CloR, a Bifunctional Non-heme Iron Oxygenase Involved in Clorobiocin Biosynthesis*

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Florence Pojer‡, Rainer Kahlich§, Bernd Kammerer§, Shu-Ming Li‡, and Lutz Heide‡¶

From the ‡Universität Tübingen, Pharmazeutische Biologie, Auf der Morgenstelle 8, 72076 Tübingen, Germany and the ¶Universität Tübingen, Institut für Pharmakologie und Toxikologie, Abteilung Klinische Pharmakologie, Otfried-Müller-Str. 45, 72076 Tübingen, Germany

The aminocoumarin antibiotics novobiocin and clorobiocin contain a 3-dimethylallyl-4-hydroxybenzoate (3DMA-4HB) moiety. The biosynthesis of this moiety has now been identified by biochemical and molecular biological studies. CloQ from the clorobiocin biosynthetic gene cluster in Streptomyces roseochromogenes DS 12976 has recently been identified as a 4-hydroxyphenylpyruvate-3-dimethylallyltransferase. In the present study, the enzyme CloR was overexpressed in Escherichia coli, purified, and identified as a bifunctional non-heme iron oxygenase, which converts 3-dimethylallyl-4-hydroxyphenylpyruvate (3DMA-4HPP) via 3-dimethylallyl-4-hydroxymandelic acid (3DMA-4HMA) to 3DMA-4HB by two consecutive oxidative decarboxylation steps. In 18O2-labeling experiments we showed that two oxygen atoms are incorporated into the intermediate 3DMA-4HMA in the first reaction step, but only one further oxygen is incorporated into the final product 3DMA-4HB during the second reaction step. CloR does not show sequence similarity to known oxygenases. It apparently presents a novel member of the diverse family of the non-heme iron (II) and α-ketoacid-dependent oxygenases, with 3DMA-4HPP functioning both as an α-keto acid and as a hydroxylation substrate. The reaction catalyzed by CloR represents a new pathway for the formation of benzoic acids in nature.

Novobiocin and clorobiocin contain a prenylated 4-hydroxybenzoate moiety (called Ring A) (Fig. 1A). Recently, Lafitte et al. (9) demonstrated that Ring A plays a role in the binding affinity of these antibiotics for gyrase. Knowledge of the biosynthetic pathway of Ring A may facilitate future efforts to create structural modifications of this moiety by combinatorial biosynthesis.

The dimethylallyl moiety of Ring A is derived from the methyl erythritol-4-phosphate (MEP) pathway (10). The 4-hydroxybenzoic acid (4HB)3 moiety can be formed in nature by at least three well-established mechanisms: (a) the direct conversion of chorismic acid to 4HB by chorismate pyruvate-lyase (11); (b) the removal of acetyl-CoA from the side chain of 4-coumaroyl-CoA by an oxidative reaction mechanism, analogous to the β-oxidation of fatty acids, resulting in 4-hydroxybenzoyl-CoA (12, 13); and (c) the removal of a C2 unit from the side chain of a β-hydroxy-β-phenylpropionate derivative via a retro-aldol reaction; the resulting benzaldehyde derivate can subsequently be oxidized to the corresponding acid (14, 15).

In novobiocin biosynthesis, feeding experiments with isotope-labeled precursors showed that 4-hydroxyphenylpyruvate and tyrosine are efficiently incorporated into Ring A (10, 16), ruling out the chorismate pyruvate-lyase reaction as the principal source of this compound. The aminocoumarin moiety of novobiocin (Ring B) is formed from tyrosine via the intermediate β-hydroxytyrosyl-S-NO2H. Chen and Walsh (17) suggested that Ring A may also be formed from this intermediate, following the reaction mechanism shown in Fig. 2A, i.e. by a retro-aldol reaction to 4-hydroxybenzaldehyde, oxidation to the acid and prenylation. 3-Prenylation of 4HB has a well-established precedent in ubiquinone biosynthesis (18).

Cloning and sequencing of the biosynthetic gene clusters of novobiocin, clorobiocin, and coumermycin A1 (Fig. 1B) (19) first appeared to support this hypothesis. Three genes were identified in the novobiocin and clorobiocin clusters for which no homologues existed in the coumermycin cluster. We speculated that these genes might be involved in the biosynthesis of Ring A (which is absent in coumermycin A1). These genes were: (a) cloR and novR, which showed sequence similarity to putative aldolases; CloR was indeed shown to be involved in Ring A biosynthesis by a gene inactivation experiment (19); (b) cloF and novF, which show sequence similarities to dehydrogenases; and (c) cloQ and novQ, which did not show sequence similarities to known genes in the data base. We recently

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†To whom correspondence should be addressed. Tel.: 49-7071-297-2460; Fax: 49-7071-295-250; E-mail: heide@uni-tuebingen.de.

The abbreviations used are: 4HB, 4-hydroxybenzoic acid; 4HPP, 4-hydroxyphenylpyruvate; 3DMA-4HPP, 3-dimethylallyl-4-hydroxybenzoate; 3DMA-4HMA, 3-dimethylallyl-4-hydroxymandelic acid; 3DMA-4HB, 3-dimethylallyl-4-hydroxybenzaldehyde; 3DMA-4HBAL, 3-dimethylallyl-4-hydroxybenzoate; IPTG, isopropyl-1-thio-β-d-galactopyranoside; GST, glutathione S-transferase; LC, liquid chromatography; MS, mass spectrometry; MS-MS, tandem mass spectrometry; HPLC, high performance liquid chromatography; HmaS, 4-hydroxymandelate synthase; DMAPP, dimethylallyl diphosphate.
Figure 1. A, structures of the aminocoumarin antibiotics. B, map of the biosynthetic gene cluster of the aminocoumarin antibiotics: novobiocin (top), clorobiocin (middle), and coumermycin A₁ (bottom).

Figure 2. Previous hypotheses for the formation of the 3-dimethylallyl-4-hydroxybenzoate moiety (Ring A) of novobiocin and clorobiocin.
confirmed that CloQ codes for a prenyltransferase (20). The substrate of this enzyme, however, was none of the intermediates discussed above but, rather, 4-hydroxyphenylpyruvate (4HPP, Fig. 2B). The product of the reaction, i.e. 3-dimethylallyl-4HPP, was easily degraded to 3-dimethylallyl-4-hydroxybenzaldehyde (3DMA-4HBAL), suggesting a biosynthesis of Ring A by the mechanism depicted in Fig. 2B, i.e. via oxidation of 3DMA-4HBAL to the corresponding acid. 

In the present study, we investigated the conversion of 3DMA-4HPP to Ring A and the role of CloR in this reaction sequence. Unexpectedly, the results showed that the biosynthesis of Ring A follows neither of the two mechanisms depicted in Fig. 2. Rather, the conversion of 3DMA-4HPP to Ring A proceeds in two oxidative decarboxylation steps, via 3-dimethylallyl-4-hydroxybenzaldehyde (3DMA-4HMA) as intermediate, and is catalyzed by the bifunctional non-heme iron oxygenase CloR. This represents a new pathway for the formation of benzoic acids in nature.

**EXPERIMENTAL PROCEDURES**

**General Chemicals**—4-Hydroxyphenylpyruvate (4HPP), D- and L-mandelic acid, D-4-hydroxyxymandelic acid, and 4-hydroxyphenyllactic acid were purchased from Sigma. β-(R,S)-Hydroxy-L-tyrosine and DMAPP were kindly provided by Dr. K.-H. van Pée (Dresden, Germany) and Dr. K. Yazaki (Kyoto, Japan), respectively. 3-Dimethylallyl-4-hydroxybenzaldehyde (3DMA-4HBAL) was synthesized as described by Glusenkamp and Buchi (21). Ring A (3-dimethylallyl-4-hydroxybenzoic acid) was prepared as described previously (22). Oxygen ⁸⁰O₂ (99%) was purchased from Campreo Scientific (Berlin, Germany).

**DNA Manipulation**—DNA manipulations and standard genetic techniques in *Escherichia coli* were carried out as described by Sambrook and Russell (23). DNA fragments were isolated from agarose gels using a NucleoSpin 2 in 1 extraction kit (Macherey-Nagel). DNA was digested with BamHI and XhoI, gel purified and ligated into the same site of pGEX-4T-1 (Amersham Biosciences) to give cloR-pGEX4T1. The plasmid was transformed into *E. coli* strain BL21(DE3) for overexpression of CloR as GST (glutathione S-transferase)-tagged protein.

**Overexpression and Purification of CloR Protein**—*E. coli* BL21(DE3)/cloR-pGEX4T1 cells were grown in 100 ml of LB medium containing 50 µg/ml carbenicillin at 15 °C until an A₅₅₀ of 0.6 was reached (~20 h). IPTG was added to a final concentration of 250 µM. After 24 h at 15 °C, the cells were harvested by centrifugation (10 min at 5,000 × g) and resuspended in phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) containing 1 mg/ml lysozyme (76,344 units/mg, Fluka). Resuspended cells were broken by sonication (Branson sonifier, 10-min sonification). Cell debris was removed by centrifugation (30 min at 20,000 × g). Purification of CloR as a GST fusion protein and subsequent cleavage of the GST tag by thrombin treatment were carried out according to the manufacturer’s instructions (Amersham Biosciences) using glutathione-Sepharose 4B and the batch method.

**Overexpression and Purification of CloQ**—Construction of vector cloQ-pGEX4T1, overexpression in *E. coli* BL21(DE3), and purification of CloQ were carried out as described in Pojer et al. (20).

**Protein Analysis**—Standard protein techniques were used as described in previous studies (24, 25). The molecular mass of native CloR was determined by gel filtration on a HiLoad 26/60 Superdex 200 column that has been equilibrated with 50 mM Tris-HCl buffer (pH 8.0), containing 150 mM NaCl. The column was calibrated with dextran blue 2000 (2000 kDa), aldolase (158 kDa), albumin (67 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa) (Amersham Biosciences). DNA fragments were isolated from agarose gels using a NucleoSpin 2 in 1 extraction kit (Macherey-Nagel). DNA was digested with BamHI and XhoI, gel purified and ligated into the same site of pGEX-4T-1 (Amersham Biosciences) to give cloR-pGEX4T1. The plasmid was transformed into *E. coli* strain BL21(DE3) for overexpression of CloR as GST (glutathione S-transferase)-tagged protein.

**Overexpression and Purification of CloQ**—Construction of vector cloQ-pGEX4T1, overexpression in *E. coli* BL21(DE3), and purification of CloQ were carried out as described in Pojer et al. (20). A reaction mixture (50 µl) containing 75 mM Tris-HCl (pH 7.5), 0.25 mM 4HPP, 0.5 mM DMAPP, 2.5 mM MgCl₂, and purified CloQ, was incubated at 30 °C for 1 h. This reaction produces about 7.5 nmol of 3DMA-4HPP.

**Incubation of Holo-CloR with CloQ Reaction Products**—3DMA-4HPP, the substrate for CloR reaction, was obtained by incubating CloQ with 4HPP and DMAPP as described in Pojer et al. (20). After 24 h at 15 °C, the reaction mixture (50 µl) containing 75 mM Tris-HCl (pH 7.5), 0.25 mM 4HPP, 0.5 mM DMAPP, 2.5 mM MgCl₂, and purified CloQ, was incubated at 30 °C for 1 h. This reaction produces about 7.5 nmol of 3DMA-4HPP.
Holo-CloR was reconstituted by preincubation of apo-CloR (~4 μg) with 1 mM FeSO₄ for 20 min at room temperature. Reaction conditions for the incubation of holo-CloR with 3DMA-4HPP were as described for HmaS (26, 27) with minor modifications. The reaction (150 μl) contained 50 μl of the CloQ reaction mixture (final concentration of 3DMA-4HPP, ~50 μM), 0.2 M Tris-HCl (pH 7.5), 80 mM potassium phosphate

Fig. 5. ¹⁸O₂ labeling experiment with the bifunctional oxygenase CloR. A, molecular ions of 3DMA-4HMA and 3DMA-4HB resulting from 3DMA-4HPP under unenriched (left) and ¹⁸O₂-enriched (right) atmospheres. Fragmentation patterns are indicated. B, molecular ions of 3DMA-4HB resulting from 3DMA-4HMA under unenriched (left) and ¹⁸O₂-enriched (right) atmospheres. Fragmentation patterns are indicated. C, schematic summary of the incorporation of ¹⁸O₂ into 3DMA-4HB (Ring A) during the CloR reaction.

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Expression and Purification of CloR—We decided to express CloR as a glutathione S-transferase (GST) fusion protein rather than as an His-tagged protein, because CloR showed sequence similarity to class II aldolases, and these enzymes require zinc for their activity (31). A purification using the His-tagged protein can lead to a complete loss of the aldolase activity due to interactions between the hexahistidyl tag and the metal ion (Zn$^{2+}$). E. coli cells harboring CloR expression constructs yielded only insoluble protein when grown at temperatures of 20 °C or higher. To obtain soluble CloR-GST fusion protein, cells were cultured at 15 °C and induced with 250 μM IPTG. After purification, GST was cleaved from CloR by thrombin treatment and removed. This procedure resulted in apparently homogenous CloR protein as judged by SDS-PAGE (Fig. 3). The molecular mass observed in SDS corresponded to the calculated mass of the protein (30.5 kDa). A protein yield of 1 mg of pure CloR per liter of culture was obtained. By using gel chromatography, the molecular mass of native CloR was determined as 124.5 kDa showing that the protein was tetrameric in solution.

Characterization of the Reaction Products of the CloR Reaction—To investigate the catalytic activity of CloR, we first produced 3DMA-4HPP (Fig. 2B), the putative substrate of CloR, by incubation of 4-hydroxyphenylpyruvate (4HPP) with DMAPP and the prenyltransferase CloQ. Subsequently, CloR and different cofactors, e.g. Zn$^{2+}$ and NADH (32), were added to the reaction mixture. However, no formation of 4-hydroxybenzaldehyde (3DMA-4HBAL) or Ring A could be detected by HPLC.

A more sensitive analysis using a radioactive assay with 4HPP and (1-14C)DMAPP as substrates, however, revealed the presence of a small amount of a new radioactive compound (termed product X), with a retention time of 18.4 min in HPLC (data not shown). This product was absent if heat-denatured CloR was used, or if 4HPP or DMAPP were omitted from the prenylation assay. This indicated that the new metabolite X was derived enzymatically from 3DMA-4HPP.

In the biosynthesis of chloroeremomycin in Amycolatopsis orientalis, 4HPP is converted to 4-hydroxymandelate acid under catalysis of the non-heme iron dioxygenase HmaS (= ORF21) (26, 27). As described by Hubbard et al. (26), HmaS and similar enzymes need to be activated by preincubation with an excess of Fe$^{2+}$ immediately before incubation and Fe$^{3+}$, generated by oxidation, has to be reduced to Fe$^{2+}$ by ascorbic acid to maintain an active enzyme. Although CloR did not show sequence similarity to HmaS or other non-heme iron-dependent enzymes, we decided to test CloR under similar conditions. After preincubation of CloR for 20 min with 1 mM FeSO$_4$, ascorbic acid and enzymatically generated 3DMA-4HPP were added. After incubation for 1 h, the products of the reaction were analyzed by HPLC (Fig. 4). Under these conditions, formation of the new product X was ~15-fold higher than in the absence of Fe$^{2+}$ and ascorbate. Furthermore, an additional product was

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detected that showed the same retention time as an authentic Ring A standard. LC-MS-MS analysis in comparison with an authentic reference compound confirmed that this latter product was indeed Ring A (m/z: 205, 161, 150, 106).

When product X was isolated by HPLC and incubated with holo-CloR in the presence of ascorbic acid, again the formation of Ring A was clearly demonstrated by LC-MS-MS analysis (data not shown), proving that product X was an intermediate in the formation of Ring A.

LC-MS-MS analysis of product X revealed that this compound showed the calculated mass ([M-H]− = 235) of 3-di-methylallyl-4-hydroxymandelic acid (3DMA-4HMA). Furthermore, it showed the characteristic fragmentation pattern of a 4-hydroxymandelic acid derivative (m/z: 235 [M−1]−, 191 [M−44]−, and 189 [M−46]−); the same pattern was observed from an authentic 4-hydroxymandelic acid (m/z: 167 [M−1]−, 123 [M−44]−, and 121 [M−46]−). Therefore, CloR catalyzes two consecutive reactions (Fig. 4): first, the conversion of 3DMA-4HPP to 3DMA-4-hydroxymandelic acid (3DMA-4HMA), and second, the conversion of 3DMA-4HMA to 3DMA-4-hydroxybenzoic acid, i.e. Ring A.

CloR was found to be specific for 3DMA-4HPP and 3DMA-4HMA as substrates. No product formation was observed with the non-prenylated substrates 4-hydroxyphenylpyruvate or DL-4-hydroxymandelic acid, nor with L-mandelic acid, l-mandelic acid, β-hydroxytyrosine, or 4-hydroxyphenyllactic acid. Additional experiments confirmed that the prenyltransferase CloQ (20) specifically prenylated 4HPP and was not able to react with DL-4-hydroxymandelic acid or 4-hydroxybenzoic acid (data not shown).

Replacement of Fe3+ with other monovalent or divalent metal ions (1 mM Cu++, Zn++, Mg++, or Mn++) resulted in almost complete (95–98%) loss of enzymatic activity of CloR, as described previously for non-heme iron oxygenases (33). Many non-heme iron-dependent oxygenases require, besides Fe3+, an α-ketoacid as cofactor, e.g. α-ketoglutaric acid (27). However, this activity of CloR was not stimulated by addition of α-ketoglutarate.

Purified CloR was a colorless protein. UV-visible spectrometry showed an absorption maximum at 283 nm but no absorption in the visual range. Therefore, CloR is not a heme protein, as are, e.g., the cytochrome P450 monoxygenases.

Investigation of the Reaction Mechanism of CloR—The requirement of CloR for Fe3+ and ascorbate suggested that it belongs to the non-heme iron oxygenases (33–35). To confirm whether indeed molecular oxygen was the substrate of the CloR reaction, and whether one or both oxygen atoms of O₂ were incorporated into the product, we carried out isotope-labeling experiments with 18O₂. Incorporation of the label was analyzed by LC-MS-MS analysis.

In the first experiment, CloR (after preincubation with Fe3+) was incubated with 3DMA-4HPP and ascorbate in an 18O₂ atmosphere. A control incubation was carried out in the usual 16O₂ atmosphere. The reaction products 3DMA-4HMA and 3DMA-4HB were separated by HPLC and analyzed by MS-MS analysis. Fig. 5A shows the molecular ions obtained in a usual atmosphere and in 18O₂ atmosphere. Unlabeled 3DMA-4HMA showed the molecular ion at [M−H]− = 235. In contrast, most of the 3DMA-4HMA produced under the 18O₂ atmosphere showed the molecular ion at [M−H]− = 239, proving the incorporation of two 18O atoms. The position of the label was revealed by MS-MS analysis: the decarboxylation product (m/z = 193) was 46 Da smaller than the parent compound, proving that one of the 18O atoms had been incorporated into the carboxyl group of 3DMA-4HMA. The corresponding keto compound arising from oxidation (m/z = 191) was 2 Da larger than the corresponding ions of the unlabeled 3DMA-4HMA, indicating that the other 18O₂ had been incorporated into the α-hydroxyl group. For the molecular ion of 3DMA-4HMA, also a minor peak at [M−H]− = 237 was detected (Fig. 5A), resulting from the incorporation of a single 18O atom into the product. This shows a certain dilution of the label and has been reported previously for HmaS as well as for other non-heme iron oxygenases (27). This dilution has been suggested to result from an exchange of a presumed Fe3+O intermediate with water (27). The product mixture also contained some unlabeled 3DMA-4HMA ([M−H]− = 235), most likely due to the presence of residual 16O₂ in the incubation vial.

In addition to 3DMA-4HMA, the CloR reaction also produced 3DMA-4HB. Incorporation of two 18O atoms into this product was demonstrated by the molecular ion at [M−H]− = 209, in
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comparison to [M-H]− = 205 for the unlabeled compound (Fig. 5A). As expected, both these labeled oxygens were located in the carboxyl group (Fig. 5A).

For a second labeling experiment, the intermediate 3DMA-4HMA was first produced in unlabeled form and isolated by HPLC. This compound was then incubated in an 18O2 atmosphere with CloR (preincubated with Fe2+) and ascorbate. The resulting 3DMA-4HB was analyzed by LC-MS-MS (Fig. 5B), and this clearly showed the incorporation of one 18O atom into the carboxyl group of the product, as demonstrated by the molecular ion at [M-H]− = 207, and the decarboxylation product at m/z = 161 (Fig. 5B).

Fig. 5C summarizes the results of the 18O2 incorporation experiments. Two 18O atoms are incorporated in the first reaction step, and one 18O atom is incorporated in the second reaction step. The other 18O atom involved in the second step is most likely converted to water (see “Discussion”).

**DISCUSSION**

In the present study, CloR was identified as a bifunctional oxygenase that converts 3-dimethylallyl-4-hydroxyphenylpyruvate (3DMA-4HPP) in two consecutive reaction steps to 3-dimethylallyl-4-hydroxybenzoate (3DMA-4HB), i.e. to the Ring A moiety of clorobiocin. An 18O2 labeling experiment unequivocally confirmed that molecular oxygen is used as substrate by CloR. The purified CloR protein does not contain a heme prosthetic group, and its activation by Fe2+ and ascorbate indicated that it belongs to the non-heme iron oxygenase.

The first reaction catalyzed by CloR, i.e. the conversion of 3DMA-4HPP to 3-dimethylallyl-4-hydroxymandelic acid (3DMA-4HMA) has a well-established precedent in the HmaS reaction in chloroeremomycin biosynthesis. HmaS belongs to the iron(II)- and α-ketoacid-dependent dioxygenases (reviewed in Refs. 33, 35, 36). These enzymes utilize O2 and an α-ketoacid as cosubstrates. During the reaction, the α-ketoacid loses CO2, and the keto function is oxidized to a carboxyl group by introduction of one of the oxygen atoms of O2. The other oxygen may be used for a hydroxylation reaction, exemplified, e.g. by the prolyl 3-hydroxylase reaction. However, iron(II)- and α-ketoacid-dependent oxygenases have been shown to catalyze not only hydroxylations but a wide range of diverse oxidative transformations, including epoxidations, desaturations, ring formation, and ring expansion reactions. Some of these enzymes are bifunctional (e.g. deacetoxy-deacetylcephalosporin synthase) or even trifunctional (e.g. clavaminic acid synthase or thymine hydroxylase), catalyzing several consecutive oxidative transformations within a single biosynthetic pathway.

In the conversion of 4-hydroxyphenylpyruvate (4HPP) to homogentisate by 4HPP dioxygenase, 4HPP serves as a substrate for hydroxylation and at the same time as α-ketoacid cosubstrate. CloR (in its first reaction step) and HmaS carry out a very similar reaction as 4HPP dioxygenase but hydroxylate the benzylic position of the substrate instead of the phenyl ring. However, although HmaS shows obvious sequence similarity to 4HPP dioxygenase, CloR does not.

The second reaction step catalyzed by CloR is the conversion of 3DMA-4HMA to 3DMA-4HB. It is tempting to speculate that this reaction may involve hydroxylation of the α-position of 3DMA-4HMA, resulting in a α,α-gem-diol, which eliminates water to give the corresponding α-ketoacid, i.e. 4-hydroxybenzoylformate. Oxidative decarboxylation of this compound would result in the final product, 3DMA-4HB. Thereby, the overall reaction mechanism would be similar to that of the first reaction and resemble that of other iron(II)- and α-ketoacid-dependent dioxygenases.

However, LC-MS-MS analysis did not confirm the presence of 4-hydroxybenzoylformate in the incubation mixture, and the exact mechanism of the CloR reaction remains speculative at present. It is believed that the reactions catalyzed by iron(II)- and α-ketoacid-dependent oxygenases involve a reactive FeIV=O species (35, 37). Whether this is true for CloR remains to be shown.

The different groups of iron(II)- and α-ketoacid-dependent oxygenases possess little overall sequence similarity to each other (35, 36), and it is therefore not surprising that CloR does not show sequence similarity to known members of this family. However, a data base search reveals that CloR does show significant similarity to several proteins of so far unknown function, deduced from genome sequences of different microorganisms. These may possibly represent enzymes of similar function as CloR.

The common structural motif of iron(II)- and α-ketoacid-dependent enzymes is the so-called 2-His-1-carboxylate facial triad (36, 38). It consists of two histidyl groups and one glutaryl or asparagyl residue, which together anchor the iron atom in the active site of the enzyme. Comparison of the primary sequence of CloR with that of NovR as well as with the sequences of six of the data base entries of unknown function with high sequence similarity to CloR (data not shown) identifies His-161, His-176, His-178, His-241, and Asp-170 of CloR as strictly conserved residues. These amino acids may be candidates for a potential 2-His-1-carboxylate facial triad of this oxygenase.

The involvement of CloR in the biosynthesis of the prenylated 4-hydroxybenzoate moiety of clorobiocin was proven in vivo by a gene inactivation experiment (19). The present results now allow the formulation of a detailed hypothesis for the formation of this moiety of clorobiocin, as shown in Fig. 6. CloQ is a prenyltransferase, which converts 4HPP to 3DMA-4HPP (20). CloR converts the CloQ reaction product to Ring A, as demonstrated in the present study. CloF shows sequence similarity to prephenate dehydrogenases and is therefore likely to produce 4HPP as the substrate for CloQ, similar to ORF1 of the chloroeremomycin biosynthetic gene cluster (39). Notably, the coumermycin cluster does not contain a CloF homologue (Fig. 1B).

Radioactive feeding experiments with the novobiocin precursor (40) showed that [U-14C]-tyrosine was incorporated preferentially into Ring B, whereas [U-14C]-4HPP was incorporated preferentially in Ring A. This may suggest that a primary metabolic prephenate dehydrogenase provides tyrosine for the formation of Ring B, whereas CloF supplies 4HPP for Ring A biosynthesis, and that cross-talk exists between both pathways probably via a transaminase reaction (Fig. 6).

Chen and Walsh (17) have previously speculated that Ring A and Ring B of novobiocin may both be produced via β-hydroxy-tyrosyl-S-NovH as a common precursor. Our studies on CloQ and CloR (Refs. 19 and 20 and the present study), however, now establish that these two aromatic moieties of clorobiocin (and very likely those of novobiocin as well) are produced by two independent pathways (Fig. 6). The discovery of CloR adds a new interesting member to the diverse family of the non-heme iron oxygenases and demonstrates the existence of a new pathway for the formation of benzoic acids in nature.

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