Enzymatic Halogenation

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Changing the Regioselectivity of the Tryptophan 7-Halogenase PrnA by Site-Directed Mutagenesis**

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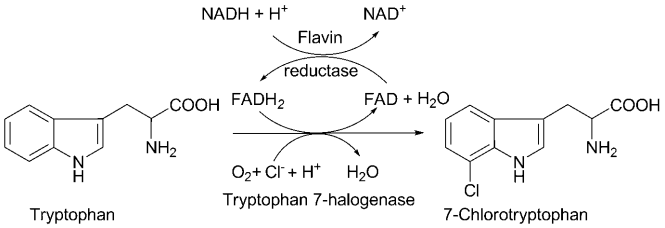
Dedicated to Professor Franz Lingens on the occasion of his 85th birthday

For many years, haloperoxidases were the only type of halogenating enzymes known.[1,2] Haloperoxidases (heme- and vanadium-containing) catalyze the formation of hypohalous acids,[3,4] which diffuse out of the active site and then react with substrate. Perhydrolases catalyze the formation of peracids, which react outside of the active site with halide ions to form hypohalous acids.[5] In both cases the actual halogenation step initiated by haloperoxidases and perhydrolases is a nonenzymatic step consistent with the lack of substrate specificity and regioselectivity seen with these enzymes. The structures of many halogenated metabolites suggested that there are naturally occurring halogenating enzymes that have a high degree of substrate specificity and are capable of regioselective halogen incorporation. The halogenated indole (or tryptophan) derivatives serve as an elegant demonstration system since a series of derivatives can be isolated in which each individual position of the indole ring system has a halogen substituent.[6] This clearly shows that halogenating enzymes with regioselectivity for each of the positions of the indole ring system must exist. The first halogenase found to catalyze the regioselective chlorination or bromination of tryptophan was the tryptophan 7-halogenase PrnA involved in pyrrolnitrin biosynthesis.[7] PrnA was identified as a flavin-dependent halogenase requiring a flavin reductase as a second enzyme component.[8] This flavin reductase produces $FADH_2$ from flavin adenine dinucleotide (FAD) and reduced nicotinamide adenine dinucleotide (NADH; Scheme 1). $FADH_2$ is bound by PrnA where it reacts with molecular oxygen to form a flavin hydroperoxide.[9] A single chloride ion is bound close to the isoalloxazine ring of the FAD (Figure 1) and attacks the flavin hydroperoxide leading to the formation of hypochlorous acid.[10] However, since the substrate tryptophan is bound about 10 Å away from the isoalloxazine ring,



Scheme 1. Reaction catalyzed by the two-component system of the flavin-dependent halogenases.

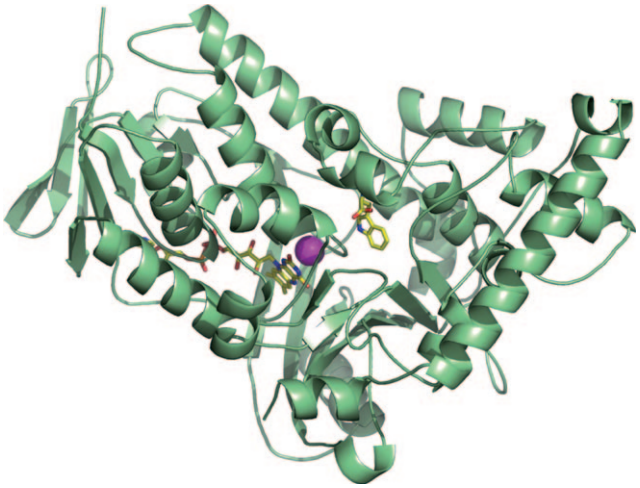


Figure 1. Active site of PrnA showing the binding of the substrate tryptophan. The isoalloxazine ring of FAD is to the left and the substrate tryptophan to the right. The chloride ion bound near the isoalloxazine ring of FAD is shown as a sphere between the isoalloxazine ring and the substrate.

the hypochlorous acid is guided through a "tunnel" towards the substrate. In this process, a serine residue (S347), which is located halfway between the isoalloxazine ring and the substrate, seems to be involved. A lysine (K79) and a glutamate residue (E346) are located close to the substrate, and both are absolutely required for enzyme activity (Figure 2).[10,11] The lysine residue is suggested to react with the hypochlorous acid to form a chloramine as the halogenating intermediate.[9] Flecks et al.[11] suggested that a concerted interaction of hypochlorous acid with the lysine and the glutamate residue should increase the electrophilicity of the chlorine species and in addition ensure the correct positioning of the chlorine species for the regioselective incorporation of chlorine into the indole ring of tryptophan.

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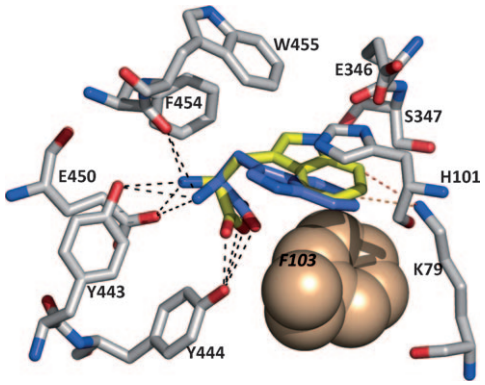

Figure 2. Model of the active site of PrnA (gray C, blue N, red O). The suggested change in substrate orientation for the PrnAF103A variant relative to that in PrnA can be seen, which allows chlorination of the 5-position by the PrnAF103A variant. The mutated residue F103 is shown as a space-filling model. The substrate bound by native PrnA is shown in yellow and the suggested binding by the mutant is shown in blue. The positions of the indole ring that are halogenated by the native or mutant enzyme are connected to the ε-amino group of lysine by dashed lines. Interactions of the amino and carboxylic acid groups of tryptophan with the enzyme are also shown by dashed lines.

Comparison of the amino acid sequences of flavin-dependent halogenases showed that the lysine residue is absolutely conserved, whereas the glutamate residue is conserved only in tryptophan halogenases. In the three-dimensional structure of the tryptophan 5-halogenase PyrH from the biosynthesis of pyrroindomycin the positions of the amino acid residues involved in catalysis as well as the isoalloxazine ring are identical to their positions in the tryptophan 7-halogenases PrnA und RebH.[9,12,13] Thus, regioselectivity must be regulated by the orientation of the substrate in the active site. This was confirmed by comparison of the three-dimensional structure of the substrate (tryptophan) complexes of tryptophan 7-halogenase PrnA with that of the tryptophan 5-halogenase PyrH. In PrnA, the 7-position of the indole ring points into the tunnel and is the only position assessable to the chlorine species. The other reactive positions of the indole ring are shielded by large aromatic amino acids (W455 and F103), which sandwich the substrate between them. The carboxylic acid and the amino group of tryptophan interact with two tyrosine residues and one glutamate residue. In addition, the NH group of the indole forms a hydrogen bond with a peptide-bond oxygen atom, which results in the 7-position of the indole ring pointing into the tunnel. In the tryptophan 5-halogenase PyrH, the 5-position of the indole ring is exactly at the same position as the 7-position in PrnA. The different orientation of the substrate is controlled by a number of changes in the residues that surround the indole ring, notably Y454 and F94. The binding modes of PrnA and PyrH are mutually exclusive.[12]

PrnA had previously been shown to catalyze the chlorination of a number of indole derivatives other than tryptophan. However, in all cases these substrates were chlorinated in the 2-position or in the 2- and 3-positions of the indole ring. If one looks at these experiments along with the structural data (not available at the time) it is evident that these substrates were not properly bound in the active site and thus chlorination occurred at the positions activated for electrophilic attack,[14] similar to the situation with the haloperoxidases.

We wished to make use of our understanding of the structure of the tryptophan halogenases to alter the regioselectivity of the chlorination of tryptophan. The obvious starting point was the large aromatic amino acid residues that sandwich and shield the indole ring, H101, F103, and W455 (Figure 2). Each was mutated to an alanine residue, and the double-mutant W455A and F103A was constructed (but this turned out to be inactive). The determination of the kinetic data of the purified His-tagged enzyme variants showed that the kinetic properties of PrnAF103A are quite similar to those of the His-tagged form of the wild-type enzyme (Table 1). However, the kinetic values (K_m and k_{cat}) for the other two enzyme variants were quite different from those of PrnA. While k_{cat} for PrnAH101A and PrnAW455A was decreased by a factor of 5.4 and 6.5, respectively, K_m was increased by a factor of 36 for both enzyme variants. The increase in the K_m values of both mutants supports the structural data that H101 and W455 are important for substrate binding. Analysis of the assay mixtures of the reactions catalyzed by PrnAH101A and PrnAW455A only showed 7-chlorotryptophan as the reaction product. However, HPLC analysis of the reaction mixture of PrnAF103A showed 7-chlorotryptophan as the main product with a shoulder indicating the formation of a second product. The separation of the main product from the second, unknown product was more pronounced when bromine was used as the halogenating substrate instead of chlorine. Therefore a large-scale reaction in the presence of bromide was performed, and HPLC analysis showed that the two products formed in a ratio of about 2:1. According to HPLC–MS analysis, both products are monobrominated tryptophan. ^{1}H NMR analysis of the purified products revealed that the main product is 7-bromotryptophan and the new product was identified as 5-bromotryptophan.

Table 1: K_m and k_{cat} values of His-tagged wild-type PrnA and of some mutant forms.

Enzyme variant	K_m [μM]	k_{cat} [min^{-1}]
HisPrnA	49.9 ± 5.2	6.79 ± 0.27
HisPrnAF103A	198.6 ± 27.3	3.99 ± 0.21
HisPrnAH101A	1785.1 ± 117.6	1.26 ± 0.05
HisPrnAW455A	1814.2 ± 382.1	1.05 ± 0.12
HisPrnAF103AW455A	inactive	inactive

The exchange of the large amino acid F103 for the smaller alanine apparently allows the substrate to adopt a different conformation when bound to the protein. Electrophilic aromatic substitution by *N*-chlorolysine[9] requires a specific orientation of the indole ring. As both PyrH and PrnA anchor the amine and carboxylate group in a similar orientation we predict that the F103A mutant binds tryptophan in the "normal" PrnA mode but also in a manner very similar to that observed in PyrH. *N*-Chlorolysine requires a specific distance and E346 a specific orientation. The unchanged residues that bind the amino and carboxylate groups would seem likely to

fix their position. In PrnA, Y444 and N459 prevent the tryptophan substrate from adopting the same orientation as that seen in PyrH. We predict that the PrnA mutant F103A allows the tryptophan to rotate to approximately (but displaced from) the orientation seen in PyrH. We have assumed the carboxylate and amine groups remain essentially in the same position as in the native enzyme, thus the energetic minima of the torsion angles of the tryptophan amino acid displaces the indole ring away from the exact position seen before (in both PyrH and PrnA). This position would normally clash with F103, but the mutation to the smaller Ala permits this. In Figure 2, a hand-built model of the active site is presented showing the possible change in the orientation of the substrate for halogenation in the 5-position compared to halogenation in the 7-position. As this orientation is displaced relative to the ideal geometry at the active sites, we suggest that this accounts for the preference shown for the 7-bromotryptophan product. Once the halogen is added to the ring, the halogenated substrate would no longer fit in the active site, rationalizing the absence of any dibrominated product.

This work is the first demonstration that the regioselectivity of tryptophan halogenases can be engineered by site-directed mutagenesis. In this first example a mixture of products halogenated at two different positions were obtained at a rate comparable to that of the native enzyme. Since mimicking the strategy used by nature to change the position of the desired site of halogenation is possible, future experiments can now be designed to improve the regioselectivity and truly harness the biosynthetic potential of halogenases.

Experimental Section

The His-tagged PrnAH101A, PrnAF103A, and PrnAW455A single mutants were constructed by overlap extension polymerase chain reaction using pUC-*prnA* as the template.[10,15] The primers used for the construction of the mutants are shown in the Supporting Information. The fusion fragments were ligated into pBluescript II SK (+) and introduced into *E. coli* by electroporation.

For the construction of the PrnAF103AW455A double mutant, the fragments containing the mutated genes were isolated from the respective pBluescript II SK (+) derivatives using *Eco*130I and *Bsh*TI. All mutants were confirmed by DNA sequencing. For expression, the halogenase genes were ligated into the *E. coli–Pseudomonas* shuttle vector pCIBHis[16] and introduced into *Pseudomonas fluorescens* BL915 ΔORF 1–4.

His-tagged enzymes were purified by chromatography on nickel-chelating sepharose FF. The halogenating activity was determined according to the method described previously: 1 U of halogenating activity is defined as 1 μmol product formed per minute.[8,17] The detailed composition of the reaction mixture can be found in the Supporting Information. After incubation at 30 °C for 30 min, the reaction was stopped by boiling for 5 min and the assay mixture was analyzed by HPLC. For the determination of K_m and k_{cat} values, tryptophan concentrations were varied between 0.025 and 2 mM. Experiments were conducted in quadruplicate. The calculated rates were analyzed by several linear and nonlinear regression analysis methods. The presented K_m and k_{cat} values were determined by a hyperbola fit function (Michaelis–Menten equation) approximated by 30–50 cycles of 200 Levenberg–Marquardt iterations.[18]

The composition of the reaction mixture for the large-scale preparation for the identification of the reaction products formed by the PrnAF103A variant is described in the Supporting Information. After incubation at 30 °C for 4 h, the reaction was stopped by boiling in a water bath for 5 min. Protein was removed by centrifugation, and the resulting supernatant was loaded onto a solid-phase extraction column (Strata C 18-E, 1000 mg, Phenomenex). The column had been equilibrated with methanol and water. After washing with 10% methanol, the halogenated substances were eluted with 100% methanol. The eluates of ten large-scale preparations were concentrated in vacuo. The two halogenated species were separated and purified by HPLC (LiChrospher 100 RP-18, 5 μm, 250 × 4 mm, methanol/water 40:60, 0.1% TFA (v/v), flow rate: 1.0 mL min^{-1}). The reaction products were identified by ^{1}H NMR spectroscopy and ESI-MS (see the Supporting Information).

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