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An Enzymatic Pathway for the Biosynthesis of the Formylhydroxyornithine Required for Rhodochelin Iron Coordination

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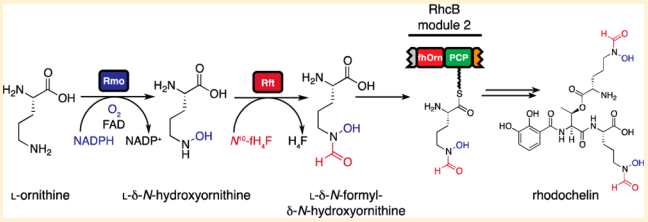
Supporting Information

ABSTRACT: Rhodochelin, a mixed catecholate−hydroxamate type siderophore isolated from *Rhodococcus jostii* RHA1, holds two L-*δ*-*N*-formyl-*δ*-*N*-hydroxyornithine (L-fhOrn) moieties essential for proper iron coordination. Previously, bioinformatic and genetic analysis proposed *rmo* and *rft* as the genes required for the tailoring of the L-ornithine (L-Orn) precursor [Bosello, M. (2011) *J. Am. Chem. Soc. 133*, 4587−4595]. In order to investigate if both Rmo and Rft constitute a pathway for L-fhOrn biosynthesis, the enzymes were heterologously produced and assayed *in vitro*. In the presence of molecular oxygen, NADPH and FAD, Rmo monooxygenase was able to convert L-Orn into L-*δ*-*N*-hydroxyornithine (L-hOrn). As confirmed in a coupled reaction assay, this hydroxylated intermediate serves as a substrate for the subsequent N^{10}-formyl-tetrahydrofolate-dependent (N^{10}-fH$_4$F) Rtf-catalyzed formylation reaction, establishing a route for the L-fhOrn biosynthesis, prior to its incorporation by the NRPS assembly line. It is of particular interest that a major improvement to this study has been reached with the use of an alternative approach to the chemoenzymatic FolD-dependent N^{10}-fH$_4$F conversion, also rescuing the previously inactive CchA, the Rft-homologue in coelichelin assembly line [Buchenau, B. (2004) *Arch. Microbiol. 182*, 313−325; Pohlmann, V. (2008) *Org. Biomol. Chem. 6*, 1843−1848].



The production and the secretion of siderophores is the most efficient iron-scavenging strategy used by environmental and pathogenic bacteria to mobilize iron from iron-depleted microbial niches. After secretion out of the extracellular space, the ferric iron−siderophore complex is selectively and actively imported into the intracellular space. Subsequently to its release, the reduced ferrous iron is then channeled to its dedicated cellular destinations, to be used in many essential biochemical processes.[1] Although all siderophores perform the same biological function, they display great chemical diversity, in both assembly strategy and iron coordination.[2] Regarding the siderophore biosynthesis, two different classes can be distinguished: NRPS-dependent and NRPS-independent.[3,4] On the other hand, the iron-binding property relies on the coordination by three major functional groups: cathecholates, hydroxamates, and carboxylates, which can be combined to generate mixed type siderophores, further increasing the iron coordination diversity.[5]

Hydroxamate-coordinating moieties are ordinarily found in both NRPS-dependent and NRPS-independent siderophores. They mainly derive from the hydroxylation of the side chain amino group of lysine and ornithine or from the primary amino group of putrescine. This modification reaction is catalyzed by a homologous group of enzymes belonging to the *N*-hydroxylating flavoprotein monooxygenases (NMO), which have been extensively characterized on both the biochemical and structural level, and even as potential drug target candidates against life-threatening microbial infections.[6,7] IucD (*Escherichia coli*), SidA (*Aspergillus fumigatus*), PvdA (*Pseudomonas aeruginosa*), CchB (*Streptomyces coelicolor*), and EtcB (*Saccharopolyspora erythraea*) are members of this enzyme family.[8−13] Once the hydroxylated products have been synthetized, the monomers can be either directly incorporated into their corresponding assembly pathways or further modified prior to assembly on their respective side chains by acetylation or formylation.[6] As a model for the acetylation reaction, the Mcd enzyme from the erythrochelin gene cluster has been recently characterized, demonstrating the transfer of an acetyl group from malonyl- or acetyl-CoA donor to the side chain of an L-hOrn residue.[13] A similar study aimed at the characterization of the putative formyltransferase CchA, proposed to be involved in the formylation of L-hOrn in the coelichelin biosynthesis gene cluster, was not able to successfully confirm its function (Figure 1). Nevertheless, an analogous "hydroxylation first" model for the biosynthesis of the nonproteinogenic amino acid L-fhOrn has been inferred, based on the substrate specificity of the ornithine monooxygenase CchB.[12]

Rhodochelin is a mixed-type catecholate−hydroxamate siderophore isolated from *Rhodococcus jostii* RHA1 (Figure 1). It possesses a unique branched structure with an unusual ester bond between an L-fhOrn moiety and the side chain of a threonine residue. Rhodochelin biosynthesis is accomplished by three distantly located gene clusters, implying an unprece-

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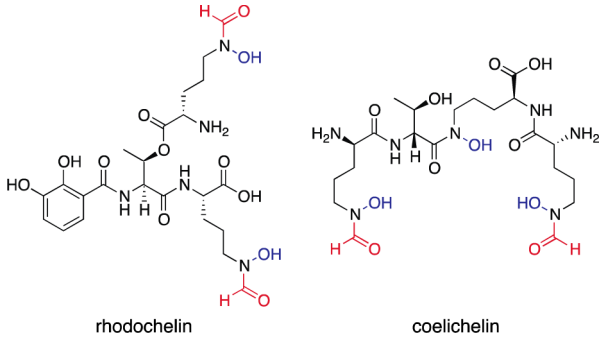

Figure 1. Chemical structures of rhodochelin and coelichelin with highlighted *δ-N*-ornithine modifications involved in Fe^{3+} coordination (hydroxylation blue, formylation red).

dented and uncharacterized cross talk mechanism between them. In particular, *rmo* and *rft* are located in close proximity of a silent NRPS and encode for an ornithine monooxygenase and a formyltransferase, respectively. They are homologous to coelichelin's *cchB* and *cchA*, and their essentiality for rhodochelin biosynthesis has previously been proven through the construction of their respective isogenic deletion strains.[14] Curiously, rhodochelin and coelichelin share another interesting feature regarding the incorporation of the L-fhOrn moiety by their respective NRPS assembly machineries. Despite the presence of only one adenylating-domain with proper L-fhOrn specificity, the non-proteinogenic amino acid is incorporated twice into the peptide, following a so-called "module skipping" mechanism.[15]

In the present work, the biochemical characterization of the recombinant Rmo and Rft is presented. For both enzymes, the substrate specificity has been investigated, together with the determination of the kinetic parameters for the L-Orn tailoring modifications, confirming their proposed biochemical functions. Finally, in a coupled reaction assay, the capability of Rmo and Rft to generate the hydroxamate-containing L-fhOrn from the L-Orn precursor has been investigated, establishing a model for the generation of the non-proteinogenic amino acid, prior to its incorporation by the peptide assembly line.

EXPERIMENTAL PROCEDURES

Isolation of Genomic DNA. *Rhodococcus jostii* RHA1 was maintained on agar slants as described before.[14] For DNA isolation, 5 mL of liquid culture was harvested by centrifugation, and the pellet was washed with 1 mL of water. The cell pellet was resuspended in 500 μL of lysis buffer (100 mM TRIS, 50 mM EDTA, 1% (w/v) SDS, pH 8), and acid-washed glass beads were added to a final volume of 1.25 mL. The mixture was vortexed for 2 min, and the recovered supernatant was transferred into a new microfuge tube. 275 μL of 7 M ammonium acetate pH 7 was added, and the solution was incubated at 65 °C for 5 min and then further on ice for 5 min. 500 μL of chloroform was added, and the mixture was vortexed for 2 min. Following a centrifugation step at 13 000 rpm for 5 min, the recovered aqueous phase was added to 800 μL of ice-cold isopropanol. The genomic DNA was precipitated by centrifugation (5 min, 4 °C, 13 000 rpm), prior to 5 min incubation on ice. Subsequently, the pellet was washed with 500 μL of ice-cold 70% ethanol solution, dried, and resuspended in 50 μL of EB buffer (10 mM TRIS, pH 8.5).

Construction of Recombinant Expression Vectors. The *rmo* and *rft* were amplified from genomic DNA using the primers listed in Table 1, with Phusion High-Fidelity DNA Polymerase (NEB) following the manufacturer's protocol with modifications for GC-rich DNA templates (5% DMSO final). Amplicons were purified in accordance with QIAgen gel extraction purification kit instructions, digested with the corresponding endonucleases (NEB), and cloned into pET28a-(+) (Novagen) with T4 ligase (NEB). The construct was used to transform *E. coli* TOP10 cells (Invitrogen), and after verification of the correct fragment insertion (sequencing by GATC Biotech), *E. coli* BL21 (DE3) (Novagen) was transformed for subsequent expression.

Expression and Purification of the Recombinant Proteins. Expression and purification of the recombinant Rmo, Rft, and CchA were performed as follows.[12] An overnight culture was diluted 1/100 in LB medium (supplemented with 50 μg/mL kanamycin) and grown at 25 °C at 230 rpm until $OD_{600} \sim 0.5$ was reached. Protein expression was induced with IPTG (50 μM), followed by incubation at 25 °C for 4 h. Cells were harvested by centrifugation (6000 rpm, 4 °C, 15 min), resuspended in HEPES A buffer (50 mM HEPES, 300 mM NaCl, pH 8), and frozen at −20 °C until further processing. Cells were lysed via French press (SLM Aminco), and after a centrifugation step (17 000 rpm, 4 °C, 30 min), the cleared lysate was applied to a Ni-NTA column using an ÄktaPrime system (Amersham Pharmacia Biotechnology). The elution was carried out using a linear gradient from 3 to 50% HEPES B buffer (50 mM HEPES, 250 mM imidazole, 300 mM NaCl, pH 8) in 30 min, followed by a linear increase to 100% B in 10 min. Protein-containing fractions were analyzed by SDS-PAGE, pooled, dialyzed against 25 mM HEPES, 100 mM NaCl, pH 7.5 buffer, and concentrated with Amicon Ultra-15 concentrators (Millipore). Final protein concentration was determined by Bradford colorimetric assay using a BSA calibration curve.[16] Protein aliquots were flash-frozen in liquid nitrogen and stored at −80 °C.

Analytical Size-Exclusion Chromatography. 25 μL of a 50 μM Rmo or Rft solution was analyzed using a Superdex 200 5/150 GL column combined with an ÄktaPurifier system (Amersham Pharmacia Biotechnology) equilibrated with 25 mM TRIS, 150 mM NaCl, pH 8 buffer. Protein elution was monitored at 280 nm. Aldolase (158 kDa), ovalbumin (43 kDa), ribonuclease (13.7 kDa), and aprotinin (6.5 kDa) were used as standards to determine the molecular weight of the proteins in solution.

Table 1. List of Primers Used in This Study

primer name	restriction site	sequence (5′−3′)
rftF	*NdeI*	GGAATTCCATATGAGAGTCGCCACACTCGGATATC
rftR	*NotI*	ATAAGAATGCGGCCGCTCAGCTGAGGTAGCCGCCG
rmoF	*NdeI*	GGAATTCCATATGAGTGAATCGCCGGAAACGGTCG
rmoR	*NotI*	ATAAGAATGCGGCCGCTCATCTCGCCTCGCTTGTCGCATAC

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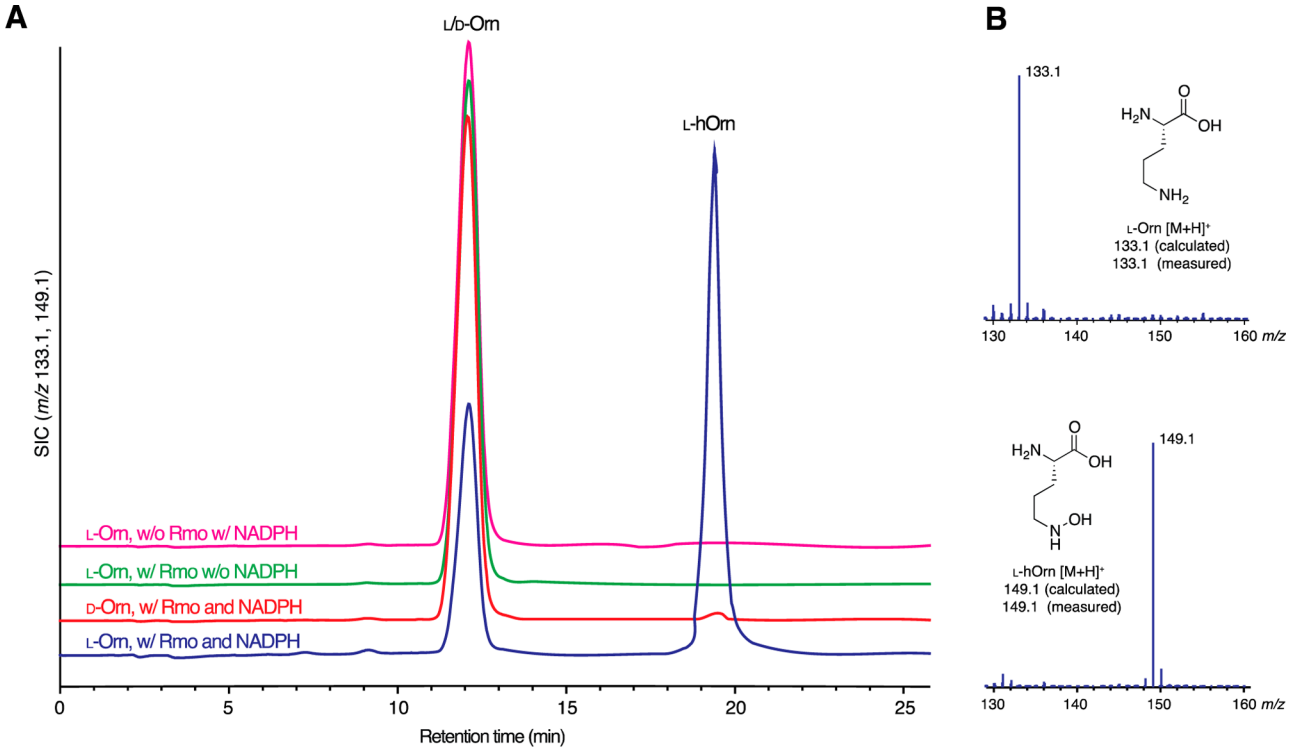

Figure 2. Rmo-mediated L-Orn hydroxylation. (A) HPLC-MS single-ion chromatogram (SIC) of the hydroxylation assays is shown: in the presence of the L-Orn substrate and the reducing cosubstrate NADPH, Rmo catalyzes the conversion of L-Orn to L-hOrn (blue trace). The control reactions evidence that Rmo is unable to hydroxylate D-Orn (red trace) and that the reaction does not proceed if either NADPH or the enzyme is missing (green and purple traces, respectively). (B) Chemical structures and observed ESI-MS spectra of the L-Orn substrate and the L-hOrn product.

Rmo-Mediated L-Orn Hydroxylation. A 50 μL reaction contained the following: 100 mM TRIS pH 8, 1 mM L-Orn, 2 mM NADPH, 25 μM FAD, and 20 μM Rmo. Reaction controls included the absence of cosubstrate NADPH or enzyme as well as a panel of different amino acids tested for substrate specificity (Supporting Information Table 1). Reactions were incubated for 4 h at 30 °C and stopped by the addition of 2 μL of formic acid and analyzed via HPLC-MS (see below).

Determination of the kinetic parameters for the Rmo-dependent hydroxylation was performed maintaining the NADPH and FAD concentration at 0.5 mM and 20 μM, respectively, and varying L-Orn substrate concentration between 0.10 and 20 mM. Reactions were started by the addition of Rmo to a final concentration of 5 μM and stopped with 2 μL of formic acid after 2.5 min incubation. Product formation was quantified via HPLC-MS, using a L-hOrn calibration curve. All reactions were perfomed in triplicate.

Synthesis of the Formyl-Donor Cosubstrate Intermediate N^5,N^{10}-methenylH_4F. In the first half-reaction, 7 mg of N^5-fH_4F was dissolved in 1.5 mL of water and was converted to N^5,N^{10}-methenylH_4F by dropwise addition of 0.1 M HCl, until pH 1.9 was reached. The solution was then brought to a final volume of 2.2 mL with water and further incubated at room temperature for 4 h. The formation of N^5,N^{10}-methenylH_4F resulted in a color change of the solution, from colorless to pale yellow, and was further verified via ESI-MS measurements (Supporting Information Figure S1). The obtained compound (~6 mM final concentration) was aliquoted and stored at −20 °C until usage.

***In Situ* N^{10}-fH_4F Conversion and L-hOrn Formylation Assay.** The transformylation reaction assay was setup in a 50 μL volume, in the presence of 50 mM HEPES buffer pH 7.5, 1 mM L-hOrn, 1.5 mM N^5,N^{10}-methenylH_4F, and 25 μM Rft (or CchA). Prior to the addition of the amino acid and the enzyme, the cosubstrate intermediate was preincubated in the reaction buffer at 30 °C for 30 min, to permit the final pH-dependent conversion to N^{10}-fH_4F. After the addition of the substrate and the enzyme, the reaction was allowed to proceed for 4 h at 30 °C, then stopped by the addition of 2 μL formic acid, and analyzed via HPLC-MS. L-hOrn was synthesized according to a protocol previously described.[17]

The kinetic parameters for Rft-mediated transformylation were determined by maintaining the N^5,N^{10}-methenylH_4F concentration at 1.5 mM and varying the L-hOrn between 0.25 and 15 mM. Prior to the beginning of the reaction, the final N^5,N^{10}-methenylH_4F conversion to N^{10}-fH_4F was allowed as described. Reactions were started by adding Rft to a final 5 μM concentration and stopped with 2 μL of formic acid after 5 min incubation. Product formation was quantified via HPLC-MS. All reactions were performed in triplicate.

Coupled L-Orn Hydroxylation and Formylation. In a similar fashion, coupled enzymatic assays containing both enzymes have been performed in HEPES buffer, where the N^{10}-fH_4F cosubstrate has first been generated as described, followed by the addition of all the other components needed for the reaction. A typical assay in HEPES buffer contained 1 mM L-Orn, 2 mM NADPH, 1.5 mM N^5,N^{10}-methenylH_4F, 40 μM FAD, 25 μM Rmo, and 25 μM Rft. The reaction was carried out for 4 h, stopped with 2 μL of formic acid, and analyzed via HPLC-MS.

HPLC-MS Analysis of the Enzymatic Assays. All enzymatic assays were analyzed on a Hypercarb column (Thermo Fisher) combined with an Agilent 1100 HPLC system connected to an ESI-MS detector (Agilent 1100 MSD).

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Table 2. Kinetic Parameter for Rmo-Mediated L-Orn Hydroxylation and Comparison with Other Homologues L-Orn Monooxygenases Involved in Siderophore Biosynthesis

enzyme	siderophore	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} mM^{-1})
Rmo[a]	rhodochelin	1.6 ± 0.2	0.2331 ± 0.008	0.15
EtcB[b] (13)	erythrocelin	0.286 ± 0.035	0.3267 ± 0.0005	1.14
CchB[b] (12)	coelichelin	3.6 ± 0.58	0.290 ± 0.01	0.081
VbsO[b] (18)	vicibactin	0.305 ± 0.024	1.80 ± 0.03	5.90
PvdA[a] (11)	pyoverdin	0.60 ± 0.07	0.400 ± 0.05	0.67
SidA[a] (9)	ferrichrome	1.70 ± 0.06	0.4833 ± 0.005	0.284
SidA[a] (10)	ferrichrome	0.58 ± 0.07	0.611	1.0

[a]Steady state parameters for hydroxylated product formation. [b]Steady state parameters for coupled NADPH oxidation assay.

20 mM aqueous NFPA (solvent A) and acetonitrile (solvent B) were used, employing a linear gradient from 0% to 15% B within 25 min, followed by a linear increase to 100% B in 2 min and finally holding B for an additional 3 min. The flow rate was set to 0.2 mL/min and the column temperature to 20 °C. The elution was monitored in single-ion mode (SIM). Additional high-resolution FTICR-MS (HR-MS) measurements were carried out on a LTQ-FT instrument (Thermo Fisher Scientific).

RESULTS

Biochemical Characterization of L-Orn Hydroxylation. In a previous study, it has been demonstrated that *rmo* encodes for a putative L-Orn monooxygenase involved in rhodochelin biosynthesis, since its deletion from the chromosome results in an abolished siderophore production. *rmo* is located in a cryptic cluster (rhodochelin cluster 2), whose product has only been postulated on the basis of the predicted A-domain specificities and the presence of a second ORF encoding for a formyltransferase function (*rft*, see further).[14]

A bioinformatic analysis showed that Rmo belongs to the class of NADPH/FAD-dependent and, when compared to already characterized homologues (CchB, EtcB, PvdA, SidA, and IucD), it displays an overall sequence conservation, especially on the sites involved in substrate, NADPH, and FAD binding (Figure S2).[6,8,9,11−13] *rmo* was amplified from the *R. jostii* RHA1 chromosome and cloned into the pET28a(+) expression vector. The recombinant protein was heterologously produced in *E. coli* as an N-terminal His-tag fusion and purified via Ni-NTA affinity chromatography. The enzyme was purified in the *apo* form, without bound FAD cofactor (Figure S3). The estimated molecular mass of Rmo in solution was calculated to be 220 kDa, suggesting the enzyme to adopt a tetrameric quaternary structure (Figure S4).

In order to investigate if Rmo is able to catalyze the conversion of L-Orn to L-hOrn, substrate and enzyme were incubated in the presence of the NADPH cosubstrate and the FAD cofactor. After a 4 h incubation, HPLC-MS analysis revealed 65% conversion of L-Orn (t_R 12.1 min, m/z 133.1 $[M + H]^+$ observed, m/z 133.1 $[M + H]^+$ calculated) to L-hOrn (t_R 19.4 min, m/z = 149.1 $[M + H]^+$ observed, m/z 149.1 $[M + H]^+$ calculated) in the presence of the enzyme and molecular oxygen (Figure 2). HR-MS analysis confirmed the identity of L-hOrn (m/z 149.0916 $[M + H]^+$ observed, 149.0921 $[M + H]^+$ calculated). No enzymatic conversion was observed if either Rmo or NADPH was omitted or if NADH was used as the reducing cosubstrate. In addition, the enzyme showed exclusive substrate specificity toward L-Orn over a panel of different amino acids (Supporting Information Table 1).

Kinetic parameters for Rmo-mediated L-Orn hydroxylation were determined monitoring the product formation and plotting the starting velocity as a function of the increasing substrate concentration. The kinetic parameters were determined using a Michaelis−Menten equation plot and were calculated to an apparent K_M = 1.6 ± 0.2 mM and k_{cat} = 0.2331 ± 0.008 s^{-1}, resulting in a catalytic efficiency k_{cat}/K_M = 0.15 s^{-1} mM^{-1} (Figure S5). When compared with other L-Orn monooxygenase homologues, the obtained values are in accordance with previously published results, despite the fact that Rmo does not possess similarly high catalytic efficiency as EtcB or VbsO (Table 2).[13,18]

In conclusion, Rmo represents a typical member of the NADPH/FAD-dependent monooxygenases required for the *δ*-*N*-hydroxylation of L-Orn or L-Lys side chains associated with the biosynthesis of hydroxamate-type siderophores.[6]

Biochemical Characterization of the L-hOrn Formylation. Similarly to *rmo*, *rft* is essential for rhodochelin biosynthesis.[14] A bioinformatic analysis showed that Rft exhibits an overall sequence conservation compared to the proposed formyltransferases CchA and AmcP, putatively involved in the generation of the formyl-derived iron-coordinating hydroxamate moieties in coelichelin and amychelin, respectively.[15,19] Additionally, the sequence homology also extendes to the N-terminal domain of ArnA (a bifunctional enzyme required for the generation of a lipid A analogue essential for polymixine resistance in *Escherichia* and *Salmonella* spp.) and to the endogenous and essential bacterial methionyl-$tRNA^{fMet}$-formyltransferase.[20,21] In a derived phylogenetic tree, these sequences clearly clusters into different clades, according to their different substrate specificities (Figure S6).

Additionally, Rft shares a bimodular organization with the above-mentioned enzymes (Figure S7).[22] The N-terminal subdomain displays typical elements for tetrahydrofolate binding enzymes: the catalytic Asn, His, Asp triad, and the N^{10}-fH_4F "SLLP" binding motif. These conserved residues are also found in the formyltransferases FxbA and PvdF (associated with the biosynthesis of L-fhOrn in exochelin and pyoverdine systems), the N-terminal formylation domain of the initiation module of the linear gramicidine NRPS LgrA1, and the glycinamide ribonucleotide formyltransferases (GARF) family proteins, although in this latter case, additional structural differences and less sequence homology have already been reported (Figure S8).[21,23−26] On the other hand, the overall sequence homology between Rft and its above-discussed closest homologues decreases through the C-terminal subdomain, which seems not to be involved in catalysis and, as demonstrated for the methionyl-$tRNA^{fMet}$-formyltransferase, could be associated with proper substrate recognition.[23]

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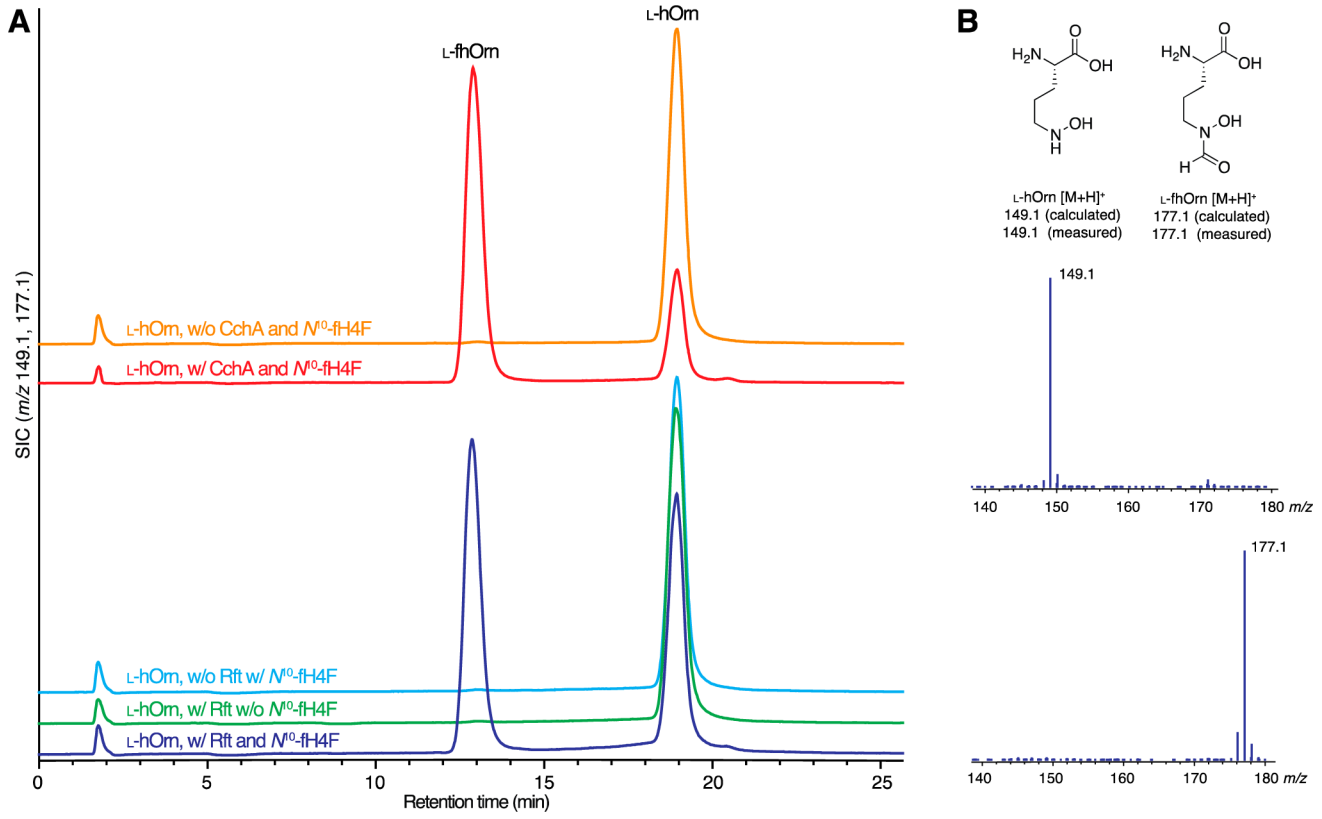

Figure 3. Rft- and CchA-mediated L-hOrn formylation. (A) Lower traces: HPLC-MS single-ion chromatogram (SIC) of the formyltransferase reaction assay is shown: in the presence of the L-hOrn substrate and the *in situ* generated N^{10}-fH$_4$F formyl-donor cosubstrate, Rft catalyzes the conversion of L-hOrn to L-fhOrn (blue trace). Control reactions were carried out in the absence of the donor cosubstrate or the enzyme (green and light blue traces, respectively). Upper traces: a similar assay performed in the presence or the absence of CchA (red and orange traces, respectively) rescues the enzymatic activity of this Rft homologue from the coelichelin biosynthesis gene cluster, which was previously reported to be inactive. (B) Chemical structures and observed ESI-MS spectra of the L-hOrn substrate and the L-fhOrn product.

In order to investigate the role of Rft in L-fhOrn biosynthesis, the corresponding gene was amplified and cloned in pET28a-(+) expression vector. The recombinant protein was purified as an N-terminal His-tag fusion and tested for *in vitro* activity (Figure S9). The required N^{10}-fH$_4$F cofactor was generated *in situ* from its N^5,N^{10}-methenylH$_4$F intermediate, through a 30 min preincubation in the assay buffer. In the presence of L-hOrn and N^{10}-fH$_4$F, Rft was able to transfer the formyl group from the donor cosubstrate to the side chain of the hydroxylated amino acid. After a 4 h incubation, HPLC-MS analysis revealed 55% conversion of L-hOrn (t_R 18.9 min, m/z 149.1 $[M + H]^+$ observed, m/z 149.1 $[M + H]^+$ calculated) to L-fhOrn (t_R 12.9 min, m/z = 177.1 $[M + H]^+$ observed, m/z 177.1 $[M + H]^+$ calculated; Figure 3). L-fhOrn identity was confirmed by HR-MS analysis (m/z 177.0871 $[M + H]^+$ observed, 177.0870 $[M + H]^+$ calculated). No conversion was observed in the absence of enzyme or cosubstrate. In addition, in similar assay conditions, Rft is unable to formylate L-Orn or both L-Orn and L-hOrn if N^5-fH$_4$F was used as the donor cofactor (data not shown).

Previous attempts to characterize Rft using the N^{10}-fH$_4$F cosubstrate generated via a chemoenzymatic synthesis approach (chemical conversion of H$_4$F to N^5,N^{10}-methyleneH$_4$F in the presence of formaldehyde, followed by a FolD-catalyzed regiospecific oxidation/cyclohydrolysation) always resulted in no detectable enzymatic activity (data not shown).[27] Similar results were obtained during the characterization of CchA, which left unclear whether the enzyme was purified in a soluble but inactive form or was rendered inactive by the incubation in the reaction assay.[12] Therefore, on the basis of the obtained results for Rft, CchA was expressed, purified, and assayed for enzymatic activity. As shown in Figure 3, CchA was able to catalyze the conversion of L-hOrn to L-fhOrn (75% after 4 h incubation).

The kinetic parameters of Rft tranformylation were determined monitoring the conversion of substrate to product and plotting the starting velocities as a function of the increasing substrate concentration. Surprisingly, the experimental data could be fitted best using a sigmoidal curve, indicative of an allosteric kinetic mechanism, rather than the typical rectangular hyperbola characteristic of classical Michaelis–Menten kinetic (Figure S10).[28] Normalized parameter values were calculated through the nonlinear Hill-fit regression and were found to be equal to a V_{max} = 0.078 ± 0.001 μmol L^{-1} s^{-1}, a $K_{0.5}$ = 1.2 ± 0.1 mM, and n = 2.7 ± 0.2. A value of the Hill parameter n greater than 1 is indicative of a positive cooperative mechanism. To further clarify the origin of this cooperative behavior, analytical size exclusion chromatography was employed to elucidate the oligomeric state of Rft.[29] As shown in Figure S4, the estimated molecular weight of Rft was 146 kDa, suggesting the enzyme to adopt a tetrameric quaternary structure in solution.

L-fhOrn Coupled Enzymatic Biosynthesis. In order to verify whether Rmo and Rft were able to act in tandem to generate L-fhOrn from the L-Orn substrate, a similar assay to the Rft-dependent L-hOrn transformylation was set up, where

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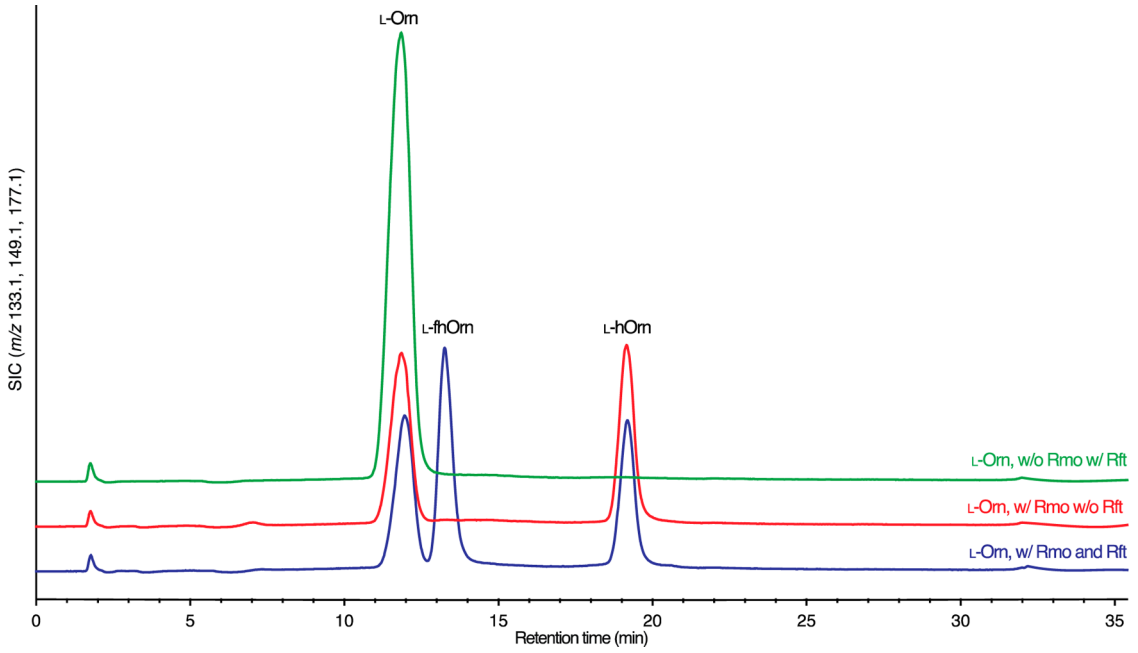

Figure 4. HPLC-MS single-ion chromatogram (SIC) of the coupled enzymatic biosynthesis of L-fhOrn from its L-Orn precursor in the presence of the Rmo monooxygenase and the Rft formyltransferase (blue trace). If Rft is omitted, the reaction stops generating only the L-hOrn intermediate (red trace); if Rmo is excluded, no conversion at all is observed (green trace).

first the N^{10}-fH$_4$F cosubstrate was generated *in situ*, followed by the addition of all the remaining components needed for the enzymatic tandem conversion to L-fhOrn. Comparison of HPLC-MS traces showed the substrate conversion to the L-hOrn intermediate and the L-fhOrn product if both enzymes were present or, as expected, only to L-hOrn if Rft was omitted. On the other hand, if Rmo was missing, no substrate conversion was observed (Figure 4).

DISCUSSION

The use of hydroxamate groups as iron-coordinating moieties is a shared strategy employed by both NRPS-dependent and NRPS-independent enzymes for siderophores biosynthesis.[4] In both cases, it generally requires the hydroxylation of the lateral amino group of a basic amino acid (lysine or ornithine), followed by the additional transfer of an acetyl or formyl group to the secondary amine intermediate, generating the functional hydroxamate moiety. This building block is subsequently incorporated by the NRPS-dependent or NRPS-independent assembly enzymes into the siderophore peptide scaffold.[6,30] In this study we report the biochemical characterization of Rmo and Rft, the tailoring enzymes required for the biosynthesis of the L-fhOrn moieties found in rhodochelin, the siderophore isolated from *R. jostii* RHA1.[14]

On the basis of a bioinformatic analysis, Rmo was predicted to belong to the *N*-hydroxylating flavoprotein monooxygenases (NMO), of which different homologues have previously been characterized through extensive biochemical and structural studies.[6,9−13,18] The recombinant enzyme was purified in the *apo* form, without bound FAD cofactor. The crystal structure of PvdA (Rmo homologue from *P. aeruginosa*) demonstrates that the FAD binding site is located in close proximity to the surface of the enzyme. Therefore, the loss of the flavin cofactor during purification is not unexpected.[31] As other members of the NMO family, Rmo shows an exclusive preference toward its cognate amino acid substrate and the reducing cosubstrate NADPH. In addition, likewise SidA, Rmo adopts a tetrameric quaternary structure in solution, which does not alter the catalytic properties of the single subunits, resulting in a classical Michaelis−Menten kinetic.[9]

The biochemical characterization of Rft represents the first *in vitro* study of a tailoring formyltransferase involved in the biosynthesis of formyl-based iron-coordinating hydroxamate moieties. In fact, previous attempts to elucidate the role of homologous and analogous enzymes (Ccha, PvdF, and FxbA) were not successful or relied on the use of genetic strategies employing indirect detection methods.[12,24,25] Additionally, in contrast to the endogenous methionyl-tRNA^{fMet}-formyltransferase, ArnA, and the F-domain embedded within the LgrA1 initiation module, Rft catalyzes the formylation of a secondary amine, whereas the previously mentioned enzymes modify a primary amino group.[20,21,26]

The use of an alternative strategy for the generation of the unstable, and thus commercially unavailable, N^{10}-fH$_4$F cosubstrate, namely the *in situ* transformation of N^5,N^{10}-methenylH$_4$F to the desired N^{10}-fH$_4$F, through preincubation in the assay buffer, has proved to be successful, resulting in enzymatically active cofactor. Likewise, this strategy was applied to the hitherto inactive CchA, promoting the conversion of L-hOrn to L-fhOrn, implying a similar biosynthetic mechanism for the same iron-coordinating group in the siderophore coelichelin.[12]

Rft kinetic characterization indicates a positive cooperative effect, possibly resulting from the fact that, in solution, the enzyme adopts a tetrameric quaternary structure. In this context, the conserved presence of the C-terminal subdomain (which does not feature any catalytic residues) could be seen as a modification of the original tRNA binding function of the methionyl-tRNA^{fMet}-formyltransferase to an oligomerization and/or allosteric role in Rft.[23] In addition, the positive cooperative control mechanism could improve the overall biosynthesis of the non-proteinogenic L-fhOrn amino acid (and

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thus its incorporation by the NRPS assembly line), providing an enhancement for siderophore biosynthesis under the restrictive iron-limiting growing conditions.

In conclusion, on the basis of the results of the coupled assay and the additional inability of Rmo to hydroxylate L-fOrn and of Rft to transformyate L-Orn, a model for the biosynthesis of the formyl-based hydroxamate-containing siderophores could be proposed, according to the so-called "hydroxylation first" mechanism, recently described for the acetyl-based hydroxamates.[13] Initially, the L-Orn side chain amino group is hydroxylated by a NMO enzyme, and then the newly modified L-hOrn could be either incorporated by the NRPS assembly line (coelichelin) or further modified by formylation, leading to the generation of iron-coordinating L-fhOrn (coelichelin or rhodochelin).

■ ASSOCIATED CONTENT

Supporting Information

Substrate specificity table for Rmo-mediated hydroxylation, reaction scheme, and ESI-MS measurements for N^5,N^{10}-methenylH_4F intermediate synthesis, SDS-PAGE of purified recombinant enzymes, analytical size exclusion chromatography, and results of the bioinformatics analysis and kinetic parameter determination for both Rmo and Rft. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

A-domain, adenylation domain; BSA, bovine serum albumin; CoA, coenzyme A; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; ESI-MS, electrospray ionization mass spectrometry; F-domain, formylation domain; FAD, flavin adenine dinucleotide; FTICR-MS, Fourier transform ion cyclotron resonance mass spectrometry; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl β-D-1-thiogalactopyranoside; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; L-Orn, L -ornithine; L-fOrn, L-δ-*N*-formylornithine; L-fhOrn, L-δ-*N*-formyl-δ-*N*-hydroxyornithine; L-hOrn, L-δ-*N*-hydroxyornithine; LB, Luria–Bertani; MS, mass spectrometry; N^5-fH_4F, N^5-formyl-tetrahydrofolate; N^5,N^{10}-methenylH_4F, N^5,N^{10}-methenyl-tetrahydrofolate; N^5,N^{10}-methyleneH_4F, N^5,N^{10}-methylene-tetrahydrofolate; N^{10}-fH_4F, N^{10}-formyl-tetrahydrofolate; NMO, *N*-hydroxylating flavoprotein monooxygenase; Ni-NTA, Ni-nitrilotriacetic acid; NFPA, nonafluoropentanoic acid; NRPS, nonribosomal peptide synthetase; ORF, open reading frame; PCP, peptidyl carrier protein; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SIC, single-ion chromatogram; SIM, single-ion mode; TRIS, tris(hydroxymethyl)aminomethane.

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