyt b_5. Since the 5′-deazaFAD reductase reacts with two of its redox partners in a manner indistinguishable from that of the native reductase, it is assumed that the mutant and wild-type reductases will also react with its physiologic redox partner, cyt P450, in a similar manner.

SUMMARY

The preparation and characterization of a membrane-bound form of cyt P450 reductase capable of transferring a single electron from the $FMNH_2$ to two of its redox partners, namely, cyts b_5 and *c*, have been described. The FMN domain of this mutant reductase preparation functions in a manner indistinguishable from the FMN domain in the native reductase based on its ability to be reduced by dithionite and oxidized by oxygen and cyts b_5 and *c*. However, because the 5′-deazaFAD analogue is not active in redox reactions under our experimental conditions, the reactions of the FMN domain can be deconvoluted from those of the FAD domain. An investigation of the reaction of the 5′-deazaFAD reductase with its in vivo redox partner cyt P450 is currently in progress.

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