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Enzymatic Generation of the Antimetabolite γ,γ-Dichloroaminobutyrate by NRPS and Mononuclear Iron Halogenase Action in a Streptomycete

Masashi Ueki,[1,2,3] Danica P. Galonić,[2,3] Frédéric H. Vaillancourt,[2,3,4] Sylvie Garneau-Tsodikova,[2,5] Ellen Yeh,[2] David A. Vosburg,[2,6] Frank C. Schroeder,[2] Hiroyuki Osada,[1] and Christopher T. Walsh[2,*]

[1] Antibiotics Laboratory
RIKEN Institute
2-1 Hirosawa
Wako
Saitama 351-0198
Japan

[2] Department of Biological Chemistry
and Molecular Pharmacology
Harvard Medical School
240 Longwood Avenue
Boston, Massachusetts 02115

Summary

Four adjacent open reading frames, *cytC1–C4*, were cloned from a cytotrienin-producing strain of a *Streptomyces* sp. by using primers derived from the conserved region of a gene encoding a nonheme iron halogenase, CmaB, in coronamic acid biosynthesis. CytC1–3 were active after expression in *Escherichia coli*, and CytC4 was active after expression in *Pseudomonas putida*. CytC1, a relatively promiscuous adenylation enzyme, installs the aminoacyl moieties on the phosphopantetheinyl arm of the holo carrier protein CytC2. CytC3 is a nonheme iron halogenase that will generate both γ-chloro- and γ,γ-dichloroaminobutyryl-*S*-CytC2 from aminobutyryl-*S*-CytC2. CytC4, a thioesterase, hydrolytically releases the dichloroaminobutyrate, a known streptomycete antibiotic. Thus, this short four-protein pathway is likely the biosynthetic source of this amino acid antimetabolite. This four-enzyme system analogously converts the *proS*-methyl group of valine to the dichloromethyl product regio- and stereospecifically.

Introduction

In recent studies on the biosynthesis of the coronamic acid moiety, 1-amino-1-carboxy-2-ethyl cyclopropane, of the pseudomonal phytotoxin coronatine, we have determined that the cyclopropane ring is constructed by a cryptic chlorination pathway [1]. Among the novel enzymatic features are a nonheme mononuclear iron halogenase (CmaB) that acts at the unactivated CH_3 of an L-*allo*-Ile moiety while it is tethered in thioester linkage to the pantetheinyl arm of a carrier protein, CmaD [1]. The γ-chloro-aminoacyl thioester then is acted on by CmaC, which generates an α-carbanion equivalent for intramolecular displacement of the γ-Cl, producing the cyclopropyl aminoacyl scaffold still tethered to the prosthetic arm of the carrier protein. The last step is hydrolytic release of the coronamic acid, mediated by the thioesterase CmaT [2].

The ansa-bridged cytotrienin (Figure 1) [3], produced by a soil bacteria *Streptomyces* sp. strain, contains a 1-aminocyclopropane-1-carboxylate (ACC) moiety, reminiscent of coronamic acid. Feeding studies show that the ACC moiety in this producer is derived from the aminobutyryl moiety of L-methionine [4]. In plants, ACC is biosynthesized from *S*-adenosylmethionine by the pyridoxal phosphate (PLP)-dependent ACC synthase [5], and this product serves as a precursor to the plant hormone ethylene [6]. However, no such pathway has been detected in prokaryotic microorganisms [4]. To evaluate whether similar logic and enzymatic machinery to that of coronamic acid biosynthesis is in play for ACC biogenesis in the cytotrienin producer, we have used PCR primers homologous to the conserved sequences in the CmaB halogenase gene to look for a comparable ORF. Herein, we report the cloning and sequencing of four adjacent ORFs, named *cytC1–C4*, as well as their heterologous expression and functional characterization. CytC1 is a free-standing adenylation enzyme for amino acids, including aminobutyrate. CytC2 is a 10 kDa peptidyl carrier protein that can be primed with phosphopantetheine and loaded with various amino acids. CytC3 is a mononuclear nonheme iron halogenase that can doubly chlorinate aminobutyryl-*S*-CytC2 as well as valyl-*S*-CytC2 at the unactivated γ-carbon. CytC4 is a thioesterase and cleaves the product amino acid from the pantetheine tether. The net action of these four tandemly encoded proteins is the generation of the known *Streptomyces* antimetabolite γ,γ-dichoroaminobutyrate [7]. Despite additional sequencing and cloning efforts, no cyclopropane-forming homolog has yet been detected in the cytotrienin producer.

Results

The CytC Gene Cluster

A gene fragment homologous to *cmaB* [1, 8] was used to clone out genes involved in the loading and halogenation of amino acids in this producer. This cloning strategy identified a DNA fragment containing four tandemly arranged genes, termed *cytC1*, *cytC2*, *cytC3* (the gene homologous to *cmaB*), and *cytC4* (Table 1). All of the protein products represent different nonribosomal peptide synthetase (NRPS) domains. CytC1 is predicted to function as an adenylation (A) domain, CytC2 as a phosphopantetheine (PP)-binding (PCP) domain, CytC3 as an aliphatic halogenase, and CytC4 as a thioesterase (TE) domain (Figure 2A). CytC3 is homologous to the

*Correspondence: christopher_walsh@hms.harvard.edu
[3] These authors contributed equally to this work.
[4] Present address: Department of Biological Sciences, Research and Development, Boehringer Ingelheim (Canada) Ltd., Laval, Quebec H7S 2G5, Canada.
[5] Present address: Department of Medicinal Chemistry in the College of Pharmacy and Life Sciences Institute, University of Michigan, Ann Arbor, Michigan 48109.
[6] Present address: Department of Chemistry, Harvey Mudd College, Claremont, California 91711.

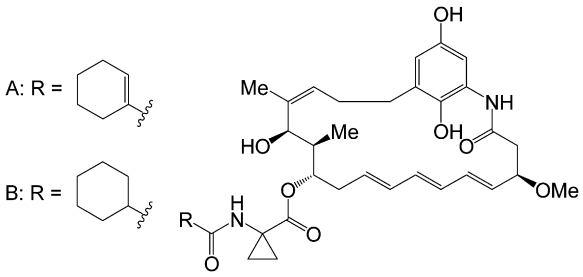

Figure 1. Cytotrienin A and Cytotrienin B

small but growing class of nonheme iron halogenases [1, 9–12]. It possesses the key active site residues characteristic of this class of enzyme (Figure 2B). The typical carboxylate ligand of nonheme iron hydroxylases is replaced by an alanine, the key feature of the nonheme iron halogenases. The replacement of the carboxylate ligand by an alanine allows for the binding of exogenous chloride to the iron, as shown in the crystal structure of the syringomycin halogenase SyrB2 [12, 13].

Substrate Specificity of CytC1

Most A domains in NRPS modules are highly specific for one amino acid. For example, CmaA, which is an A and T didomain protein involved in coronamic acid biosynthesis, has high specificity for L-*allo*-Ile and accepts other aliphatic amino acids with less than 8% efficiency when compared to L-*allo*-Ile [1]. Several A domains that activate aliphatic amino acid substrates have a slightly broader specificity. For example, LicC from lichenysin synthetase accepts not only L-Ile, but also L-Leu and L-Val [14]. It is known that the substrate specificity of NRPS A domains is determined by the 8 residues around the substrate-binding pocket [15]. No direct similarity of these key residues in CytC1 with other A domains was observed. However, this set is most similar to residues in those A domains that activate L-Val, L-Ile, and L-Leu. CytC1 might therefore prefer aliphatic amino acids.

The His-tagged CytC1 was overproduced in *Escherichia coli* as a soluble protein and was purified by using the Ni-NTA resin. The first half-reaction of CytC1, the reversible formation of aminoacyl-AMP, was followed by amino acid-dependent exchange of $^{32}PP_i$ into ATP (Table 2). CytC1 activated different amino acids in the following order of specificity: L-Val > ACC > D-Aba > D-Val > 4-Cl-L-Aba > L-Aba > L-*allo*-Ile ~ D-Cys > L-Cys > L-Ile ~ L-Leu. Only two other proteinogenic amino acids, L-Thr and L-Ala, were slightly activated by CytC1 (23% and 5% of the L-Aba rate, respectively). All other proteinogenic amino acids were tested and showed rates of 0.1%–0.8% when compared to L-Aba. The surprising ability of both D-Val and D-Aba to be activated to the aminoacyl-AMP as well or even better than the L-isomer does have a precedent in the activation of both L- and D-Phe by the first module of tyrocidine synthetase [15, 16]. ACC with Cα substituents mimicking both an D and an L center has a rate of ~25% of that of the best substrate, L-Val, in this first half of the CytC1 reaction.

Loading of Amino Acids onto CytC2

The second half-reaction of CytC1 is the transfer of the activated amino acid (aminoacyl-AMP) to the phosphopantetheinyl moiety of the T domain CytC2. N-terminally His-tagged CytC2 overproduced in *E. coli* was obtained as a soluble protein and was purified by using the Ni-NTA resin. The holo (phosphopantetheinylated) form of the T domain was generated in situ via the action of phosphopantetheinyl transferase Sfp [17] on purified CytC2. Using universally labeled ^{3}H-coenzyme A, incorporation of the radiolabel into CytC2 was monitored, and it was demonstrated to reach a plateau within 5 min

Table 1. Designations and Functions of the Genes Inserted in pSPHE02

Predicted Polypeptide	Amino Acids/MW (kDa)	Predicted Function	Most Homologous Gene (Organism)	E Value	Homologous Genes in *S. avermitilis*[a]	Homologous genes in *S. coelicolor*[a]
Orf1	708/75.4	Nitrate reductase catalytic subunit	Nitrate reductase catalytic subunit (*Amycolatopsis mediterranei*)	0	SAV2330	SCO2473
Orf2	462/48.4	Nitrate extrusion protein	Putative nitrate extrusion protein (*Streptomyces avermitilis*)	e^{-109}	SAV5119	SCO2959
Orf3	329/33.9	Unknown	Hypothetical transport protein yyaM (*Bacillus subtilis*)	$3e^{-18}$	NE	NE
CytC1	524/56.8	NRPS adenylation domain	—[b]	—	—	—
CytC2	87/9.42	NRPS PP-binding domain	—[b]	—	—	—
CytC3	319/36.4	Nonheme iron halogenase	Nonheme iron halogenase SyrB2 (*Pseudomonas syringae* pv. *Syringae*)	e^{-100}	NE	NE
CytC4	244/25.9	NRPS thioesterase domain	Putative thioesterase SAV3201 (*Streptomyces avermitilis*)	$2e^{-16}$	SAV3201	SCO6287
Orf4	315/33.6	ABC-type sugar transporter	Putative transport system integral membrane protein SAV2145 (*Streptomyces avermitilis*)	e^{-114}	SAV2145	SCO6086
Orf5	284/30.6	ABC-type sugar transporter	Putative transport system integral membrane protein SCO6087 (*Streptomyces coelicolor* A3(2))	$5e^{-99}$	SAV2144	SCO6087

Based on predicted amino acid sequences, the putative functions of the predicted proteins are shown with homologous genes. NE: no homologous gene with an E value less than e^{-10}.
[a] Most homologous genes of *Streptomyces avermitilis* MA-4680 and *S. coelicolor* A3(2).
[b] Homologous genes are not listed because too many genes are found in the database.

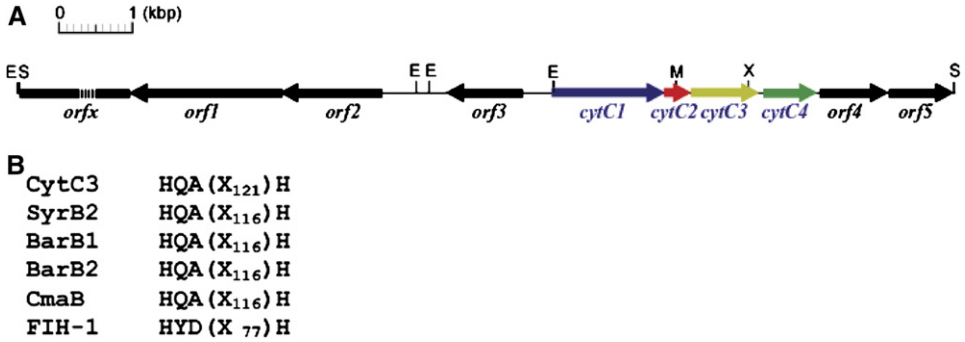

Figure 2. Overview of an Inserted Fragment in pSPHE02

(A) Nine predicted ORFs are shown with restriction sites: E, EcoRI; S, SphI; M, MluI; and X, XhoI. Four of the genes, *cytC1*, *cytC2*, *cytC3*, and *cytC4*, are part of the NRPS system. The predicted amino acid sequence of *orfx* was homologous to NADPH-dependent nitrate reductases, although several nucleotides remain unknown (indicated with a broken line). The proposed function of each ORF is given in Table 1.

(B) Sequence alignment of CytC3 with other known iron/αKG-dependent halogenases and one hydroxylase showing only the iron coordination motif. SyrB2 is involved in syringomycin biosynthesis [9], BarB1 and BarB2 are involved in barbamide biosynthesis [10], CmaB is involved in coronamic acid biosynthesis [1], and FIH-1 [32] is a nonheme iron/αKG asparaginyl hydroxylase. Ala120 in CytC3 is strictly conserved in the halogenase class and replaces the conserved aspartate ligand in the hydroxylases.

when 7 mol% Sfp was used. Therefore, apo-CytC2 could be efficiently modified by Sfp to form holo-CytC2.

The holo-CytC2 peak was observed at 13.8 min by HPLC. In the presence of CytC1 and various amino acids, a new peak was observed at ~13.2 min. To confirm that the new peak corresponded to aminoacyl-CytC2, the peak was fractionated and analyzed by MALDI-TOF MS. Apo- and holo-CytC2 were also analyzed by this method. Apo-CytC2 was observed at *m/z* 11454.0, and the holo form at *m/z* 11794.0, consistent with the theoretical molecular weight (MW) of N-terminally His-tagged protein lacking the first methionine (expected MW 11,453.2 Da), which is commonly removed during overproduction in *E. coli*. Holo-CytC2 was observed at 340 Da higher than the apo form, and various aminoacylated forms were observed (for example, L-Val-CytC2: calculated 11,893.8, found 11,895.0; L-Thr-CytC2: calculated 11,895.8, found 11,896.1; L-Leu-CytC2: calculated 11,907.9, found 11,906.3).

Mono- and Dihalogenation of L-Aba and L-Val by CytC3

To evaluate the activity of CytC3, the enzyme was removed from anaerobic storage just prior to incubations and added to aminoacyl-*S*-CytC2. In the presence of the three cosubstrates (O_2, αKG, and chloride), halogenation activity was observed with L-Aba-*S*-CytC2 and L-Val-*S*-CytC2 as substrates. Given that the product amino acid is covalently tethered to CytC2, detection of product involved the previously described release of the aminoacyl moieties by enzymatic treatment with a thioesterase [9]. As shown in Figure 3A, the enzymatic incubation of L-[^{3}H]Aba-*S*-CytC2 with CytC3 (CytC3:CytC2 ratio of 0.8:1) gave both 4-Cl-L-[^{3}H]Aba and 4,4-diCl-L-[^{3}H]Aba product-derived isoindoles (Figure 3A, trace b). Incorporation of chloride into the products was shown by using radioactive [^{36}Cl] for both 4-[^{36}Cl]-L-Aba and 4,4-di[^{36}Cl]-L-Aba (Figure 3A, trace c). Incubation of CytC3 with D-Aba-*S*-CytC2, obtained via loading of CytC2 with CytC1, did not form any products, arguing in favor of stereo-specific utilization of L-Aba in this halogenation pathway. When L-[^{14}C]Val-*S*-CytC2 was incubated with CytC3 (CytC3:CytC2 ratio of 0.6:1), the major product obtained was 4,4-diCl-L-[^{14}C]Val, while only a trace amount of monochlorinated product could be detected (Figure 3B, trace b), suggesting that valine is a better substrate for the second halogenation. The identities of the dichlorinated amino acids were determined via direct NMR-spectroscopic analyses of the unfractionated product mixtures obtained from incubation of L-Aba-*S*-CytC2 and L-Val-*S*-CytC2 with CytC3 (CytC3:CytC2 ratio of 0.8:1). Direct NMR-spectroscopic analyses of crude biological extracts provide a means for their structural characterization in an unbiased and nondestructive manner and can be used to identify even minor components of complex small-molecule mixtures, often eliminating the need for fractionation and/or derivatization [18]. The NMR-spectroscopic analyses of the extracts derived from enzymatic incubation with CytC3 were based on high-resolution dqf-COSY spectra, which clearly showed crosspeaks corresponding to the proton spin systems of 4,4-diCl-L-Aba or 4,4-diCl-L-Val, as well as signals representing unreacted L-Aba or L-Val, respectively (see the Supplemental Data available with this article online). In addition, the dqf-COSY spectra of the crude product mixtures revealed glycerol as well as ATP, ADP, and AMP as major components, as could be expected from the incubation protocol. The identity of

Table 2. Apparent Steady-State Kinetic Parameters of CytC1 for Different Amino Acids Determined by ATP-$^{32}PP_i$ Exchange Assays

Substrate	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}mM^{-1}$)
L-Val	0.60 ± 0.05	17.2 ± 0.4	29 ± 3
ACC	1.65 ± 0.07	11.9 ± 0.2	7.2 ± 0.4
D-Aba	4.0 ± 0.3	17.4 ± 0.5	4.3 ± 0.4
D-Val	6.9 ± 0.5	10.7 ± 0.4	1.6 ± 0.2
4-Cl-L-Aba	7.6 ± 0.6	7.6 ± 0.3	1.0 ± 0.1
L-Aba	13 ± 1	10.7 ± 0.5	0.8 ± 0.1
L-*allo*-Ile	18 ± 3	12 ± 1	0.6 ± 0.2
D-Cys	47 ± 12	29 ± 6	0.6 ± 0.3
L-Cys	5.2 ± 0.7	2.7 ± 0.2	0.5 ± 0.1

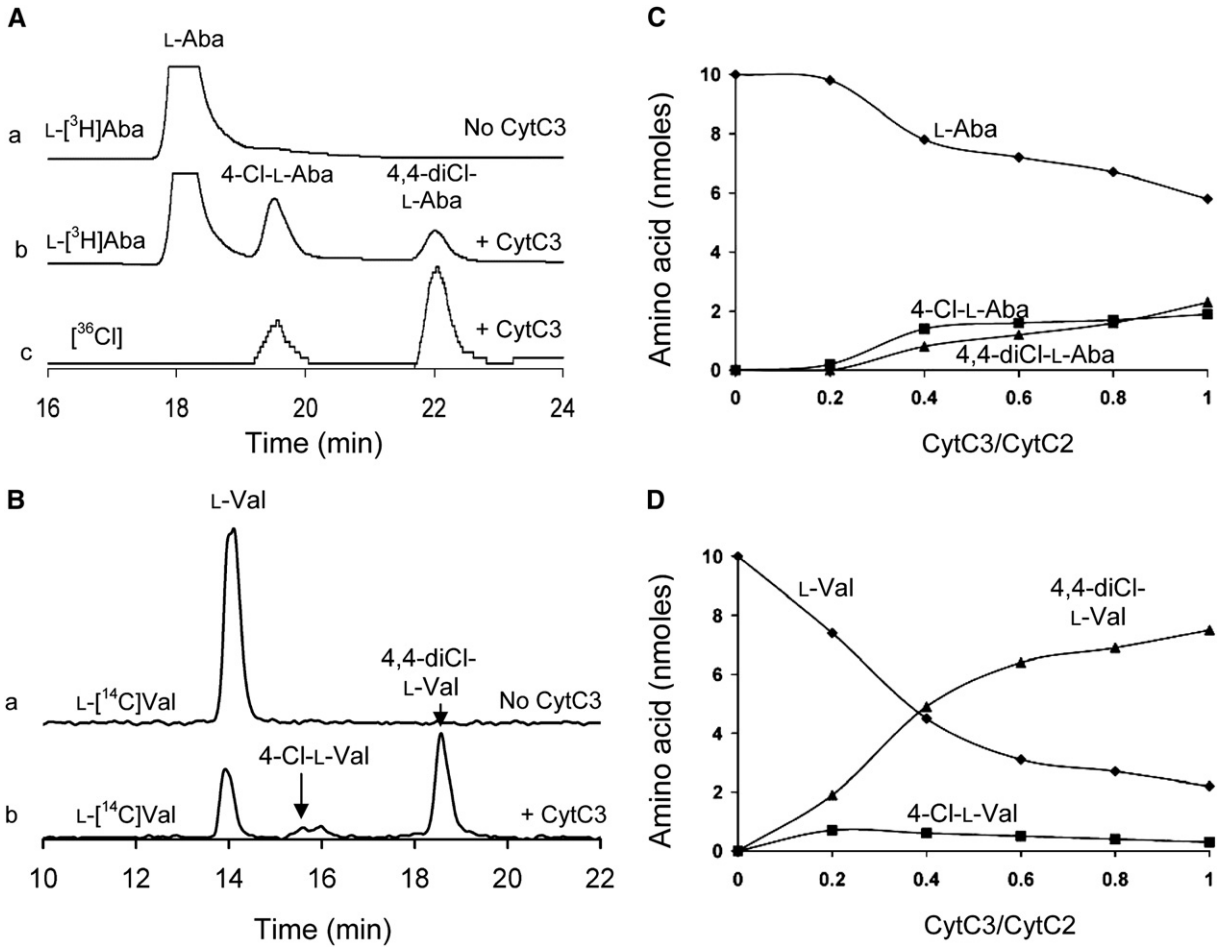

Figure 3. Biosynthesis of Chlorinated Amino Acids Catalyzed by CytC3

(A) Traces of hydrolyzed amino acid obtained after incubation of L-Aba-*S*-CytC2 with CytC3. Trace a, radio-HPLC trace of the control reaction performed with L-[^{3}H]Aba-*S*-CytC2 in the absence of CytC3. Trace b, radio-HPLC trace of the reaction performed with L-[^{3}H]Aba-*S*-CytC2, chloride, O_2, αKG, and CytC3. Trace c, radio-HPLC trace of the reaction performed with L-Aba-*S*-CytC2, O_2, αKG, and CytC3 in the presence of Na[^{36}Cl].

(B) Traces of a hydrolyzed amino acid obtained after incubation of L-Val-*S*-CytC2 with CytC3. Trace a, radio-HPLC trace of the control reaction performed with L-[^{14}C]Val-*S*-CytC2 in the absence of CytC3. Trace b, radio-HPLC trace of the reaction performed with L-[^{14}C]Val-*S*-CytC2, chloride, O_2, αKG, and CytC3.

(C) Analysis of the amount of L-Aba (diamond) consumed and the amount of 4-Cl-L-Aba (square) and 4,4-diCl-L-Aba (triangle) produced at different ratios of CytC3/CytC2.

(D) Analysis of the amount of L-Val (diamond) consumed and the amount of 4-Cl-L-Val (square) and 4,4-diCl-L-Val (triangle) produced at different ratios of CytC3/CytC2.

the dqf-COSY crosspeaks assigned to 4,4-diCl-L-Aba and 4,4-diCl-L-Val was confirmed by direct comparison with dqf-COSY spectra of authentic synthetic samples. No other spin systems representing compounds of comparable abundance were observed. 4,4-Dichlorination of both L-Aba and L-Val was further confirmed by coelution with authentic synthetic samples of 4,4-diCl-L-Val and 4,4-diCl-L-Aba, as noted in the Experimental Procedures, thus unambiguously demonstrating that both chlorines are incorporated onto the same carbon atom. The amount of mono- and dichloroamino acids produced on CytC2 and released by thioesterase action was investigated as a function of varying ratios of CytC3 to CytC2 (Figures 3C and 3D). With L-Aba-*S*-CytC2 as a substrate, a nearly constant amount of monochlorinated product was noted at ratios of 0.4:1 to 1:1. An almost linear increase in dichlorination as a function of the amount of added halogenase was observed. The efficiency of dichlorination was especially pronounced when L-Val was used as the substrate, as the monochloro derivative accumulates only to a small extent. Furthermore, incorporation of both chlorine atoms to the *pro-S* methyl carbon of the valine substrate demonstrates a remarkable regio- and stereospecificity of aliphatic halogenation catalyzed by CytC3.

The gene showing some homology to the coronamic acid cyclopropane-forming enzyme CmaC [1] was identified in the draft sequence of the *Streptomyces* sp. RK95-74 genome (A. Toyoda et al., personal communication) and cloned. The protein product was analyzed for conversion of CytC2-tethered γ-Cl-Aba to ACC. No formation of the desired product was noted (data not shown). Based on its dimeric structure (MW of 33 kDa) and homology to other members of the vicinal oxygen

Table 3. Apparent Steady-State Kinetic Parameters of CytC4 for Different Amino Acids Loaded on CytC2

Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}\mu M^{-1}$)
4-Cl-L-Aba	106 ± 7	1020 ± 50	10 ± 1
L-Aba	80 ± 7	470 ± 30	5.9 ± 0.9
ACC	73 ± 6	32 ± 2	0.44 ± 0.06
L-Val	80 ± 4	5.8 ± 0.2	0.073 ± 0.007

chelate superfamily [19] to which CmaC belongs, it is likely that this streptomycete protein is involved in an alternative epimerase reaction in the producer.

Thioesterase Activity of CytC4

N- and C-terminally His-tagged CytC4 were insoluble when overproduced in *E. coli* BL21 (DE3) by using pET28b or pET37b, respectively, under several conditions, including various incubation temperatures and media. However, overproduction of N-terminally His-tagged CytC4 in *Pseudomonas putida* KT2442 gave soluble protein. The N-terminally His-tagged CytC4 overproduced in *P. putida* was purified by Ni-NTA chromatography.

An initial evaluation of the thioesterase substrate specificity of CytC4 is shown in Table 3. CytC4 has a higher specificity for 4-Cl-L-Aba-*S*-CytC2 and L-Aba-*S*-CytC2 than ACC-*S*-CytC2. CytC4 has an 81-fold lower specificity for L-Val when compared to L-Aba, whereas CytC1 has a 36-fold higher specificity for L-Val when compared to L-Aba.

Antibiotic Activity of 4,4-DiCl-L-Aba and 4,4-DiCl-L-Val

4,4-DiCl-L-Aba (armentomycin) has previously been isolated from a *Streptomyces* species and is shown to have antibitotic activity [7]. Given that we had synthesized the dichlorinated amino acids as standards, we had sufficient material to evaluate the antibiotic activity of 4,4-diCl-L-Aba and 4,4-diCl-L-Val against *E. coli* strains W3110 and Imp^- (strain Imp^- has an improved membrane permeability). In both cases, no antibiotic activity could be detected when the strains were grown in Luria Bertani medium. However, when the strains were grown in M9 minimal media with glucose, antibiotic activity was observed. Using disc assays on plates (1 mM amino acid added to disc), zones of inhibition of 1 mm and 12 mm were observed for strain W3110 with 4,4-diCl-L-Val and 4,4-diCl-L-Aba, respectively. Zones of inhibition of 7 mm and 20 mm were observed for strain Imp^- with 4,4-diCl-L-Val and 4,4-diCl-L-Aba, respectively. This inhibition of growth could be prevented when L-Leu or L-Ile was added to the media. The minimal inhibitory concentration of 4,4-diCl-L-Aba was 128 μg/ml when evaluated in liquid media for strain W3110. For strain Imp^-, the minimal inhibitory concentration was 32 μg/ml and 512 μg/ml for 4,4-diCl-L-Aba and 4,4-diCl-L-Val, respectively. No antibiotic activity was detected for 4-Cl-L-Val and 4-Cl-L-Aba under comparable conditions.

Discussion

The existence of the highly functionalized ACC ring in the apoptosis-inducing streptomycete metabolite CytA [3], an ansa-bridged macrolactone with 1-aminocyclopropane-1-carboxylic acid, inspired a search for and the discovery of the *cytC1–4* cluster described in this work. In particular, we believed that a mononuclear nonheme iron halogenase might exist and be responsible for the generation of a 4-chloroaminobutyryl skeleton as a precursor to ACC. Cyclopropane-containing amino acids have been found in other biological contexts, most notably as free ACC for the generation of ethylene as a signaling hormone in plants [6], but also as the ethyl-substituted ACC (coronamic acid) in the phytotoxin coronatine, produced by several *Pseudomonas syringae* strains during growth on plants [1, 20, 21]. While in plants the ACC biosynthetic pathway starts with *S*-adenosylmethionione and is catalyzed by a PLP-dependent ACC synthase [5], no homologous enzyme was found in prokaryotic microorganisms [4]. The strategy for enzymatic formation of the aminocarboxycyclopropyl ring of coronamic acid is distinct [1]. Rather than using a PLP-mediated amino acid aldimine as the precursor to a stabilized C2 carbanion, this enzymatic strategy relies on the generation of an aminoacyl thioester to enable stabilization of C2 carbanion. The required leaving group on the γ-carbon of the L-*allo*-Ile-*S*-CmaD substrate is incorporated via chlorination of the C4 methyl by a nonheme iron halogenase, CmaB [1].

Based on the premise that *Streptomyces* could follow a similar pathway to that of *Pseudomonas syringae* for the generation of the cyclopropane moiety, primers derived from the *cmaB* DNA sequence were designed and used to probe the genomic DNA of the CytA producer *Streptomyces* sp. RK95-74 for homologs. The four-gene cluster *cytC1–C4* was discovered, with CytC1 homologous to free-standing A domains, CytC2 to free-standing T domains, CytC3 to nonheme iron halogenases, and CytC4 to thioesterases. Thus, the CytC1, CytC2, CytC3, and CytC4 sequences suggest an NRPS logic, where an amino acid is selected and activated as an aminoacyl-AMP (A domain), loaded covalently onto a T domain that has been posttranslationally primed with phosphopantetheine to create the aminoacyl-*S*-T domain intermediate. This intermediate is then modified by a tailoring enzyme prior to hydrolysis by a thioesterase. The sequestration of a fraction of the pool of a proteinogenic amino acid as a covalent aminoacyl-*S*-protein species may be a general strategy by which producer organisms make nonproteinogenic amino acids for use in secondary metabolism as well as activate them for incorporation into NRPS assembly lines [22].

The key enzyme in this study is an αKG-dependent nonheme iron halogenase CytC3. This enzyme was purified anaerobically and reconstituted with Fe(II). It was tested in the halogenation of L-Aba-*S*-CytC2 and L-Val-*S*-CytC2. These substrates were generated quantitatively by the action of CytC1 on primed CytC2. The formed products were released by enzymatic thioester bond cleavage by using a thioesterase, derivatized on the amino group with a fluorescent probe, chromatographed by HPLC, and compared to authentic standards. Using this method, four different chlorinated amino acids were shown to be formed by the CytC3 halogenase: 4-Cl-L-Aba, 4,4-diCl-L-Aba with L-Aba-*S*-CytC2 as

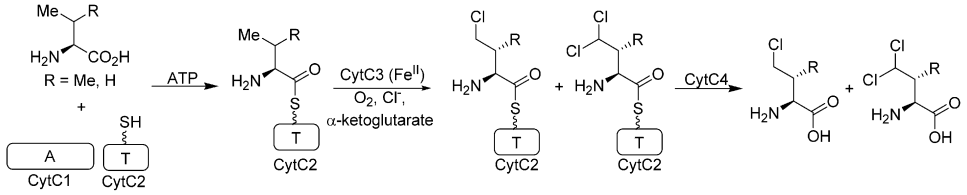

Figure 4. CytC3-Catalyzed Formation of Chlorinated Amino Acids in Soil *Streptomyces* sp.

the substrate, and 4-Cl-L-Val and 4,4-diCl-L-Val from L-Val-*S*-CytC2 (Figure 4). Interestingly, 4,4-diCl-L-Aba (called armentomycin) was previously isolated from a *Steptromyces* species and was shown to have antibiotic activity [7]. The antibiotic activity was confirmed in this study, and 4,4-diCl-L-Aba was shown to inhibit the biosynthesis of L-Leu and L-Ile because the antibiotic activity could be prevented when either of these two amino acids was added to the media. These results, together with strong sequence homology of CytC3 with other known halogenases [12, 23], including key active site residues (Figure 2B), validate that the CytC3 is a new, to our knowledge, member of the nonheme iron halogenase family. This class of enzyme requires O_2, chloride, and αKG for their activity. The crystal structure of SyrB2 showed that the chloride is bound to the iron(II) of the enzyme at the beginning of the catalytic cycle [13]. Recent kinetic and spectroscopic studies revealed that hydrogen atom abstraction from the substrate is carried out by an Fe(IV)oxo intermediate, resulting in the generation of the γ-$CH_2^{\bullet}$ radical in the substrate and the Cl-Fe(III)-OH species [13] (D.P.G., E.W. Barr, C.T.W., J.M. Bollinger, Jr., and C. Krebs, unpublished data). It is postulated that subsequent transfer of the chlorine atom to the organic radical leads to the generation of the C-Cl bond in the product.

It is an open question whether the γ-chloroaminobutyryl moiety is cyclized to ACC in the producer organism or if CytC1–4 are operating to make and release the free mono- or dichloroamino acids, some of which serve as antimetabolites. The producer streptomycete has not yet been successfully transformed with DNA to allow knockout of any of the genes in the *cytC1–C4* cluster to evaluate if cytotrienin formation is abrogated (M.U., unpublished data). No protein with a function homologous to CmaC (the cyclopropane-forming enzyme in the coronamic acid cluster) was detected in the *cytC* gene cluster or elsewhere in the producer genome (M.U. and A. Toyoda, unpublished data).

In sum, an NRPS-inspired route for the formation of chlorinated amino acids in *Streptomyces* has been described. This system consists of four genes: a promiscuous adenylation enzyme, CytC1; the peptidyl carrier protein CytC2; the nonheme iron halogenase CytC3; and the thioesterase CytC4. The formed mono- and dichloro intermediates can either be utilized as antimetabolites or perhaps serve as precursors to ACC derivatives by the action of a cyclopropane-forming enzyme yet to be found at a different locus in the genome.

Significance

To our knowledge, this work represents the first cloning and analysis of a biosynthetic gene cluster responsible for the formation of γ-chlorinated amino acids in an actinomycete. The CytC cluster is composed of four ORFs: CytC1, an adenylation enzyme; CytC2, a thiolation protein; CytC3, a nonheme iron halogenase; and CytC4, a thioesterase. The adenylation enzyme was able to activate various amino acids and load them onto CytC2, and the CytC3 halogenase was able to both mono- and dichlorinate L-Aba and L-Val. 4,4-DiCl-L-Aba (armentomycin) was already shown to be a naturally occurring antibiotic, an activity that was confirmed in this study. Engineering of this pathway could enable the production of a wide variety of regio- and stereospecifically chlorinated amino acids.

Experimental Procedures

Chemicals

L-[^{14}C]Val (250 mCi/mmol) and [^{36}Cl]NaCl (16 mCi/g Cl) were purchased from American Radiolabeled Chemicals, Inc. L-[^{3}H]Aba (1 Ci/mmol) was purchased from Moravek Biochemicals, Inc., and [^{32}P]pyrophosphate was purchased from Perkin Elmer Life Sciences, Inc. Thrombin was purchased from Novagen. Norcoronamic acid and 4-Cl-L-Val were synthesized as described previously [1]. 4,4-Dichloro-2-aminobutyric acid (4,4-diCl-Aba) was prepared by a known procedure [24]. 4-Chloro-2-aminobutyric acid (4-Cl-Aba) and 4,4-diCl-L-Val were synthesized as described below. All other chemicals were of analytical grade.

NMR Spectroscopy

All analyses were carried out by using a VARIAN INOVA 600 MHz NMR spectrometer, equipped with a 5 mm inverse-detection HCN probe. NMR spectra were acquired at 25°C, by using the standard pulse sequences provided by VARIAN. Spectra of crude enzymatic samples were acquired by using Shigemi tubes and dissolving the sample in 220 μl D_2O. For the dqf-COSY spectra, the following parameters were used: acquisition time, 0.6 s; relaxation delay, 1 s; and 64 increments per ppm of sweep width. Phase cycling was used for coherence selection, and gradients were used for homospoil during the relaxation delay. Relevant sections of the dqf-COSY spectra are shown in the Supplemental Data.

Chemical Synthesis of 4-Cl-Aba

Boc-Hse-O^{t}Bu was prepared by following the method of Perich [25]. CCl_4 (194 μl, 0.35 mmol), followed by a solution of Ph_3P (92.4 mg, 0.35 mmol) in CH_2Cl_2 (0.5 ml), was added to a cooled (0°C) solution of Boc-Hse-O^{t}Bu (48.5 mg, 0.176 mmol) in anhydrous CH_2Cl_2 (1.5 ml). The solution was warmed to room temperature for 30 min, then heated in a 40°C oil bath for 15 hr. The reaction mixture was then concentrated in vacuo, triturated with 5:1 hexanes:Et_2O, and filtered through a cotton plug. The filtrate was concentrated in vacuo and chromatographed on silica (7:1 hexanes:Et_2O) to give Boc-(4-Cl)Abu-O^{t}Bu (35.0 mg, 68% yield) as a colorless oil that forms needles when stored at −20°C; R_f = 0.2 (5:1 hexanes:Et_2O); ^{1}H-NMR (500 MHz, $CDCl_3$); δ = 5.17 (br, 1H), 4.29 (m, 1H), 3.58 (m, 2H), 2.28 (m, 1H), 2.09 (m, 1H), 1.46 (s, 9H), 1.44 (s, 9H); ^{13}C-NMR (125 MHz, $CDCl_3$); δ = 171.2, 155.6, 82.6, 80.1, 52.4, 40.9, 36.1, 28.5, 28.2. Boc-(4-Cl)Abu-O^{t}Bu (90.3 mg, 0.307 mmol) was dissolved in trifluoroacetic acid (1.0 ml) and left at room temperature for 3 hr. Volatiles were removed with a stream of nitrogen. Exhaustive concentration in vacuo gave a tan solid (77.0 mg, quantitative yield).

4-Cl-Aba was conveniently stored as its trifluoroacetic acid salt; one equivalent of NaOH was added prior to enzymatic assays. ^{1}H-NMR (500 MHz, D_2O); δ = 4.13 (app t, 1H, *J* = 6.7 Hz), 3.66 (m, 2H), 2.36 (m, 1H), 2.21 (m, 1H); ^{13}C-NMR (125 MHz, D_2O); δ = 171.8, 51.0, 40.6, 32.9.

Chemical Synthesis of 4,4-DiCl-L-Val

(2S,4R)-2-Amino-3-Methyl-4-Oxo-Butyric Acid

Oxalyl chloride (34 μl, 0.39 mmol) was added dropwise to a cooled (−78°C) solution of DMSO (55.2 μl, 0.78 mmol) in anhydrous CH_2Cl_2 (2.3 ml). The resulting solution was stirred at this temperature for 20 min. A cooled (−78°C) solution of (3*R*,4*S*)-*N*-(*tert*-Butyldimethylsilyl)-3-methyl-azetidin-2-one-4-carboxylic acid *tert*-butyl ester (22.5 mg, 0.078 mmol) [1] in anhydrous CH_2Cl_2 (6.9 ml) was canula transferred to the reaction mixture. The resulting white suspension was stirred at −78°C for 20 min, and Et_3N (162 μl, 1.17 mmol) was subsequently added. The resulting solution was warmed up to −40°C and was stirred at this temperature for 15 min. Water (2 ml) was added, and the reaction mixture was warmed up to room temperature and partitioned between CH_2Cl_2 (100 ml) and saturated aqueous ammonium chloride solution (50 ml). The organic layer was washed with saturated aqueous sodium bicarbonate (50 ml), followed by saturated aqueous sodium chloride solution (50 ml), dried (Na_2SO_4), and concentrated in vacuo. The residue was purified by silica gel flash chromatography (5:1 hexanes:EtOAc) to provide (2*S*,4*R*)-2-amino-3-methyl-4-oxo-butyric acid (19.0 mg, 85%) as a white solid; R_f = 0.66 (5:1 hexanes:EtOAc); ^{1}H-NMR (500 MHz, $CDCl_3$); δ = 9.74 (s, 1H), 5.36 (d, *J* = 8.30 Hz, 1H), 4.69 (dd, *J* = 7.81, 3.91 Hz, 1H), 3.09 (m, 1H), 1.50 (s, 9H), 1.48 (s, 9H), 1.17 ppm (d, *J* = 7.33 Hz, 3H).

(2S,4R)-2-Amino-4,4-Dichloro-3-Methyl-Butyric Acid

A solution of hydrazine hydrate (65 μl, 1.32 mmol) in MeOH (400 μl) was stirred at room temperature in the presence of powdered molecular sieves (95 mg) for 35 min. A solution of (2*S*,4*R*)-2-amino-3-methyl-4-oxo-butyric acid (19.0 mg, 0.066 mmol) in MeOH (900 μl) was added dropwise, and the resulting suspension was stirred at room temperature for 2 hr. The resulting solution was filtered, sieves were washed with Et_2O (3 × 1.5 ml), and the filtrate was concentrated in vacuo and then under high vacuum to provide the crude hydrozone.

Triethyl amine (41.0 μL, 0.3 mmol) was added to a solution of dry copper (II)-chloride (78.0 mg, 0.58 mmol) in anhydrous MeOH (500 μl), and the resulting suspension was stirred at room temperature for 10 min. The reaction mixture was subsequently cooled to 0°C, and a solution of crude hydrazone in dry MeOH (750 μl) was added dropwise. The reaction mixture was stirred at this temperature for 1 hr, and then at room temperature for 50 min. A 3.5% solution of aqueous ammonia (2 ml) was then added, and the reaction mixture was partitioned between CH_2Cl_2 (75 ml) and saturated aqueous sodium chloride solution (30 ml). The organic layer was dried (Na_2SO_4) and concentrated, and the residue was purified by silica gel flash chromatography (7:1 hexanes:EtOAc) to provide (2*S*,4*R*)-2-amino-4,4-dichloro-3-methyl-butyric acid (2.3 mg, 10%) as a white solid; R_f = 0.34 (7:1 hexanes:EtOAc); ^{1}H-NMR (500 MHz, $CDCl_3$); δ = 5.94 (d, *J* = 5.40 Hz, 1H), 5.18 (br. s, 1H), 4.48 (br. s, 1H), 2.59 (br. s, 1H), 1.55 (s, 9H), 1.51 (s, 9H), 1.24 (d, *J* = 7.81, 3.91 Hz, 1H), 3.09 (m, 1H), 1.50 (s, 9H), 1.48 (s, 9H), 1.24 ppm (d, *J* = 6.84 Hz, 3H).

4,4-Dichloro-L-Val·TFA

Trifluoroacetic acid was added to a solution of Boc-(4,4-diCl)Val-O^{t}Bu (1.6 mg, 0.005 mmol) in CH_2Cl_2, and the resulting solution was stirred at room temperature for 15 hr. The reaction mixture was concentrated in vacuo to provide (2S,4R)-4,4-diCl-Val·TFA (1.3 mg, 93%) as a yellowish oil. ^{1}H-NMR (500 MHz, D_2O); δ = 6.28 (d, *J* = 8.30 Hz, 1H), 4.21 (d, *J* = 3.42 Hz, 1H), 2.61 (m, 1H), 1.32 ppm (d, *J* = 6.84 Hz, 3H).

Strains and Culture Conditions

Streptomyces sp. RK95-74, the CytA producer, was cultured in Bennet's broth medium at 30°C on rotary shaker at 200 rpm. *E. coli* TOP10 (Invitrogen) and *E. coli* BL21 (DE3) (Invitrogen) were grown in Luria-Bertani (LB) medium at appropriate temperatures. Ampicillin (120 μg/ml) and kanamycin (50 μg/ml) were used for the selection of *E. coli* transformants, and tetracycline (20 μg/ml) was used for selections of *E. coli* and *P. putida* KT2442 carrying the pVLT31 vector [26]. *E. coli* W3110 (ATCC 27325) and *E. coli* Imp$^-$ [27] were used to investigate the antibiotic activity of chlorinated amino acids in LB and M9 minimal media.

DNA Manipulation and Southern Hybridization

Agarose gel electorophoresis and restriction enzyme digestion were performed by following standard procedure [28]. DNA probes for Southern hybridization were labeled with alkaline phosphatase, and hybridization signals were generated by using the AlkPhos Direct Labeling and Detection System with CDP-Star (GE Healthcare) according to the manufacturer's instructions.

Computational Analyses

Alignment of primary sequence data was performed by using the AssemblyLIGN (Oxford Molecular Group) run on a Macintosh computer. ORFs were predicted by using the FramePlot ([29]; http://watson.nih.go.jp/~jun/cgi-bin/frameplot.pl). Basic local alignment search tool (BLAST) analyses were performed at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/).

Cloning of the DNA Fragment Containing the CytC Cluster

A designated PCR primer pair was used to amplify a fragment homologous to a portion of *cmaB* from the total DNA of RK95-74. The pair consisted of CMB1F (5′-ACCTACGACCGSCACCTSG AC-3′; S = G or C) and CMB2R (5′-GCCTGGTGCCAGTCSGTSCC-3′). These primers were designed based on alignment analysis of CmaB (NCBI protein ID: AAQ93485), BarB1 (AAN32975), and BarB2 (AAN32976), which are involved in barbamide biosynthesis by the cyanobacteria *Lyngbya majuscula*, and SyrB2 (AAD50521), which is involved in syringomycin biosynthesis by *Pseudomonas syringae* pv. *syringae*. PCR with this primer pair was performed, and the amplified fragments (~160 bp) were purified in agarose gel and ligated into the TA cloning vector pGEM-T Easy (Promega). Sequence analysis and its predicted amino acids showed that the fragment had 66% identity to CmaB and 77% identity to SyrB2. Using this fragment as a probe, southern hybridization against completely digested total DNA of strain RK95-74 with several restriction enzymes was performed with nylon transfer membrane Hi-bond N+ (GE Healthcare) and the AlkPhos detection kit (GE Healthcare) according to the manufacturer's instructions. The probe hybridized to 15–20 kbp SphI-digested fragments. The fragments were extracted from agarose gel, followed by ligation into pUC19. About 300 clones were probed by using the alkaline phosphophatase-labeled fragment. One of clones, SPHE02, was hybridized with the labeled probe, and the plasmid pSPHE02 was extracted from the clone. The inserted fragment was then sequenced. Automated DNA sequencing was performed on ABI Prism 3100 and 3730 DNA sequencers at the DNA sequencing facility of the Dana-Farber Cancer Institute (Boston, MA).

Subcloning, Overproduction, and Purification of N-Terminal His-Tagged CytC1 and CytC2

Two sets of PCR primer pairs were used to amplify *cytC1* and *cytC2* genes from pSPHE02. One set consisted of CYTC1F (5′-GGTTTC CCCATATGCTGCTGCAATC-3′; restriction site is underlined) and CYTC1R (5′- CCCAAGCTTCATCATGAACGTGCCACC-3′) to amplify *cytC1*, and the other set consisted of CYTC2F (5′- GGTGGCACGCA TATGACGGGACAGC-3′) and CYTC2R (5′-CCCAAGCTTCTACTAC TTGGTGGACCG-3′) to amplify *cytC2*. These primers were designed to introduce an NdeI restriction site on the forward strand and a HindIII site on the complementary strand into amplified fragments. After PCR amplification with *Pfu* Turbo DNA polymerase, resulting products of the ~1.6 kbp *cytC1* and the ~290 bp *cytC2* were purified by agarose gel electrophoresis and were digested with NdeI and HindIII. The digested fragments were purified as described above and ligated into NdeI- and HindIII-digested pET28b. After transformation into *E. coli* TOP10 (Invitrogen), the expression vectors were purified and inserts were sequenced by using T7 promoter primers to confirm sequences. The vectors were transformed into *E. coli* BL21 (DE3), and resulting transformants were grown in LB medium supplemented with 50 μg/ml kanamycin. Six liters of medium were inoculated with 60 ml of an overnight culture and were incubated at 37°C to an OD_{600} = 0.7. The incubation temperature was lowered

to 25°C for 1 hr prior to induction of overexpression by the addition of 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were grown for an additional 15 hr at 25°C, harvested by centrifugation (10 min at 6,000 × g), and resuspended in 40 ml of 25 mM Tris-HCl (pH 8.0), 400 mM NaCl, 10% glycerol. Cell lysis was performed by two passages through a French press at 15,000 psi. Cell lysates were clarified by centrifugation (30 min at 15,000 × g) and were applied to 2 ml Ni-NTA agarose resin (Qiagen). After gentle shaking for 2 hr at 4°C to bind the desired His-tagged protein, the resin was washed with 10 ml lysis buffer and then eluted with a step gradient of lysis buffer containing increasing amounts of imidazole (2, 6, 20, 40, 60, 200, and 500 mM). CytC1 eluted in the 20 and 40 mM imidazole fractions, and CytC2 eluted with 60 and 200 mM imidazole. Fractions containing CytC1 or CytC2 were dialyzed overnight against 50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM DTT, 10% glycerol; frozen in liquid nitrogen; and stored at −80°C. Protein concentrations were determined by the Bradford method [30]. Alternatively, the protein was purified in the apo form and was reconstituted as previously described [10] after the gel filtration step.

Subcloning, Overproduction, and Purification of CytC3

A pair of PCR primers was used to amplify the *cytC3* gene from pSPHE02. The pair consisted of CYTC3F (5′-GAGAATCCATATGACCACTGTGAGC-3′) and CYTC3R (5′-CCCAAGCTTCATCATGAACGTGCCACC-3′). The amplified product (~1.0 kbp) was digested with NdeI and HindIII and was cloned into pET28b. Transformants of *E. coli* BL21(DE3) expressing *cytC3* were prepared as described above. When OD_{600} = 0.6, overproduction of CytC3 was induced by the addition of 150 μM IPTG for 15 hr at 15°C. Six liters of culture were grown and resuspended in 40 ml of 20 mM 4-(2-hydroxyethyl)-1-piperazinepropanesulfonic acid (HEPPS) (pH 8.0), 300 mM NaCl, 5 mM imidazole. After two successive passages through a French press at 15,000 psi, the lysate was placed under a stream of argon and was clarified by centrifugation at 15,000 × g for 30 min. After this step, all handling of CytC3 was performed anaerobically in an Mbraun Labmaster glovebox as previously described [9]. The supernatant was added to 3 ml Ni-NTA agarose resin and was stirred for 1 hr. The resin was washed with four column volumes of 20 mM HEPPS (pH 8.0), 300 mM NaCl, 30 mM imidazole, and bound protein was eluted with 20 mM HEPPS (pH 8.0), 100 mM NaCl, 200 mM imidazole. The eluted fractions were concentrated by using an Amicon ultrafiltration membrane (Millipore) and were buffer exchanged with 20 mM HEPPS (pH 8.0), 80 mM NaCl. $CaCl_2$ (2 mM) was added, and the solution was incubated for 3 hr at 19°C with thrombin (1 U:10 mg thrombin:CytC3). The protein solution was then diluted 1:1 with 20 mM Tris (pH 8.0) and was loaded onto a MonoQ 10/100GL column (GE Healthcare) equilibrated with 20 mM Tris (pH 8.0) (multiple runs were performed). CytC3 was eluted by using a NaCl gradient of 100–250 mM over 15 column volumes at a flow rate of 4 ml/min. CytC3 eluted at ~200 mM NaCl. CytC3 was then concentrated and incubated with 2 mM αKG, 600 μM $Fe(NH_4)_2(SO_4)_2$, and 1 mM DTT, and it was then loaded onto a 26/60 Superdex 75 column equilibrated in 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) (pH 7.5). Eluted CytC3 (monomer) was concentrated, frozen in liquid nitrogen, and stored at −80°C. Protein concentration was determined by the Bradford method [30], and the iron concentration of CytC3 was determined by using Ferene S [31].

Subcloning, Overproduction, and Purification of His-Tagged CytC4

N- and C-terminal His-tagged CytC4 were completely insoluble when expressed in *E. coli* BL21(DE3). A plasmid to overexpress CytC4 in *Pseudomonas* was constructed. A pair of PCR primers was used to amplify the *cytC4* gene from pSPHE02. The pair consisted of CYTC4F (5′-GGAGGTACATATGACGCCCCGTCCC-3′) and CYTC4R (5′-CCCGAAGCTTCATCAAGGAGTACGGCCG-3′). The amplified product (~750 bp) was digested with NdeI and HindIII and was cloned into pET28b. The resulting plasmid, pET28b-CytC4, was digested with XbaI and HindIII, and the ~850 bp fragment was ligated into XbaI- and HindIII-digested pVLT31. After tranformation into *E. coli* TOP10, clones carrying pVLT31-CytC4 were selected on LB agar plates containing 20 μg/ml tetracycline. An overnight culture of *P. putida* KT2442 was pelleted at 5,000 rpm, and cells were washed twice with cold, sterile 1 mM HEPES (pH 7.0). A total of 0.5 μl pVLT31-CytC4 was added to the cell pellet, and the mixture was transferred into a 1 mm-gapped Gene Pulser Cuvette (Bio-Rad). Electroporation was conducted by using a Gene Pulser II at 400 Ω, 25 μF, and 1.25 kV, followed by immediate addition of 250 μl SOC medium. After incubation of treated cells at 30°C for 1 hr, cells were selected on LB agar plates containing 20 μg/ml tetracycline. A 60 ml overnight culture of the transformant was transferred to 6 liter LB medium supplemented with 89 mM potassium phosphate buffer (pH 7.00) and 20 μg/ml tetracycline. When OD_{600} = 0.6, overexpression was induced by the addition of 1 mM IPTG. After induction at 25°C for 15 hr, cells were pelleted by centrifugation. Purification of CytC4 was performed as described for CytC1 and CytC2.

ATP-$^{32}PP_i$ Exchange Assay

ATP-$^{32}PP_i$ exchange reactions were carried out at 25°C in 100 μl mixtures containing 75 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM tris(2-carboxyethyl)phosphine (TCEP; pH 7.0), 5 mM ATP, 1 mM $Na^{32}PP_i$ (5 mCi/mol, Perkin Elmer), 1 μM CytC1, and various concentrations of amino acids. The reactions were initiated by the addition of CytC1 and were quenched at appropriate time points by the addition of 500 μl quenching solution (4.46% tetrasodium pyrophospate and 3.5% perchloric acid) with 1.6% activated charcoal. After vortexing vigorously, the charcoal was washed twice with quenching solution not containing charcoal, was resuspended in 500 μl sterile water, and was transferred to 7 ml Ultima Gold scintillation fluid (Perkin Elmer). Radioactivity bound to charcoal was counted by using a LS6500 scintillation counter (Beckman Coulter).

HPLC Analyses of Different Forms of CytC2

Purified CytC2 was 4′-phosphopantetheinylated in a reaction containing 75 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 5 mM TCEP (pH 7.0), 100 μM Coenzyme A (CoA), 15 μM CytC2, and 1.0 μM Sfp. After incubation at room temperature for 1 hr, aminoacylation of the resulting holo-CytC2 was initiated by mixing with an equal volume of a solution containing 75 mM HEPES (pH 8.0), 10 mM $MgCl_2$, 5 mM TCEP (pH 7.0), 10 mM ATP, and various concentrations of amino acids and CytC1. After appropriate incubation times, the reaction mixtures were analyzed by HPLC (Beckman System Gold) with a Vydac Protein and Peptide column (4.6 mm × 250 mm) at a flow rate of 1.0 ml/min by using the following conditions: A solvent, 0.1 % trifluoroacetic acid (TFA) in water; B solvent, acetonitrile, 20 min linear gradient of 30%–60% B in A. Aminoacylated-*S*-CytC2 eluted at 13.2 min, and holo-CytC2 eluted at 13.8 min. To assay CytC4, rates were determined by evaluating the concentration of holo-CytC2 formed after the hydrolysis of aminoacyl-*S*-CytC2.

CytC3 Activity Assays

The reaction catalyzed by CytC3 was investigated by incubating the enzyme with loaded CytC2. Loaded CytC2 was prepared by incubating holo-CytC2 (80–100 μM) with 15–17.5 μM CytC1, 1.5–2.5 mM amino acid, and 2.5 mM ATP for 60 min, followed by incubation with 2 mM αKG and CytC3 for 60 min. Chloride (2 mM) was present in the reaction mixture. The resulting reactions were transferred to 0.5 ml Ultrafree centrifugal devices; excess amino acids was removed; and protein-bound amino acids were hydrolyzed, derivatized, and analyzed by HPLC as previously described [9]. In all cases, coelution with authentic standards was achieved.

Supplemental Data

Supplemental Data include the dqf-COSY spectra of synthetic and enzymatic dichloro-Aba and -Val and are available at http://www.chembiol.com/cgi/content/full/13/11/1183/DC1/.

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