Domain Characterization of Cyclosporin Regio-Specific Hydroxylases in Rare Actinomycetes

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Introduction

Bacterial cytochrome P450 hydroxylase (CYP) belongs to the superfamily of heme-containing monoxygenases, which catalyze the biotransformation of natural products such as steroids, fatty acids, polyketides, and xenobiotic compounds [6, 7, 12, 13]. Bacterial CYP is known to transfer a single CYP-bound oxygen atom along with a single hydrogen atom from NAD(P)H, which is regenerated by the ferredoxin (FD)-ferredoxin reductase (FDR) electron transfer system, to its substrate [4, 8, 11]. The catalytic abilities of CYPs have gained attention owing to their superior regio- and stereo-selectivities and thus hold potential for the hydroxylation of natural and synthetic compounds with diverse structures via CYP-driven bioconversion processes.

Several CYPs in various actinomycetes have been shown to produce pharmacologically useful secondary metabolites [2, 14]. CYPs detected in rare actinomycetes such as Sebekia benihana are known to introduce hydroxyl groups to secondary metabolites at certain locations [5, 13]. Among them is cyclosporin A (CsA) produced from Tolypocladium inflatum, which receives a hydroxyl group at its 4th amino acid, methyl leucine (γ-hydroxy-N-methyl-L-Leu4-CsA, 4HCsA). Although S. benihana CYP also introduces a hydroxyl group at the 9th methyl leucine of CsA (γ-hydroxy-N-methyl-L-Leu9-CsA, 9HCsA), it demonstrates a preference for the 4th amino acid position. Another actinomycete named Pseudonocardia autotrophica also introduces a hydroxyl group at the 4th or 9th methyl leucine, but it shows preference for the 9th methyl leucine position (LG Household and Health Care. 2007. Korean Patent...
We previously identified two CsA-specific CYPs: *S. benihana* CYP-sb21 preferentially introduces a hydroxyl group at the 4\(^{th}\) methyl leucine of CsA, whereas *P. autotrophica* CYP-pa1 prefers the 9\(^{th}\) methyl leucine of CsA [1, 9, 10]. To identify which domain determines regio-selectivity between these two CYP genes, we divided the two CYP genes into four domains, followed by hybrid CYP gene construction by domain swapping with each other. Each hybrid CYP was then introduced into a deletion mutant of CYP-sb21 (S. benihana ∆CYP-sb21) or CYP-pal (P. autotrophica ∆CYP-pa1) in order to determine the domain responsible for CsA regio-selectivity.

## Results

### Amino Acid Sequence Alignment and Domain Swapping Between CsA-Specific CYPs

We performed amino acid comparison between the two CsA-specific CYPs in order to select locations for division of the CYP-sb21 and CYP-pa1 domains (Fig. 1A). Amino acid comparison detected 55% similarity, and chosen sequences showed significant differences or amino acid insertion compared with the control group. Using SWISS-MODEL, other CYPs with known 3D structures were compared with these two CYPs in order to select a template CYP, which was identified as vitamin D3 hydroxylase from *P. autotrophica*. In comparing the expected 3D structures of the two CYPs with that of vitamin D3 hydroxylase (Fig. 1B), the CYP-sb21 and CYP-pa1 genes were subdivided into four domains. The CYP-sb21 gene was divided into 1–135, 136–225, 226–301, and 302–410, whereas the CYP-pa1 gene was divided into 1–134, 135–228, 229–304, and 305–412 (Fig. 1A).

To determine whether or not a specific domain affects regio-selectivity during hydroxylation of CsA, we altered the four domains of the two CYP genes. Using the In-fusion cloning method (Fig. 1C), a total of 14 hybrid CYP genes were generated in the actinomycete chromosome integration pMMBL005 vector, and the nomenclature was as follows: CYP-SP001 ~ CYP-SP007 and CYP-PS001 ~ CYP-PS007 (Fig. 1D). Each hybrid CYP sequence was analyzed to confirm the domain changes. To determine whether or not CsA is hydroxylated by hybrid CYPs, each hybrid CYP gene was introduced into S. benihana ∆CYP-sb21 or P. autotrophica ∆CYP-pa1 by the *E. coli*-actinomycetes interspecies conjugation method.

### CsA Hydroxylation by Hybrid CYPs

To determine whether or not regio-selectivity of hydroxylation differs between wild-type and hybrid CYPs, recombinant strains along with *S. benihana* and *P. autotrophica* wild-type strains were tested for CsA bioconversion. Since bioconversion rates for all recombinant *S. benihana* ∆CYP-
sb21 strains were too low to compare regio-selectivities (data not shown). *S. benihana* ΔCYP-sb21 was identified as an inappropriate host for hybrid CYP bioconversion analysis. In the case of recombinant *P. autotrophica* ΔCYP-pa1 strains, two recombinant strains (*P. autotrophica* ΔCYP-pa1/pCYP-SP007 and *P. autotrophica* ΔCYP-pa1/pCYP-PS002)
exhibited significant bioconversion activities (Fig. 2A). The 9HCsA/4HCsA ratio for *P. autotrophica* ΔCYP-pa1/pCYP-SP007 was 1.6, which was similar regio-selectivity as the *P. autotrophica* ΔCYP-pa1/pCYP-pa1 control (Fig. 2B). On the other hand, *P. autotrophica* ΔCYP-pa1/pCYP-pPS002 showed a 9HCsA/4HCsA ratio of 4.3, indicating stronger preference for 9HCsA over CYP-pa1 (Fig. 2B). This result suggests that the region critical for CsA regio-specific hydroxylation to 9HCsA is located in the 2nd domain of CYP-pa1 (135–228).

**Identification of the Regio-Selectivity Region in the Second Domain**

To determine which section of the 2nd domain (135–228) affects regio-selectivity, amino acid comparison was performed on the 2nd domains of CYP-sb21 and CYP-pa1. The two sections showing the largest differences were selected (Fig. 3A). We then produced an additional four hybrid CYP genes after altering the two sections of CYP-sb21 and CYP-pa1 and named them CYP-SP008, CYP-SP009, CYP-PS008, and CYP-PS009. To determine whether
or not CsA is hydroxylated by each hybrid CYP gene, the hybrid CYP gene construct was introduced into \textit{P. autotrophica} \(\Delta\text{CYP-pa1}\), and bioconversion analysis was conducted. There was not much difference in bioconversion or regioselectivity between \textit{P. autotrophica} \(\Delta\text{CYP-pa1}/\text{pCYP-SP009}\) and \textit{P. autotrophica} \(\Delta\text{CYP-pa1}/\text{pCYP-sb21}\) (Fig. 3B). On the other hand, \textit{P. autotrophica} \(\Delta\text{CYP-pa1}/\text{pCYP-SP008}\) showed approximately 52% more bioconversion than \textit{P. autotrophica} \(\Delta\text{CYP-pa1}/\text{pCYP-sb21}\), and the 4HCsA/9HCsA ratio was 7.5 (Fig. 3C). Compared with \textit{S. benihana} wild-type, \textit{P. autotrophica} \(\Delta\text{CYP-pa1}/\text{pCYP-SP008}\) showed approximately 27% more bioconversion of CsA to 4HCsA than 9HCsA (Fig. 3C), implying the 2nd domain of CYP-sb21 also induced conversion of CsA to 4HCsA (136-225 of CYP-sb21).

\textbf{Fig. 3.} Identification of the regio-selectivity region in the second domain. (A) Amino acid sequence alignment between 2nd domains in CYP-sb21 and CYP-pa1. Swapping residues are indicated by boxes. (B) HPLC profile of CsA bioconversion in \textit{P. autotrophica} wild-type and \textit{P. autotrophica} recombinant strains. (C) Bioconversion yields in \textit{P. autotrophica} wild-type and \textit{P. autotrophica} recombinant strains.
**Discussion**

The potential of hydroxylated CsA as a potential hair growth stimulator has been proven to be 100 times more effective than the existing product minoxidil, in the context of a mice skin graft experiment. The position of CsA hydroxylation is very important, since 4HCsA shows 10 times less immunosuppressive activity than 9HCsA (LG Household and Health Care. 2007. Korean Patent 100865210000, LG Household and Health Care. 2008. Korean Patent 1006816700000), implying that CsA hydroxylation regio-selectivity might be important to the potential development of hydroxylated CsA as a hair growth stimulator with fewer side effects.

Here, we identified the domain of CYP that determines region-selectivity for CsA by the domain switch method. Using amino acid sequence comparison and 3D structure estimation, CYP-sb21 and CYP-pa1 were divided into four domains, which were then switched with each other. Fourteen mutant CYPs with switched domains were introduced into S. benihana ΔCYP-sb21 and P. autotrophica ΔCYP-pa1, and bioconversion experiments for CsA were conducted. In the bioconversion experiment, the 2nd domain of CYP-pa1 showed regio-selectivity in P. autotrophica ΔCYP-pa1/pCYP-P5002 with a 9H/C 4H ratio of 4.3. In other words, mutant CYP containing the 2nd domain of CYP-sb21 preferentially converted CsA to 9HCsA. To better identify the 2nd domain affecting regio-selectivity, amino acid sequence comparison was performed on the 2nd domains of CYP-sb21 and CYP-pa1. The 4H/C 9H ratio in P. autotrophica ΔCYP-pa1/pCYP-SP008 was 7.5, indicating a preference for 4HCsA over CYP-sb21 as well as 27% higher conversion yield than S. benihana wild type. These results imply that the 2nd domain of Csa-specific CYP plays a critical role in CsA regio-selectivity, thereby setting the stage for biotechnological application of CsA regio-selective hydroxylation.

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**References**


