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Halogenation of Unactivated Carbon Centers in Natural Product Biosynthesis: Trichlorination of Leucine during Barbamide Biosynthesis

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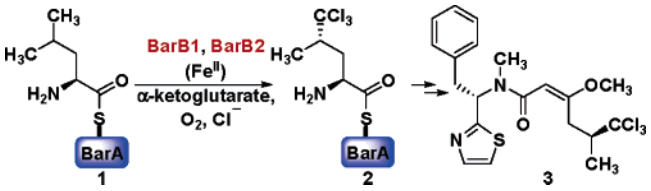
Halogenation is a common structural feature of more than 4500 natural products of both marine and terrestrial origin.[1] This biosynthetic modification has a profound influence on the biological activity of the resulting compounds.[2] Recently, our laboratory described a novel class of halogenating enzymes capable of carrying out halogenations at aliphatic carbon centers of peptidyl carrier protein-linked amino acid residues.[3] These enzymes, represented by SyrB2 and CmaB, are nonheme Fe^{II} halogenases that require oxygen, α-ketoglutarate (α-KG), and chloride for their activity. In analogy to related Fe^{II}-α-KG-dependent hydroxylases,[4] it is postulated that the initial generation of a high-valent oxoiron species (Fe^{IV}═O) is followed by hydrogen atom abstraction from an aliphatic carbon center in the substrate and subsequent chlorination of the substrate radical.[5] Herein, we report that the trichlorination of the C5 methyl group of L-leucine attached to a peptidyl carrier protein BarA in the biosynthesis of barbamide is carried out by tandem action of two nonheme Fe^{II} halogenases, BarB1 and BarB2.

Barbamide (**3**, Scheme 1) is a potent molluscicidal agent isolated from the marine cyanobacterium *Lyngbya majuscula*.[6] An intriguing structural feature of this natural product is the presence of a trichloromethyl moiety. Feeding experiments with isotopically labeled precursors have established that the trichloromethyl group is biosynthetically derived from the *pro-R* methyl group of L-leucine.[7] Furthermore, high incorporation of labeled exogenous [2-^{13}C]-5,5,5-trichloroleucine implies a direct role for trichloroleucine as an intermediate in the biosynthesis of barbamide.[8] Analysis of the barbamide gene cluster revealed the presence of only two candidate halogenase genes, *barB1* and *barB2*, whose putative products are predicted to be highly similar to syringomycin halogenase SyrB2,[3,9] making triple halogenation of the unactivated methyl group of leucine an interesting biosynthetic problem.

To study the trichlorination event, synthetic genes (DNA 2.0, Menlo Park, CA) encoding for the peptidyl carrier protein BarA (T domain), the two putative halogenases BarB1 and BarB2, and the adenylation (A) domain BarD optimized for *E. coli* overexpression were obtained. All four proteins were overproduced in *E. coli* and purified as N-His-tagged fusions. The 12.9 kDa BarA was obtained in its apo form in a yield of 15 mg/L of culture, while overexpression of the 60.3 kDa BarD yielded 4 mg/L. The apo BarA (100 μM) was subsequently post-translationally modified with the phosphopantetheine arm through in situ incubation with coenzyme A (200 μM) and Sfp (2.5 μM),[10] providing the desired holo protein. To minimize any adventitious deactivation of oxygen-labile putative nonheme Fe^{II} halogenases, crude cell lysates containing His-tagged BarB1 and BarB2 were kept under nitrogen and purified in an anaerobic glovebox as apo proteins. Proteolytic removal of BarB1 and BarB2 His tags was followed by further purification by gel filtration chromatography and reconstitution with Fe^{II} in the presence of α-KG and chloride to provide the desired holo BarB1 (yield 0.4 mg/L, iron content 115−130%) and holo BarB2 (yield 1.2 mg/L, iron content 70%).

Scheme 1



In trans loading of holo BarA with L-[^{14}C]Leu (2 mM) was accomplished by incubation with BarD (20 μM) and ATP (2 mM). L-[^{14}C]Leu-*S*-BarA (**1**, Scheme 1) was incubated with reconstituted BarB1 (40 μM) and BarB2 (40 μM) either separately or in the presence of both enzymes, together with α-KG (2 mM) and chloride (∼15 mM), and incubated for 1 h at room temperature. The modified amino acids were released by treatment of T domain-tethered products with the type II thioesterase TycF[11] and further converted to isoindole derivatives as previously described.[3] Products were identified by radio-HPLC coelution with isoindole-derivatized authentic standards of (2*S*,4*S*)-5-chloroleucine, (2*S*,4*S*)-5,5-dichloroleucine, and (2*S*,4*S*)-5,5,5-trichloroleucine (Figure 1Aa).[8,12]

When L-[^{14}C]Leu-*S*-BarA was presented to BarB1 in the presence of O_2, Cl^-, and α-KG, only trace amounts of trichloroleucine and no mono- or dichloroleucine were observed (Figure 1Ab), suggesting that leucine-loaded BarA is a poor substrate for this halogenase. In contrast, when halogenation of the L-[^{14}C]Leu-*S*-BarA was examined in the presence of BarB2, (2*S*,4*S*)-5,5-dichloroleucine was formed as the major product, accompanied by a small amount of (2*S*,4*S*)-5,5,5-trichloroleucine (Figure 1Ac). Interestingly, no (2*S*,4*S*)-5-monochloroleucine accumulated in this experiment. When both BarB1 and BarB2 were incubated with the leucine-loaded T domain, both (2*S*,4*S*)-5,5-di- and (2*S*,4*S*)-5,5,5-trichloroleucine were detected (Figure 1Ad). An increase in the intensity of the trichloroleucine peak was noted when compared to incubation with BarB2 alone. A further increase in the production of trichloroleucine was observed when BarB1 was added to the L-[^{14}C]Leu-*S*-BarA that had been preincubated with BarB2 (Figure 1Ae). In the absence of its preferred substrate, it is believed that BarB1 undergoes inactivation via either Fe^{II} oxidation[13a] or radical-induced self-hydroxylation,[13b,c] two common uncoupling pathways. We postulate that these modes of deactivation are responsible for the decrease in the amount of trichloroleucine product in the assay where both halogenases are present from the very beginning of incubation (Figure 1Ad) when compared to pregeneration of dichloroleucine−BarA prior to the addition of BarB1 (Figure 1Ae). Collectively, these data suggest that BarB2 is an efficient dihalogenating enzyme working twice in trans on the L-Leu-*S*-BarA to yield (2*S*,4*S*)-5,5-

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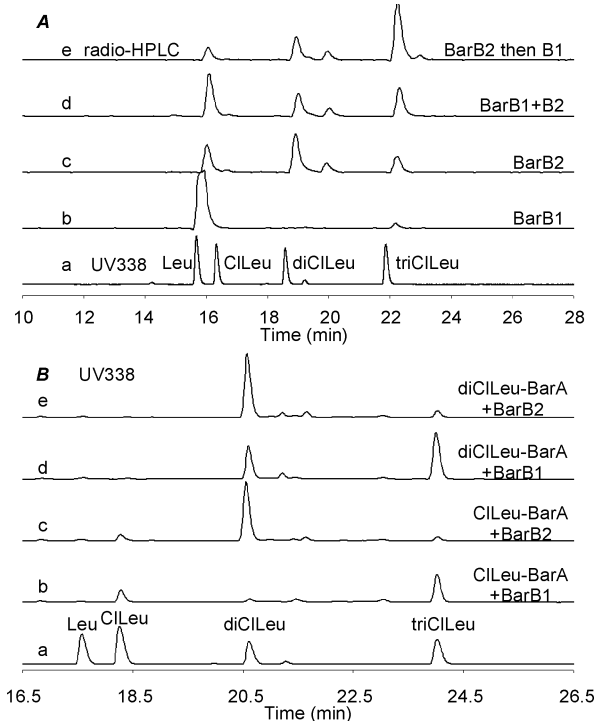


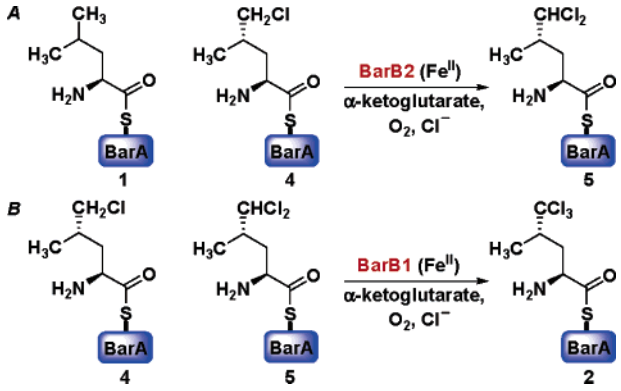
Figure 1. Analysis of the reactions catalyzed by BarB1 and BarB2. (A) UV 338 nm traces of standards (a); ^{14}C radio-HPLC traces of products obtained in L-[^{14}C]Leu-*S*-BarA incubation with BarB1 (b), BarB2 (c), BarB1 and BarB2 (d), BarB2, followed by BarB1 (e). The 0.4 min shift between retention times of trace a (UV) and b−e (radioactivity) is caused by the positioning of the two detectors. (B) UV 338 nm traces of standards (a), products of (2*S*,4*S*)-5-chloro-Leu-*S*-BarA incubation with BarB1 (b) and BarB2 (c), and products of (2*S*,4*S*)-5,5-dichloro-Leu-*S*-BarA incubation with BarB1 (d) and BarB2 (e).

dichloro-Leu-*S*-BarA. The inefficiency of BarB2 in catalyzing trichlorination is overcome by the presence of the second halogenase, BarB1, which converts dichloroleucine-loaded BarA to the trichloroleucine derivative.

To further investigate the substrate specificity of each of the halogenating enzymes, holo BarA was loaded with authentic (2*S*,4*S*)-5-mono- and (2*S*,4*S*)-5,5-dichloroleucine in the presence of BarD. The resulting aminoacyl-*S*-BarA derivatives were separately incubated with each halogenase in the presence of O_2, α-KG, and Cl^- and products analyzed as described previously (Figure 1B). While BarB1-catalyzed chlorination of monochloroleucyl-*S*-BarA provided (2*S*,4*S*)-5,5,5-trichloroleucine (Figure 1Bb and Supporting Information), BarB2-catalyzed halogenation of this substrate led to the formation of dichloroleucine as the major product (Figure 1Bc). When (2*S*,4*S*)-5,5-dichloro-Leu-*S*-BarA was subjected to enzymatic halogenation with BarB1, formation of (2*S*,4*S*)-5,5,5-trichlorinated product was observed (Figure 1Bd). In contrast, dichloroleucine-loaded BarA is not a good substrate for BarB2 (Figure 1Be). Together, these data suggest that BarB2 not only dichlorinates BarA-tethered L-leucine **1** but also converts monochloro-Leu-*S*-BarA **4** to dichloroleucine derivative **5** (Scheme 2A). Inefficient trichlorination by this enzyme is circumvented by the presence of the second halogenase BarB1, able to convert both mono- and dichloro derivatives **4** and **5** to (2*S*,4*S*)-5,5,5-trichloro-Leu-*S*-BarA **2** (Scheme 2B).

In summary, in vitro reconstitution of the formation of the trichloroleucine moiety has been achieved. It was demonstrated that

Scheme 2



the triple chlorination of the unactivated methyl group of BarA-tethered L-leucine substrate is mediated by the tandem action of two nonheme Fe^{II} halogenases, BarB1 and BarB2, establishing complementary roles of these two enzymes in the generation of (2*S*,4*S*)-5,5,5-trichloroleucine in barbamide biosynthesis and setting up the system for subsequent mechanistic analysis.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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