NADH-Dependent Halogenases Are More Likely To Be Involved in Halometabolite Biosynthesis Than Haloperoxidases**

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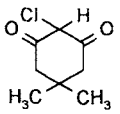
The detection of chloroperoxidase from the fungus *Caldariomyces fumago*[1] and the development of a simple spectrophotometric assay[2] for the detection of halogenating enzymes based on the synthetic compound monochlorodimedone (**1**) as organic substrate resulted in the subsequent isolation of a number of haloperoxidases from different organisms. All these enzymes produce hypohalogenic acid, which is the actual halogenating agent. Thus, halogenation catalyzed by haloperoxidases lacks substrate and regiospecificity.[3-4] However, investigations of the biosynthetic pathways of different halometabolites have shown that biological halogenation must be specific.[3, 5] Furthermore, the formation of fluorinated metabolites by haloperoxidases is difficult to explain, as fluoride cannot be oxidized in the haloperoxidase reaction.[6]



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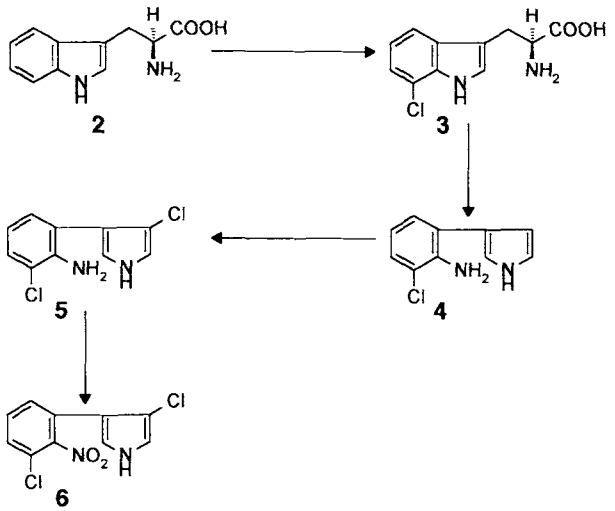
Recently, genetic investigations showed that haloperoxidase-type enzymes are definitely not involved in the biosynthesis of chlorotetracycline and pyrrolnitrin.[7-8] These results raise some interesting questions. What other type of halogenating enzymes could exist, and how can they be detected? It had always been assumed that the enzyme oxidizes the halide ion and that the oxidized halide reacts with the organic substrate. However, why couldn't the enzyme first react with the organic substrate in a way that would make it suitable for nucleophilic attack by the halide ion itself?

Apparently all groups working on enzymatic halogenation have ignored the fact that, if they were looking for specific enzymes, they should use the natural substrates for these enzymes and not a substrate like **1**. One reason that this approach was ignored is the lack of knowledge about the structure of these substrates. Thus, prior to the use of a "natural" substrate it had to be established that this compound actually is halogenated in vivo.

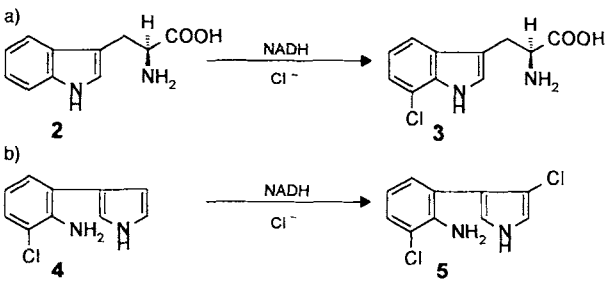
Tryptophan (**2**) would be such a substrate, if the chlorination of **2** to 7-chlorotryptophan (**3**) is the first step in the biosynthesis of the antifungal antibiotic pyrrolnitrin (**6**, Scheme 1).[9] To check this hypothesis, the growth medium of a mutant of *Pseudomonas fluorescens* blocked in the second step of pyrrolnitrin biosynthesis was analyzed. The isolated **3** was identified as the L-isomer by employing D- and L-amino acid oxidases. Thus, chlorination of the L-isomer of **2** was identified as the first step



Scheme 1. Pathway for pyrrolnitrin (**6**) biosynthesis based on the isolation of metabolites and cloning of the biosynthetic genes [9,11].

in pyrrolnitrin biosynthesis by *P. fluorescens*, and this strain therefore must contain an enzyme that catalyzes the specific chlorination of the L-isomer of **2** to the L-isomer of **3**. A second chlorination occurs later, where monodechloroaminopyrrolnitrin (**4**) is chlorinated to aminopyrrolnitrin (**5**, Scheme 1).[9]

Using the L-isomer of **2** as the substrate and a *Pseudomonas fluorescens* mutant that lacked all chromosomal pyrrolnitrin-biosynthesis genes but harbored the gene for the first step on a plasmid, we searched for tryptophan halogenase activity by means of an HPLC assay. As we did not know what kind of cofactor, if any, would be needed, a number of different cofactors were tested. The chlorinated **3** could only be detected when NADH was added to the cell-free extract (Scheme 2a).



Scheme 2. NADH-dependent chlorination of a) L-tryptophan (**2**) to 7-chloro-L-tryptophan (**3**) catalyzed by tryptophan halogenase and b) monodechloroaminopyrrolnitrin (**4**) to aminopyrrolnitrin (**5**) by monodechloraminopyrrolnitrin halogenase.

Attempts to chlorinate **2** with haloperoxidases only resulted in the formation of the oxindole derivative of tryptophan and not in a halogenated product.[10] Chemical chlorination of **2** in the 7-position is also not possible. The enzymatic chlorination of **2** can therefore be regarded as a specific and unusual reaction, although there are a number of naturally produced antibiotics that are derived from **3**.[5]

The addition of NADH to cell-free extracts of a *P. fluorescens* pyrrolnitrin-deletion mutant containing the gene coding for the enzyme that catalyzed the second chlorination step on a plasmid was also necessary for activity. Thus the halogenase chlorinating **4** to **5** is also NADH-dependent (Scheme 2b). As the oxidation of halide ions in an NADH-dependent reaction can be ruled

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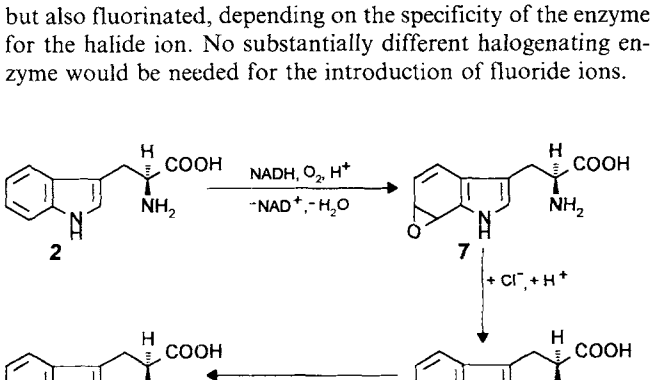
out, the NADH-dependence could be explained by the involvement of oxygen leading to the intermediate formation of an epoxide (**7**). From this enzyme-bound epoxide a halohydrin (**8**) could be formed by the incorporation of halide ion with subsequent specific removal of water (Scheme 3). Thus the organic substrate can not only be chlorinated, brominated, and iodated, but also fluorinated, depending on the specificity of the enzyme for the halide ion. No substantially different halogenating enzyme would be needed for the introduction of fluoride ions.



Scheme 3. Hypothetical mechanism for the NADH-dependent halogenation. Compounds **7** and **8** would probably not be released by the enzyme.

No enzymatically formed products other than **3** or **5** could be detected in the enzyme assays. The enzymes are specific, and tryptophan halogenase does not catalyze the chlorination of **4** or vice versa. Comparison of the amino acid sequences of the two halogenases showed no sequence homology. However, monodechloroaminopyrrolnitrin halogenase was found to be homologous to the halogenase that catalyzes the chlorination in 7-chlorotetracycline biosynthesis.[7, 11]

The newly detected NADH-dependent halogenases, in contrast to haloperoxidases, have at least some of the properties expected of specifically halogenating enzymes. It was always difficult to understand why halogenation by haloperoxidases in vitro produces halocompounds that could not be found in the organisms from which these haloperoxidases had been isolated. It is very likely that haloperoxidases are not the halogenating enzymes involved in the specific biosynthesis of halometabolites. For these reactions NADH-dependent halogenases are much better candidates. Thus, in organisms where discrepancies arise between the halometabolites produced in vivo and the products formed in vitro, the involvement of haloperoxidases should be reinvestigated and NADH-dependent halogenases sought.

Experimental Section

For the isolation of **3**, bacteria were grown in 2 L of glycerol minimal medium [9] in the presence of 8.5×10^{-5} M kanamycin and 6.2×10^{-5} M tetracycline at 30 °C for two days. As **3**, like **2**, is metabolized by a number of different pathways, it was not expected to accumulate in large amounts. Therefore the growth medium was adjusted to pH 5.3 with 1 N HCl and loaded onto an Amberlite CG-120I cation exchange column with a column volume of 25 mL, equilibrated with 0.35 M citrate buffer (pH 5.3). After elution with 1% aqueous ammonia the fractions (2.4 mL) were neutralized with 1 M acetic acid and analyzed by HPLC [12]. Fractions containing **3** were pooled, concentrated under vacuum, and desalted over a Sephadex G-10 column (column volume 5 mL). The fractions (1 mL) were analyzed by HPLC and those containing **3** were pooled and concentrated. The isolated compound was identified by comparison of its retention time, UV spectra, and mass spectra with those of authentic, synthesized **3** [13].

Oxidation of isolated **3** to 3-(3-indolyl)-pyruvic acid by amino acid oxidases was performed in a total volume of 75 µL in 0.2 M Tris/HCl buffer (pH 8.3) in the presence of 9×10^{-4} units of catalase. The reactions were started by addition of 5×10^{-1} units of D-amino acid oxidase or 5×10^{-2} units of L-amino acid oxidase. The assays were incubated at 30 °C with continuous shaking. Samples (20 µL) were analyzed at the start of the reaction, after 60, and after 90 min.

For detection of the halogenase activities, a *Pseudomonas fluorescens* mutant that lacked all pyrrolnitrin-biosynthesis genes but contained the genes for tryptophan or monodechloroaminopyrrolnitrin halogenase on a plasmid was grown for three days as described for the isolation of **3**. Cells were harvested by centrifugation, resuspended in 2 mL g^{-1} cells volume of 100 mM ammonium acetate buffer (pH 6.8) and disrupted by sonication at 2 °C for 6 min. The cell debris was removed by centifugation, and the crude extracts were used for analysis of the halogenase activities. The assays used contained the following components: 200 µL of crude extract, 20 mM NaCl, 0.2 mM **2** or **4** as the organic substrate, 2 mM cofactor, and 0.1 mM ammonium acetate buffer (pH 6.8) in a total volume of 1 mL. After incubation for 16 h at room temperature the reaction was stopped by boiling in water for 5 min in the case of tryptophan halogenase. After removal of precipitated protein by centrifugation, the assay mixture (20 µL) was analyzed by HPLC [12]. With **4** as the substrate, the assay mixture was extracted with 2 × 0.5 mL ethyl acetate. After evaporation of the organic solvent, the extracted compounds were redissolved in methanol (50 µL), and a 20 µL sample of the solution was analyzed by HPLC [14]. The products were identified by comparison of their retention times, HPLC-UV spectra, HPLC-MS spectrum in the case of **3**, and GC-MS spectrum in the case of **5** with those of authentic samples [13,15].

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[1] P. D. Shaw, L. P. Hager, *J. Am. Chem. Soc.* **1959**, *81*, 6527–6528.
[2] L. P. Hager, D. R. Morris, F. S. Brown, H. Eberwein, *J. Biol. Chem.* **1966**, *241*, 1769–1777.
[3] K.-H. van Pée, *Annu. Rev. Microbiol.* **1996**, *50*, 375–399.
[4] M. C. R. Franssen, *Biocatalysis* **1994**, *10*, 87–111.
[5] G. W. Gribble, *Prog. Chem. Org. Nat. Prod.* **1996**, *68*, 119–166.
[6] S. L. Neidleman, J. Geigert, *Biohalogenation: Principles, Basic Roles and Applications*, Ellis Horwood, Chichester, **1986**.
[7] T. Dairi, T. Nakano, K. Aisaka, R. Katsumata, M. Hasegawa, *Biosci. Biotechnol. Biochem.* **1995**, *59*, 1099–1106.
[8] S. Kirner, S. Krauss, G. Sury, S. T. Lam, J. M. Ligon, K.-H. van Pée, *Microbiology* **1996**, *142*, 2129–2135.
[9] K.-H. van Pée, O. Salcher, F. Lingens, *Angew. Chem.* **1980**, *92*, 855-856; *Angew. Chem. Int. Ed. Engl.* **1980**, *19*, 828–829.
[10] M. Morrison, G. R. Schonbaum, *Annu. Rev. Biochem.* **1976**, *45*, 861–888.
[11] P. E. Hammer, D. S. Hill, S. T. Lam, K.-H. van Pée, J. M. Ligon, *Appl. Environ. Microbiol.*, **1997**, *63*, 2147–2154.
[12] HPLC analysis of **3**: LiChrospher 100, RP-18, 5 µm, 250 mm × 4.6 mm, MeOH/H_2O/trifluoroacetic acid 40/60/0.1, 1 mL min^{-1}. UV detection at 280 nm; retention time of the isolated compound, the enzymatically produced product, and authentic **3** is 13.7 min.
[13] K.-H. van Pée, O. Salcher, F. Lingens, *Liebigs Ann. Chem.* **1981**, 233–239.
[14] HPLC for the analysis of **4**: LiChrospher 100, RP-18, 5 µm, 250 × 4.6 mm, MeOH/H_2O 60/40, 0.8 mL min^{-1}, UV detection at 280 nm; retention time of the enzymatically produced product and of **4** is 10.0 min.
[15] K.-H. van Pée, O. Salcher, P. Fischer, M. Bokel, F. Lingens, *J. Antibiotics* **1983**, *36*, 1735–1742.

  0570-0833/97/3618-2013 $ 17.50+.50/0