Genetic Variants in the Putidaredoxin–Cytochrome P-450$_{cam}$ Electron-Transfer Complex: Identification of the Residue Responsible for Redox-State-Dependent Conformers†

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ABSTRACT: Camphor is hydroxylated in *Pseudomonas putida* by a three-component system comprised of an oxidase, cytochrome P-450$_{cam}$, and a two-protein electron-transfer chain, putidaredoxin and putidaredoxin reductase [Tyson et al. (1972) *J. Biol. Chem. 274*, 5777–5784]. The enzymatic removal of putidaredoxin's C-terminal tryptophan is known to cause a much reduced rate of enzymatic activity in the reconstituted camphor hydroxylase system [Sligar et al. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3906–3910]. To further study the role of tryptophan in the association and/or electron-transfer reactions of putidaredoxin, the gene coding for the iron–sulfur protein was altered so that the tryptophan codon was either deleted or replaced by Phe, Tyr, Asp, Leu, Val, or Lys. Although the initial evaluation of these variant proteins [Davies et al. (1990) *J. Am. Chem. Soc. 112*, 7396–7398] showed much reduced velocities of electron transfer between P-450$_{cam}$ and the nonaromatic C-terminal proteins, the relative contributions of the binding specificity and intracomplex electron-transfer rates were not addressed. We report here a complete kinetic characterization of these proteins where the dependence of the rate constant on the putidaredoxin concentration was used to determine the intracomplex electron-transfer rate constants and the association energies for all the putidaredoxins in both oxidation states. The sum of forward and reverse intracomplex electron-transfer rate constants varies from 4.90 s^{-1} for the Lys C-terminal variant to 172 s^{-1} for the native protein. Differences in the behavior of the variant proteins are most striking when comparing the cytochrome P-450$_{cam}$ association energies with reduced putidaredoxins. The presence of a C-terminal aromatic residue is required for a relatively high cytochrome P-450$_{cam}$ affinity of the reduced relative to the oxidized protein. The desolvation of putidaredoxin's C-terminal residue is discussed as a possible explanation for this behavior.

The camphor monooxygenase cytochrome P-450$_{cam}$[1] and its redox partners putidaredoxin and putidaredoxin reductase were originally isolated from a *Pseudomonas putida* strain found in sewage sludge. In this organism, camphor catabolism is a sufficient source of both carbon and energy (Bradshaw et al., 1959). The P-450$_{cam}$ monooxygenation cycle requires that Pd reduce, in one-electron steps, both the ferric and oxygen-bound forms of the P-450$_{cam}$ enzyme. Pd transfers an electron to P-450$_{cam}$ in a bimolecular complex where electron transfer is the rate-limiting step at room temperature (Pederson et al., 1976; Hintz & Peterson, 1981), but association is rate-limiting in cryosolvent (Hui Bon Hoa et al., 1978). Unlike the reduction of the oxygen-bound form of P-450$_{cam}$, the reduction of ferri-P-450$_{cam}$ can be made to proceed to a stable intermediate. In the absence of oxygen, Pd reduces P-450$_{cam}$ reversibly to the ferrous form of the protein. Without oxygen, but in the presence of CO, ferrous P-450$_{cam}$ forms a CO adduct in what is essentially an irreversible reaction. The rate of putidaredoxin's reduction of ferric P-450$_{cam}$ has been measured in the presence of CO where the observed electron-transfer rate reached a maximum at approximately 30 s^{-1} with a half-saturation concentration of less than 5 μM protein (Pederson et al., 1976; Hintz & Peterson, 1981). In summary, the Pd–ferri/ferro-P-450$_{cam}$ electron-transfer reaction represents a well-defined physiological process where both the reactant and product states are in equilibrium.

The energetics of the Pd–P-450$_{cam}$ couple depend on the heme environment and interprotein association effects. In the absence of camphor, the sixth ligand of the heme is water or hydroxide which produces a low-spin complex (Griffith & Peterson, 1975). When camphor is bound in the active site, water access to the heme is limited, producing a five-coordination complex that is ~95% high-spin (Fisher & Sligar, 1985a). The dehydration of the camphor binding pocket decreases the polarity of the heme environment, causing a 133-mV increase in P-450$_{cam}$'s reduction potential (Fisher & Sligar, 1985b). The association of Pd and P-450$_{cam}$ is also important in determining the driving force of electron transfer. Direct measurement (Sligar & Gunsalus, 1976) and kinetic modeling (Hintz et al., 1982) of the Pd–P-450$_{cam}$ association reaction show that reduced Pd binds P-450$_{cam}$ more tightly than the oxidized protein. The complex stabilization energy between reduced Pd and ferri-P-450$_{cam}$ is reflected in an increased reduction potential for Pd (Sligar & Gunsalus, 1976). This asymmetry in association energies is also seen kinetically. The electron-transfer equilibrium constant found as a ratio of the rate constant for Pd oxidation (determined in the presence of CO) and the rate constant for Pd reduction (determined as the difference in rate constants measured with and without CO) indicates that the free energy difference

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[1] Abbreviations: P-450$_{cam}$, cytochrome P-450 camphor hydroxylase; Pd, putidaredoxin; meV, millielectron volt(s); where 1 meV = 0.09649 kJ/mol or 0.02362 kcal/mol. The variant putidaredoxins are described by the C-terminal residue number 106 followed by the three-letter code for the substituted amino acid. The variant where the tryptophan has been genetically deleted is referred to as 106 Del. The references to camphor are specific for (1*R*)-(+)-camphor.

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between the two one-electron-reduced complexes is less than predicted from the redox states of the free proteins (Pederson et al., 1976).

A key residue in determining putidaredoxin's redox behavior is its C-terminal tryptophan. The initial work showing the importance of this residue was accomplished by enzymatically removing tryptophan from Pd and observing that the protein supported a much decreased activity in the reconstituted camphor hydroxylase system (Sligar et al., 1974). More recently, recombinant DNA technology was used to alter putidaredoxin's genetic code so that the tryptophan was either deleted or changed to Phe, Tyr, Asp, Leu, Val, or Lys. Preliminary experiments with the C-terminal variant proteins were inconclusive. Flash-photolysis kinetic experiments showed apparently large differences in the P-450$_{cam}$ association energies of the reduced Pd reactions while fluorescent titrations of the oxidized complex implied that each Pd binds P-450$_{cam}$ with similar energy (Davies et al., 1990). Clearly, a more complete kinetic analysis is required.

Reported herein are kinetic measurements of the association and intracomplex electron-transfer rate constants for putidaredoxin's oxidation of reduced P-450$_{cam}$ and putidaredoxin's reduction of oxidized P-450$_{cam}$. Stopped-flow spectrophotometry was used to observe the rates of redox equilibration between the proteins at different concentrations of Pd. By recording rates where Pd exhibited both first- and zero-order concentration dependence, the data could be fitted to a kinetic equation relating the decay constants to the association and intracomplex electron-transfer rates. The energetic equivalents of the association and electron-transfer constants were sufficient to construct a four-state free-energy profile for each Pd. It will be seen that the major difference in the behavior of the different C-terminal putidaredoxins is in the relative affinity of reduced Pd for oxidized P-450$_{cam}$.

EXPERIMENTAL PROCEDURES

Site-directed mutants of Pd were generated as originally described by Davies et al. (1990). The first cloning of Pd was reported by Koga et al. (1989) followed by Peterson et al. (1990) and Romeo et al. (1991). The proteins used in these experiments were derived from fermentations of separate *Escherichia coli* TB-1 strains carrying the variant Pd genes inserted into a pUC vector (Perron-Yanisch et al., 1985). The details of the genetic constructions and protein purification will be published elsewhere. The UV–visible spectra of the variant putidaredoxins were identical to those of the native protein with the exception of the loss in absorbance at 280 nm due to the absence of tryptophan. EPR spectra of the 106 Val protein showed *g*-values identical to native Pd, indicating that the iron–sulfur center is not altered in the variant proteins.

The following equipment was used in the kinetic experiments. The pneumatic and flow-cell (2-cm path length) portions of the stopped-flow were purchased from Kinetic Instruments. The light source was a 24-V 150-W tungsten–halogen lamp run at 20 V using a home-made direct-current source with less than 0.05% ripple. The light source was focused through a Beckman DU monochromator with the opening and exit slit set at 0.5 mm. The photomultiplier, current to voltage converter, and high-voltage source were a single unit from Photon Technology International Inc. (01-612). The voltage signal was digitized on a Nicolet 3091 oscilloscope and transferred to a PC where the data were converted to absorbance and analyzed using the computer program KinFit from OLIS Inc.

An experimental temperature of 20 °C was obtained using a Haake F3 push–pull water bath where the tank was purged constantly with house nitrogen. The flow apparatus from the drive syringes to the stop syringe was immersed in thermostated water. Instrumental anaerobic conditions were obtained by placing the entire stopped-flow mixing unit in a Coy anaerobic chamber with constant catalytic removal of oxygen. A fiber-optic cable connecting the monochromator to the observation cell as well as other electrical, gas, and coolant lines was fitted through the wall of the chamber. Sufficient anaerobiosis was assumed if, on mixing a 2 to 1 ratio of reduced Pd to oxidized P-450$_{cam}$, the post-P-450$_{cam}$ reductive absorbance was stable indefinitely.

For all experiments, the electron-transfer reaction was monitored at 450 nm where the absorptive properties of both Pd and P-450$_{cam}$ change in opposite directions on reduction. At 450 nm, the change in absorbance due to electron transfer is at its maximum for the system on either side of the P-450$_{cam}$ Soret peak which was avoided to minimize the overall absorbance. Electron-transfer experiments at other wavelengths gave identical results but with lower signal to noise ratios.

Sample preparation was accomplished as follows. Non-protein solutions with the exception of aqueous NADH and dithionite were evacuated for at least 1 h at ~1 torr with subsequent argon purging for 0.5 h. The solutions were then moved into the anaerobic chamber where they were allowed to equilibrate with stirring for at least 24 h. The dithionite solution was made by dissolving the solid sodium salt in anaerobic water inside the chamber. Proteins for use in the kinetic experiments were treated as follows. Less than 30 mg of Pd was concentrated in a Centricon to ~1 mM and loaded onto a 30-mL Bio-Rad P4 gel filtration column equilibrated with 50 mM potassium phosphate, pH 7.0, and 20 mM β-mercaptoethanol. The protein was collected and again concentrated to ~1 mM. The Pd, ~8 mg of 300 μM P-450$_{cam}$, and ~25 mM NADH were placed in separate vials, each with a sealable evacuation nipple and septal cap. The solutions were gently degassed by gradually applying a vacuum of ~1 torr until no bubbles were evident for at least 5 min. A positive argon pressure was applied through the septal cap, and the solutions were moved into the anaerobic chamber.

After all the required solutions were assembled in the anaerobic chamber, the concentrations of the protein, NADH, and dithionite solutions were measured by removing diluted samples (sealed in the case of dithionite) using a Cary 219 spectrophotometer. The extinction coefficients used for NADH and dithionite were 6.23 mM^{-1} cm^{-1} at 340 nm and 8.00 mM^{-1} cm^{-1} at 316 nm (Dixon, 1971), respectively. The Pd and P-450$_{cam}$ extinction coefficients used were those reported in Gunsalus and Wagner (1978). The following five solutions were prepared in falcon tubes: (1) 4.0 μM P-450$_{cam}$, 1.0 mM camphor (10 mM stock), and 10 mM potassium phosphate, pH 7.0 (500 mM stock); (2) 4.0 μM P-450$_{cam}$, 4.0 μM dithionite, 1.0 mM camphor, and 10 mM potassium phosphate, pH 7.0; (3) X μM Pd, where X is twice the concentration intended for the ferro-P-450$_{cam}$–oxidized Pd electron-transfer experiment, and 10 mM potassium phosphate, pH 7.0; (4) X μM Pd, where X is twice the concentration intended for the reduced Pd–ferr-P-450$_{cam}$ electron-transfer experiment, 10 mM potassium phosphate, pH 7.0, a 4 to 1 equiv ratio of NADH to Pd, and a trace (~0.01 μM) of putidaredoxin reductase; (5) 10 mM potassium phosphate, pH 7.0. Dithionite and NADH/putidaredoxin reductase act to reduce P-450$_{cam}$ (Hintz & Peterson, 1980) and Pd, respectively. The amount of stock potassium phosphate buffer added to arrive at the final concentration was corrected for

the amount of phosphate added with the stock protein solutions. The Pd concentration ranges were chosen from preliminary data so that both zero- and first-order effects would be observed.

Once prepared, the five solutions were placed in the open-temperature bath to equilibrate. The NADH reduction of Pd was allowed to occur for a minimum of 2 h although most of the reaction was complete within 20 min. The dithionite reduction of P-450$_{cam}$ was allowed to occur for a minimum of 3 h. Experiments were begun by first filling the two 1-mL drive syringes with one of either set of previously prepared solutions: reduced Pd and oxidized P-450$_{cam}$ or oxidized Pd and reduced P-450$_{cam}$. Six kinetic traces (~0.2-mL total volume per shot) were acquired at the initial Pd concentration (30 psi drive force either held until after the reaction was complete or, if the reaction was slow, released in 25 ms). The filling and recording processes were repeated 5 or 6 times using progressively larger phosphate blank (solution 5) dilutions of the prepared Pd solution.

RESULTS

In anaerobiosis, partially reduced solutions containing both Pd and P-450$_{cam}$ equilibrate to redox equilibrium. From any starting point, the rate of progress to equilibrium is a function of all the kinetic rate constants describing the two free protein and two bound protein states. At different concentrations of Pd, observed changes in the range of relaxation times depend most heavily on the rate constants describing the association of the reactant proteins and the forward electron transfer of the reactant complex. The rate of forward electron transfer depends heavily on the concentration of the reactant complex and therefore is very sensitive to changes in the concentrations of the proteins in their initial redox states. Because the protein present at a lower concentration acts as a limiting reagent, the concentration of product complex depends little on the initial concentration of the protein in excess. Because of these considerations, the Pd–P-450$_{cam}$ electron transfer can be approximately described by a three-state system where the final state is the sum of the bound and unbound product proteins. Using a three-state model, expressions exist for extracting kinetic constants from sets of relaxation times measured at different concentrations of protein.

The three-state model appropriate to the Pd–P-450$_{cam}$ enzyme system is shown in eq 1. In this equation, Pd and

$$A + B \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} C \underset{k_{-2}}{\overset{k_2}{\leftrightarrow}} D \qquad (1)$$

P-450$_{cam}$ are the associating species A and B. Species C corresponds to the reactant complex, and D represents the bound and unbound product proteins. The kinetic constants k_1, k_{-1}, and k_2 correspond to the actual kinetic constants defining the electron-transfer reaction. The kinetic constant k_{-2} is the observed electron-transfer rate constant supported at the concentration of product Pd generated as the reaction progresses to equilibrium. By evaluating the Pd–P-450$_{cam}$ reaction for net electron transfer in both directions, the association and electron-transfer rate constants for the four-state system can be determined.

The reaction progress of any three-state system is defined by a spectrum of two relaxation times. The relaxation time dependence on the kinetic parameters defined in eq 1 has been derived by a number of authors (Bernasconi, 1976; Connors, 1990). However, the relaxation time expressions for a two-step system can be significantly simplified if the association step occurs much faster than the reaction step. In native and variant Pd concentration ranges with a measurable zero-order Pd concentration dependence, the rates of electron transfer between the putidaredoxins and P-450$_{cam}$ exhibit single-exponential decays due to the relatively slow electron transfer compared to association (Hintz & Peterson, 1981). A representative kinetic trace for 3.0 μM reduced 106 Tyr and 2.0 μM oxidized P-450$_{cam}$ is shown in Figure 1. The kinetic trace shows the decrease in absorbance associated with the differential absorptive properties of reactant compared to the product Pd–P-450$_{cam}$ states. The single-exponential fit of the data supports the assertion that the spectrum of two relaxation times collapses to a single decay.

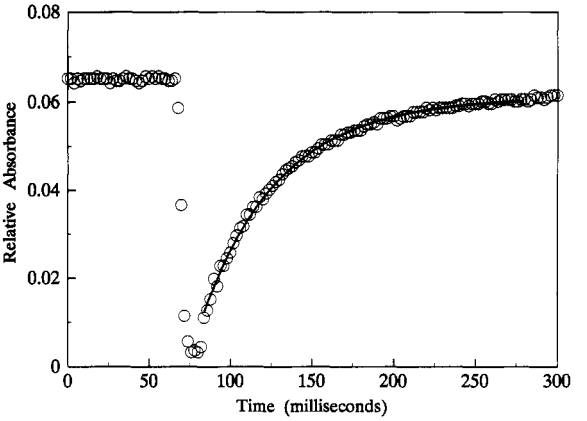

FIGURE 1: Stopped-flow experiment showing pre- and posttrigger records: 3.0 μM reduced 106 Tyr and 2.0 μM oxidized P-450$_{cam}$. Flow through the system ceased at 80 ms. The circles represent every fifth experimental point, and the line through the points represents a single-exponential fit of the data. The absorbance values were determined by setting the dark current to zero and choosing the maximum observed photomultiplier current as I_o where $A = \log (I_o/I)$.

Equation 2 is the expression for the reciprocal relaxation time (σ^{-1}) in terms of the kinetic constants defined in eq 1.

$$\sigma^{-1} = \frac{k_2}{1 + \{k_{-1}/(k_1[\text{Pd} + \text{P-450}_{cam}])\}} + k_{-2} \qquad (2)$$

The applicability of eq 2 depends on the appropriateness of the "linearization approximation" used in its derivation. The linearization approximation discounts higher order reaction progress terms in the differential rate equations by assuming that the square of a small change is insignificant. Without the linearization approximation, the relaxation time for the slow second step of a two-step reaction can be expressed by eq 3. The parameter r in eq 3 is first-order in X, making the

$$dX/dt = \sigma^{-1}X(1 + r) \qquad (3)$$

reaction second-order in X overall. The value of r is a measure of the degree to which the higher order term affects the relaxation time, σ. The parameter is a function of the concentrations and equilibrium constants describing the reaction. Expressions for r appropriate to many three-state systems have been derived (Bertigny et al., 1983). Using the fit parameters as estimates of the association and equilibrium constants for the Pd–P-450$_{cam}$ system, r was seen to be small when the reaction is pseudo-first-order or where the electron-transfer reaction was thermodynamically unfavorable. The maximum value of r for any reported experiment was 0.13 although most of the values were much less.

The relaxation equation's independent variable is the sum of the concentrations of the unbound reactants at equilibrium.

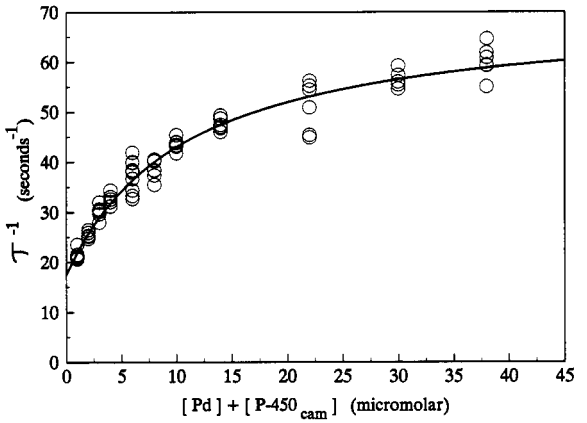

FIGURE 2: Plot of the relaxation time versus the sum of approximate concentrations of the unbound reactant 106 Tyr variant and P-450$_{cam}$ ([Pd] + [P-450$_{cam}$]). The circles represent the individual relaxation time measurements, and the line is a multiple linear regression fit of eq 2 in the text.

With the exception of the reduced aromatic C-terminal putidaredoxins, the concentrations of either the oxidized or the reduced putidaredoxins were at least 5 times greater than the concentration of the reaction limiting P-450$_{cam}$. Under these conditions, the summed equilibrium concentrations of Pd and P-450$_{cam}$ were approximated by subtracting the concentration of P-450$_{cam}$ (2 μM in all experiments) from the concentration of Pd. This approximation assumes that the reactions progressed nearly to completion as would be expected at large concentration excesses of reduced or oxidized Pd compared to the available P-450$_{cam}$. The concentration variable for the reduced aromatic C-terminal Pd data could not be estimated using this approximation because of the lower experimental ratios of Pd to P-450$_{cam}$. In the reduced aromatic C-terminal experiments, the data were treated by fitting the relaxation times versus concentration as with the other data sets plus a single iteration where the amounts of free reactant Pd and P-450$_{cam}$ were calculated using the initial fit association constant and the fraction of P-450$_{cam}$ that was either reduced or oxidized. The fraction of P-450$_{cam}$ reduced could be estimated from the amplitude of the reaction progress curves. The relaxation times were refit using the new concentration values to give the constants reported for the aromatic C-terminal protein reactions.

Multiple linear regression fits of eq 2 were made using the observed relaxation times at different concentrations of the native and variant putidaredoxins in both redox states. The fit parameters are estimates of the association and electron-transfer constants appropriate to the particular experimental reactions. Figure 2 shows a plot and fit of individual experiment relaxation times for oxidation of the 106 Tyr variant. The reciprocal relaxation time determined from the data in Figure 1 is shown as one of the low-concentration points in Figure 2. Full data sets for native or a variant putidaredoxins consisted of measured relaxation times between 4 and 12 Pd concentrations for both net oxidation and reduction of each Pd. Table I shows the association and kinetic data for all the variant and native putidaredoxins. Not shown in Table I are the observed reverse electron-transfer rate constants. In each case, the observed constants were consistent with the expected rates considering the association and electron-transfer rate measured from the opposite direction.

DISCUSSION

Much has been written about a possible role for aromatic residues in mediated protein electron-transfer events (Pielak et al., 1985; Axup et al., 1988; Everest et al., 1991). These reports suggest that π-electron systems are little if any more effective than other covalent linkages in forming electron-transfer pathways. The data presented here for the reduction of cytochrome P-450$_{cam}$ by putidaredoxin suggest that aromaticity at the carboxy terminus of Pd is not contributing significantly in the actual electron-transfer step. An estimate of electronic coupling from the reaction rate constants requires comparison of the rate constants at similar electron-transfer driving forces and constant nuclear reorganizational energy (Marcus & Sutin, 1985). For redox partners which associate prior to electron transfer, the driving force for the reaction depends both on the reduction potentials of the free species and on any differences in the association free energies for the one-electron-reduced complexes. In other words, the more tightly bound a complex, the less energetically favorable will be its oxidation or reduction.

Primarily because of differences in association energy, the driving forces for the native and variant Pd–P-450$_{cam}$ complex are scattered between +62 and −109 mV. For the purpose of evaluating the rate constants and, by implication, the electronic coupling between the proteins, putidaredoxin oxidation or reductions over similar driving forces should be compared. For example, the driving forces for native Pd reduction and 106 Del oxidation are +62 and +78 mV, respectively. The native putidaredoxin's support of an intercomplex electron-transfer rate constant 4.2 times greater at a slightly lower driving force is consistent with the native protein having a somewhat greater degree of electronic coupling between the metal centers. Likewise, comparison of the rate constants for 106 Tyr oxidation, 60 s^{-1}, and 106 Leu oxidation, 38 s^{-1}, at similar driving forces, +25 and +82 mV, respectively, leads to the conclusion that the 106 Tyr variant is better coupled to P-450$_{cam}$. A comparison of the two variant aromatic C-terminal proteins shows that both the driving forces and the rate constants for 106 Phe reduction and 106 Tyr oxidation are similar, 29 mV, 57 s^{-1} and 25 mV, 60 s^{-1}, respectively. This observation is consistent with these two proteins having similar electronic coupling. With the exception of the 106 Lys variant, comparisons across the range of variant proteins lead to the conclusion that the aromatic C-terminal putidaredoxins support at most 5-fold higher rate constants than do the nonaromatic C-terminal proteins. This effect could be either conformational or electronic in the sense that

Table I: Equilibrium Binding and Kinetic Constants for the Putidaredoxin/P-450$_{cam}$ Electron-Transfer Complexes[a]

	Pd^{red} + P-450^{ox}		Pd^{ox} + P-450^{red}	
putidaredoxin	k_2 (s^{-1})	K_d (μM)	k_2 (s^{-1})	K_d (μM)
106 Trp	14 ± 2.3	1.6 ± 1.1	160 ± 22	88 ± 24
106 Phe	18 ± 0.85	4.7 ± 1.0	57 ± 33	190 ± 150
106 Tyr	60 ± 1.5	8.5 ± 1.2	22 ● 4.4	47 ± 33
106 Del	38 ± 3.0	81 ± 13	1.7 ± 0.11	36 ± 11
106 Asp	42 ± 2.1	54 ± 16	0.80 ± 0.056	25 ± 15
106 Leu	38 ± 2.8	72 ± 12	1.5 ± 0.72	88 ± 76
106 Val	66 ± 15	220 ± 66	1.8 ± 0.89	136 ± 106
106 Lys	4.8 ● 1.8	710 ± 330	0.064 ± 0.013	42 ± 33

[a] K_d is defined as the product of the concentrations of free Pd and P-450$_{cam}$ divided by the concentration of the interprotein complex. The numbers to the right of the ± signs are standard deviations. Standard deviations for a multiple linear regression fit are derived from the contributions of each constant parameter to the differences between the calculated and observed dependent variables (Bevington, 1969). Large deviations were found primarily in the data set where the zero- or first-order Pd contributions were difficult to obtain. In other words, there was shallow curvature in the dependence of relaxation time on concentration whenever the process was dominated by zero-order effects such as the oxidation of native Pd or first-order effects as in the oxidation of 106 Lys.

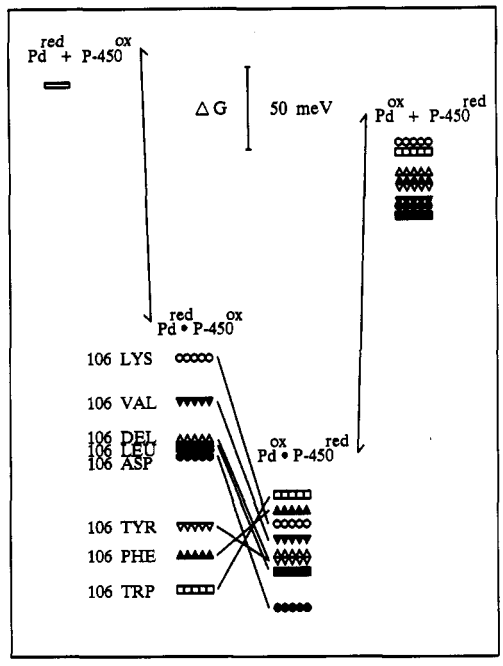

FIGURE 3: Free energy profiles showing the energetic relationships between the free and associated one-electron-reduced Pd–P-450$_{cam}$ states. The reduced aromatic C-terminal Pd association to oxidized P-450$_{cam}$ is more favorable than the association reactions of either the reduced nonaromatic C-terminal putidaredoxins or the oxidized putidaredoxins.

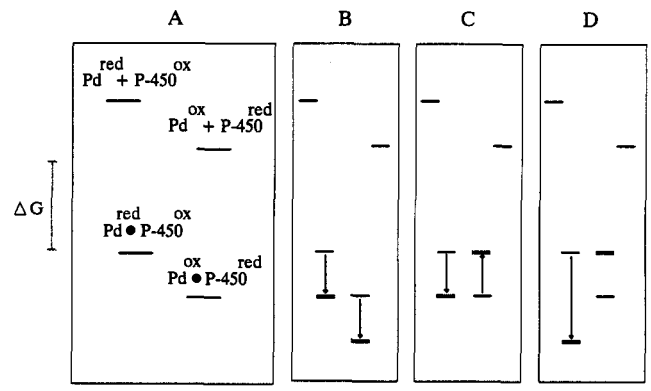

FIGURE 4: Diagrammatic explanation of the large P-450$_{cam}$ association energy found in the aromatic C-terminal putidaredoxins. (A) Free energy profile representative of the nonaromatic C-terminal putidaredoxins. Both one-electron-reduced complexes associate with similar energy. (B) Effect of aromatic residue desolvation on free energy levels from profile A. (C) Effect of decreased dielectric environment in the presence of positive charge on free energy levels from profile A: reciprocal stabilization of reduced Pd and destabilization of oxidized Pd. (D) Summed effects of desolvation (B) and dielectric (C) would tend to stabilize the complex involving reduced Pd but have little effect on the free energy of the complex involving oxidized Pd.

aromatic resides may be particularly good residues with which to build electron-transfer pathways (Beratan et al., 1990).

The 106 Lys variant displays an electron-transfer rate constant an order-of-magnitude lower than do the other putidaredoxins. This slow electron-transfer rate constant is consistent with the 106 Lys variant forming an altered and inefficient electron-transfer complex with P-450$_{cam}$. Indeed, it is possible to imagine a range of electron-transfer complex conformations with the aromatic C-terminal putidaredoxins forming the most favorable complex, the nonaromatic non-Lys C-terminal putidaredoxins forming a less favorable complex, and the Lys C-terminal putidaredoxin forming the least favorable complex. With the present data, it is impossible to distinguish between the electronic or conformational explanations. Nonetheless, these data are not consistent with the Pd–P-450$_{cam}$ electron-transfer mechanism proposed by Baldwin et al. (1991) which postulated a covalent "switch" mechanism for the Pd C-terminal tryptophan residue.

A more striking difference in the behavior of the native and variant putidaredoxins is the especially tight affinity of the reduced aromatic C-terminal proteins for P-450$_{cam}$. This effect is clearly shown in a four-state free energy profile (Figure 3) where the free energy parameters for the bound and unbound proteins are derived from the kinetic data. The parameters calculated from the relaxation experiments can be related directly to the free energy of association and the free energy of electron transfer [$RT \ln [k_{forward}(Pd^{ox})/k_{forward}(Pd^{red})]$]. It should be noted that the relative free energies of the dissociated reduced Pd and oxidized P-450$_{cam}$ states are not the same for different putidaredoxins. The initial free energy levels are shown as equal to highlight differences in the reduced Pd affinity.

In addition to the relationships shown, the energy levels for the unbound protein states are related by the sum of the reduction potentials of the two proteins. Taking into account differences in the reduction potentials of the variant putidaredoxins (Davies et al., 1990), a predicted free energy difference between the two free protein states can be determined independently of the kinetic data. The predicted followed by the measured differences in the free energy states are given for each Pd: 106 Trp, 63 meV, 40 meV; 106 Phe, 66 meV, 64 meV; 106 Tyr, 69 meV, 69 meV; 106 Del, 71 meV, 58 meV; 106 Asp, 89 meV, 81 meV; 106 Leu, 65 meV, 87 meV; 106 Val, 68 meV, 79 meV; 106 Lys, 53 meV, 38 meV. The at most 23-meV difference between the predicted and measured free energy differences is due to summed errors in quantitating the association energies and intracomplex electron-transfer rates.

Figure 3 shows that the association energies of the reduced aromatic C-terminal putidaredoxins are much larger than the association energies for any other Pd. A model describing this behavior would necessarily explain the origin of the aromatic C-terminal protein's association energy dependence on redox state. In general, a contribution to association energy from an aromatic residue is expected to come from desolvation of the residue in the complex. The transfer of all or part of the side chains of Trp, Phe, or Tyr from water to a more hydrophobic environment is energetically favorable (Richards, 1977; Sharp et al., 1991). Also important would be the desolvation energetics of any other protein surface that is dehydrated along with the residue side chain. For example, the exclusion of solvent from the environment of the metal center could change the relative energies of the redox states.

A plausible model for linking redox state and association energy supposes that the extent of the residue's desolvation in the interprotein complex depends on the redox state of Pd. In such a case, the nuclear reorganizational energy (Marcus & Sutin, 1985) associated with electron transfer would include the differential solvation of the residue. An observation that does not support an oxidation-state dependence on the microenvironment of 106 Trp is the apparent similarity of fluorescent decay kinetics for both Pd redox states (Stayton & Sligar, 1991). It is possible, however, that the distribution or the very nature of the states changes, when Pd is bound to P-450$_{cam}$. Another model that would explain the electron-transfer data reported herein does not rely on a redox-state-dependent difference in the solvation of the residue. The partial desolvation of a residue in the binding cleft could shield the

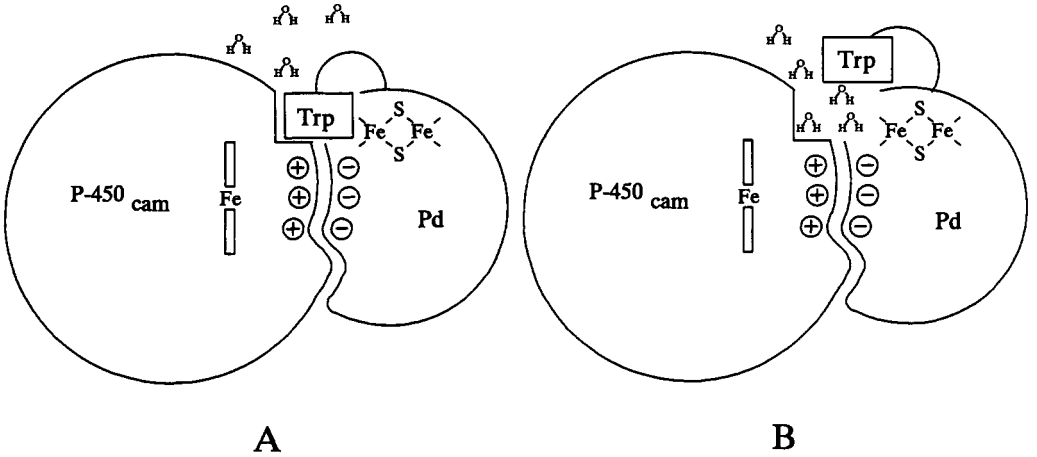

FIGURE 5: Schematic representation of two possible tryptophan conformations in the Pd–P-450$_{cam}$ electron-transfer complex. The differential solvation model for the redox-state-dependent association energy supposes that tryptophan is less solvated in the reduced Pd complex with P-450$_{cam}$ (panel A) and more solvated in the oxidized Pd complex with P-450$_{cam}$ (panel B). In contrast, the charge-dielectric model supposes that tryptophan is desolvated to the same extent when Pd is either oxidized or reduced (panel A). This model relies on significant shielding of the iron–sulfur center from solvent, thereby increasing the magnitude of electrostatic effects from nearby charges. The proximity of the positive potential surface of P-450$_{cam}$ would stabilize putidaredoxin's reduced state.

iron–sulfur center from solvent. The accompanying decrease in the dielectric constant for the region surrounding the iron–sulfur center would increase the magnitude of any existing electrostatic interactions. In the complex, the immediate presence of the positive potential surface constituting P-450$_{cam}$'s Pd binding site (Stayton et al., 1989; Stayton & Sligar, 1990) would tend both to stabilize the reduced redox state and to destabilize the oxidized redox state. In other systems, the charge distribution and dielectric around redox centers are known to affect the relative energies of redox states (Kassner, 1972; Caffery & Cusanovich, 1991; Rodgers & Sligar, 1992). This electrostatic charge effect would necessarily occur in conjunction with the stabilization of both one-electron-reduced complexes by the partial desolvation of the aromatic residue. The combination of both phenomena would tend to stabilize greatly the complex between P-450$_{cam}$ and reduced Pd but would have little effect on the stability of the complex when Pd is oxidized. This model is presented schematically in Figure 4. If this model is correct, the similar association energies for both redox states in the nonaromatic C-terminal proteins could be explained by the residue's inability to shield the iron–sulfur center from solvent, thereby minimizing the electrostatic interactions with nearby charges.

From the present experimental results, we are not able to determine which, if either, model is most appropriate. An important point to be made about the two models is that they are not mutually exclusive. Indeed, the differential solvation and charge effect models can be thought of as end points on a continuum. A cartoon representing the two models is shown in Figure 5. Of the nonaromatic C-terminal proteins, the only one to show significant redox-dependent association energy is the Lys variant. The Lys variant binds P-450$_{cam}$ much less tightly when the variant is in its reduced form. This protein represents a special case in that it has markedly reduced electron-transfer rate constants, perhaps implying an altered conformation in the complex. The Val, Leu, Asp, and deletion variants show no significant redox dependence in the energy of their association reactions. That the Val and Leu residues do not enhance the binding of reduced Pd to P-450 is probably an indication that they reside in conformations that are not affected in complex formation or that they are too small to shield the iron–sulfur center from solvent. Other independence experiments are supportive of tryptophan undergoing desolvation in the electron-transfer complex. In unbound Pd, the tryptophan fluorescence emission maximum is indicative of a high degree of solvent exposure (~358 nm) (Stayton & Sligar, 1991). Also, carboxypeptidase A digestion of tryptophan is inhibited in the presence of P-450$_{cam}$ (Sligar et al., 1974). Tryptophan is apparently solvent-exposed until association with P-450$_{cam}$, where protease access is limited.

Another point of consideration is the effect of ionic strength on the relative association energies. The maximum electron-transfer rate for Pd oxidation shows little dependence on ionic strength (Hintz & Peterson, 1981). In the present study (data not shown), the observed electron-transfer rate constants at a constant concentration of Pd and P-450$_{cam}$ depended inversely on the ionic strength of the buffer in a manner similar to other systems (Pan et al., 1990; Mauk et al., 1982). Because many of the aliphatic variant proteins bind loosely to P-450$_{cam}$, the ionic strength was minimized to give the maximum zero-order Pd contribution at low Pd concentrations. The limiting factor in lowering the ionic strength was the requirement for potassium in camphor binding to P-450$_{cam}$. The presence of camphor is essential to raise the reduction potential of P-450$_{cam}$ to a range where Pd reduction is significant (Sligar & Gunsalus, 1976). Buffer conditions of 10 mM potassium phosphate, pH 7.0, and 500 μM camphor are sufficient to saturate the enzyme with camphor. The important question is whether the relative binding free energies of the native and variant putidaredoxins change with increasing ionic strength such as that found in vivo. In answer, the electron-transfer rate constants divided by the association constants for the reduced putidaredoxins scale roughly with the steady-state rates for the full catalytic cycle acquired at 50 mM potassium phosphate and 150 mM KCl.

In summary, the identity of putidaredoxin's C-terminal residue was found to be important in the protein's association behavior with P-450$_{cam}$. The aromatic C-terminal proteins were unique in associating more tightly in the reduced state. The mechanism whereby the C-terminal aromatic residues could exert their influence is either a redox-state-dependent differential solvation of the residue or a combination of desolvation and shielding effects. Considering the physiologic behavior of Pd, the stabilization of a reactant electron-transfer complex favors the capacity to maintain a high electron flux at low concentrations of reduced Pd. The shift in the equilibrium position of electron transfer is probably unimportant in vivo because subsequent reactions such as oxygen

binding (Peterson et al., 1972) pull the catalytic cycle to completion. Taken as a whole, the presence of an aromatic residue at putidaredoxin's C-terminus has a dramatic effect on the performance of Pd as an electron carrier.

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Registry No. P-450, 9035-51-2; Trp, 73-22-3; camphor hydroxylase, 9030-82-4.