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Redox control of the P450cam catalytic cycle: effects of Y96F active site mutation and binding of a non-natural substrate

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Spectroelectrochemistry measurements are used to demonstrate that active site mutation and binding of an non-natural substrate to P450cam (CYP101) reduces the shift in the redox potential caused by substrate-binding, and thereby results in slower catalytic turnover rate relative to wild-type enzyme with the natural camphor substrate.

Bacterial cytochrome P450cam (CYP101), the best-characterized monooxygenase enzyme system, catalyses hydroxylation of camphor exclusively at the 5-*exo* position with almost 100% coupling to the camphor substrate and the NADH reducing equivalents. A key role in substrate orientation and binding is played by the active site tyrosine Y96, which forms a hydrogen bond with camphor carbonyl. Elimination of hydroxyl by Y96F mutation has been shown to improve acceptance of non-natural hydrophobic substrates, but with reduced coupling efficiency, product stereospecificity and turnover rate.[1–3] Harnessing P450 oxygenating ability towards substrates of industrial importance could lead to biocatalytic syntheses of novel compounds.

Oxygenation consists of several steps including two single electron transfers from the iron–sulfur protein putidaredoxin (Pdx). The catalytic cycle is initiated upon binding of substrate, causing the expulsion of active site water and spin conversion of heme iron from low to high. This spin shift is related to a positive shift in enzyme formal reduction potential ($E^{0\prime}$), which enables the first electron transfer from Pdx.[4,5] This step is rate limiting for the catalytic cycle when Pdx is present in stoichiometric excess. As with other heme-thiolate proteins (NO-synthase, chloroperoxidase), $E^{0\prime}$ of cytochromes P450 is a characteristic parameter of various catalytic cycle intermediates, and significantly influences catalytic turnover.[6]

Despite general agreement about the direction of the potential shift and its relation to the direction of electron flow in the catalytic cycle of wild-type P450cam, there is large variation in reported values of $E^{0\prime}$. For substrate-free P450cam, $E^{0\prime}$ has been reported to range from −0.270 to −0.330 V (*vs.* NHE).[4,5,7,8] Upon saturation with camphor, it shifts to more positive values ranging from −0.138 to −0.207 V.[5,7–10] Experimental uncertainties in the methods used to determine $E^{0\prime}$ have been implicated for such wide variation.[11] Since $E^{0\prime}$ = −0.228 V for Pdx,[12] only camphor-bound form of wild-type P450cam is thermodynamically competent to accept electrons from Pdx. Formal potential data is absent for the important Y96F mutant of P450cam.[3] Accurate measurements of $E^{0\prime}$ will help understand the kinetics of initial electron flow in the various P450–substrate combinations, and more generally, provide added insight about heme protein structure–function relationships.[6]

We have reexamined the thermodynamic charge transfer equilibrium of P450cam and its active site mutant, Y96F. Formal potentials were determined prior to and following the binding of camphor or styrene using a thin layer spectroelectrochemical method. The method allows reliable Nernst titration of the enzyme solution while approaching redox equilibrium from both sides, thereby assuring the existence of thermodynamic reversibility.[12]

Cytochrome P450cam and the Y96F mutant were expressed in recombinant *E. coli* and purified as previously described.[3a] Solutions contained 20 μM protein, 0.2 M KCl, 50 mM TRIS/HCl (pH = 7.4) and 13 μM Phenosafranine as a redox mediator and an internal standard. For substrate bound measurements, pure styrene or camphor (Aldrich Chemical)† were introduced at saturating concentrations. $E^{0\prime}$ was quantified by spectroelectrochemical titration using a thin layer cell and Chem2000 fiber optic spectrometer (Ocean Optics, Dunedin, FI). Nano-crystalline Sb-doped tin oxide electrodes, prepared according to ref 14 were mounted to form a 0.2 mm gap and served as a working electrode. The airtight cell also contained Pt wire counter electrode, Ag/AgCl reference (Microelectrodes, Inc.) and provisions for solution and Ar gas delivery. Solution absorption spectra for several wavelength channels were recorded during the roundtrip potential scans (0.2 to −0.6 to 0.2 V, at 0.1 mV s^{-1}) at 5 or 10 mV intervals. Average values from the

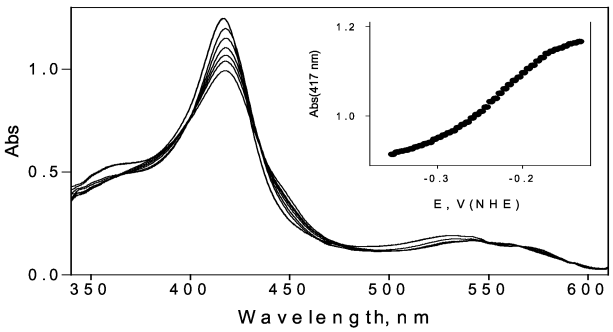

Fig. 1 Absorption spectra of P450cam Y96F substrate-free mutant during redox titration at 20 °C in a thin layer cell. Optical pathlength 5 mm. Plot of absorptivity *vs.* potential at 417 nm is shown in the inset.

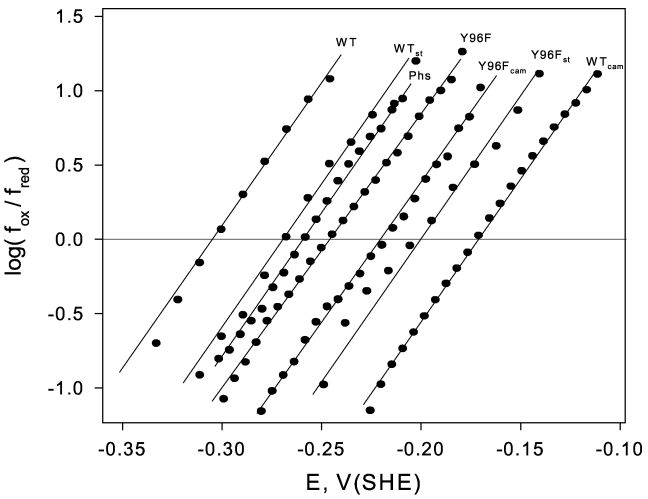

Fig. 2 Nernst plots of purified cytochrome P450cam and Y96F mutant with and without camphor or styrene as substrates. Phenosafranine (Phs) is included for comparison. Fractions of the reduced species were calculated from the absorbance values at 417 nm (substrate-free proteins) or 390 nm (in the presence of substrates), after subtraction of the phenosafranine absorbance.

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Table 1 Formal potential ($E^{0\prime}$) and substrate-induced shift ($\Delta E^{0\prime}$) for P450cam proteins as determined from the Nernst plots shown in Fig. 2. NADH turnover rates are a measure of relative overall enzymatic activity

	$E^{0\prime}$/V *vs.* NHE		$\Delta E^{0\prime}$/V		NADH turnover rate/ nmol (nmol P450)$^{-1}$ s^{-1}	
Enzyme state	WT	Y96F	WT	Y96F	WT	Y96F
Substrate-free	−0.306 ± 0.01	−0.248 ± 0.02				
Camphor-bound	−0.170 ± 0.005	−0.224 ± 0.01	0.136 ± 0.01	0.024 ± 0.02	852 ± 24[13]	29[2]
Styrene-bound	−0.265 ± 0.02	−0.204 ± 0.02	0.041 ± 0.02	0.044 ± 0.03	56 ± 7[11]	130 ± 15[11]

cathodic and anodic scans were used for $E^{0\prime}$ determination. Values of $E^{0\prime}$ were corrected relative to the Phenosafranine potential (−0.257 V *vs.* NHE).

Fig. 1 illustrates the change in the absorption spectrum with applied potential for the substrate-free Y96F mutant. The low spin state Soret band at 417 nm loses intensity upon protein reduction, and it was used for potential titration of the substrate-free proteins (inset of Fig. 1). Absorption spectra were stable for several hours at room temperature and could be slowly cycled several times between fully reduced and fully oxidized states. Data in Table 1 were collected during the first potential cycle well before P420 formation was indicated by absorption spectra (prominent δ band at 367 nm and Soret band shift to 422 nm).[8]

Ratios of oxidized to reduced protein fractions (f_{ox}/f_{red}) are plotted in Fig. 2. The linearity in plots of log f_{ox}/f_{red} *vs.* E demonstrates nearly ideal Nernstian behavior for a single electron transfer process (slopes ≈59 mV decade^{-1}). The intersections with the abscissa give $E^{0\prime}$ values (Table 1). Our $E^{0\prime}$ values for substrate-free and camphor-bound wild type P450cam agree well with one of the earlier determinations made by the dye photoreduction method.[5] For substrate-free Y96F mutant, the potential ($E^{0\prime}$ = −0.248 V) displays a large positive shift from the value for wild type protein. Although not directly bound to the porphyrin, the substitution of tyrosine 96 by more hydrophobic residue causes destabilization of the hydrogen bond network comprised of six ordered water molecules in the active site.[15] Then, the positive shift we have seen with this mutant would be consistent with findings on model systems where the reduction of the substituent polarity on the distal porphyrin side can raise $E^{0\prime}$ by as much as 0.1 V.[6] Increase in the active site hydrophobicity also facilitates the protonation of the sixth iron ligand and may contribute to the observed $E^{0\prime}$ shift.

Comparison of the NADH turnover kinetic data with the P450cam formal potentials upon substrate binding clearly indicates that the fastest cycling occurs when substrate binding shifts $E^{0\prime}$ of P450cam to a more positive value than $E^{0\prime}$ of the Pdx, in particular when camphor binds to WT protein, or styrene to the Y96F mutant (Table 1). However, the final value of $E^{0\prime}$ after binding is as influential to achieving high turnover as is the magnitude of $\Delta E^{0\prime}$. Despite comparable magnitude in $\Delta E^{0\prime}$ when styrene binds to WT or Y96F mutant protein, the NADH turnover is significantly slower for WT protein than Y96F (Table 1). In the first case, the substrate bound protein potential is still more negative than that of Pdx ($E^{0\prime}$ = −0.228 V), while for the Y96F-styrene pair, $E^{0\prime}$ is about 20 mV more positive than the Pdx potential. This knowledge of the $E^{0\prime}$ values for the substrate free enzymes is at least as important for understanding catalytic efficiency as other factors such as geometrical compatibility of substrate–protein active site pairs.

In summary, spectroelectrochemical measurements revealed that the redox potential of styrene-bound WT cytochrome P450cam is insufficiently altered to create thermodynamically favorable conditions for the electron transfer from its redox cofactor putidaredoxin. Mutation of the active site tyrosine to phenylalanine (Y96F) shifts $\Delta E^{0\prime}$ of the substrate-free protein by about +60 mV, thus enabling electron transfer after styrene binding. Our results strongly suggest that knowledge of the absolute enzyme formal potential for both substrate-free and bound states is necessary to infer electron flow direction and rate. On the practical level, $E^{0\prime}$ assay in oxygenase enzyme systems, along with the spin shift–binding assays,[3b] could be useful for screening prospective enzyme–substrate couples.

Notes and references

† Certain commercial equipment, instruments, and materials are identified in this paper to specify adequately the experimental procedure. In no case does such identification imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the material or equipment is necessarily the best available for the purpose.

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