J|A|C|S
COMMUNICATIONS

Published on Web 10/05/2002

CYP119 Plus a *Sulfolobus tokodaii* Strain 7 Ferredoxin and 2-Oxoacid:Ferredoxin Oxidoreductase Constitute a High-Temperature Cytochrome P450 Catalytic System

Andrei V. Puchkaev,[†] Takayoshi Wakagi,[‡] and Paul R. Ortiz de Montellano*[,†]

Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94143-0446, and Department of Biotechnology, The University of Tokyo, Tokyo 113-8657, Japan

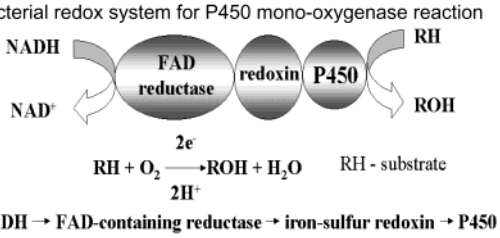
Received August 19, 2002

The utilization of enzymes, and of enzymes engineered by mutagenesis to optimize desired properties, in fine organic synthesis is of growing importance in modern biotechnology.[1] One limitation to the use of enzymes in synthesis is their instability to the organic solvents or high temperatures or both that are sometimes desirable in industrial processes. Among the enzymes with the greatest industrial potential are the cytochromes P450, as they are able to catalyze the regio- and stereospecific oxidation of even unactivated hydrocarbons. The cytochrome P450 enzymes are members of a superfamily of heme-thiolate proteins that are found in most species and are involved in the biosynthesis or catabolism of fatty acids, sterols, natural products, and a vast array of xenobiotics.[2,3] The fact that many of the reactions catalyzed by these enzymes are very difficult from an organic synthesis point of view, combined with the broad substrate specificities of many P450 enzymes, makes them highly attractive as potential biocatalysts.

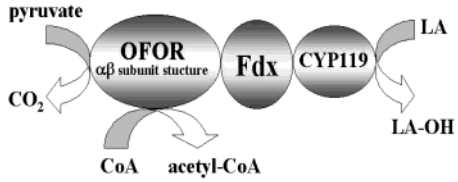
The industrial potential of cytochrome P450 enzymes has not yet been realized, however, because of limitations inherent in their catalytic requirements. One limitation has been the requirement of all known P450 systems for two reducing equivalents derived from either NADH or NADPH. The transfer of electrons from these pyridine nucleotide cofactors to the P450 is mediated either by a single flavoprotein containing FAD and FMN prosthetic groups or by an FAD-flavoprotein and an iron−sulfur Fe_2S_2 redoxin partner (Scheme 1).[4] The high cost and instability of pyridine nucleotide cofactors makes them unsuitable for biotechnological applications. A further limitation on the use of P450 enzymes in biotechnology has been their thermal instability, which seriously limits their catalytic lifetime even at temperatures below 25 °C.

Cloning from the acidothermophilic archaeon *Sulfolobus solfataricus*,[5] heterologous expression in *E. coli*, and purification of CYP119[6,7] has demonstrated the existence and viability of thermostable P450 enzymes. Although the native substrate and, more importantly, native redox partners of CYP119 are not known, the enzyme has been shown to inefficiently hydroxylate lauric acid using putidaredoxin, putidaredoxin reductase, and NADH as surrogate redox partners.[7] At least two additional thermophilic P450 enzymes are now known, although neither of them has been as well characterized as CYP119, and for neither have the high-temperature redox partners been identified.[8] The use of the nonthermostable redox partners, required by the unavailability of native partners, has limited catalytic studies of CYP119 to normal physiological temperatures. We report here the identification of a high-temperature set of redox partners from a related strain of *S. solfataricus* that both make possible the first high-temperature P450 catalytic system and circumvent the requirement for a reduced pyridine nucleotide cofactor (Scheme 2).

Scheme 1. Bacterial Redox System for P450 Monooxygenase Reaction



Scheme 2. CYP119 Reconstitution System with *Sulfolobus tokodai*i Strain 7 Ferredoxin and 2-Oxoacid:Ferredoxin Oxidoreductase as Redox Partners



2-Oxoacid:ferredoxin oxidoreductase (OFOR) catalyzes the coenzyme A-dependent oxidative decarboxylation of 2-oxoacids, typically pyruvate and 2-oxoglutarate, in archaea. OFOR from *Sulfolobus tokodaii* strain 7 has been isolated, cloned, and expressed.[9,10] A ferredoxin (Fdx) that acts as an electron acceptor in the OFOR reaction[11,12] has also been cloned from *S. tokodaii* strain 7 and has been overexpressed in *E. coli*.[13a] The decarboxylation of pyruvate and 2-oxoglutarate by the OFOR and Fdx from *S. tokodaii* strain 7 was reported earlier.[10] The involvement of a thermophilic ferredoxin in this system led us to investigate whether the OFOR and Fdx system could transfer electrons to CYP119.

Incubation of the *Sulfolobus solfataricus* CYP119 with the OFOR and Fdx from *S. tokodaii* strain 7 in the presence of lauric acid, coenzyme A, and pyruvic acid led to the consumption of pyruvic acid and hydroxylation of lauric acid (Figure 1 and Scheme 2) despite the absence of either NADPH or NADH in the incubation.[14] Furthermore, a sharp linear increase in the rate of lauric acid hydroxylation was observed with rising temperature, resulting in a 16-fold higher rate at 70 °C than at 25 °C. At temperatures above 70 °C, the hydroxylation activity rapidly declined. Two factors probably contribute to the marked temperature-dependent increase in the hydroxylation rate. The proportion of CYP119 in the high spin state, the state required for electron transfer to the iron, increases 5-fold between 25 and 70 °C.[15] Second, the activity of OFOR, which provides electrons to Fdx, is also temperature-dependent.[10] The temperature dependence of OFOR may reflect

* To whom correspondence should be addressed. E-mail: ortiz@cgl.ucsf.ecu.
† University of California.
‡ The University of Tokyo.

10.1021/ja0282036 CCC: $22.00

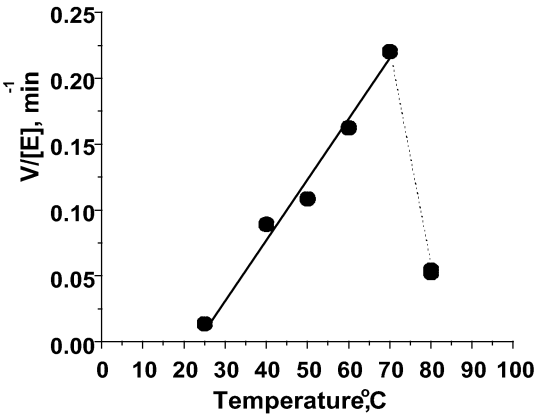


Figure 1. Dependence on temperature of the rate of lauric acid hydroxylation by CYP119 with OFOR and Fdx as redox partners. Reaction was started by addition of CoA and was carried out for 20 min at the indicated temperature. Final concentrations of reagents were: 50 mM phosphate buffer, pH 7.0; 1 μM CYP119, 3 μM OFOR, 15 μM Fdx; 0.12 mg/mL catalase; 40 μM lauric acid; 2 mM pyruvate; and 0.2 mM CoA. Product (LA-OH) formation was determined by HPLC analysis using ^{14}C-labeled lauric acid as previously reported.[7]

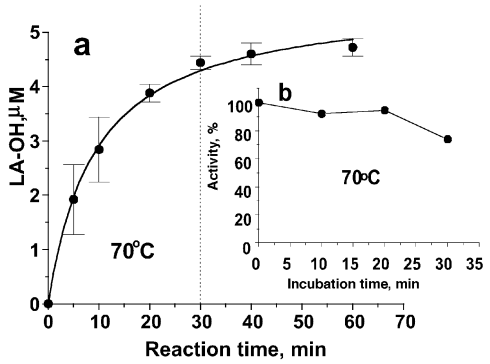


Figure 2. Time dependency of product formation. (a) Kinetic curve of product formation. Reaction conditions were the same as in Figure 1, and the temperature of the reaction was 70 °C. Vertical dotted line shows the time (~30 min) up to which the reaction system works effectively at the given temperature. (b) Dependence of relative activity on incubation time at 70 °C. Two mixtures: one consisting of CYP119, OFOR, Fdx, catalase and the other of CoA, lauric acid, and pyruvate were incubated separately, and equal aliquots from the two mixtures were mixed after fixed time intervals to assay the CYP119 activity. Final reagent concentrations and reaction time were the same as in Figure 1. Values in a are reported as the mean of triplicate determinations ±SD.

its $\alpha\beta$ subunit structure (α, 70 kDa; β, 34 kDa).[9,10] It has been shown for oligomeric indolepyruvate ferredoxin oxidoreductase from the hyperthermophile *Pyrococcus kodakaraensis* (IOR) that heterooligomerization of the IOR subunits is facilitated by increases in temperature up to 70 °C.[16] The decrease in the rate of lauric acid hydroxylation above 70 °C may stem from a similar temperature effect on OFOR oligomerization and activity.

When CYP119 incubations were carried out at 70 °C, fatty acid hydroxylation was observed for a period of approximately 30 min (Figure 2a). After 30 min, however, little hydroxylated product was formed. As the components of the reaction system were present at concentrations that would support a much higher extent of product formation, the cessation of catalysis reflects inactivation of one of the reaction components. This is confirmed by the finding that preincubation of the components for 30 min at 70 °C prior to combining them and assaying catalytic activity is well tolerated (Figure 2b). Little loss of activity is observed during the first 20 min, and only ~25% loss after 30 min. Significantly, the percent of the initial CYP119 lauric acid hydroxylation activity after 30 min at 70 °C (77%) (Figure 2b) is similar to the percent of the pyruvate decarboxylation activity of OFOR that remains after incubation for the same time at 70 °C (83%).[10] As CYP119 is stable for prolonged periods at 85 °C,[15] and the Fdx at 90 °C,[13b] the gradual inactivation of the catalytic system after 30 min (Figure 2a), the decrease of activity with incubation time (Figure 2b), and the sharp decrease in LA hydroxylation by CYP119 above 70 °C (Figure 1) reflect inactivation of OFOR rather than CYP119 or Fdx.

The present system (Scheme 2) is the first in which a P450 enzyme has been reconstituted with a protein electron donor to achieve catalysis at high temperature without a requirement for NADPH or NADH. Further work is now in progress to fully characterize and optimize this catalytic system, to identify even higher temperature partners for the P450 enzyme, and to mutate CYP119 into a practical catalytic system.

Acknowledgment. This work was supported by National Institutes of Health Grant GM25515.

References

(1) (a) Davis, B. G.; Boyer V. *Nat. Prod. Rep.* **2001**, *18*, 618. (b) Patel, R. N. *Curr. Opin. Biotechnol.* **2001**, *12*, 587. (c) Tholey, A.; Heinzle, E. *Adv. Biochem. Eng. Biotechnol.* **2002**, *74*, 1.
(2) Ortiz de Montellano, P. R. *Cytochrome P450: Structure, Mechanism and Biochemistry*, 2nd ed; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1995; p 473.
(3) Nelson, D. R.; Koymans, L.; Kamataki, T.; Stegeman, J. J.; Feyereisen, R.; Waxman, D. J.; Waterman, M. R.; Gotoh, O.; Coon, M. J.; Estabrook, R. W.; Gunsalus, I. C.; Nebert, D. V. *Pharmacogenetics* **1996**, *6*, 1.
(4) Lewis, D. F. V.; Hlavica P. *Biochim. Biophys. Acta* **2000**, *1460*, 353.
(5) Wright, R. L.; Harris, K.; Solow, B.; White, R. H.; Kennelly, P. J. *FEBS Lett.* **1996**, *384*, 235.
(6) McLean, M.; Maves, S.; Weiss, K.; Krepich, S.; Sligar, S. *Biochem. Biophys. Res. Commun.* **1998**, *252*, 166.
(7) Koo, L. S.; Immoos, C. E.; Cohen, M. S.; Farmer, P. J.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **2002**, *124*, 5684.
(8) (a) Sideso, O.; Williams, R. A. D.; Welch, S. G.; Smith, K. E. *J. Steroid Biochem. Mol. Biol.* **1998**, *67*, 163. (b) Yano, J. K.; Blasco, F.; Li, H.; Schmid, R. D.; Poulos, T. L. *12th International Conference on Cytochrome P450*; La Grande Motte, France, September 11−15 2001; Abstract P105.
(9) Zhang, Q.; Iwasaki, T.; Wakagi, T.; Oshima, T. *J. Biochem (Tokyo)* **1996**, *120*, 587.
(10) Fukuda, E.; Kino, H.; Matsuzawa, H.; Wakagi T. *Eur. J. Biochem.* **2001**, *268*, 5639.
(11) Kersher, L.; Oesterhelt, D. *Trends. Biochem. Sci.* **1982**, *7*, 371.
(12) Danson M. J. *Can. J. Microbiol.* **1989**, *35*, 58.
(13) (a) Wakagi, T.; Fujii. T.; Oshima T. *Biochem. Biophys. Res. Commun.* **1996**, *225*, 489. (b) Kojoh, K.; Matsuzawa, H.; Wakagi, T. *Eur. J. Biochem.* **1999**, *264*, 85.
(14) All thermophilic proteins in the present study were expressed, isolated and purified according to the published protocols.
(15) Koo, L. S.; Tschirret-Guth, R. A.; Straub, W. E., Moenne-Loccoz, P.; Loehr, T. M.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **2000**, *275*, 14112.
(16) Siddiqui, M. A.; Fujiwara, S.; Takagi, M.; Imanaka, T. *FEBS Lett.* **1998**, *434*, 372.

JA0282036