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DpgC Is a Metal- and Cofactor-Free 3,5-Dihydroxyphenylacetyl-CoA 1,2-Dioxygenase in the Vancomycin Biosynthetic Pathway

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Summary

3,5-Dihydroxyphenylglycine is a crucial amino acid monomer in the nonribosomal glycopeptide antibiotic vancomycin. This nonproteinogenic amino acid is constructed from malonyl-CoA by a set of four enzymes, DpgA–D, in the biosynthetic cluster. DpgC is an unusual metal-free, cofactor-free enzyme that consumes O_2 during the conversion of 3,5-dihydroxyphenylacetyl-CoA (DPA-CoA) to the penultimate intermediate 3,5-dihydroxyphenylglyoxylate (DPGx). We show that in anaerobic incubations, DpgC catalyzes the exchange of the C_2-methylene hydrogens of DPA-CoA at unequal rates, consistent with enzyme-mediated formation of the substrate-derived C_2-carbanion as an early intermediate. Incubations with $^{18}O_2$ reveal that DpgC transfers both atoms of an O_2 molecule to DPGx product. This establishes DpgC as a 1,2-dioxygenase that mediates thioester cleavage by the oxygen transfer process. These results are consistent with a DPA-CoA C_2-peroxy intermediate, followed by enzyme-directed α-peroxylactone formation and collapse by O-O bond cleavage.

Introduction

The glycopeptide antibiotic vancomycin has a disaccharide moiety attached to a highly crosslinked heptapeptide scaffold that creates the rigid architecture for recognition of *N*-acyl-D-Ala-D-Ala termini in bacterial cell walls [1]. The 25–30 biosynthetic genes for assembly of vancomycin and related glycopeptide antibiotics (including teicoplanin, chloroeremomycin, balhimycin, and A47934) are clustered together, encoding dedicated nonproteinogenic amino acid monomers, a multimodular, nonribosomal peptide synthetase assembly line, and a bevy of post-assembly line tailoring enzymes [2–6].

The heptapeptide backbone is assembled by a nonribosomal peptide synthetase (NRPS) assembly line that uses 24 domains, organized in 7 modules, distributed on 3 high-molecular weight subunits [1]. Three of the seven amino acids are nonproteinogenic phenylglycines involved in all of the rigidifying side chain crosslinks introduced into the heptapeptide scaffold (Figure 1A). Residues four and five are D-4-hydroxyphenylglycine (Hpg), synthesized in a four-enzyme sequence from chorismate [7, 8]. Remarkably, the structurally similar L-3,5-dihydroxyphenylglycine (Dpg) found at residue seven is assembled with completely different molecular logic, by the four enzymes DpgA–D, from four molecules of malonyl-CoA [9, 10]. As shown in Figure 1A, the immediate precursor of Dpg is 3,5-dihydroxyphenylglyoxylate (DPGx), acted on by the same transaminase [7] that makes Hpg. DpgA, DpgB, and DpgD combine to produce the eight-carbon 3,5-dihydroxyphenylacetyl-CoA (DPA-CoA) from four malonyl-CoAs [9, 11].

It is the task of the remaining enzyme, DpgC, to convert DPA-CoA to DPGx [9, 10]. This reaction involves two chemical steps: (1) a four-electron oxidation of the C_2-methylene group to a C_2-keto group, and (2) cleavage of the thioester to a free acid (Figure 1). We have previously established that DpgC requires molecular oxygen as a cosubstrate and that this enzyme lacks detectable redox metal or organic cofactors [9], thereby falling into a small class of metal-free and coenzyme-free oxygenases (reviewed in [12]). To gain insight into how DpgC catalyzes these two changes in an O_2-dependent conversion, we carried out both anaerobic exchange studies in D_2O and $^{18}O_2$ incorporation studies. The former establishes reversible substrate carbanion formation by the enzyme; the latter reveals that DpgC is a dioxygenase and indicates a nonhydrolytic route for cleavage of the C-S bond of DPA-CoA.

Results

DpgC Catalyzes Equilibration of DPA-CoA C_2-Hydrogens with Solvent Protons

DpgC catalyzes the conversion of the C_2-methylene group of the DPA-CoA substrate to the C_2-ketone of the DPGx product (Figure 1). The thioester linkage at C_1 should enable C_2-H cleavage in a low-energy transition state, since the resultant C_2-carbanion can be delocalized into the thioester carbonyl. To evaluate whether one or both of the C_2-methylene hydrogens can be reversibly removed as a proton by DpgC during catalysis, we examined the ability of the enzyme to equilibrate those hydrogens with solvent deuteriums in D_2O. Incubations were conducted anaerobically in the glove box to analyze whether C_2-H cleavage could be catalyzed in the absence of O_2. As shown in Figure 2A, enzyme-mediated incorporation of deuterium from D_2O can be detected by mass spectrometry by a gain of one or two mass units as the first and then second hydrogen at C_2 is equilibrated to yield mono- and dideuterated DPA-CoA. Time points were quenched by acidification, followed by the addition of hydroxylamine (NH_2OH) to cleave the thioester bond of DPA-CoA, forming 3,5-dihydroxyphenylacetyl hydroxamic acid (DPA-NHOH) (Figure 2A). The conversion of DPA-CoA to DPA-NHOH simplified mass spectrometric analysis, as the coenzyme A (CoASH) por-

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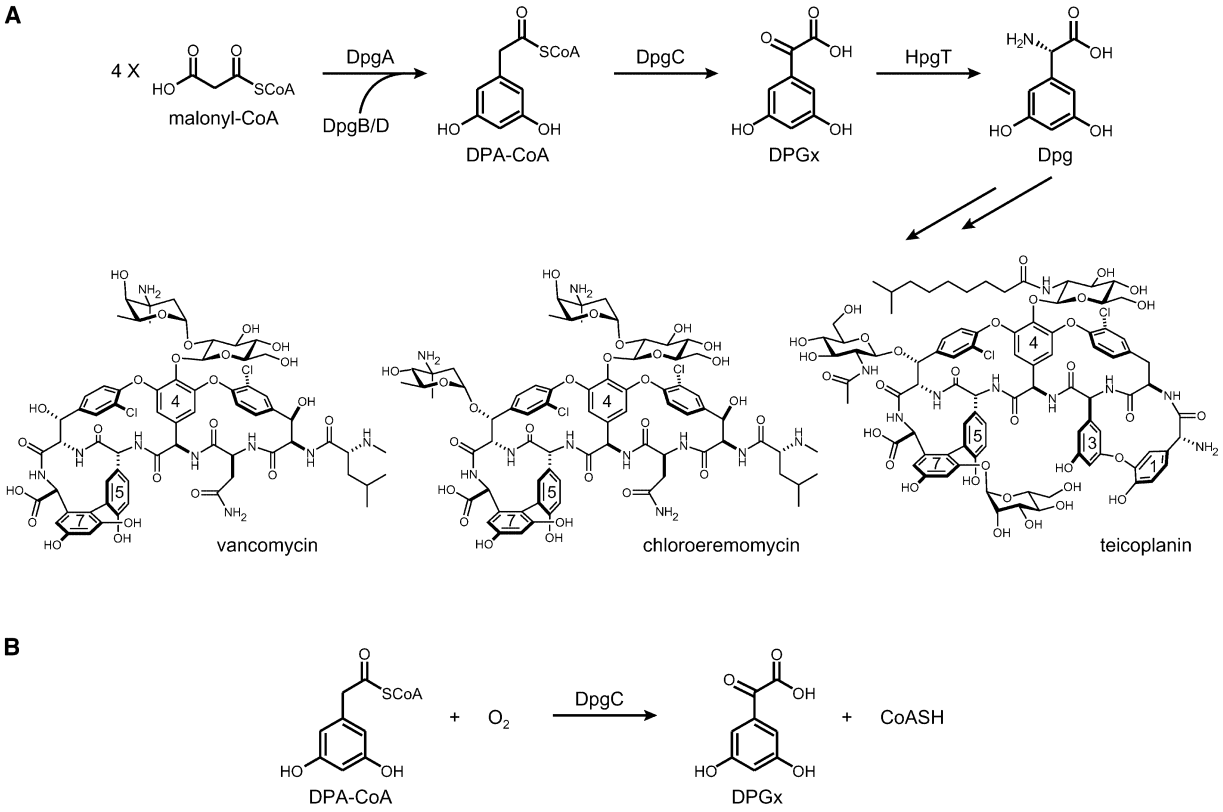

Figure 1. Biosynthesis of Dpg

(A) Biosynthetic pathway of Dpg, with the structures of some glycopeptide antibiotics that contain it. The Hpg and Dpg residues in each structure are numbered by position.

(B) Reaction catalyzed by DpgC: conversion of DPA-CoA and O_2 to DPGx and CoASH.

tion of DPA-CoA contained non-enzyme-mediated solvent-exchangeable protons (data not shown).

Figure 2B shows a time course for the appearance of the M+1 peak at 183 Da. In the absence of DpgC, no exchange was detected (data not shown), validating that the observed wash-in of solvent deuterium into DPA-CoA is enzyme dependent. An M+2 peak appeared at a slower, but real, rate (Figure 2B). The relative abundances of the different species at each time point were quantified by comparing peak heights, and rate calculations determined that DpgC catalyzes exchange of the first hydrogen at C_2 of DPA-CoA at 14.2 min^{-1} and the second hydrogen at a 5-fold slower rate (3.0 min^{-1}) (Figure 2C). The fast rate is comparable to the turnover rate of 10 min^{-1} that we previously reported for the enzyme turning over in the presence of cosubstrate O_2 [9]. Thus, the enzyme exchange reaction in the absence of O_2 is kinetically competent. This is consistent with reversible formation of the bound substrate C_2-carbanion as an early species in the reaction pathway, before O_2 involvement.

DpgC Consumes Stoichiometric Amounts of O_2 and DPA-CoA

To validate the utilization of O_2 as a cosubstrate in DpgC incubations, O_2 consumption was measured with an O_2 electrode. In the absence of DPA-CoA, there was no O_2 consumption above background by DpgC. When DPA-CoA was added and endpoint consumption of O_2 was determined by using an O_2 electrode calibrated with 2,3-dihydroxybiphenyl and 2,3-dihydroxybiphenyl 1,2-dioxygenase (DHBD), which constitute a well-coupled system [13], a stoichiometry of 0.94 $\pm$ 0.01 O_2 molecule consumed per molecule of DPA-CoA was observed, demonstrating that the utilization of DPA-CoA and O_2 by DpgC is tightly coupled. Validity of the assay was shown by coupling experiments performed with soybean lipoxygenase, cyclooxygenase I, and catalase that resulted in stoichiometries of 0.86, 1.8, and 0.49 O_2, respectively (see the Experimental Procedures). Moreover, in enzymatic reactions monitored with the O_2 electrode, no additional O_2 production was observed in the presence of excess superoxide dismutase and/or catalase. This indicates that H_2O_2 or superoxide was not leaking out of the enzyme during catalysis [14, 15] and is consistent with the proposed mechanism (see the Discussion).

Apparent steady-state kinetic parameters for DPA-CoA and O_2 were evaluated by fitting data obtained in air-saturated buffer to the Michaelis-Menten equation. The apparent K_m and k_{cat} for DPA-CoA were 5 $\pm$ 2 μM and 17 $\pm$ 1 min^{-1}, respectively. These values are comparable to those obtained by HPLC analysis, following CoASH release (K_m = 6 μM, k_{cat} = 10 min^{-1}) [9]. The

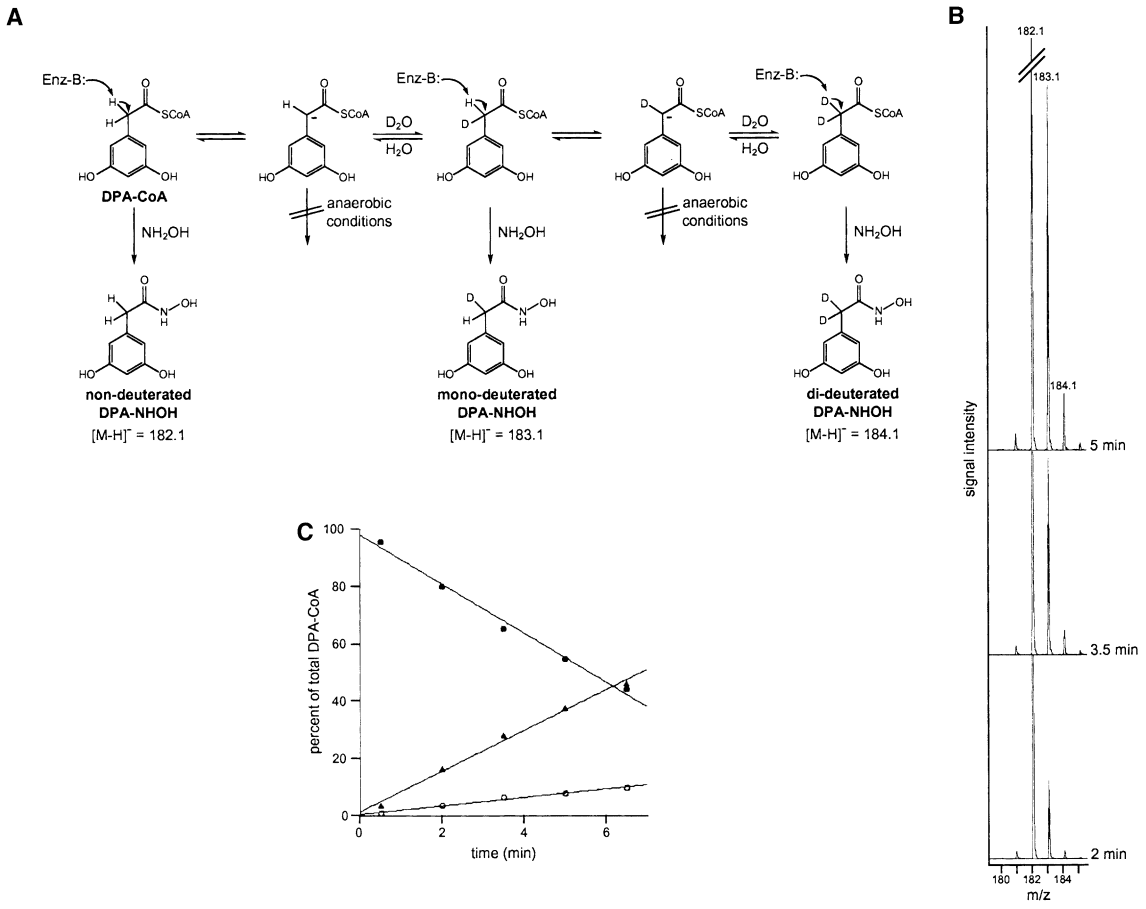

Figure 2. DpgC-Catalyzed Deuterium Exchange of DPA-CoA α-Protons

(A) Schematic of the assay for DpgC-catalyzed α-proton abstraction from DPA-CoA in D_2O under anaerobic conditions, as described in the Experimental Procedures.

(B) ESI-MS traces of time points from the assay described in (A) showing the increase in the mono- and dideuterated species with time.

(C) Representative time course of relative amounts of DPA-NHOH from the assay described in (A). Filled circle, nondeuterated; filled triangle, monodeuterated; open circle, dideuterated. The nondeuterated species decreases at 17.2 min^{-1}, the monodeuterated species increases at 14.2 min^{-1}, and the dideuterated species increases at 3.0 min^{-1}. Data shown are representative of time courses performed in duplicate. The amount of variation between separate time courses was less than 10%.

apparent K_m and k_{cat} for O_2 were 1.0 ± 0.4 mM and 56 ± 18 min^{-1}, respectively. The initial rates increased almost linearly with O_2 concentration, causing the low precision in the parameters, as no points above the K_m for O_2 could be determined.

DpgC Is a Dioxygenase

In our initial report on the activity of purified DpgC, we found that O_2 was required to detect any formation of DPGx product and proposed that the enzyme was an oxygenase [9]. To establish directly that DpgC does incorporate oxygen from O_2 into the DPGx product, we conducted incubations in 99% $^{18}O_2$, isolated the DPGx product by HPLC, and analyzed its mass spectrum. Control incubations in $^{16}O_2/H_2{}^{16}O$ gave the anticipated $[M-H]^-$ = 181 for DPGx (Table 1, column *i*, line 1). As schematized in Figure 3A, we anticipated that DPGx would exchange its C_2-keto oxygen rapidly with solvent by the indicated gem diol pathway. We validated this by placing purified DPGx from each of the four incubation conditions in either $H_2{}^{16}O$ or $H_2{}^{18}O$. The anticipated almost complete exchange-in of solvent-derived oxygen at the C_2-keto oxygen was detected via a mass shift of +2 for all products when incubated in $H_2{}^{18}O$ as compared to $H_2{}^{16}O$ (Figures 3C and 3D; Table 1, columns *i* and *ii*).

The intriguing result from this series is shown in Figure 3B, which illustrates that the product had a molecular mass of M+2 = 183 in $^{18}O_2$ incubations. Even though Figures 3C and 3D validate that any ^{18}O transferred to the C_2-ketone of DPGx product would be washed out via reversible formation of the gem diol, the retention of M+2 strongly indicates that one ^{18}O atom was in the C_1 carboxylate of DPGx. Finally, if one performed the enzymatic incubation in $^{18}O_2$ and then put the product in $H_2{}^{18}O$, an M+4 peak at 186 should predominate, from the +2 from $^{18}O_2$ and then the +2 contributed by wash-in to the keto group of DPGx from $H_2{}^{18}O$. This was the observed result, as shown in Table 1 (column *ii*, lines 3 and 4).

Table 1. Mass Distribution of the DPGx Product after Various Treatments, Demonstrating that the Source of the DPGx Carboxylate Oxygen Is O_2

	(*i*) Dissolved in $H_2{}^{16}O$			(*ii*) Dissolved in $H_2{}^{18}O$			(*iii*) Addition of HpgT		
Incubation Conditions	+0	+2	+4	+0	+2	+4	+0	+2	+4
$^{16}O_2/H_2{}^{16}O$	100%	—	—	3%	97%	—	100%	—	—
$^{16}O_2/H_2{}^{18}O$	96%	4%	—	—	95%	5%	90%	10%	—
$^{18}O_2/H_2{}^{16}O$	13%	87%	—	—	8%	92%	16%	84%	—
$^{18}O_2/H_2{}^{18}O$	4%	96%	—	—	—	100%	4%	96%	—

Assay conditions are as schematized in Figure 3A and as described in the Experimental Procedures. "—" indicates mass species not detected.

To suppress exchange of the keto oxygen and retain the initial oxygen atom in the product, we turned to in situ derivatization with sodium cyanoborohydride ($NaBH_3CN$) (Figure 4A). We determined that DpgC incubations could be conducted in the presence of $NaBH_3CN$ without drastic inhibition or inactivation of enzyme activity. With enough $NaBH_3CN$, the reaction flux could be almost quantitatively diverted to the reduced C_2-alcohol derivative, 3,5-dihydroxymandelate (Dman), which could be isolated by HPLC and subjected to mass spectrometric analysis (Figure 4A).

As shown in Figure 4B, Dman from an $^{18}O_2/H_2{}^{16}O$ incubation with $NaBH_3CN$ showed an M+4 peak as the major species, reflecting 80% abundance, and 20% of the M+2 species. This result unambiguously proves that DpgC is a dioxygenase that incorporates one oxygen atom of O_2 at C_2 and one at C_1 of the product. The 80/20 split suggests that the hydride reductive quench may not be fully efficient in competing for the ketone before gem diol formation and solvent oxygen exchange. This was corroborated by a control experiment with $^{16}O_2/H_2{}^{18}O$ in DpgC incubations with $NaBH_3CN$ (Table 2, line 2), in which 18% of the Dman reduction product was the M+2 species, consistent with an ~20% split of solvent-exchanged ketone before reduction to the alcohol and cessation of solvent wash-in. Dman does not exchange with solvent-derived oxygen, which validates the stability of both the C_2-hydroxyl and C_1-carboxylate linkages to nonenzymatic processes (data not shown).

Performance of this experiment with 50% $^{18}O_2$ and 50% $^{16}O_2$ allowed the evaluation of whether both oxygen atoms derive from the same starting O_2 molecule by determining the differential abundance of the M+2 versus M+4 peaks. As shown in Figure 4C, the distribution of the M+4 (31%) and M+2 (17%) forms of Dman is statistically consistent with both atoms of oxygen in the product deriving from a single molecule of cosubstrate O_2, with allowances for some solvent wash-in at the α-ketone before reduction and an expected small kinetic isotope effect [16]. This result is consistent with the 1:1 stoichiometry of O_2 consumption to DPA-CoA conversion to DPGx.

In Situ Coupling of DPGx to Dpg by Action of Hpg Aminotransferase

To validate that one of the ^{18}O atoms incorporated from $^{18}O_2$ into DPGx was at one of the C_1-carboxylate oxygen atoms, the keto acid product from DpgC incubations

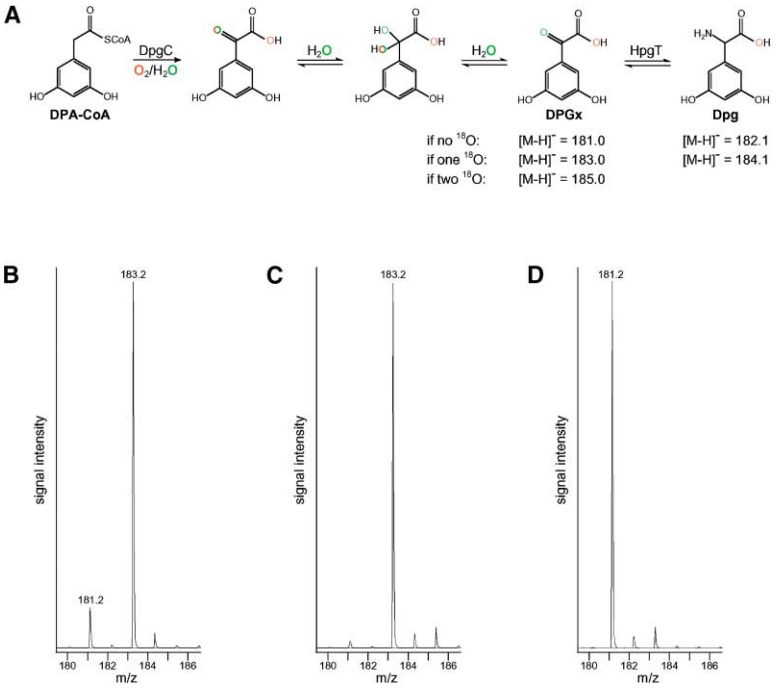

Figure 3. Source of the DPGx Carboxylate Oxygen Is from O_2

(A) Schematic of the assay analyzing oxygen incorporation into DPGx from incubations of DPA-CoA with DpgC in various combinations of $^{18}O_2$ and $H_2{}^{18}O$, as described in the Experimental Procedures. Exchange of the enzymatically formed α-keto oxygen with solvent through reversible formation of the gem diol is illustrated.

(B) ESI-MS trace of the DPGx product from a 99% $^{18}O_2/H_2{}^{16}O$ incubation, dissolved in $H_2{}^{16}O$, showing that the nonexchangeable carboxylate oxygen derives from O_2.

(C) ESI-MS trace of the DPGx product from a $^{16}O_2/H_2{}^{18}O$ incubation, dissolved in $H_2{}^{18}O$.

(D) ESI-MS trace of the same DPGx product as in (C), except dissolved in $H_2{}^{16}O$. (C) and (D) illustrate that the identity of the α-keto oxygen depends on the identity of the solvent.

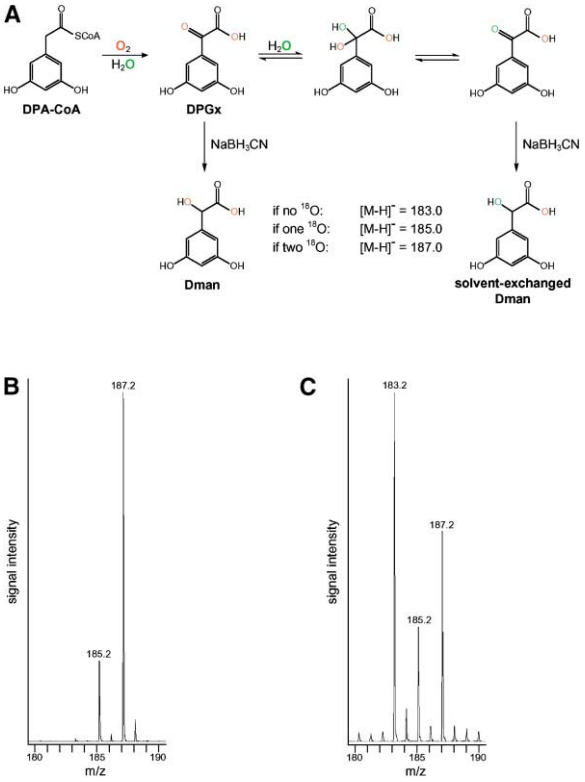

Figure 4. DpgC Is a Dioxygenase

(A) Schematic of the assay analyzing oxygen incorporation into DPGx from incubations of DPA-CoA with DpgC in various combinations of $^{18}O_2$ and $H_2{}^{18}O$, with the additional utilization of $NaBH_3CN$ to prevent solvent exchange of the enzymatically formed α-keto oxygen, as described in Experimental Procedures.

(B) ESI-MS trace of the Dman product from a 99% $^{18}O_2/H_2{}^{16}O$ incubation, showing that both the α-keto and carboxylate oxygens derive from O_2.

(C) ESI-MS trace of the Dman product from a 50% $^{18}O_2/H_2{}^{16}O$ incubation, showing that the ratio of the species is consistent with the two oxygens deriving from the same molecule of O_2.

was coupled to the Hpg aminotransferase (HpgT) [7, 17] (Figure 3A). The resultant Dpg product has lost the keto oxygen at C_2 by reductive amination via action of the pyridoxal phosphate (PLP)-containing enzyme HpgT. Any ^{18}O that remains must be in the C1-carboxylate group. As shown in Table 1 (column *iii*, lines 3 and 4), the Dpg molecules from DpgC/HpgT incubations conducted in $^{18}O_2$ did retain the M+2 peak, proving that one of the two oxygens transferred from O_2 by DpgC was indeed at C_1 of DPGx.

Table 2. Mass Distribution of the Dman after Purification in $H_2{}^{16}O$, Demonstrating that DpgC Is a Dioxygenase

Incubation Conditions	+0	+2	+4
$^{16}O_2/H_2{}^{16}O$	100%	—	—
$^{16}O_2/H_2{}^{18}O$	82%	18%	—
$^{18}O_2/H_2{}^{16}O$	—	20%	80%
$^{18}O_2/H_2{}^{18}O$	—	8%	92%
50% $^{18}O_2/H_2{}^{16}O$	52%	17%	31%
50% $^{18}O_2/H_2{}^{18}O$	34%	21%	45%

Assay conditions are as schematized in Figure 4A and as described in the Experimental Procedures. The $^{16}O_2$ and 99% $^{18}O_2$ data shown are averages of reactions performed in duplicate. The amount of variation between separate reactions was less than 2%. "—" indicates mass species not detected.

Thioester Cleavage by DpgC Is Nonhydrolytic

The presence of O_2-derived oxygen in the carboxylate of DPGx is consistent with a nonhydrolytic conversion of the thioester linkage in the substrate to the carboxylate in the product. In particular, this argues against an enzymatic four-electron oxidation at C_2 to generate 3,5-dihydroxyphenylglyoxyl-CoA as a bound intermediate that is then subjected to enzyme-catalyzed thioester hydrolysis. To address directly the question of whether DpgC shows arylglyoxyl-CoA thioesterase activity, we synthesized phenylglyoxyl-CoA. We have previously shown that phenylacetyl-CoA is a substrate for DpgC, generating phenylglyoxylate [9], and because phenylglyoxylate was available, we chose it to make the CoA-thioester.

DpgC was very sluggish as a thioesterase, accelerating the nonenzymatic rate of hydrolysis of phenylglyoxyl-CoA by no more than 2-fold under incubation conditions where it would catalyze rapid turnover of DPA-CoA to DPGx (data not shown). Even at high enzyme concentrations, there was no convincing evidence for acceleration of phenylglyoxyl-CoA hydrolysis. This failure is consistent with the notion that the α-ketoacyl-CoA is not an intermediate on the reaction pathway and that DpgC does not possess standard thioesterase activity. We have also previously observed that anaerobic incubations of DPA-CoA with DpgC led to no detectable thioester hydrolysis, confirming that oxygenation and thioester cleavage are coupled [9].

Discussion

In the biosynthesis of the vancomycin and teicoplanin classes of antibiotics, the construction of an oxidatively crosslinked, NRPS-derived heptapeptide scaffold sets the architectural constraints that allow recognition of bacterial cell wall peptidoglycan *N*-acyl-D-Ala-D-Ala termini targets [18, 19]. Enzymatic oxidation to produce the three (vancomycin) or four (teicoplanin) rigidifying crosslinks depends on the electron-rich phenolic side chains of the tyrosyl and hydroxyphenylglycyl side chains (Figure 1A) [1].

The nonproteinogenic Hpg residues in vancomycin and teicoplanin arise from the prototypic common intermediate in aromatic amino acid metabolism, chorismate, diverting flux down a pathway notable for a newly described non-heme iron oxygenase that creates 4-hydroxymandelate as an intermediate [7, 8]. In contrast, the Dpg residues are assembled by completely different logic using four enzymes, DpgA–D. Four molecules of malonyl-CoA are the source of the eight carbons tandemly elongated and regiospecifically cyclized by a type III polyketide synthase (DpgA). The aromatizing dehydration requires the help of DpgB and DpgD and yields DPA-CoA (Figure 1A) [9, 10].

The remaining enzyme, DpgC, carries out the oxidative conversion of DPA-CoA to DPGx, an enzymatic

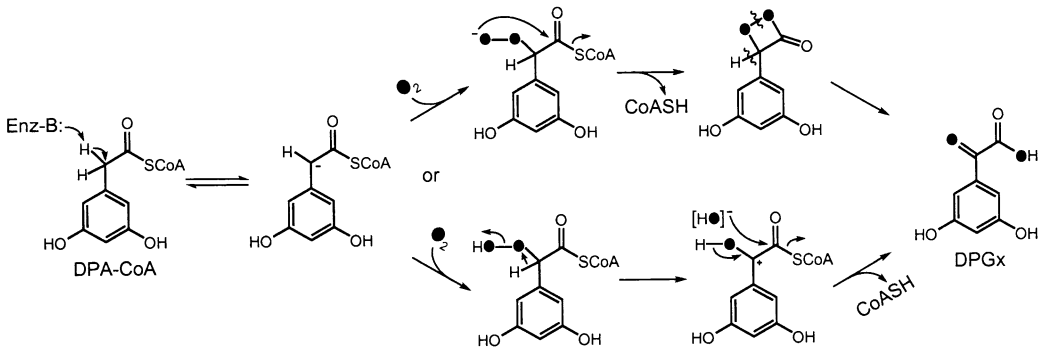

Figure 5. Two Proposed Mechanisms for the Conversion of DPA-CoA by DpgC

The upper pathway depicts an α-peroxylactone intermediate, and the lower pathway depicts a Criegee rearrangement with C-H bond migration. For clarity, the oxygen atoms deriving from O_2 are filled in.

transamination away from the nonproteinogenic amino acid monomer Dpg (Figure 1). This is a remarkable transformation. The net four-electron oxidation at C_2, as a methylene group is converted to a keto group, is reminiscent of the three-step conversion in fatty acid degradation, where a three-enzyme array of a flavoprotein, a hydratase, and an NADH reductase are employed ad seriatim [20]. Additionally, DpgC cleaves the thioester bond of DPA-CoA to the free carboxylate of DPGx. At first glance, this would seem to be a hydrolytic thioesterase reaction, but things are not what they seem with this enzyme.

First, DpgC requires O_2 for catalysis, consuming one molecule as a cosubstrate for every molecule of DPA-CoA converted to DPGx. It has neither a tightly bound redox active metal ion, nor needs one provided from solution. It also does not use a redox active cofactor such as a flavin or pterin coenzyme [9]. DpgC is therefore a member of a small family of metal-free and cofactor-free oxygenases that take advantage of the high reactivity of conjugated substrates undergoing oxygenation to produce carbanionic and/or radical intermediates that can react rapidly with O_2 [12]. These include tetracenomycin F1 monooxygenase (TcmH) and the ActVA-orf6 protein in tetracenomycin and actinorhodin biosynthesis, respectively, as well as 1*H*-3-hydroxy-4-oxo-quinoline 2,4-dioxygenase (Qdo) and 1*H*-3-hydroxy-4-oxo-quinaldine 2,4-dixoxygenase (Hod) in the bacterial degradation pathways of *N*-heteroaromatic compounds [21–23].

We anticipated that DpgC would initiate catalysis by utilizing the thioester of DPA-CoA to enable low-energy carbanion formation at C_2. This carbanion is also benzylic and so would be further stabilized by the aromatic ring. The ability of the enzyme to catalyze exchange with solvent deuteriums confirms that reversible deprotonation at C_2 is a catalytic capacity of the enzyme and likely an early step in catalysis. While chiral proton abstraction and re-addition is anticipated for an enzymatic process at the C_2-methylene group, there is clear evidence for exchange of the second methylene proton at about 20% of the rate of the first proton (Figure 2C). We do not yet know if this is chiral abstraction and stereorandom reprotonation (deuteration) or if the enzyme is only stereoselective at this relatively acidic carbon site. The observed exchange is fully dependent on the enzyme, with no detectable nonenzymatic background in the mass spectroscopic assay under the experimental conditions. That it occurs under anaerobic conditions establishes that the C_2-H cleavage can occur before any O_2 involvement.

Once formed in the DpgC active site, the DPA-CoA C_2-carbanion could be subjected to one-electron autoxidation and/or transfer to O_2 [24]. All of the metal-free, cofactor-free oxygenases use such oxidizable substrates as likely sources for the initial one-electron transfer to O_2. Then, radical recombination between the transient proposed substrate radical and the superoxide anion would yield a hydroperoxide/peroxy anion adduct (Figure 5).

To explain the observed transfer of one atom of O_2 to the carboxylate of DPGx, we suggest attack of the peroxy anion on the adjacent C_1-thioester carbonyl. This tetrahedral adduct at C_1 can ketonize with expulsion of CoASH and would constitute nonhydrolytic fragmentation of the C-S bond, with one putative route involving an enzyme-bound α-peroxylactone (Figure 5, upper pathway). Such a dioxetane intermediate is similar to that formed in the mechanism of action of luciferase [25], although there is no obvious light produced in DpgC incubations. To complete catalysis from such an intermediate, the weak O-O single bond must be fragmented, as well as the remaining C_2-H bond. This can occur through a variety of one- or two-electron routes. An alternate potential mechanism consists of a Criegee rearrangement of the hydroperoxide, with migration of the C-H bond to generate hydroxide ion, which can subsequently attack the thioester carbonyl (Figure 5, lower pathway). This mechanism requires a very shielded active site pocket to prevent exchange of the hydroxide ion. There is precedent in Baeyer-Villiger chemistry for the conversion of a hydroperoxide to a ketone through such a hydrogen migration [26]. In agreement with the data, both mechanisms show that, in $^{18}O_2$ incubations, one oxygen atom would reside at the C_2-ketone, susceptible to rapid exchange with solvent via a gem diol intermediate, and the other would be at the C_1-carboxylate.

Further studies will be required to evaluate the relative free energies of various transition states involving cleavage of each of the C_2-H bonds, to determine if radical

species can be detected, and if evidence for a cyclic peroxylactone or other intermediates can be accrued. It is evident that DpgC harnesses the reactivity of O_2 and the DPA-CoA substrate in resourceful ways, and that the path to Dpg in antibiotic biosynthesis subsumes some intriguing chemistry, both at the DpgA- and DpgC-catalyzed steps [9, 11]. The nonproteinogenic amino acid Dpg is found in other nonribosomal peptides, where the electron-rich aromatic side chain expands the range of chemistry available to these peptide scaffolds.

Significance

DpgC is a novel metal-free, cofactor-free 1,2-dioxygenase that converts 3,5-dihydroxyphenylacetyl-CoA (DPA-CoA) to 3,5-dihydroxyphenylglyoxylate (DPGx), the penultimate intermediate in the biosynthesis of 3,5-dihydroxyphenylglycine (Dpg), a crucial nonproteinogenic amino acid monomer in the glycopeptide antibiotic vancomycin. We investigated the unusual mechanism of action of DpgC, showing that the first step is likely O_2-independent α-proton abstraction, and demonstrated that both atoms from a single molecule of O_2 are incorporated into the α-ketone and carboxylate of DPGx. These results are consistent with a DPA-CoA C_2-peroxy intermediate, followed by intramolecular participation of the distal peroxide oxygen in C-S bond cleavage. In addition to the mechanistic insight that our work has provided on DpgC, the understanding of the biosynthetic pathway of Dpg provides valuable information for efforts to make novel vancomycin analogs through the reprogramming of steps for combinatorial biosynthesis in vitro and in vivo.

Experimental Procedures

Materials

99% D_2O and 95% $H_2{}^{18}O$ were purchased from Cambridge Isotope Laboratories. 99% $^{18}O_2$ was purchased from Isotec. Nonstatistically distributed 50% $^{18}O_2$ was purchased from Icon Isotopes. 2,3-Dihydroxybiphenyl was purchased from Wako Chemicals. Catalase and superoxide dismutase from bovine liver were purchased from Calbiochem. Soybean lipoxygenase, cyclooxygenase I from ovine seminal vesicles, and other chemicals were purchased from Sigma-Aldrich.

Synthesis of CoA-Thioesters

DPA-CoA was synthesized and purified as previously described [9]. Phenylglyoxyl-CoA was synthesized from phenylglyoxylate and CoASH through activation of the phenylglyoxylate to the acid chloride by following the protocol by Lai et al. [27]. It was purified in the same manner as DPA-CoA.

Cloning, Overexpression, and Protein Purification

DpgC was overexpressed and purified from *E. coli* BL21(DE3) as previously described [9]. HpgT from the complestatin producer *Streptomyces lavendulae* was cloned into the NdeI/XhoI sites of the pET-16b vector, which encodes an N-terminal His_{10} tag, as previously described [17], and overexpressed and purified by following the same procedure as for DpgC. Its identity was verified by DNA sequencing at the Molecular Biology Core Facilities of the Dana Farber Cancer Institute. Proteins were dialyzed into 25 mM Tris-HCl (pH 7.5)/100 mM NaCl/15% glycerol/1 mM DTT, flash frozen, and stored at −80°C. DHBD was purified as previously described [13].

General Anaerobic Procedures

The preparation of DpgC for experiments involving anaerobic incubations in D_2O or exposure to labeled O_2 and H_2O was performed under an inert atmosphere in a Mbraun Unilab glove box maintained at <2 ppm O_2. Aliquots of DpgC were anaerobically thawed immediately prior to use and exchanged into 20 mM Tris (pH 7.5) by gel filtration chromatography with a Biogel P6-DG desalting gel (Bio-Rad). Buffers were sparged with argon and equilibrated in the glove box for at least 24 hr prior to use. When necessary, DpgC was concentrated under a flow of nitrogen with a stirred cell concentrator equipped with a YM10 ultrafiltration disc (Millipore).

Anaerobic Incubations in D_2O

Reaction mixtures (260 μl) containing DPA-CoA (500 μM) either with or without DpgC (2.5 μM) in buffered D_2O (20 mM Tris, [pD 7.5]) were incubated under anaerobic conditions at 24°C. At 0.5, 2, 3.5, 5, and 6.5 min, 50 μl aliquots of the reaction mixtures were quenched by adding 0.5 μl 50% trifluoroacetic acid (TFA). After a 10 min incubation of each aliquot with 49.5 μl 1 M NH_2OH (pH 7.5) to cleave the thioester bond of DPA-CoA and form DPA-NHOH, an additional 7.8 μl 50% TFA was added, and the aliquots were removed from the glove box. Precipitated protein was removed by centrifugation, and DPA-NHOH was purified by HPLC using a Vydac C18 monomeric (120 Å, 4.6 × 250 mm) column, monitoring at 220 nm (1 ml/min; 0–3 min, 0% B; 3–28 min, 0%–50% B; A = H_2O, 0.1% TFA; B = acetonitrile; the same HPLC solvents are used throughout this study). Purified samples were lyophilized and dissolved in H_2O for electrospray ionization-mass spectrometry (ESI-MS) analysis at the Mass Spectrometry Facility of the Harvard Department of Chemistry and Chemical Biology. The relative abundances of the different species in each sample were determined by comparing the peak heights at the appropriate masses.

Measurement of O_2 Consumption with an O_2 Electrode

Enzymatic activity was measured by following the consumption of O_2 using a Clark-type polarographic O_2 electrode (Hansatech Instruments, Respire 1 model). The electrode signal was recorded on a computer equipped with a PCI-6023E multifunction board and Virtual Bench Data Logger (National Instruments). Data were recorded every 0.1 s, and initial velocities were determined from progress curves using Microsoft Excel. The slopes of the progress curve were calculated for all consecutive 12 s intervals.

The O_2 electrode was zeroed by adding excess sodium hydrosulfite to buffer in the reaction chamber and calibrated by using standard concentrations of 2,3-dihydroxybiphenyl and DHBD [13]. The coupling of DPA-CoA and O_2 consumption was investigated by monitoring the amount of O_2 consumed upon the addition of different amounts of DPA-CoA to the reaction mixture, with reactions initiated by injection of DpgC into the reaction chamber. Control experiments for the coupling of O_2 consumption were performed with linoleic acid and soybean lipoxygenase (1 mole O_2 consumed per mole linoleic acid; [28]), arachidonic acid and cyclooxygenase I (2 moles O_2 consumed per mole arachidonic acid; [28]), and H_2O_2 and catalase (0.5 mole O_2 produced per mole H_2O_2 consumed; [15]). The effects of catalase (1,500 U/ml) and superoxide dismutase (200 U/ml) on the reaction of DpgC with DPA-CoA were also investigated.

The apparent kinetic parameters of DpgC for DPA-CoA were investigated in air-saturated buffer using 2–100 μM DPA-CoA, and those for O_2 in the presence of 100 μM DPA-CoA were investigated using 100–750 μM O_2. The reaction buffers containing different concentrations of dissolved O_2 were prepared by vigorously bubbling with mixtures of O_2 and N_2 gases for at least 15 min prior to the experiment. Equilibrated buffer was transferred to the reaction chamber using a Hamilton gas-tight syringe while flushing the reaction chamber continuously with the gas mixture. 100% O_2 or 10% O_2 in N_2 and prepurified N_2 were mixed in the desired proportions with a gas proportioner (SpecialtyGasEquipment.com), and the concentration of dissolved O_2 in the reaction mixture was verified using the O_2 electrode. All experiments were performed in 20 mM Tris (pH 7.5) at 25°C. Steady-state rate equations were fit to data using the least squares and dynamic weighting options of LEONORA [29].

Incubations with $^{18}O_2$ and $H_2{}^{18}O$

Reaction mixtures (500 μl) containing DPA-CoA (500 μM) and DpgC (20 μM) in buffered $H_2{}^{16}O$ or $H_2{}^{18}O$ (20 mM Tris [pH 7.5]) were added to a vial under anaerobic conditions at 24°C. After tight capping of the vials with septa, the reaction mixtures were removed from the glove box and exposed to either $^{16}O_2$ or $^{18}O_2$ for 8–15 min, at which point the reactions had proceeded to completion. The reaction mixtures were lyophilized and dissolved in $H_2{}^{16}O$, protein was removed by centrifugation, and DPGx was purified by HPLC as for the D_2O samples. Purified samples were lyophilized, and either: (*i*) dissolved in $H_2{}^{16}O$; (*ii*) dissolved in $H_2{}^{18}O$; or (*iii*) incubated with HpgT (83 μM), L-Tyr (5 mM), and PLP (25 μM) for 24 hr at pH 7.5. Dpg from the samples incubated with HpgT was purified by HPLC, monitoring at 280 nm (1 ml/min; 0–10 min, 0% B; 10–25 min, 0%–50% B), lyophilized, and dissolved in $H_2{}^{16}O$. Samples were analyzed by ESI-MS, and the relative abundances of the different species in each sample were determined as for the D_2O samples.

Incubations in which $NaBH_3CN$ was used to reduce the enzymatically formed DPGx to Dman in situ were performed using the same protocol, with three modifications: (*i*) the reaction mixtures contained 40 μM of DpgC; (*ii*) anaerobic 10 mM $NaBH_3CN$ was added just before exposure to $^{16}O_2$ or $^{18}O_2$; and (*iii*) purified samples were not incubated with HpgT.

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