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Zinc-Substituted Cytochrome $P450_{cam}$: Characterization of Protein Conformers F420 and F450 by Photoinduced Electron Transfer

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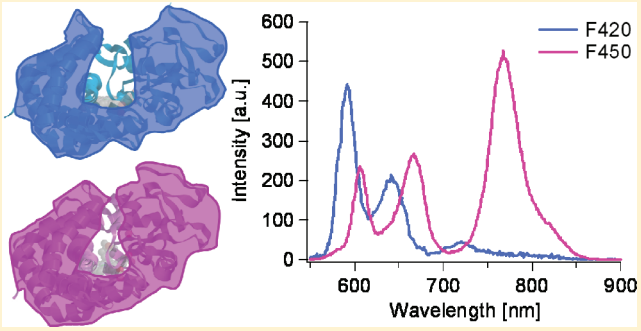
Supporting Information

ABSTRACT: Metal substitution of heme proteins is widely applied in the study of biologically relevant electron transfer (ET) reactions. It has been shown that many modified proteins remain in their native conformation and can provide useful insights into the molecular mechanism of electron transfer between the native protein and its substrates. We investigated ET reactions between zinc-substituted cytochrome $P450_{cam}$ and small organic compounds such as quinones and ferrocene, which are capable of accessing the protein's hydrophobic channel and binding close to the active site, like its native substrate, camphor. Following the substitution method developed by Gunsalus and co-workers [Wagner, G. C., et al. (1981) *J. Biol. Chem. 256*, 6262−6265], we have identified two dominant forms of the zinc-substituted protein, F450 and F420, that exhibit different photophysical and photochemical properties. The ET behavior of F420 suggests that hydrophobic redox-active ligands are able to penetrate the hydrophobic channel and place themselves in the direct vicinity of the Zn-porphyrin. In contrast, the slower ET quenching rates observed in the case of F450 indicate that the association is weak and occurs outside of the protein channel. Therefore, we conclude that F420 corresponds to the open structure of the native cytochrome $P450_{cam}$ while F450 has a closed or partially closed channel that is characteristic of the camphor-containing cytochrome $P450_{cam}$. The existence of two distinct conformers of Zn-bound $P450_{cam}$ is consistent with the findings of Goodin and co-workers [Lee, Y.-T., et al. (2010) *Biochemistry 49*, 3412−3419] and has significant consequences for future electron transfer studies on this popular metalloenzyme.



Electron transfer (ET) reactions play a crucial role in biological systems. They occur at critical steps of numerous metabolic pathways and are studied in a variety of ways. One of the methods involves photoinduced ET investigation of chemically modified proteins, which are labeled[3] or transformed[4,5] to be photoactive. Optical time-resolved methods allow one to directly probe fast kinetics that is not accessible to many other techniques. The most popular class of redox-active proteins that allow modifications facilitating the study of ET reactions consists of heme proteins such as cytochromes. They are excellent model systems because they are naturally involved in electron transport and the spectroscopic and redox properties of the heme group can be easily manipulated. Because of large spin−orbit coupling, the iron-containing heme group dissipates the excitation energy through rapid radiationless transitions that lead to extremely short excited state lifetimes in the femtosecond range.[6] Consequently, native heme proteins are not very good targets for photoinduced electron transfer investigations. To overcome this problem, various methods were developed to exchange the metal in the prosthetic group. The replacement of iron with diamagnetic zinc at the porphyrin (PP) center dramatically changes the photophysics of the enzyme. Proteins containing zinc porphyrins (ZnPP) exhibit both fluorescence (nanoseconds) and long-lived phosphorescence[7] (milliseconds at room temperature in deoxygenated solutions). Studies on Zn-substituted cytochrome *c* (ZnCC) and myoglobin (ZnMb) have shown that the exchange of iron(II) with zinc(II) porphyrin does not alter the conformation of these proteins.[8] Importantly, the singlet and triplet excited states of zinc-substituted proteins are able to accept or donate an electron from good acceptors or donors, thus maintaining the key redox properties of the native protein, naturally, with different oxidation and reduction potentials. Because of their emissive nature, they are amenable to the highly sensitive single-photon counting techniques, which permit kinetic studies over a very broad range of concentrations and time scales. Optical spectroscopy experiments with Zn-substituted cytochrome *c* and its modified variants laid the fundamental groundwork for the understanding of electron transfer dynamics in proteins.

Metal exchange has been accomplished in several other cytochromes, but the procedures are generally much more difficult and yields of the substituted protein considerably lower. The replacement of the heme moiety in cytochrome $P450_{cam}$ with ferriprotoporphyrin IX and manganese, cobalt,

Received: August 25, 2011
Revised: January 13, 2012
Published: January 17, 2012

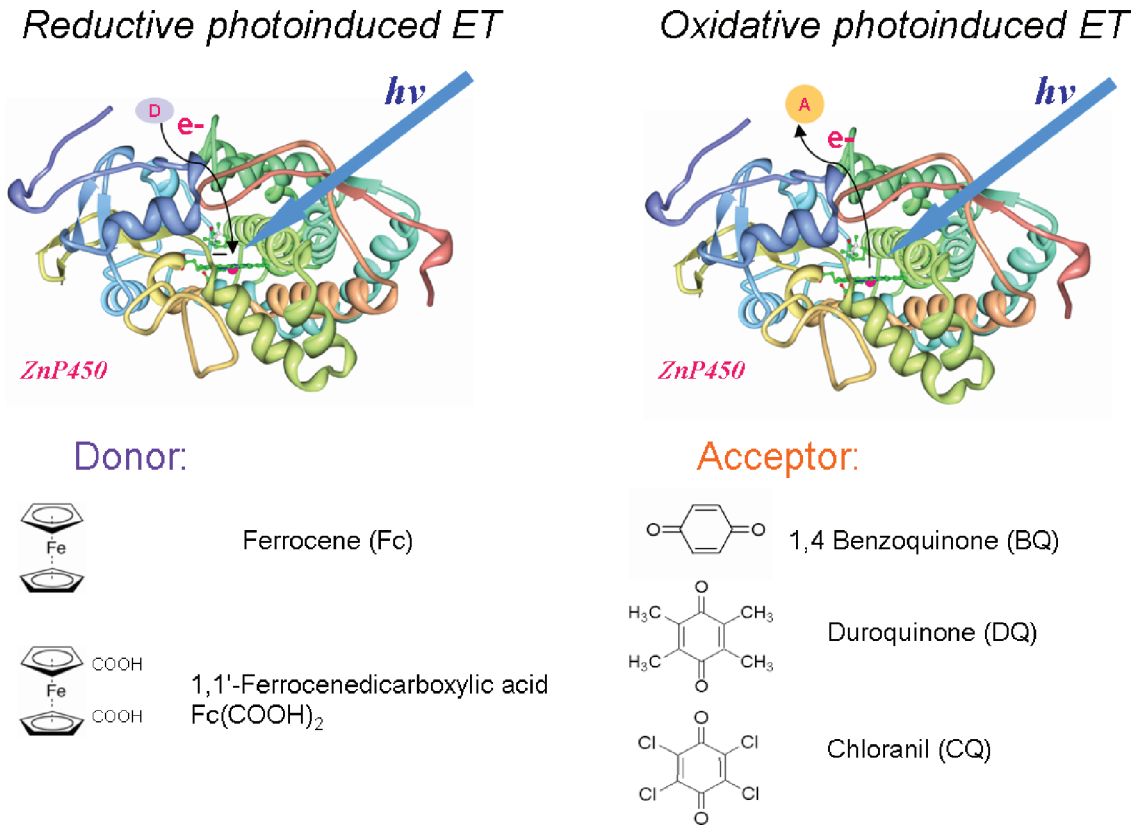


Figure 1. Small organic and organometallic donors and acceptors selected for the study of photoinduced electron transfer properties of ZnP450$_{cam}$.

and zinc substitutions of cytochrome P450$_{cam}$ were reported.[1,9−12] The primary challenge in these substitutions lies in the fact that unlike in the case of cytochrome *c* or myoglobin, the heme is not covalently bound to the backbone of cytochrome P450, yet nevertheless it appears to play an significant role in maintaining the overall structure of the properly folded enzyme and the accessibility of the substrate channel. This structural lability leads to poor substitution yields and the presence of non-native conformers in the reconstituted protein. For the same reason, the subsequent handling of the substituted protein, which is not nearly as robust as Zn-bound cytochrome *c*, also requires considerable care.

In this work, we discuss the optical spectroscopy and photoinduced electron transfer behavior of zinc-substituted cytochrome P450$_{cam}$ (ZnP450), which point to the presence of two distinct conformers of the metal-substituted protein, F420 and F450. The existence of two metastable conformations of Zn-substituted P450$_{cam}$ is significant because zinc-substituted heme proteins are so frequently used as models in the studies of electron transfer dynamics in biological systems. P450 is an important member of the heme monooxygenase family that catalyzes a vast variety of processes, including drug metabolism, carcinogenesis, degradation of xenobiotics, and biosynthesis of steroids, lipids, and secondary metabolites.[13] The camphor metabolizing cytochrome P450$_{cam}$ isolated from *Pseudomonas putida* is the most widely utilized representative of this group and very often serves as a model for other cytochromes P450. Our findings are consistent with the recent report of Goodin et al.,[2] who determined the structure of native P450$_{cam}$ in the presence and absence of camphor and demonstrated that the substrate-free protein has an open structure with the access channel exposed to the solvent.[2] Upon binding camphor, the protein adopts a tighter, closed structure, in which the access channel narrows and restricts the ingress and egress of substrates. The presence of the open conformation was previously postulated by Gray et al. and Goodin et al. on the basis of their work on the ligation of native P450$_{cam}$ to synthetic "molecular wires".[14−17] As we show below, the two conformations of ZnP450 exhibit widely differing electron transfer dynamics with the same redox partners. In the case of ZnP450_F420, the ET behavior is consistent with the binding of small hydrophobic redox partners deep inside the access channel of the enzyme, while the behavior of ZnP450_F450 suggests only a loose association with the ligand. Furthermore, form F420 exhibits the typical photophysical characteristics of other Zn-porphyrin proteins, including Zn-cytochrome *c* and myoglobin. The spectral properties and ET activity of form F450 agree very well with the observations Morishima et al., who found, rather surprisingly, that the zinc-substituted P450$_{cam}$ did not at all undergo excited state ET reactions with small organic redox ligands with dimensions similar to those of camphor.[12] We believe that the results presented here explain the unexpected behavior encountered by Moroshima and will facilitate future studies of Zn-substituted P450$_{cam}$.

The ligands for this study of photoinduced electron transfer in ZnP450 were chosen on the basis of their redox potentials and structural properties (Figure 1). The acceptors, small quinones, are involved in numerous redox reactions in natural biological systems; albeit, in nature they often carry lipophilic side chains.[18] The donors, ferrocene and its derivatives, are strong reductants known to be capable of transferring electrons to the excited states of other zinc-substituted proteins.[19] The active site of P450$_{cam}$, the heme, is ligated by a cysteine residue buried deep inside the protein pocket and surrounded by

dx.doi.org/10.1021/bi201344h | *Biochemistry* 2012, 51, 1431−1438

hydrophobic amino acid residues that are responsible for the substrate binding. The channel leading from the protein's surface to the active site near the heme is approximately 22 Å deep.[14] The hydrophobic nature and the shape of the channel favor ligand binding inside the active pocket at the very end of the entry channel. Such positioning puts the redox partners in the proximity and facilitates the electron transfer process.

$P450_{cam}$ binds not only derivatives of camphor but also several other ligands such as styrene, pyrene, ferrocene, and even large exobiotic molecules that are more than twice the size of its natural partner.[15,17,20] As demonstrated by Goodin et al., the biding of large exobiotic ligands is driven primarily by hydrophobic interactions. The direct coordination to the heme iron plays a decidedly minor role and is not necessary for docking, as evidenced by stable binding of saturated hydrocarbons such as adamantane. The size of all redox-active ligands selected for this work is comparable to that of camphor and adamantane. As a consequence, they were expected to bind to the Zn-substituted protein in a very similar fashion, deep inside the access channel, leading to efficient reductive (ferrocenes) and oxidative (quinones) electron transfer quenching of the excited state of $ZnP450_{cam}$.

EXPERIMENTAL PROCEDURES

Materials. The *Escherichia coli* vector containing CYP101 was a gift from S. G. Sligar. Culture media were prepared at Genentech's media preparation facility. Isopropyl β-D-1-thiogalactopyranoside (IPTG), dithiothreitol (DTT), and 5-aminolevulenic acid were purchased from Pro-Omega. Ni-NTA agarose was obtained from Qiagen Inc. Zinc protoporphyrin IX, *d*-camphor, β-mercaptoethanol (BME), ferrocene, 1,1′-ferrocenedicarboxylic acid, benzoquinone, chloranil, and duroquinone were purchased from Sigma Alfa Chemical Co. Deuterium oxide (99.9%) was obtained from MP Biomedicals, LLC.

Methods. Expression and purification of six-His-tagged Fe-cytochrome $P450_{cam}$ was performed following the protocols of Sligar et al.[21] The Zn-substituted cytochrome $P450_{cam}$ was prepared as described by Gunsalus et al.[1] and later by Morishima et al.[12] with small modifications at the purification stage. Briefly, the crude ZnP450 was first passed through a ProteinPak column (Waters Inc.) equilibrated against buffer containing 40 mM potassium phosphate and 0−1 mM *d*-camphor (pH 7.8) to remove any unbound porphyrin. The collected protein fractions were subsequently purified on a HiprepQ ion exchange high-performance liquid chromatography column. The sample was loaded onto the column in 40 mM potassium phosphate and 0−1 mM *d*-camphor (pH 7.8) and eluted with a linear 0 to 400 mM KCl gradient in 40 mM potassium phosphate and 0−1 mM *d*-camphor (pH 7.8). The protein was eluted with ∼150 mM KCl. Finally, the purified protein was dialyzed overnight against 40 mM potassium phosphate and 0−1 mM *d*-camphor (pH 7.4). All manipulations of the ZnP450 were performed in the dark. The F420:F450 ratio depended on the camphor concentration and increased from 1:2.8 to 1:1.4 as the concentration of the latter was lowered from 1 to 0 mM (Supporting Information).

Preparation of Samples for the ET Studies. The appropriate amount of quencher was dissolved in 40 mM potassium phosphate and 0.1 mM *d*-camphor (pH 7.4) and mixed with an equal volume of 2−10 μM ZnP450. Because of its low solubility, ferrocene (Fc) was introduced to the protein solution at a saturating concentration. To increase the quencher concentration, we first dissolved the quinones in a few microliters of DMF. In each case, the reference sample of ZnP450 was suspended in the same buffer as the samples containing the quencher. For the isotope effect experiments, the purified protein solution was concentrated to a minimal volume and then dissolved in deuterated buffer to give the final H_2O:D_2O ratio of 1:35. The F420:F450 ratio in the samples used for the electron transfer studies was kept close to 1:2.

Spectroscopic Methods. Absorption spectra were recorded using a Cary 500 Scan UV−vis NIR spectrophotometer. Fluorescence, phosphorescence, and triplet lifetime measurements were taken using a Varian Cary Eclipse fluorescence spectrophotometer following excitation at 420 or 450 nm. The differences in the photophysical properties of F450 and F420 allow one to analyze both species in the same sample by changing the excitation wavelength. If the sample containing both forms was excited at 450 nm, only the triplet state of F450 was observed, but when it was excited at 420 nm, the phosphorescence and delayed fluorescence of F420 as well as residual emission originating from F450 were seen. Because the phosphorescence band of F450 (maximum at 768 nm) overlaps with the phosphorescence band of F420 (maximum at 732 nm), the decay of ^{3}F420 was monitored at the delayed fluorescence peak at 590 nm. The solutions for all measurements were exhaustively degassed by several freeze−pump−thaw cycles that were repeated until no further change in the recorded triplet state lifetime was observed.

RESULTS

UV−vis spectra of purified ZnP450 revealed that the substituted protein exists as a mixture of two forms, F450 and F420 (Figure 2). The main product of the Zn-porphyrin

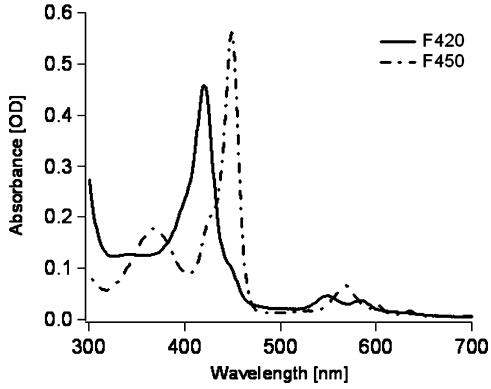


Figure 2. Absorption spectra of two fractions of zinc-substituted cytochrome $P450_{cam}$ collected at different elution times. Data for the fraction containing primarily form F420 are shown with a solid line and those for the fraction containing form F450 as a dotted line.

substitution, form F450, which was described by Morishima et al.,[12] exhibits a red-shifted Soret band at 449 nm and an intense hyperband at 367 nm. Such hyperbands are characteristic of the zinc-porphyrin derivatives after ligation with thiols (Table S1 of the Supporting Information). The second form, F420, displays the expected photophysical properties that are consistent with other zinc-substituted heme proteins, all of which have a Soret band in the vicinity of 420 nm. Gaussian line shape fitting locates the maximum of the F420 Soret band more precisely at 424 nm, i.e., remarkably close to that of ZnCC (Table 1 and the Supporting Information). Similarly, the fluorescence spectrum of F420 has the appearance that is characteristic of zinc-

dx.doi.org/10.1021/bi201344h | *Biochemistry* 2012, 51, 1431−1438

Table 1. Absorption and Emission Maxima of Zinc-Substituted Heme Proteins[a]

		absorption				Fl[b] (S)		Ph[c] (T)	S−T gap
F450[d]	nm	367	**449**	568	602	**607**	669	**768**	–
	cm^{-1}	27200	**22300**	17600	16600	**16500**	14900	**13000**	3500
F420[d,e]	nm	340	**424**	551	584	**592**	641	**723**	–
	cm^{-1}	29900	**23800**	18200	17100	**16900**	15600	**13800**	3100
ZnCC[f]	nm	346	**423**	549	584	**591**	644	**726**	–
	cm^{-1}	28900	**23600**	18200	17100	**16900**	15500	**13800**	3100
ZnMb[g]	nm	356	**428**	553	595	**595**	647	**735**	–
	cm^{-1}	28100	**23400**	18100	16800	**16800**	15500	**13600**	3200

[a]F450 and F420, the forms of zinc-substituted cytochrome P450; ZnCC, zinc-substituted cytochrome *c*; ZnMb, zinc-substituted myoglobin. The most relevant wavelengths are shown in bold. [b]Fluorescence. [c]Phosphorescence. [d]From this work. [e]F420 is more sensitive to environmental changes; few nanometer shifts (±2 nm) in absorbance spectrum were observed if either the pH or the ionic strength was changed. [f]From ref 25. [g]From ref 26.

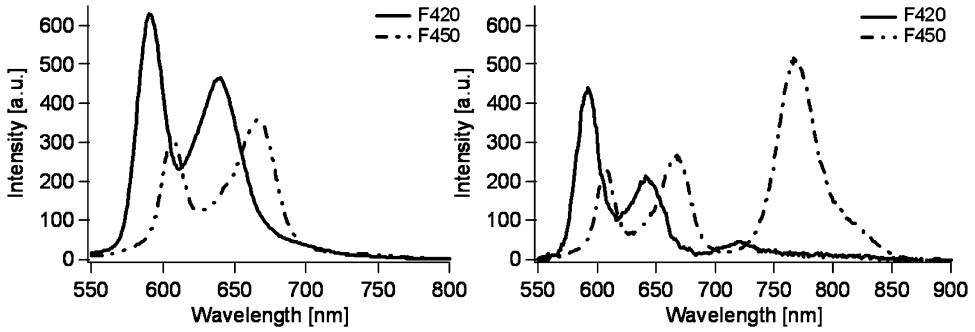


Figure 3. Fluorescence (left) and phosphorescence (right) spectra of $ZnP450_{cam}$ fractions collected at different elution times. Emission spectra of the fraction containing primarily form F420 excited at 420 nm are shown as solid lines, and emission spectra of the fraction containing form F450 excited at 450 nm are shown as dotted lines.

substituted heme proteins, with maxima at 592 and 646 nm. In contrast, the fluorescence of F450 is considerably red-shifted, with maxima at 606 and 667 nm (Figure 3). Both forms exhibit delayed fluorescence. F420 has weak phosphorescence at 723 nm with a long lifetime of approximately 7−14 ms, depending on the preparation and experimental conditions, that is also characteristic for other zinc-substituted heme proteins.[22] The phosphorescence of form F450 at 768 nm is much more intense, probably at least in part as a result of the large S_1-T_1 gap and hence slower repopulation of the S_1 state, which at room temperature is a significant decay channel for Zn-porphyrins. Excitation at 450 nm produces exclusively the F450 T_1 state, and as a result, the phosphorescence decay is purely monoexponential, with a τ_{F450} of 1.67 ms (Figure 4). Because of the spectral overlap, excitation at 420 nm always leads to a mixture of the F420 and F450 triplet states. As a consequence, the resulting phosphorescence decay is biexponential and consists of a major 10 ms component that belongs to form F420, as well as a minor short-lived F450 contribution with the aforementioned τ_{F450} of 1.67 ms. The unusually short triplet state lifetime at room temperature and the large S−T splitting found for form F450 are not consistent with other Zn-substituted cytochromes. In contrast, the S−T gap and the triplet lifetime of form F420 are very similar to these of ZnCC (Table 1).

We determined that the initial F420:F450 ratio depends on the concentration of camphor during the exchange of the prosthetic group; however, under all tested conditions, the F450 is more abundant (Supporting Information). Goodin et al.[2] similarly observed that redissolving a crystal of the "open conformation" of native $P450_{cam}$ yielded a mixed solution of

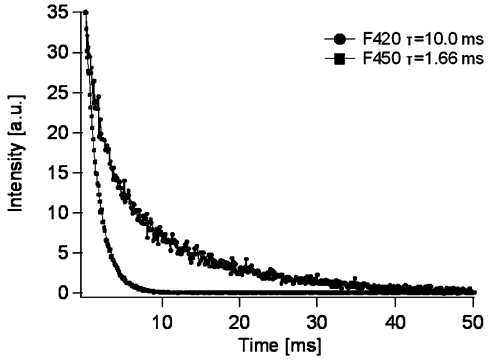


Figure 4. Triplet state lifetimes measured on a 1:2 mixture of forms F420 and F450 of $ZnP450_{cam}$ excited at 420 nm (black circles) and 450 nm (gray squares) and monitored at 590 and 768 nm, respectively. The amplitudes at time zero were normalized.

P420 and P450. The resulting equilibrium was shifted if additional camphor was introduced into the solution.

To learn more about the conformers of $ZnP450_{cam}$, we investigated the magnitude of the solvent deuterium−hydrogen isotope effect on the triplet lifetime. Because the prosthetic group in $P450_{cam}$ is located deep inside the protein channel, the magnitude of the isotope effect should reflect the ligand and solvent accessibility of the zinc porphyrin. Upon H_2O to D_2O exchange, an increase in the triplet lifetime was observed for both forms (Figure 5 and Table 2). The decay of ^{3}F450 was somewhat less affected (k_{H_2O}/k_{D_2O} = 1.2) than that of ^{3}F420 (k_{H_2O}/k_{D_2O} = 1.3), suggesting that the active site of F420 is more readily accessible to the solvent. This can mean that either the substrate channel in F450 is partially collapsed or

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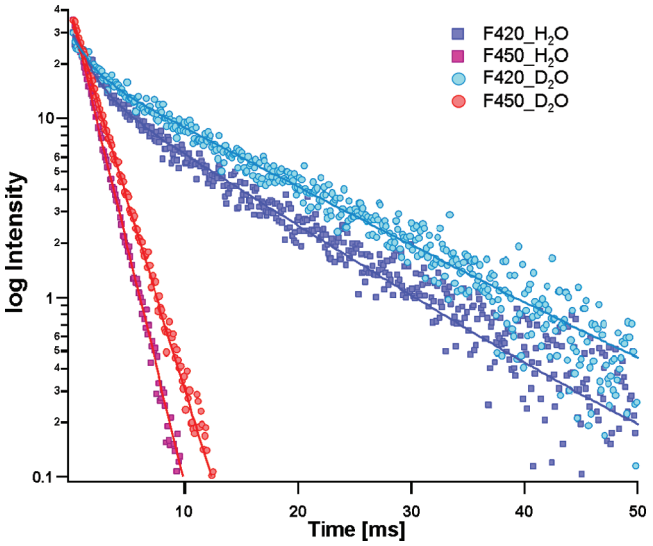

Figure 5. Normalized decay kinetics of ^{3}ZnP450$_{cam}$ in H_2O and D_2O solutions of 40 mM KP_i and 0.1 M camphor (pH 7.4 or pD 7.4), excited at 420 and 450 nm. As explained in the text, the F420 triplet state decay traces contain a small contribution from F450 at the early times.

Table 2. Triplet State Decay Rates and Deuterium Isotope Effects Measured for Both Forms of ZnP450$_{cam}$

	k_{H_2O} (s^{-1})	k_{D_2O} (s^{-1})	k_{H_2O}/k_{D_2O}
F450	602	484	1.24
F420	96	73	1.32

that direct access to the porphyrin is blocked by an axial ligand coordinated to the Zn center. The previously mentioned large bathochromic shift and the appearance of an intense hyperband in F450 are also consistent the presence of a strongly coordinating ligand in the axial position. It should be mentioned that in the case of both F420 and F450 the magnitude of the isotope effect is smaller than that when the zinc porphyrin is fully exposed to the solvent, as observed by Winkler et al.,[23] who for chemically denatured Zn-cytochrome *c* obtained a k_{H_2O}/k_{D_2O} of 1.5. This implies that in the case of both F420 and F450 the Zn-porphyrin moiety is buried within the protein and that it is unlikely that either form of the Zn-subsituted P450$_{cam}$ is appreciably unfolded.

Having demonstrated the existence of two forms of ZnP450$_{cam}$, we used photoinduced electron transfer to evaluate the redox activity and ligand binding ability of F420 and F450. Because depending on the redox properties of its partner, ^{3}ZnP450$_{cam}$ can act either as an electron donor or as an acceptor, it was possible to examine the transfer of electrons from and to the triplet state of the protein. The general reaction scheme for the oxidative (1) and reductive (2) electron transfer quenching of the triplet state is shown below:

$$^3\text{ZnP450} + \text{A} \rightarrow \text{ZnP450}^{+\bullet} + \text{A}^{-\bullet} \quad (1)$$

$$^3\text{ZnP450} + \text{D} \rightarrow \text{ZnP450}^{-\bullet} + \text{D}^{+\bullet} \quad (2)$$

The selected acceptors, benzoquinone, duroquinone, and chloranil, form complexes with the protein and efficiently quench the phosphorescence of ^{3}F450 and ^{3}F420 (Table 3 and Figure 6). Time-resolved photon counting measurements show that for all the acceptors the quenching is much more rapid for

Table 3. Thermodynamic Parameters and Measured Electron Transfer Reaction Rates of Both Forms of ^{3}ZnP450$_{cam}$

	E^{red} (V)[a]	$\Delta G°$	k_{ET} (s^{-1})
F420 + DQ	−0.49[b]	−0.43	2.0×10^3
F450 + DQ		−0.32	4.0×10^2
F420 + BQ	−0.16[b]	−0.75	5.9×10^3
F450 + BQ		−0.64	8.2×10^2
F420 + CQ	0.32[b]	−1.08	1.6×10^4
F450 + CQ		−0.97	7.0×10^3
	E^{ox} (V)[a]	$\Delta G°$	k_{ET} (s^{-1})
F450 + Fc	0.55[c]	0.24	0
F420 + Fc		0.13	3.6×10^3
F450 + Fc(COOH)$_2$	0.64[d]	0.33	0
F420 + Fc(COOH)$_2$		0.22	2.0×10^2

[a]Potentials vs the normal hydrogen electrode. [b]From ref 27. [c]From ref 28. [d]From ref 29.

F420 than for F450. In both cases, the measured rates follow the expected driving force dependence and increase with an increasing free energy, suggesting that the ET reactions lie in the Marcus "normal region". Additionally, the presence of the acceptors causes changes in the protein absorption spectrum around 420 nm, which reflect the alteration of the immediate vicinity of the porphyrin (Figure S6 of the Supporting Information). The changes are accompanied by a major decrease in the fluorescence intensity of F420 but a smaller decrease in the case of F450 (Figure 7 and Table 4). The possibility that the observed reduction in fluorescence is due to Förster energy transfer can be excluded because quinones do not absorb light at the emission wavelengths of F420 or F450. It is much more plausible that the lowering of the fluorescence yield is caused by the fast transfer of electrons from the singlet state, which effectively competes with the intersystem crossing. Because of the limited time resolution of our photon counting setup, we were not able to fully resolve the fluorescence quenching dynamics, which in the case of F420 occurs on a subnanosecond time scale.

In addition to the oxidative ET quenching by the quinones, we also investigated the reductive quenching of the F420 and F450 triplet states by strong electron donors. The interaction of the photoexcited F420 with ferrocene and its more soluble derivative, ferrocenedicarboxylic acid, results in efficient electron transfer and rapid quenching of the protein triplet state (Table 3 and Figure S7 of the Supporting Information). In contrast, no quenching at all was observed for ^{3}F450 in the presence of the same electron donors. At first, it might seem that these results could be explained by the difference in the driving force between the ^{3}F450 and ^{3}F420 reactions (both reductive ET reactions are slightly endothermic). However, the driving force in the ^{3}F420 + Fc redox pair is nearly identical to that in the F450 + Fc(COOH)$_2$ pair. While in the first case the ET reaction is quite efficient, in the second electron transfer does not proceed at all. This indicates that not only the thermodynamic parameters but also other factors, such as the active site accessibility and the distance between the Zn-porphyrin and the redox partner, play a role in the quenching of the triplet state of the ZnP450 conformers. Indeed, if we use the Marcus equation to evaluate the D−A distance between the ferrocene and the zinc porphyrin in F420, we obtain separations of 7.6 and 8.3 Å for ferrocene and Fc(COOH)$_2$, respectively. Naturally, such estimates are very sensitive to the

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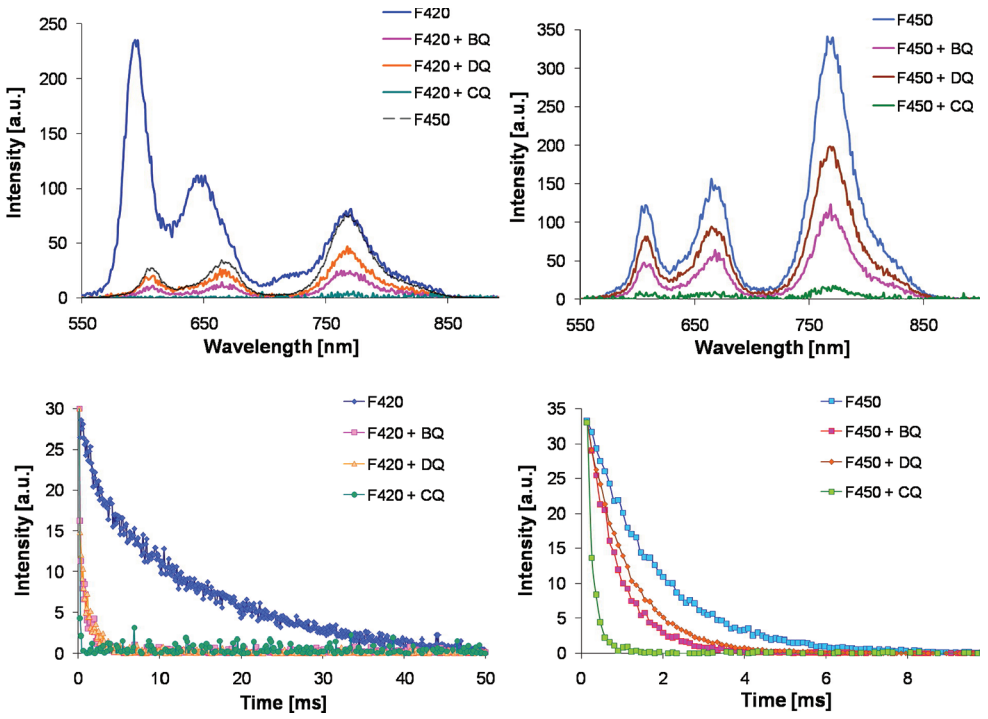

Figure 6. Phosphorescence spectra and normalized decay profiles of ZnP450 (1×10^{-6} M) in the presence of quinones (5×10^{-4} M) excited at 420 (left) and 450 nm (right) at pH 7. Note that the phosphorescence of F420 is almost completely quenched in the presence of acceptors and that the residual emission observed upon 420 nm excitation (top left) has the characteristics of that of ^{3}F450 (dashed line).

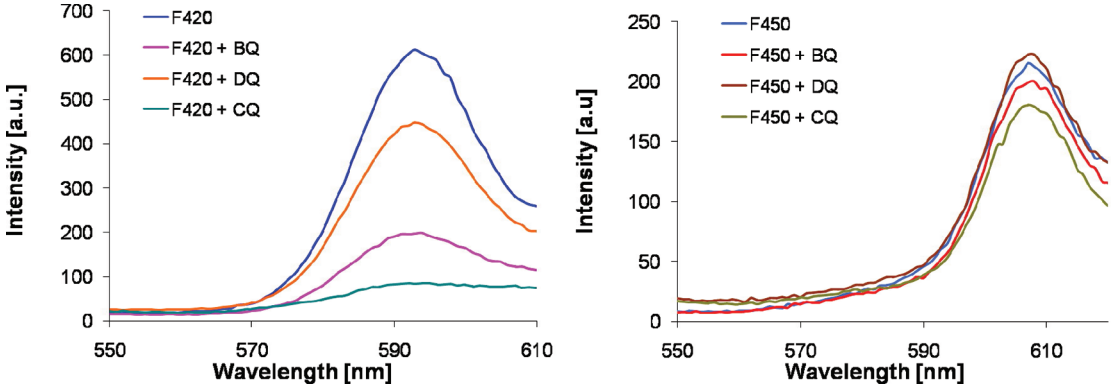

Figure 7. Fluorescence spectra of ZnP450$_{cam}$ (1×10^{-6} M) excited at 420 (left) and 450 nm (right) in an aerated solution in the presence of quinones (5×10^{-5} M) at pH 7.4.

Table 4. Normalized Fluorescence Intensities of F420 and F450 in the Presence of Quinones

	I/I_0		I/I_0
F420	1	F450	1
F420 + DQ	0.74	F450 + DQ	1.03
F420 + BQ	0.34	F450 + BQ	0.92
F420 + CQ	0.15	F450 + CQ	0.84

driving force (Table 3), as well as the values of the total reorganization energy (λ_{tot}) and the distance attenuation factor (β), which in our calculation were set to 1.1 eV and 1.1 Å^{-1}, respectively. Nevertheless, it is worth pointing out that Fülöp et al.[24] determined the crystal structure of an interesting post-translationally modified P450$_{cam}$ bearing a covalently tethered ferrocene moiety, which was shown to fold deep into the substrate channel of the enzyme. The closest edge of ferrocene in Fülöp's construct was 5.3 Å from the heme group,[1] corresponding to the Fe−Fe distance of approximately 7 Å, i.e., in remarkably good agreement with our rough estimate based on the kinetics of the reductive electron transfer reaction measured for the ferrocene−ZnP450_F420 complex.

DISCUSSION

Spectroscopic investigation of carefully and repeatedly purified ZnP450 revealed the existence two forms of substituted cytochrome P450$_{cam}$, F450 and F420, which possess different photophysical and photochemical properties. Conformer F450, which has been the subject of an earlier electron transfer study, has an abnormal spectrum containing a red-shifted Soret band and a strong hyperband.[12] The hyperband is characteristic of coordination of sulfur to ZnPP and is indicative of either cysteine or a spurious thiol ligated to the ZnPP in F450. The short lifetime of the T_1 state of F450 is also consistent with strong axial ligation. In contrast, variant F420 exhibits the

dx.doi.org/10.1021/bi201344h | *Biochemistry* 2012, 51, 1431−1438

classical spectroscopic properties and long triplet excited state lifetime that are typical of other zinc-substituted heme proteins such as Zn-cytochrome *c* and myoglobin. Our photoinduced electron transfer experiments suggest that F420 has an open conformation capable of sequestering small hydrophobic ligands. The difference in the ET behavior is most striking in the case of ferrocene, which is the bulkiest and most hydrophobic of the redox partners that were studied. The oxidation of ferrocene is mildly endoergic for both F420 and F450 triplet states; in the case of F420, it proceeds with a rate of 3.6×10^3 s^{-1}, while in the case of F450, it cannot be detected at all. Similarly, only F420 undergoes substantial fluorescence quenching in the presence of quinones, showing that in the case of this conformer the electron transfer reaction competes effectively with the short, nanosecond lifetime of the S_1 state. Such rapid quenching requires proximity between the redox partners. While it is difficult to draw definitive conclusions about the structure of forms F420 and F450 on the basis of optical spectroscopy and time-resolved electron transfer experiments, the existence of two conformers with district spectroscopic signatures is consistent with the recent findings of Goodin et al.,[2] who determined X-ray structures of two forms of native cytochrome $P450_{cam}$. The results of our time-resolved ET emission quenching experiments with $ZnP450_{cam}$ correlate form F420 with the substrate free, open, and flexible conformation of native $P450_{cam}$, while form F450 appears to be the analogue of the closed conformer of the native enzyme.

In the context of the spectroscopic data presented here and Goodin's structural determination, it appears that the photo-induced electron transfer work of Morishima focused on F450, i.e., the "closed" form of $ZnP450_{cam}$. This explains why it was not possible to observe ET emission quenching in the presence of organic ligands, which on the basis of their size were ideally suited to enter the hydrophobic channel of $P450_{cam}$ and bind with the modified enzyme.[12] The selection of form F450 in that early electron transfer study is understandable, because it is more abundant under most preparation and purification conditions. Indeed, the authors of this paper initially also made numerous unsuccessful attempts to bind organic ligands to F450.

We believe that this work will be very useful for researchers who are interested in employing metal substitution in their studies of electron transfer in cytochrome P450. The electron transfer results clearly identify F420 as the open form of metal-substituted $P450_{cam}$, which is capable of binding small hydrophobic ligands and positioning them in the immediate vicinity of the active site of the modified enzyme.

■ ASSOCIATED CONTENT

Supporting Information

A list of absorption maxima of metal porphyrins and forms of metal-substituted cytochrome P450 in the presence of thiol ligands, evolution of the absorption spectra upon addition of quinones, results of reductive excited state quenching of $ZnP450_{cam}$ in the presence of electron donors, and estimated redox potentials of $ZnP450_{cam}$. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

The support of this work by Genentech, Inc., through an unrestricted grant to P.P., and the access to the protein expression facilities of the Protein Engineering Department at Genentech, Inc., provided through the courtesy of Dr. Kurt Deshayes, are gratefully acknowledged. The instrumentation used in the course of this work was funded in part by NSF-CRIF Grant 342432 and the U.S. Department of Energy Office of Basic Energy Sciences Grant DE-FG02-06ER15828, as well as by Rutgers University.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Lars Gundlach for his assistance with setting up the time-resolved optical experiments.

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