Purification and Partial Characterization of Tryptophan 7-Halogenase (PrnA) from *Pseudomonas fluorescens***

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Dedicated to Professor Franz Lingens on the occasion of his 75th birthday.

The formation of natural organic halocompounds is not unusual.[1] Contrary to what is known about the metabolites, there is a huge lack of knowledge concerning the mechanisms for the formation of these compounds. For many years, haloperoxidases were thought to be responsible for the incorporation of halogen atoms into organic molecules.[2, 3] Haloperoxidases catalyze the incorporation of halogen atoms through the oxidation of the halide ions iodide, bromide, and chloride with the help of hydrogen peroxide. The enzyme-bound intermediates formed during this reaction are very unstable and hypohalous acid ultimately forms, which leads to unspecific halogenation reactions which are not in agreement with expectations from enzyme-catalyzed reactions.[4, 5] Nonetheless, there are situations in which unspecific halogenation reactions play important roles in nature, such as the defence mechanism of mammals against infections. Here, for example, myeloperoxidase has an important function.[6] For the specific formation of complex halogenated natural compounds, however, such a mechanism is not very useful.[3] Hints towards a different mechanism for the formation of halogenated compounds in nature were obtained during the investigation of the biosynthesis of barbamide, which is synthesized by the cyanobacterium *Lyngbya majuscula*. These results point to a radical mechanism for the formation of the trichloromethyl group of barbamide.[7, 8]

Molecular genetic investigations on the biosyntheses of the antibiotics 7-chlorotetracycline and pyrrolnitrin have shown that haloperoxidases are not involved in the formation of these halometabolites.[9–11] During the elucidation of the pyrrolnitrin biosynthesis, the existence of halogenating enzymes, which require NADH for the catalysis of halogenation reactions, was detected.[12] One of the two enzymes involved in pyrrolnitrin biosynthesis catalyzes the regioselective chlorination of tryptophan **1** to 7-chlorotryptophan **2** (see Scheme 1). This tryptophan 7-halogenase (Trp-7-Hal), PrnA, was now purified and partially characterized.

Purification to homogeneity (Figure 1) was achieved by ion exchange, hydrophobic-phase column chromatography, and molecular sieve chromatography (Table 1).

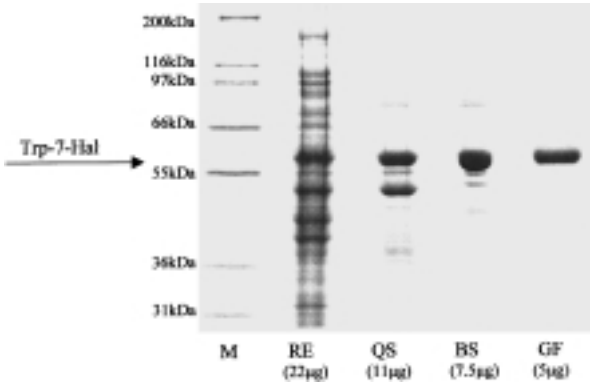

Figure 1. Sodium dodecylsulfate/polyacrylamide gel electrophoresis of the different purification steps for PrnA. M: marker proteins; RE: crude extract; QS: Q-sepharose pool; BS: butyl sepharose pool; GF: gel filtration pool; the amount of protein loaded on each lane is given in brackets.

Table 1. Purification scheme for PrnA (see Figure 1); activities (in units U [mmol min^{-1}]) were determined after addition of reductase. Without reductase, fractions after elution from the molecular sieve column did not show any activity.

Purification	Total protein [mg]	Activity [mU]	Specific activity [μU mg^{-1}]	Recovery [%]	Enhancement
Crude extract	2789.9	96.3	34.5	100.0	1.0
QS pool	221.3	92.6	418.4	96.2	12.1
BS pool	90.2	47.53	527.0	49.4	15.3
GF pool	12.5	9.12	730.0	9.5	21.2

In the first step of the purification, a protein component was partially removed which is absolutely necessary for activity (Figure 2). This protein component is a flavin reductase that reduces FAD to $FADH_2$ with the help of NADH. The FAD required for this reaction is still present after the first chromatographic step; it was, however, also removed during the later purification.

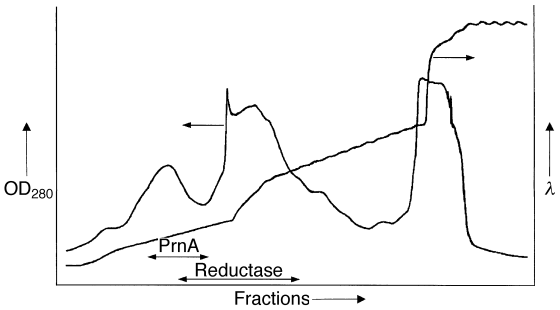

Figure 2. Elution profile from ion-exchange chromatography. QS pool: PrnA-containing fractions which were used for further purification; activator-component-pool: reductase-containing fractions. OD_{280} = optical density at 280 nm.

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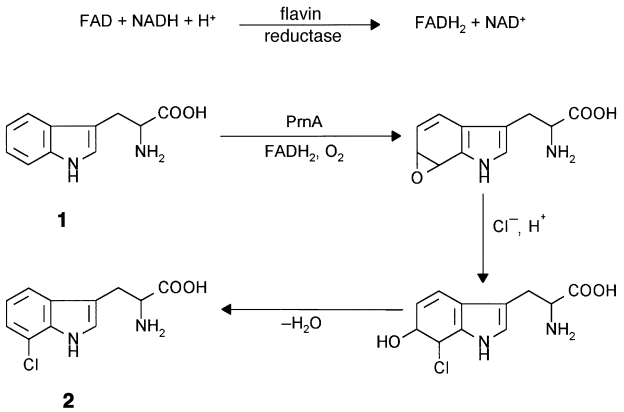
The necessity of a second component was a surprise since, according to molecular genetic data, it had to be assumed that no second protein component is necessary.[11] Obviously, the gene for this second component is not part of the biosynthetic cluster, which consists of four genes (*prnA – D*). The flavin reductase is an unspecific enzyme because the reductase from the *Pseudomonas* strain can be substituted by flavin reductases from other bacteria, such as SsuE, the flavin reductase component of the alkanesulfonate monooxygenase from *E. coli*[13] or by NADH oxidase from *Thermus thermophilus*.[14] Although flavin mononucleotide (FMN) is the better substrate for these flavin reductases, only FAD is accepted for the halogenation reaction. Obviously, tryptophan 7-halogenase is specific for $FADH_2$.

The possibility of substitution of the flavin reductase from *P. fluorescens* with a flavin reductase from *E. coli* also explains why *E. coli*, which only contains the four genes of the pyrrolnitrin biosynthetic gene cluster on a plasmid without the reductase gene, can synthesize pyrrolnitrin.[11] The other NADH/FAD-dependent halogenase thus far known, monodechloroaminopyrrolnitrin 3-halogenase (PrnC), is also a two-component system and requires a flavin reductase as the second component.

According to the hypothetical mechanism of these halogenases, the incorporation of the chloride ion requires the activation of the organic substrate by oxygen.[12] The oxygen dependence could be demonstrated by incubation under anaerobic conditions. In the absence of oxygen, no halogenating activity could be detected. The absolute requirement for oxygen, FAD, and NADH for the reaction very strongly points towards a monooxygenase-type reaction, as has been postulated earlier.[12] In the case of these monooxygenases,[15–17] a flavin reductase forms $FADH_2$ when treated with FAD and NADH, which together with oxygen then forms a flavin hydroperoxide. This flavin hydroperoxide can then attack double bonds and lead to the formation of an epoxide. By the regioselective incorporation of chloride, a halohydrin is thereby formed. The specific removal of water results in formation of the chlorinated product (Scheme 1). Recently, the genes of such halogenases have also been detected in other halometabolite-producing bacteria.[18–21] This mechanism is clearly one of the main mechanisms for the specific formation of halometabolites.



Scheme 1. Hypothetical reaction mechanism of the $FADH_2$-dependent tryptophan 7-halogenase reaction.

Experimental Section

Purification of tryptophan 7-halogenase: *P. fluorescens* BL915ΔORF1-4 harboring the tryptophan 7-halogenase gene on a plasmid [pPEH14-(*prnA*)] was used.[11] After 3 d incubation in a corn-molasses medium at 30 °C, the cells were collected by centrifugation, washed, resuspended in 100 mM potassium phosphate buffer (pH 7.2), and disrupted by ultrasonication. After the removal of insoluble cell components by centrifugation, the crude extract was dialyzed against 10 mM potassium phosphate buffer (pH 7.2) and passed through an ion-exchange column (Q-sepharose FF, Pharmacia). Elution was achieved with a linear NaCl gradient (0 – 0.5 M). Fractions that contained PrnA, which was detected by sodium dodecylsulfate/polyacrylamide gel electrophoresis (SDS-PAGE), were pooled (QS pool in Table 1). The flavin reductase eluted slightly later in the gradient and partially overlapped with PrnA (see Figure 2). Flavin reductase now free of PrnA was pooled separately and dialyzed against a 10 mM potassium phosphate buffer. For further purification, 1 M ammonium sulfate was added to the PrnA pool, which was then passed through a butyl sepharose 4 FF packed column. Elution was achieved with a linear descending gradient of ammonium sulfate (1 – 0.5 M). Fractions that contained PrnA, detected by SDS-PAGE analysis, were pooled (BS pool), concentrated by ultrafiltration, and loaded onto a HiLoad 16/60 Superdex 200 column. Elution was achieved with a 10 mm potassium phosphate buffer containing 1 M ammonium sulfate. Fractions were analyzed by electrophoresis and those containing PrnA were pooled (GF pool), dialyzed against 10 mm potassium phosphate buffer with 25 % glycerol, and concentrated by ultrafiltration.

Determination of halogenating activity: 55 μL of protein solution containing PrnA was incubated together with 110 μL of protein solution which contained either reductase (3.5×10^{-4} units SsuE from *E. coli*) or NADH oxidase (7×10^{-4} units from *Thermus thermophilus*), 10 μM FAD, 100 mM NaCl, 0.6 mM **1**, and 2.4 mM NADH in a total volume of 200 μL in 10 mM potassium phosphate buffer (pH 7.2). After incubation at 30 °C for 6 h, the reaction was stopped as described previously and analyzed by HPLC.[12]

Enrichment of PrnC: *P. fluorescens* BL915ΔORF1-4 harboring the monodechloroaminopyrrolnitrin 3-halogenase gene on a plasmid [pPEH14(*prnC*)] was used.[11] Cell incubation and preparation of the crude extract was performed as described for PrnA. Enrichment of PrnC was performed by ammonium sulfate precipitation at 25 – 45 % saturation.

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Enantioselective Intramolecular [2+2]-Photocycloaddition Reactions in Solution**

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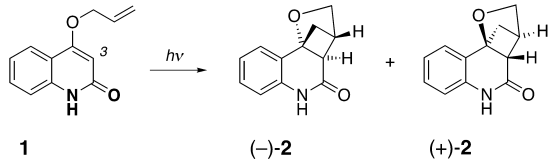
Dedicated to Professor Horst Kessler on the occasion of his 60th birthday

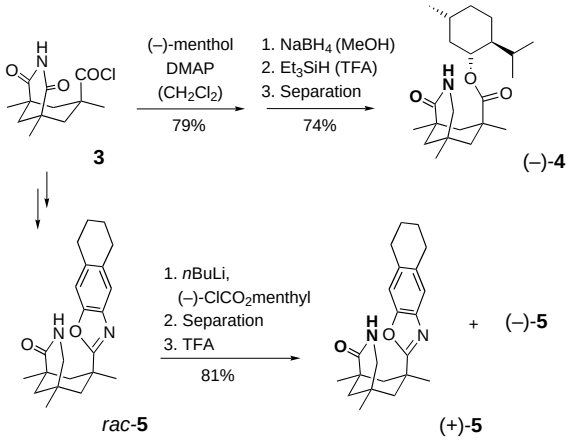
There are various approaches to obtain enantiomerically pure or enriched products from prochiral substrates by photochemical reactions in solution.[1] Whereas chiral auxiliaries which are covalently bound to one of the substrates have been successfully employed in many cases,[2, 3] chiral complexing agents which bind one substrate and thereby induce face discrimination have only been used to a limited extent.[4–8] Enantiomeric excess (*ee*) values achieved in C–C bond forming reactions based on the latter method have been moderate at best. In the solid phase however, complexes of an achiral substrate and a chiral complexing agent have been used more frequently and some excellent enantioselectivities have been recorded.[9, 10] We now report on [2+2]-photocycloaddition reactions in the presence of a chiral host, which proceeded in toluene with high enantioselectivity. Hydrogen bonds facilitate the binding of the prochiral substrate to the host and the transmission of the chiral information.

Our concept is based on the fact that prochiral lactams can coordinate to a chiral amide through their oxygen atom which acts as hydrogen acceptor and their nitrogen-bonded hydrogen atom which acts as hydrogen donor. We have recently shown that certain chiral amides derived from Kemp's triacid (1,3,5-trimethylcyclohexan-1,3,5-tricarboxylic acid) show low self-association and are therefore able to bind prochiral lactams efficiently.[11] A prochiral lactam bound to such a host is expected to undergo a stereoselective reaction. For our studies we have selected the 2-quinolone **1**, which upon irradiation with UV light cleanly yields the chiral cyclobutane (−)-**2** and its enantiomer (+)-**2** by an intramolecular [2+2]-photocycloaddition.[12] The simple diastereoselectivity of the reaction is high; only a single diastereoisomer is formed, as shown in Scheme 1.



Scheme 1. The intramolecular [2+2]-photocycloaddition reaction of the prochiral substrate **1**.

As host compounds, the bicyclic lactams (−)-**4** and (+)-**5** were prepared (Scheme 2). Association of compound **1** to the lactam unit through the atoms shown in bold would lead to an



Scheme 2. The synthesis of the enantiomerically pure host compounds (−)-**4**, (+)-**5**, and (−)-**5**. The transformation of acid chloride **3** into the racemic host *rac*-**5** is discussed in the text. (See also ref. [13]). DMAP = 4-dimethylaminopyridine, TFA = trifluoroacetic acid.

enantioface differentation in the course of the photocycloaddition, favoring enantiomer (−)-**2** (*Re* attack at carbon atom C-3). For the synthesis of host (−)-**4**, acid chloride **3**[13] was treated with enantiomerically pure (−)-menthol. Diastereoselective reduction of one carbonyl group and subsequent separation of diastereoisomers yielded compound (−)-**4** in enantiomerically pure form. The racemic benzoxazole *rac*-**5** was prepared from chloride **3** in 71 % yield by employing the corresponding *ortho*-aminophenol 3-amino-5,6,7,8-tetrahydro-2-naphthol[14] as the nucleophile according to a known procedure.[13] The resolution of enantiomers was performed by chromatographic separation of the corresponding *N*-menthyloxycarbonyllactam and its diastereoisomer. Removal of the chiral alkoxycarbonyl group yielded the enantiomerically pure host (+)-**5**[15] and its enantiomer (−)-**5**.

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