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DOI 10.1016/j.chembiol.2012.04.017

SUMMARY

Opium poppy (Papaver somniferum) produces medicinally important benzylisoquinoline alkaloids, including the analgesics codeine and morphine, in the morphinan pathway. We aligned three dioxygenases that were recently discovered in P. somniferum and subsequently identified the nonconserved regions. Two of these enzymes, codeine O-demethylase (PsCODM) and thebaine O-demethylase (PsT6ODM), are known to facilitate regioselective O-demethylation in morphinan biosynthesis. We systematically swapped the residues that were nonconserved between the PsCODM and PsT6ODM sequences to generate 16 mutant PsCODM proteins that could be overexpressed in Escherichia coli. While wild-type PsCODM can demethylate both codeine and thebaine, one engineered PsCODM mutant selectively demethylates codeine. Use of this reengineered enzyme in the reconstitution of morphine biosynthesis could selectively disable a redundant pathway branch and therefore impact the yields of the downstream products codeine and morphine in subsequent metabolic engineering efforts.

INTRODUCTION

Opium poppy produces an array of medicinally important benzylisoquinoline alkaloids, including the analgesics codeine 10 and morphine 11 (Figure 1) (Facchini, 2001; Facchini et al., 2005). The biosynthesis of these alkaloids commences with the condensation of dopamine 1 and 4-hydroxyphenylacetaldehyde 2 to form (S)-norcooclaurine 3, which is further modified to form (S)-reticuline 4, the pivotal biosynthetic intermediate of all benzylisoquinoline alkaloids. (S)-reticuline 4 is subsequently converted to thebaine 5, the intermediate at the entry point of the morphinan alkaloid pathway. Two biosynthetic routes have been proposed for the conversion of thebaine 5 to morphine 11 (Hagel and Facchini, 2010). In the first route (route A; see Figure 1), thebaine 6-O-demethylase (PsT6ODM) demethylates thebaine 5 at the 6 position to form neopinone 7, which spontaneously isomerizes to form codeinone 8. Codeinone 8 is then enzymatically reduced to yield codeine 10. Codeine O-demethylase (PsCODM) demethylates codeine 10 at the 3 position to yield morphine 11. Alternatively, in the second route (route B; see Figure 1), PsCODM demethylates thebaine 5 at the 3 position to form oripavine 6. PsT6ODM catalyzes the second demethylation of oripavine 6—this time at the 6 position—to form morphinone 9, which is then reduced to form morphine 11. Morphinan alkaloids thebaine 5, oripavine 6, codeine 10, and morphine 11 all accumulate in Papaver somniferum, suggesting that both routes are operative in vivo (Facchini, 2001; Facchini et al., 2005). Here we demonstrate how exploiting natural enzyme variation through systematically mixing and matching the nonconserved amino acid regions of two demethylases (PsCODM and PsT6ODM) has led to an enzyme with new specificity; a PsCODM mutant that is highly selective for codeine. The unique selectivity of the reengineered demethylase enzyme may allow us to explore how closing the metabolic valve of route B and redirecting substrate exclusively through route A (Figure 1) will impact downstream product yields in this branched natural product pathway.

RESULTS

Design of P. somniferum PsCODM Mutants

A clustal alignment of PsCODM, PsT6ODM, and PsDIOX2 (a P. somniferum 2-oxoglutarate/Fe(II)-dependent dioxygenase for which the native substrate has not yet been identified) revealed five specific regions where these dioxygenases differ significantly at the amino acid level: A1 (residues 145–149), A2 (residues 150–152), B (residues 334–336), C1 (residues 338–346), and C2 (residues 343–346). Lacking a crystal structure of a P. somniferum dioxygenase, we built a homology model of PsCODM based on the crystal structure of Arabidopsis thaliana anthocyanin synthase (AtANS), a structurally characterized 2-oxoglutarate/Fe(II)-dependent dioxygenase with moderate amino acid sequence similarity to PsCODM (32% identity at the amino acid level) (Schwede et al., 2003; Wilmouth et al., 2002). We employed SWISS-MODEL, an automated protein homology-modeling server, to generate the model (Figure 2) (Schwede et al., 2003). Key residues likely to be involved in Fe(II)/2-oxoglutarate binding are highlighted in the Supplemental Information (Figure S1A available online). The five regions—grouped as follows, A1 and A2 (residues 145–152); B (residues 334–336); and C1 and C2 (residues 338–346)—were then mapped on to this homology model, where they appeared to be located proximal to the anthocyanin binding site (Figure 2).

Chemistry & Biology 19, 674–678, June 22, 2012 ©2012 Elsevier Ltd All rights reserved
Initial protein expression screening revealed that all PsT6ODM mutants are expressed at low levels. Therefore, in this study, we focused our efforts on developing engineered PsCODM enzymes. We systematically replaced the native PsCODM sequence with the corresponding sequence from PsT6ODM at the five nonconserved regions using standard site-directed mutagenesis. In total, we constructed 16 PsCODM mutants (Table 1).

**Heterologous Expression of* P. somniferum* PsCODM Mutants and In Vitro Activity Assay of PsCODM Mutants**

Dr. Jillian Hagel and Professor Peter Facchini (University of Calgary, Calgary, Alberta, Canada) provided *Escherichia coli* expression plasmids pQEDIOX1 and pQEDIOX3, which contain the open reading frames of *P. somniferum* T6ODM and CODM, respectively. Primers to design the mutant constructs are listed in the Supplemental Information (Table S1). We adapted heterologous expression conditions for *E. coli* from a previously reported protocol (Hagel and Facchini, 2010). Protein expression of the majority of PsCODM mutants was robust (Figure S1B). Only the A1 A2 BC1 C2 mutant (S149M L151F S152T mutant; the asterisk designates the mutated region) was expressed at low levels, perhaps due to improper folding. We screened each of the mutant PsCODM enzymes with substrates thebaine 5 and codeine 10 at a concentration of 0.25 mM. Detailed assay conditions are provided in the Experimental Procedures.

Product formation was monitored using liquid chromatography-mass spectrometry (LC-MS). LC-MS chromatograms of representative in vitro enzymatic assays of wild-type and mutant PsCODM enzymes are shown in the Supplemental Information (Figure S2). These endpoint assays indicated that the majority of the mutants lost O-demethylase activity toward both thebaine 5 and codeine 10 substrates. Out of the total of 16 mutants, only two, A1 A2 BC1 C2 (S149M L151F S152T mutant; the asterisk designates the mutated region) was expressed at low levels, perhaps due to improper folding. We screened each of the mutant PsCODM enzymes with substrates thebaine 5 and codeine 10 at a concentration of 0.25 mM. Detailed assay conditions are provided in the Experimental Procedures.

**Figure 2. Homology Models of PsT6ODM and PsCODM Based on the Crystal Structure of AtANS**

PsT6ODM is indicated in green, and PsCODM is indicated in pink. The models were created using SWISS-MODEL. Regions A1 and A2 (residues 145-152) are shown in red, B (residues 334-336) is in yellow, and C1 and C2 (residues 338-346) are in blue.

See also Figures S1, S2, S3, and S4 and Tables S1 and S2.
PsCODM and PsCODM A1 A2 B* C1 C2 mutant thebaine demethylation products suggests that the A1 A2 B* C1 C2 mutant retained regiospecificity for the 3 position of thebaine 5 (Figure S2). In contrast, the A1 A2 B C1* C2 mutant displayed only negligible O-demethylation activity for thebaine 5 but selectively turned over codeine 10 (Figure S2). Since codeine 10 only contains a methoxy group at the 3 position, the C3 regioselectivity of the A1 A2 B C1* C2 mutant is also clearly unchanged from the wild-type PsCODM.

Competitive assay conditions with both thebaine 5 (0.25 mM) and codeine 10 (0.25 mM) were also employed to assess the activity of PsCODM mutants (Figure 3). Morphine 11 and oripavine 6 products formed at a 4:1 ratio when wild-type PsCODM was subjected to these assay conditions. Similarly, the A1 A2 B* C1 C2 mutant also yielded morphine 11 and oripavine 6 at approximately a 4:1 ratio, though notably at lower concentrations than the wild-type enzyme, indicating lower catalytic efficiency for the mutated enzyme. However, while the A1 A2 B C1* C2 mutant produced only negligible amounts of oripavine 6, morphine 11 was produced at levels similar to those observed with wild-type enzyme, confirming the stringent selectivity of the A1 A2 B C1* C2 mutant.

Steady-State Kinetic Analyses of Wild-Type P. somniferum PsCODM and Mutant A1A2BC1*C2

We measured the steady-state kinetic parameters of wild-type PsCODM and mutant PsCODM A1A2BC1*C2 by monitoring the rate of product formation. In total, we measured kinetic parameters for the following combinations: (1) wild-type PsCODM with codeine (kcat/KM = 9.54 x 10^{-5} s^{-1}μM^{-1} and KM = 72.3 ± 33.4 μM); (2) wild-type PsCODM with thebaine (kcat/KM = 1.52 x 10^{-6} s^{-1}μM^{-1} and KM = 216 ± 76.2 μM); and (3) PsCODM mutant A1A2BC1*C2 with codeine (kcat/KM = 3.62x10^{-6} s^{-1}μM^{-1} and KM = 99.0 ± 22.4 μM). Notably, the mutant enzyme formed only negligible amounts of the thebaine demethylation product after 4 hr even when substrate concentrations were as high as 2,000 μM. Steady-state kinetic data are shown in the Supplemental Information (Figure S3 and Table S2).

**DISCUSSION**

Here we have altered the substrate specificity of PsCODM, an enzyme involved in the biosynthesis of morphinan alkaloids. We identified regions for mutation by comparing three highly similar dioxygenases from *P. somniferum*, two of which have defined and distinct substrate and regioselective preferences. By scanning the aligned sequences we identified five nonconserved regions, A1, A2, B, C1, and C2 (Figure 2 and Figure S1A), that we speculated could confer either the substrate or regioselectivity of the dioxygenases in *P. somniferum*. To test this hypothesis, we systematically swapped the amino acid sequences of the A1, A2, B, C1, and C2 regions from thebaine 6 O-demethylase (PsT6ODM) into PsCODM. While most of the interchanges of these regions yielded PsCODM mutants that were catalytically inactive, replacement of the C1 region of PsCODM with the C1 region of PsT6ODM led to a switch from a wild-type enzyme that accepts two substrates—thebaine 5 and codeine 10—to a mutant enzyme that is selective for codeine 10 exclusively. This PsCODM mutant, A1 A2 B C1* C2, contains only four amino acid changes from wild-type PsCODM: E338G, I340L, L341V, and K342E.

Although structural information is not yet available for these demethylases, we could build a homology model for both wild-type and A1 A2 B C1* C2 mutant PsCODM based on the anthocyanidin synthase enzyme docked with thebaine 5 (Figure S4). While these computational results must be interpreted with caution, docking studies suggest that the binding orientation of thebaine differs substantially between the wild-type enzyme and the A1 A2 B C1* C2 mutant (Figure S4). It is interesting that the homology model (Figure 2) also predicts that the C1 region switches from an alpha helix in the wild-type enzyme to a random coil in the codeine-specific mutant, suggesting that the mutation in the C1 region introduces changes to the PsCODM secondary structure. The mutation in the C1* region appears to prevent productive binding of the thebaine 5 substrate without greatly altering the catalytic efficiency for codeine 10 (kcat/KM = 9.54 x 10^{-7} s^{-1}μM^{-1} for wild-type, kcat/KM = 3.62 x 10^{-7} s^{-1}μM^{-1} for mutant).

At the outset of this study, we hypothesized that the amino acid differences between PsCODM, a C3 O-demethylase, and PsT6ODM, a C6 O-demethylase, would control the distinct regioselectivity of the demethylation reactions. Therefore, we expected that swapping the non-conserved amino acid regions of these two enzymes would alter the regioselectivity of the mutated enzymes. However, the A1 A2 B C1* C2 PsCODM mutant retained C3 regioselectivity. Instead, the mutations altered the substrate selectivity in an unpredictable manner. PsT6ODM turns over thebaine 5 and oripavine 6, and PsCODM turns over thebaine 5 and codeine 10. Despite both wild-type enzymes accepting thebaine 5, the introduction of residues from PsT6ODM into PsCODM at the C1 position (Figure 2) yields a mutant PsCODM enzyme that is selective for codeine 10. This change in substrate specificity does not readily correlate with the substrate specificity of the parent wild-type enzymes. Particularly in the absence of experimental structural data, it is difficult...
to rationalize what structural changes these mutations confer to PsCODM and how these changes impact substrate specificity. Nevertheless, while this work highlights the difficulty of rational, structure-based protein design, we successfully demonstrate how sequence alignment of enzymes with subtle differences in specificity can be used to readily generate swapped sequences that are functionally distinct from naturally occurring wild-type variations.

One goal of metabolic engineering is the removal of shunt or redundant pathways that adversely impact production yields of a desired compound (Pickens et al., 2011). This A1 A2 B C1* C2 PsCODM mutant may fulfill an engineering function by providing a means to shut off a redundant route (route B in Figure 1) in morphinan biosynthesis. Because A1 A2 B C1* C2 PsCODM fails to demethylate thebaine 5 to form oripavine 6, the first committed step of route B, replacement of wild-type PsCODM with the A1 A2 B C1* C2 mutant would presumably force the morphine pathway to proceed via route A. Notably, growers in India, a major cultivator of licit opium, inadvertently counterselected a starting point for understanding how the subtle sequence differences of highly similar enzymes can impact substrate and regioselectivity.

**EXPERIMENTAL PROCEDURES**

**Construction of PsCODM Mutant Expression Plasmids**

pQEDIOX1 (pQET6ODM) and pQEDIOX3 (pQECODM), which contain the open reading frames of PsT6ODM and PsCODM, respectively, were provided by Professor Peter Facchini and Dr. Jillian Hagel (University of Calgary). To obtain the 16 CODM mutants (Table 1), site-directed mutagenesis (SDM) was performed using the Stratagene QuikChange Site-Directed Mutagenesis kit. SDM primers are listed in the Supplemental Information (Table 1). The PsCODM mutant constructs were sequenced to verify the DNA sequence and were subsequently transformed for expression into E. coli (strain SG13009 [QIAGEN]) via electroporation using standard protocols.

**Heterologous Expression of PsCODM Mutants**

A single transformed E. coli colony (strain SG13009 [QIAGEN]) was inoculated in 10 ml Luria-Bertani (LB) media supplemented with kanamycin (0.05 g/l) and ampicillin (0.1 g/l) and incubated overnight at 37°C with shaking at 225 rpm.
Subsequently, 800 ml LB media supplemented with kanamycin (0.05 g/l) and ampicillin (0.1 g/l) was inoculated with an overnight culture (8 ml) and incubated at 37°C with shaking at 225 rpm until reaching an optical density 800 (OD_{600}) of 0.6. Protein expression was induced by the addition of isopropyl β-D-galactopyranosidase (IPTG; final concentration 0.3 mM). Following induction, cells were incubated at 6°C with shaking at 225 rpm for 24 hr. Cells were harvested by centrifugation and lysed by sonication. The hexa-histidine-tagged PsCODM mutants were purified using Talon cobalt affinity column (Clontech) using the manufacturer’s protocols. Eluted enzyme was subsequently buffer-exchanged into Tris buffer (100 mM Tris-HCl, pH 7.4, 10% [v/v] glycerol, and 14 mM 2-mercaptoethanol) and immediately assayed for activity. These enzymes were not stable upon extended storage.

In Vitro Activity Assay of PsCODM Mutants
The in vitro assay protocol was adapted from a previous report (Hagel and Facchini, 2010). Briefly, the assay for 2-oxoglutarate/Fe(II)-dependent dioxygenase activity was performed using a 100 μl reaction mixture of 100 mM Tris-HCl (pH 7.4), 10% (v/v) glycerol, 14 mM 2-mercaptoethanol, 0.25 mM alkaid(s), 10 mM 2-oxoglutarate, 10 mM sodium ascorbate, 0.5 mM FeSO_{4}, and 1 μM purified enzyme. Assays were carried out for 4 hr at 30°C. Aliquots (25 μl) were quenched in 1 ml methanol containing yohimbine (500 nM) as an internal standard. The samples were centrifuged in a microcentrifuge (13,000 rpm, 5 min) to remove particulates and then analyzed by LC-MS. Samples were ionized by ESI with a Micromass LCT Premier TOF Mass Spectrometer. The LC was performed on an Acquity Ultra Performance BEH C18, 1.7 μm, 2.1 x 100 mm column on a gradient of 10%–90% acetonitrile/water (0.1% formic acid) over 5 min at a flow rate of 0.6 ml/min. The appearance of morphine 11 and oripavine 6 was monitored by peak integration and normalized to the internal standard. All chemicals were obtained from a commercial source (Sigma Aldrich).

Steady-State Kinetic Assay of Wild-Type CODM and A_1 A_2 B C_1 C_2 Mutant
Assay components were used in the following final concentrations: pH 7.4 Tris-HCl (67 mM) containing 10% (v/v) glycerol and 14 mM 2-mercaptoethanol, α-ketoglutarate (6.7 mM), sodium ascorbate (6.7 mM), iron (II) sulfate (333 μM), and enzyme wild-type (4.1 μg/ml) or Enzyme Mutant (18 μg/ml), in an assay volume of 150 μl. The enzyme concentrations were estimated by Bradford assay. Assays were initiated by the addition of iron sulfate and conducted at 30°C. Aliquots (25 μl) were quenched every 30 min from 1 to 3 hr with 975 μl methanol containing 500 mM ajmaline as an internal standard. Prior to analysis, methanol quenched samples were centrifuged in a microcentrifuge at 13,000 rpm for 5 min to remove any particulates. Liquid chromatography was performed on an Acquity Ultra Performance BEH C18, 1.7 μm, 2.1 x 100 mm column. The gradient was 10%–90% acetonitrile over 4 min with water and 0.1% formic acid in water as the second solvent. The flow rate was 0.5 ml/min. We performed electrospray ionization (ESI) with a Micromass LCT Premier TOF Mass Spectrometer in positive ionization V-mode. Product accumulation was measured to determine the kinetic parameters. A standard curve for morphine, the demethylation product of codeine, was constructed to determine the kinetic parameters of codeine demethylation. However, oripavine, the demethylation product of thebaine, was unavailable; therefore, hydromorphone, which was available, was used as a surrogate. Plotted experimental data were fit to a Michaelis-Menten curve using SigmaPlot version 9.0. Experiments were duplicated or triplicated to ensure reproducibility (Table S2).

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and two tables and can be found with this article online at doi:10.1016/j.chembiol.2012.04.017.

ACKNOWLEDGMENTS
We thank Dr. Jillian Hagel and Professor Peter Facchini (University of Calgary) for providing E. coli expression plasmids pQE60IX1 and pQE80IX3, which contain the open reading frames of PsT6ODM and CODM, respectively, as well as for helpful discussions. W.S.G. gratefully acknowledges a National Science Foundation predoctoral fellowship. Funding was provided by the John Innes Centre, and start-up funds were provided by the University of East Anglia. S.O.C. is supported by a Synergy faculty position at the John Innes Centre and the University of East Anglia.

Received: March 21, 2012
Revised: April 18, 2012
Accepted: April 23, 2012
Published: June 21, 2012

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