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Archives of Biochemistry and Biophysics 445 (2006) 138–146
ABB
www.elsevier.com/locate/yabbi

Heterologous expression, purification, and properties of human cytochrome P450 27C1

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Received 27 October 2005, and in revised form 4 November 2005
Available online 28 November 2005

Abstract

Cytochrome P450 (P450) 27C1 is one of the "orphan" P450 enzymes without a known biological function. A human P450 27C1 cDNA with a nucleotide sequence modified for *Escherichia coli* usage was prepared and modified at the N-terminus, based on the expected mitochondrial localization. A derivative with residues 3–60 deleted was expressed at a level of 1350 nmol/L *E. coli* culture and had the characteristic P450 spectra. The identity of the expressed protein was confirmed by mass spectrometry of proteolytic fragments. The purified P450 was in the low-spin iron state, and the spin equilibrium was not perturbed by any of the potential substrates vitamin D_3, 1α- or 25-hydroxy vitamin D_3, or cholesterol. P450s 27A1 and 27B1 are known to catalyze the 25-hydroxylation of vitamin D_3 and the 1α-hydroxylation of 25-hydroxy vitamin D_3, respectively. In the presence of recombinant human adrenodoxin and adrenodoxin reductase, recombinant P450 27C1 did not catalyze the oxidation of vitamin D_3, 1α- or 25-hydroxy vitamin D_3, or cholesterol at detectable rates. P450 27C1 mRNA was determined to be expressed in liver, kidney, pancreas, and several other human tissues.

Keywords: Cytochrome P450; P450 27C1; Adrenodoxin; Cholesterol; Vitamin D; Expression in *Escherichia coli*

Cytochrome P450[1] enzymes are involved in the oxidation of a large variety of xenobiotic chemicals including drugs, carcinogens, pesticides, etc. [2]. However, more than one-half of the human P450s have characterized roles in the metabolism of endogenous chemicals or vitamins [3]. Approximately, 13 of 57 human P450 genes do not have any defined functions in the oxidation of endogenous or xenobiotic chemicals, and these can accordingly be termed "orphans" [4].

One approach to the characterization of the P450 orphans is the heterologous expression of large amounts of the proteins to facilitate studies on involvement in reactions, using approaches focused on candidate substrates inferred from knowledge about related P450s and also broader searches [4]. One of the orphan P450s is 27C1, which has no reported information at this time concerning its site of expression, or even if it is expressed [4]. On the basis of the sequence similarity with P450s 27A1 and 27B1, this might be expected to be a mitochondrial protein with possible catalytic activity toward Vit D_3 or its hydroxylated forms [3,5–10].

We developed conditions for the high-level expression of a modified version of human P450 27C1 in *Escherichia coli* and its purification. The distribution of P450 27C1 mRNA in human tissues was analyzed, and the possibility that P450 27C1 is involved in Vit D_3 or cholesterol metabolism was examined.

Materials and methods

Chemicals

Oligonucleotides for cDNA synthesis and N-terminal modifications were purchased from Operon (Huntsville,

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[1] *Abbreviations used:* Adr, NADPH-adrenodoxin reductase; Adx, adrenodoxin; LB, Luria–Bertani medium; NTA, nitrilotriacetate; OH, hydroxy; $(OH)_2$, dihydroxy; P450, cytochrome P450 (also termed "heme-thiolate protein P450" [1]); PAGE, polyacrylamide gel electrophoresis; PCA, polymerase cycling assembly; SDS, sodium dodecyl sulfate; TB, Terrific broth (medium); Vit D_3, cholecalciferol (vitamin D_3).

0003-9861/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.abb.2005.11.002

AL, USA) in either 96-well format, at 10-nmol scale in wet form (normalized to 100 μM each), or tube format, at 50 nmol scale. All oligonucleotides were of salt-free quality and used directly without further purification. Vit D_3, 25-OH Vit D_3, 1α, 25-$(OH)_2$ Vit D_3, and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO, USA), and 1α-OH Vit D_3 was obtained from Calbiochem-EMD Biosciences (La Jolla, CA, USA). [4-^{14}C]Cholesterol (53 mCi/mmol) was purchased from Perkin-Elmer (Boston, MA, USA). All other reagents and solvents were obtained from general commercial suppliers and used without further purification.

Optimization of the P450 27C1 nucleotide sequence for heterologous expression and oligonucleotide design for the synthesis of P450 27C1 cDNA

Automatic codon optimization and oligonucleotide design for polymerase chain reaction (PCR)-based gene synthesis were performed using DNAWorks 2.4 [11], developed by the Center for Information Technology, National Institutes of Health, Department of Health and Human Services (http://molbio.info.nih.gov/dnaworks).

The amino acid sequence and the native cDNA sequence information for human P450 27C1 were obtained from D. R. Nelson's P450 homepage (http://drnelson.utmem.edu/human.P450.seqs.html) and assembled from GenBank sequence AC027142. An *Eco*RI restriction site was selected from the native cDNA sequence and used to manually separate the full-length gene into two synthons encoding amino acids 6–288 (Synthon 1) and 289–542 (Synthon 2). In addition, a $(His)_5$ tag was added to the C-terminal to facilitate purification. A series of overlapping 40- to 50-mers were prepared for each synthon (see Supplementary Material), based on the DNAWorks results (vide supra).

The codons were optimized automatically to suit the codon preference bias of *E. coli*. The codon frequency threshold was set to be 20% in the program. Overlap melting temperatures were designed to be 65 ± 3 °C. The 5′- and 3′-flanking sequences for Synthon 1 were 5′-TAGG AGGT*CATATG*GCTTTATTAGCACGTATT-3′ (sense) and 5′-*GAATTC*TGC-3′ (sense) respectively, which contained an *Nde*I restriction site and AT enrichment for the codons of the first seven amino acids on the 5′ end and an *Eco*RI restriction site on the 3′ end. The flanking sequences for Synthon 2 were 5′-CGC*GAATTC*TGC-3′ (sense) and 5′-TAA*TCTAGA*GGTA-3′ (sense), which contained an *Eco*RI restriction site on the 5′ end and an *Xba*I restriction site on the 3′ end. Other than that, all DNA sequences were designed to lack the *Nde*I, *Xba*I, and *Eco*RI sites needed for subsequent manipulation.

PCR-based gene synthesis

Ten microliters of each of the oligonucleotide components (normalized to 100 μM) of Synthons 1 and 2 were added into two separate pools and diluted to 2 μM each.

Polymerase cycling assembly was carried out in a 50-μl reaction mixture: 2.5 U *PfuUltra* High-Fidelity DNA polymerase (Stratagene, La Jolla, CA, USA), 0.2 mM each dNTP, and 5.0 μl of 10× *PfuUltra* buffer. Thermal cycling began with a 5-min denaturing step at 95 °C and was followed by 25 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s, with a final extension time of 10 min.

Polymerase chain reaction amplification mixtures (50 μl) contained 2.5 U of *PfuUltra* High-Fidelity DNA polymerase, 0.2 mM each dNTP, 5.0 μl of 10× *PfuUltra* buffer, 1 μl of each PCA product, and 0.4 μM of each of the outermost oligonucleotides. The thermal cycling parameters were essentially the same as for PCA, except that the number of cycles was raised to 30.

The 866- and 796-bp amplified PCR fragments of Synthons 1 and 2 were purified by agarose gel electrophoresis and double digested with *Nde*I and *Eco*RI and with *Xba*I and *Eco*RI, respectively. Both fragments with cohesive ends were ligated together in a "monocistronic" pCW vector (not coding for NADPH-P450 reductase [12]) and digested with *Nde*I and *Xba*I. The ligation mixture was transformed into *E. coli* DH5α competent cells and selected on LB plates with 50 μg/ml ampicillin. The plasmids were purified using a QIAprep Miniprep kit (Qiagen, Valencia, CA, USA) and screened by restriction digest analysis with *Nde*I and *Xba*I. The plasmids (construct 1) that yielded 1.6 kb fragments were sequenced in both the sense and the antisense strands in the Vanderbilt facility with an Applied Biosystems Model 3700 fluorescence sequencing unit using a Taq dye terminator kit (PE Applied Biosystems, Foster City, CA, USA).

N-Terminal modifications

N-Terminal mutations were introduced into native construct (pCW 27C1) by PCR-based mutagenesis. The N-terminal segment of 27C1 was amplified between the *Nde*I and the *Eco*RI sites using 5′ PCR primers containing the desired mutations and a complementary 3′ PCR primer (5′-GCAGAATTCGCGCCACGGCTTTGGGATAAAT GGACGTAAC-3′), which was also the 3′ outmost oligonucleotide of Synthon 1. The mutagenic primers for constructs 2 and 3 were 5′-GTCTGCCACATATGGCTCG TGCAGAAGATAAAGGTGCAGGCCGTCCAGGTA G-3′ and 5′-GTG GTGGTCATATGGCTGGTCCACGT AGTCTGGCGCATGCCAGGTCCAC-3′, respectively. *PfuUltra* High-Fidelity DNA polymerase was used for the PCR amplification at an annealing temperature of 60 °C. The products were purified by agarose gel electrophoresis, double digested with *Nde*I and *Eco*RI and ligated with the 5.8 kb fragment derived from the digestion of construct 1 with the same enzymes. The modifications were confirmed by sequencing the open reading frame regions of the new constructs.