Production of Polyketides by a Novel P-450 Aryl Coupling Catalyst

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Research aims

The genus *Streptomyces* comprises Gram-positive, soil-dwelling, filamentous bacteria with a complex life cycle similar to that of fungi. In addition to the complex morphological differentiation, *Streptomyces* is also characterized by its ability to produce a wide variety of secondary metabolites including pharmaceutically useful compounds, such as antibiotics, anti-tumor agents and immunosuppressors. We have recently characterized a P-450 gene, named P-450mel, which is responsible for 1,4,6,7,9,12-hexahydroxyperylene-3,10-quinone (HPQ) synthesis in *S. griseus*.

In this report, we describe *in vitro* reconstitution of the P-450mel catalyst with spinach ferredoxin-NADP⁺ reductase/ferredoxin. P-450mel catalyzed oxidative biaryl coupling of 1,3,6,8-tetraydroxynaphthalene (THN), which was formed from five molecules of malonyl-CoA by the action of RppA¹, to yield HPQ. We then investigated the substrate specificity of P-450mel. P-450mel accepted flavilolin as a substrate to give dimmers of flavilolin, whereas several dihydroxy naphthalenes and emodin anthrone were not accepted under the condition used in this study.

Methods

**Production and purification of P-450mel.** The nucleotide sequence (TGACGA) covering the TGA stop codon of P-450mel was changed to TCGAG to create an XhoI site by PCR with primer I: 5'-GGCGGATCCCATCGAGAACACCTCG-3' (the italic letters indicate an EcoRI site, and the boldface letters indicate the start codon of P-450mel) and primer II: 5'-GGCGGATCCATCGAGGCATGTGACCCGGC-3' (the italic letters indicate an EcoRI site and the underline indicates an XhoI site). The amplified 1.2-kb fragment was cloned between the NdeI and XhoI sites of pET26b, resulting in pET26b-P-450mel-Xho. The amplified sequence under the control of the T7 promoter in pET26b thus constructed would direct the synthesis of P-450mel-Leu-Glu-His6. P-450mel purified from E. coli was an active form, because the purified enzyme had an absorbance at 450 nm on binding CO (results not shown).

For production of P-450mel with a His-tag at its C-terminus, *E. coli* BL21 (DE3) harboring pET26b-P-450mel was grown at 37°C for 2 h in M9 medium containing 100 mM FeSO₄ and 100 µg/ml of ampicillin, followed by addition of 80 µg/ml of 5-aminolevulinic acid hydrochloride and 100 µM isopropyl-β-D-thiogalactopyranoside (IPTG). After further incubation at 22°C for 1 day, the cells were collected by centrifugation. A crude cell-lysate was prepared by sonication and removal of cell debris by centrifugation at 10,000 g for 30 min. P-450mel was purified by using a Ni-nitrilotriacetic acid column (Qiagen), according to the manual from the manufacturer. Protein concentrations were measured with a Bio-Rad protein assay kit using bovine serum albumin as a standard. The sodium dithionite-reduced carbon monoxide difference spectrum of the purified P-450mel was measured by the method described by Omura and Sato².

**In vitro reconstitution of P-450mel activity.** The reactions, containing 100 mM sodium phosphate (pH 7.3), 1 mM dithiothreitol, 1 mM EDTA, 10% glycerol, 1 mM NADPH, 0.5 U of spinach ferredoxin-NADP⁺ reductase, 40 µg spinach ferredoxin, and 23.4 µg of P-450mel, 400 µM of THN, were performed in a total volume of 500 µl. Ferredoxin and ferredoxin-NADP⁺ reductase from spinach were purchased from Sigma. THN was synthesized according to the method of Ichinose et al.³. The reactions were carried out at 30°C for 30 min and terminated by adding 50 µl of 6 M HCl, and extracted with 200 µl of ethylacetate. The organic layer was collected and evaporated and the residual material was dissolved in 20 µl of methanol for HPLC analysis. Conditions of HPLC are as follows: ODS-80T column (4.6×150 mm, Tosoh), maintained at 40°C, was eluted with 25 mM KH₂PO₄ (pH 4.7) containing 13% CH₃CN at a flow rate of 1 ml/min.

Results

**In vitro analysis of aryl coupling of THN by P-450mel.** For convenient purification of P-450mel, we placed its coding sequence under the control of the T7 promoter in pET26b and introduced it into *E. coli* BL21 (DE3). Plasmid pET26b-P-450mel thus constructed would direct the synthesis of P-450mel-Leu-Glu-His₆. P-450mel purified from the soluble fraction using histidine-bind resin gave a single protein band of about 42 kDa, as determined by SDS-polyacrylamide gel electrophoresis. P-450mel produced in *E. coli* was an active form, because the purified enzyme had an absorbance at 450 nm on binding CO (results not shown).

For reconstitution of the P-450mel activity for *in vitro* enzyme assay, we used the ferredoxin and ferredoxin-NADP⁺ reductase from spinach, as was successfully used for reconstitution of a soluble cytochrome P-450soy from *S. griseus*⁴. Incubation of the reconstituted system with THN gave two products, as analyzed by HPLC (Fig. 1A). The product at retention time 9 min was identified as HPQ by its
co-migration with authentic HPQ, which was prepared from *S. lividans* harboring pNF1 (Fig. 1B). The product at retention time 6.2 min appeared to be pseudoHPQ (Fig. 3A), a dimeric form of THN, because it had a molecular mass of 382, which is equal to \(2Mw/11002\) Da of THN (Mw 192), as revealed by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry analysis (data not shown). The *in vitro* formation of HPQ from THN by the action of P-450mel was consistent with the *in vivo* observation that simultaneous overexpression of rppA and P-450mel in *S. lividans* led to accumulation of HPQ.

Insignificant amounts of HPQ and pseudoHPQ were observed in the control incubations with a boiled enzyme (Fig. 1C) and without the redox-partners (Fig. 1D), indicating that these products were also formed non-enzymatically as a result of free radical-induced oxidation of THN, but at a lesser rate. We then performed the reaction using pseudoHPQ, which was recovered from the reaction of THN, as a substrate to clarify whether both steps of sequential oxidation of THN leading to HPQ were catalyzed by P-450mel. P-450mel rapidly consumed pseudoHPQ to yield HPQ (Fig. 1E), although pseudoHPQ was extremely unstable and underwent spontaneous intramolecular aryl coupling to yield HPQ in a control reaction containing a boiled P-450mel enzyme (Fig. 1F). These results suggest that P-450mel catalyzes sequential oxidation of THN, which is intermolecular aryl coupling of THN and intramolecular aryl coupling of resultant pseudoHPQ, to yield HPQ (Fig. 2).

**Substrate specificity of P-450mel**. The aryl coupling activity of P-450mel toward several aromatic compounds was investigated. P-450mel did not react with 1-naphthol, 2-naphthol, 1,3-dihydroxynaphthalene (1,3-DHN), 1,4-DHN, 1,5-DHN, 1,6-DHN, 1,7-DHN, 2,3-DHN, 2,6-DHN, 2,7-DHN, emodin, and emodin anthone. In contrast, P-450mel readily reacted with flavilolin to give dimmers of flavilolin. Interestingly, we have recently found that MomA, which constitutes an operon with RppA and P-450mel, catalyzes oxidation of THN to give flavilolin. This finding suggests that, in some *Streptomyces* other than *S. griseus*, flavilolin, but not THN, is the physiological substrate of P-450mel. Taken all together, our results suggest that P-450mel possesses a strict specificity toward physiological substrates, THN and flavilolin.

Fig. 1. HPLC analysis of *in vitro* products. *A*, The products from THN by the reconstituted P-450mel system were analyzed by simultaneously measuring the absorbances at 254 nm (in red) and 440 nm (in black). *B*, Authentic HPQ prepared from *S. lividans* harboring pNF1 was coinjected with the products from the P-450 reaction on THN. *C*, As a negative control, P-450mel was boiled before use for the reaction on THN. *D*, As a negative control, ferredoxin and ferredoxin-NADP+ reductase were removed from the reaction. *E*, The products from pseudoHPQ by the P-450mel reaction were analyzed. The peaks indicated with asterisks, derived spontaneously from pseudoHPQ were not identified. *F*, As a negative control, P-450mel was boiled before use for the reaction on pseudoHPQ.

Fig. 2. Proposed HPQ melanin biosynthetic pathway in *S. griseus*. 
Conclusion

The present study has demonstrated that P-450mel mediates the biaryl coupling of THN without inserting an oxygen atom into the product, HPQ. The reaction mechanism is best explained by a radical coupling, as follows. One electron oxidation of THN affords a phenolate radical and subsequent intermolecular radical coupling furnishes the aryl-aryl bond in a regiospecific manner. Resultant pseudoHPQ is subjected to further intramolecular aryl coupling that elaborates HPQ (Fig. 2).

References