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The *Sulfolobus solfataricus* electron donor partners of thermophilic CYP119: an unusual non-NAD(P)H-dependent cytochrome P450 system☆

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Abstract

CYP119 from *Sulfolobus solfataricus* is the first well-characterized thermophilic cytochrome P450 enzyme. The endogenous substrate for this enzyme is not known but it hydroxylates lauric acid in a reaction supported by surrogate mesophilic electron donors. However, reconstitution of a high-temperature catalytic system requires identification of the normal thermophilic electron donor partners of CYP119. Here, we describe cloning, expression in *Escherichia coli*, and characterization of the requisite electron donor partners from *S. solfataricus*. One is a thermostable ferredoxin and the second a 2-oxoacid-ferredoxin oxidoreductase that utilizes pyruvic acid rather than NAD(P)H as the source of reducing equivalents. CYP119 is the only cytochrome P450 to date known to obtain electrons from a non-NAD(P)H-dependent protein. The two thermophilic partners have been used to reconstitute a catalytic system that hydroxylates lauric acid at 70 °C, and the optimal conditions for this system have been defined. This first high-temperature in vitro catalytic system represents an important step in the development of industrially relevant catalysts.
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Cytochrome P450 enzymes utilize molecular oxygen to generate a reactive species that is capable of promoting reactions as difficult as the regio- and stereospecific insertion of an oxygen atom into a hydrocarbon C–H bond to give a chiral alcohol [1]. The activated oxidizing species is believed to be a ferryl [formally (Fe(V)=O)] complex in which the oxygen is bound to the iron of the prosthetic heme group of the enzyme [1,2]. Formation of the ferryl species from molecular oxygen requires two reducing equivalents, which formally bring molecular oxygen to the level of hydrogen peroxide [3]. The two required electrons are normally provided by either NADH or NADPH through the action of protein partners that uncouple the pyridine nucleotide electrons and deliver them to the P450 heme group one at a time. Three classes of electron donor systems have so far been shown to be involved in supplying electrons to P450 enzymes. One class, composed of a flavoprotein and an iron–sulfur protein, is exemplified by adrenodoxin and adrenodoxin reductase, or putidaredoxin and putidaredoxin reductase [4,5]. The second class is represented by NADPH–cytochrome P450 reductase, a protein that contains two flavin prosthetic groups [6,8]. The third class of proteins resembles cytochrome P450 reductase but is distinct from it in that the flavoprotein is part of the same polypeptide as the P450 heme domain. The first member of this third class was CYP102 ($P450_{BM-3}$) [9],

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but at least one additional member has been found [7]. Recent studies have found a different fusion construct in which the P450 heme domain is fused to a ferredoxin domain, which in turn is reduced by a separate ferredoxin reductase [10], and a protein with a flavoprotein and iron–sulfur ferredoxin fused to the heme domain [11]. Despite these variations, these systems are unified by the fact that they all utilize pyridine nucleotides as the ultimate source of the reducing equivalents.

Two thermophilic cytochrome P450 enzymes have been characterized [12–19]. CYP119 from *Sulfolobus sulfotaricus* has been shown to oxidize fatty acids when catalysis is supported by a surrogate (mesophilic) electron donor system consisting of putidaredoxin and putidaredoxin reductase [14]. The crystal structure of CYP119 has been determined and the protein has been shown to undergo an unusually large active site conformational change upon binding of imidazoles of different sizes [17]. The native substrate for CYP119, if any, is unknown. The structure of the thermophilic P450 enzyme from *Thermus thermophilus*, CYP175A1, which appears to be a carotene hydroxylase [19], has also been determined [20]. The native redox partners of both of these thermophilic enzymes have remained obscure despite their potential utility in the biotechnological reconstitution of high-temperature P450-based catalytic systems.

In a preliminary communication, we reported that CYP119 readily accepts electrons from a surrogate electron donor system isolated from *Sulfolobus* sp. strain 7, a distinct strain of *Sulfolobus* [21]. This system was highly unusual in that the ultimate source of electrons was neither NADH nor NADPH, but rather a 2-oxoacid such as pyruvic acid [21]. Here we report identification, isolation, and characterization of the native electron donor partners of CYP119 in *S. solfataricus* itself. As found earlier for the *Sulfolobus* sp. strain 7 surrogate partners, the electron supply system consists of a ferredoxin and a heterodimeric protein that utilizes pyruvic acid as the ultimate source of electrons. However, there are significant differences between the electron transfer proteins from *S. solfataricus* and *Sulfolobus* sp. strain 7. These results establish an entirely new class of electron donors for P450 enzymes and make possible the reconstitution of a high-temperature P450 catalytic system.

Materials and methods

Materials

Genomic DNA from *S. solfataricus* P2 (DSM 1617) was purchased from ATCC (Manassas, VA). *Escherichia coli* strain BL21(DE3) Gold plysS was from Novagen (La Jolla, CA). Q-Sepharose Fast Flow, Butyl-Sepharose, and Superdex-75 were purchased from Pharmacia LKB Biotechnology (Piscataway, NJ). All restriction enzymes were from New England Biolabs (Beverly, MA). *Pfu* Turbo DNA polymerase was obtained from Stratagene (La Jolla, CA). Plasmid pT-groE was kindly provided by Prof. Shunsuke Ishii (Tsukuba, Japan) [22]. [1-^{14}C]Lauric acid (55 mCi/mmol) was purchased from American Radio Chemicals (St. Louis, MO). Water was purified by the Milli-Q purification system (Millipore). All other chemicals in this study were analytical grade.

Molecular cloning and expression in E. coli of the ferredoxin gene from S. solfataricus P2

The ferredoxin structural gene was amplified by PCR from genomic DNA using a 5′-sense oligonucleotide primer containing an *Nde*I restriction site and a 3′-reverse complementary primer with a *Bam*HI site (see Table 1). The PCR product was digested with *Nde*I and *Bam*HI, and ligated to the expression vector pET-11a carrying a T7 promoter (Novagen). The coding region of the construct was sequenced to ensure that no error had been introduced into the coding region by the PCR amplification. A pET-11a plasmid with the Fdx-*Ss*[1] encoding gene was transformed in *E. coli* BL21 (DE3) Gold plysS competent cells. The other steps of expression and purification were similar to those reported for purification of ferredoxin from the thermophilic archaeon *Sulfolobus* sp. strain 7. This involved Q-Sepharose FF, Butyl-Sepharose, and Superdex-75 (Pharmacia) chromatographic steps for purification of the protein [23].

Construction of the expression plasmid for the 2-oxoacid:ferredoxin oxidoreductase from S. solfataricus P2

The α- and β-subunits of OFOR-*Ss* were cloned separately. The genes for the α- and β-subunits were amplified by PCR from genomic DNA using a 5′-sense oligonucleotide primer containing an *Nde*I restriction site and a 3′-reverse complementary primer with an *Spe*I restriction site (see Table 1). The PCR products were digested with *Nde*I and *Spe*I, and ligated into the expression vector pET-17b (Novagen) carrying a T7 promoter. The coding regions of the constructs were sequenced to ensure that no error was introduced in the PCR amplification step.

The structural gene of the β-subunit with a ribosome binding site of pET-17b was amplified from the β-subunit/pET-17b plasmid using a 5′-sense oligonucleotide primer containing an *Spe*I restriction site and a 3′-reverse complementary primer containing a 6-His region

[1] *Abbreviations used:* Fdx-*Ss*, ferredoxin from *Sulfolobus solfataricus* P2; Fdx-*St*, ferredoxin from *Sulfolobus* sp. strain 7; OFOR-*Ss*, 2-oxoacid:ferredoxin oxidoreductase from *Sulfolobus solfataricus* P2; SDS, sodium dodecyl sulfate; TPP, thiamine pyrophosphate.

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