

FADH₂-Dependence of Tryptophan 7-Halogenase

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Received: January 21, 2005; Accepted: March 29, 2005

Dedicated to Prof. Franz Lingens on the occasion of his 80th birthday.

Abstract: Tryptophan 7-halogenase (Trp 7-hal) catalyses the regioselective chlorination and bromination of tryptophan. For halogenating activity, Trp 7-hal requires FADH₂ produced from FAD and NADH by a flavin reductase, halide ions (chloride or bromide), molecular oxygen and tryptophan as the organic substrate. Investigations of the flavin dependence showed that purified Trp 7-hal itself does not contain flavin. Keeping the Trp 7-hal separated from the flavin reductase during the reaction revealed that Trp 7-hal can use diffusible FADH₂ produced by a flavin reductase showing that direct contact between the halogenase and the flavin reductase is not absolutely necessary. Thus, the reaction also proceeds when chemically

reduced flavin is used. For the catalytic regeneration of FADH₂, the organometallic complex (pentamethylcyclopentadienyl)rhodium-bipyridine $[\{\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})\}]^{2+}$ can be employed as the redox catalyst with formate as the electron donor. With this chemoenzymatic system about 85% yields of the product formed by the two-component enzyme system consisting of Trp 7-hal and a flavin reductase were obtained.

Keywords: enzymatic halogenation; FADH₂ regeneration; flavoenzyme; monooxygenase; organometallic catalyst

Introduction

Living organisms have an enormous potential for the formation of halogenated organic compounds.^[1] In contrast to chemical synthesis of organohalogenes, enzyme-catalysed halogenation reactions occurring in biosynthetic pathways proceed under mild conditions and with very high regioselectivity and substrate specificity. For almost 40 years, it was assumed that biological halogenation reactions are catalysed by haloperoxidases. However, halogenation reactions catalysed by haloperoxidases lack regioselectivity and substrate specificity. This is due to the formation of free hypochlorite or hypobromite, respectively, as the actual halogenating agent of haloperoxidases. A few years ago, a new type of halogenating enzymes was detected. In contrast to haloperoxidases, halogenation reactions catalysed by these halogenases proceed with very high regioselectivity and substrate specificity.^[2]

During the last few years, it became evident that these halogenases are the major type of halogenating enzymes involved in the biosynthesis of halogenated com-

pounds.^[3] Whereas a number of three-dimensional structures of haloperoxidases are available and their reaction mechanisms have been elucidated, hardly anything is known about the reaction mechanism of the new type of halogenases.

Tryptophan 7-halogenase (Trp 7-hal) from the pyrrol-nitrin-producing soil bacterium *Pseudomonas fluorescens* is the only halogenase whose purification in active form has been published up to now. This enzyme catalyses the incorporation of the halogen atoms chlorine and bromine into the 7-position of its natural substrate tryptophan in the presence of oxygen and an additional enzyme component, a flavin reductase. This flavin reductase produces the reduced flavin (FADH₂) required by the halogenase.^[4]

The flavin reductases present in the halogenase producer can be substituted by other flavin reductases, like the flavin reductase from the ribonucleotide reductase complex (Fre) from *E. coli*^[4] suggesting that a specific interaction between the reductase and the halogenase is not necessary. This is analogous to the situation found in a group of monooxygenases which are also a

two-component system.^[4–7] In the case of these enzymes, it is believed that they use freely diffusible flavin. Interestingly, the genes of these monooxygenases and the flavin reductases are clustered, which does not seem to be the case with the halogenases.^[4,5]

The amino acid sequences of these monooxygenases show hardly any similarities to those of the halogenases. Even an absolutely conserved motif for a nucleotide-binding site (GxGxxG) near the amino terminus of the halogenases is only present in a few of these monooxygenases. This nucleotide-binding site could be a binding site for FADH₂ or an additionally required NADH.

For the elucidation of the reaction mechanism, the interplay of the two protein components with respect to the FADH₂ transfer and the NADH requirement was investigated in detail.

Results and Discussions

Direct Interaction of the Flavin Reductase and the Halogenase Component

To investigate whether a direct contact between the flavin reductase and the halogenase is necessary, the two protein components were separated by a dialysis membrane. Even under these conditions, chlorination of tryptophan occurred, although only with about 60% (130 μ U compared to 220 μ U of halogenating activity) of the efficiency seen under normal conditions. From corresponding results obtained for the 4-hydroxyphenylacetate monooxygenase, Galan et al.^[9] have concluded that direct contact between the reductase and the monooxygenase is not necessary and that this monooxygenase is able to bind freely diffusible FADH₂. The result obtained with Trp 7-hal separated from the flavin reductase suggests a similar interpretation for the halogenase system.

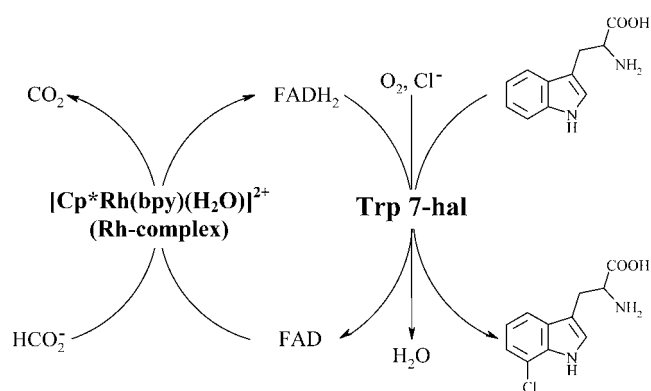
Although the separation of the two enzymes showed that direct contact of the two protein components is not absolutely required for the halogenation reaction to proceed, it did not show whether additional NADH was required by the halogenase. This question could be unequivocally answered by the use of chemically reduced FAD, since in this experiment NADH was not present at all. When chemically reduced FAD (80 μ M) was added to the halogenase assay under anaerobic conditions at room temperature and then oxygen was allowed to slowly diffuse into the solution, chlorination of tryptophan (8.6 μ M) occurred. Since FADH₂ is consumed through the very rapid, spontaneous reaction with molecular oxygen to 4 α -flavin hydroperoxide which then decomposes into FAD and H₂O₂^[10] the reaction stops after a short period of time whereby only about 10% of the reduced flavin were used for the halogenation reaction. The results obtained by separating

the two enzymes and by the use of chemically synthesised FADH₂ clearly prove that the halogenase can use freely diffusible FADH₂ for the catalysis of the chlorination of tryptophan. When FAD was substituted by FMN, no reaction occurred, showing that the halogenase is specific for FADH₂.

Halogenation using an FADH₂ Regeneration System

The results described above suggested that it might be possible to establish a continuous reaction system on the basis of a non-enzymatic FADH₂ regeneration. For this purpose an enzyme-independent *in situ* regeneration system consisting of the organometallic (pentamethylcyclopentadienyl)rhodium-bipyridine complex $\{[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}\}$ as the catalyst and formate as the electron donor was used.^[11] In this system, by which the flavin reductase as well as NADH could be substituted, FADH₂ is provided for the halogenase by the reaction of the organometallic catalyst (Scheme 1).

In comparison with the two-component enzyme system flavin reductase/tryptophan halogenase, the amount of product obtained in the chemoenzymatic assay was about 85% when catalyst concentrations of 0.2–0.4 mM were used. The addition of flavin reductase to the chemoenzymatic system had no influence on the product formation, also suggesting that the role of the flavin reductase used (Fre) was just that of a FADH₂ provider. The product formation decreased with catalyst concentrations below 0.2 mM from 53 mM 7-chlorotryptophan at 0.2 mM catalyst concentration to 38 mM 7-chlorotryptophan at 50 μ M catalyst concentration, indicating that FADH₂ became rate-limiting. The reduction in product formation in the chemoenzymatic system compared to the enzymatic two-component system is partially due to the fact that the use of formate in the chemoenzymatic system leads to a decrease in Trp 7-hal activity. Formate concentrations above 200 mM even result in total inhibition of Trp 7-hal. Additionally,



Scheme 1. The chemoenzymatic system of Trp 7-hal and the cofactor regeneration complex $[\text{Cp}^*\text{Rh}(\text{bpy})(\text{H}_2\text{O})]^{2+}$.

the substrates for Trp 7-hal, tryptophan and chloride, also have a negative influence on the rate of cofactor regeneration by the organometallic complex.

Although these results leave no doubt that Trp 7-hal can use freely diffusible FADH₂ and that direct interaction between the flavin reductase and Trp 7-hal is not absolutely necessary, it is difficult to believe that *in vivo* such a “waste” of *in situ* formed flavin reduction equivalents should occur. The very low turnover number of Trp 7-hal of 6.4 h⁻¹ in the enzymatic two-component system with both enzymes in the same solution indicates that this *in vitro* system is far from optimal. In this system, incubation for 6 h would result in the formation of 1 mM FADH₂ which could lead to the formation of 1 mM 7-chlorotryptophan, however, only 200 μM 7-chlorotryptophan are formed indicating that 80% of the formed FADH₂ are lost through autooxidation; in the chemoenzymatic system even 90% are lost (Figure 1). This corresponds to a loss of energy that would be extremely uneconomical for living cells. Thus, a direct transfer from the FADH₂-producing enzyme, the flavin reductase, to the FADH₂-consuming enzyme, the halogenase or a monooxygenase, for example, through the formation of an enzyme complex, seems much more effective. Such a protein-protein interaction was thought to occur in the case of the FADH₂-dependent 4-hydroxyphenylacetate monooxygenase (HpaB) from *E. coli* W (ATCC 11105) and the corresponding flavin reductase (HpaC). However, the search for the existence of a protein-protein complex using size-exclusion chromatography under anaerobic conditions has not provided conclusive proof for the existence of such a complex.^[6] Also the search for a potential enzyme complex between the styrene monooxygenase (StyA) and the flavin reductase (StyB) from *Pseudomonas* sp. VLB120 via ultracentrifugation gave no indications for such a protein interaction in FADH₂ transfer.^[8] Only in the luciferase/flavin reductase system from *Vibrio harveyi*, could an FMNH₂-tunnel system in the donor-acceptor enzyme complex be shown to exist *in vitro* and *in vivo*.^[12]

Another possibility would be that a third, yet unknown, component functions as an FADH₂-shuttle and thus could guarantee the safe and protected transport of the reduction equivalents. However, so far there are no data available to support this assumption, either on a genetic or on an enzymatic basis.

Hypothetical Reaction Mechanism of the Two-Component Halogenase System

Based on the data obtained for the interaction of the flavin reductase and Trp 7-hal and the resulting FADH₂ dependence a monooxygenase-like mechanism can be postulated for Trp 7-hal (Scheme 2). In a first reaction, preceding the actual halogenation reaction, FADH₂ is produced by a flavin reductase with oxidation of

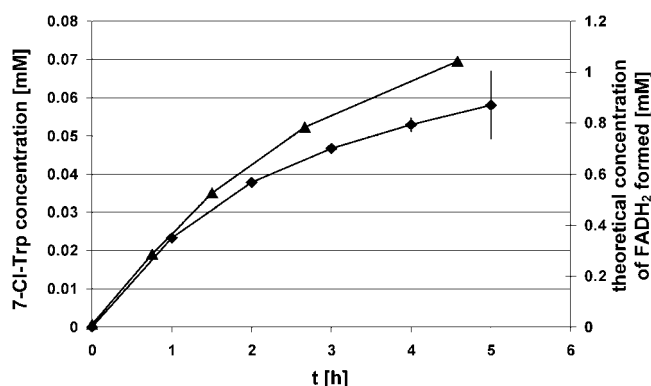
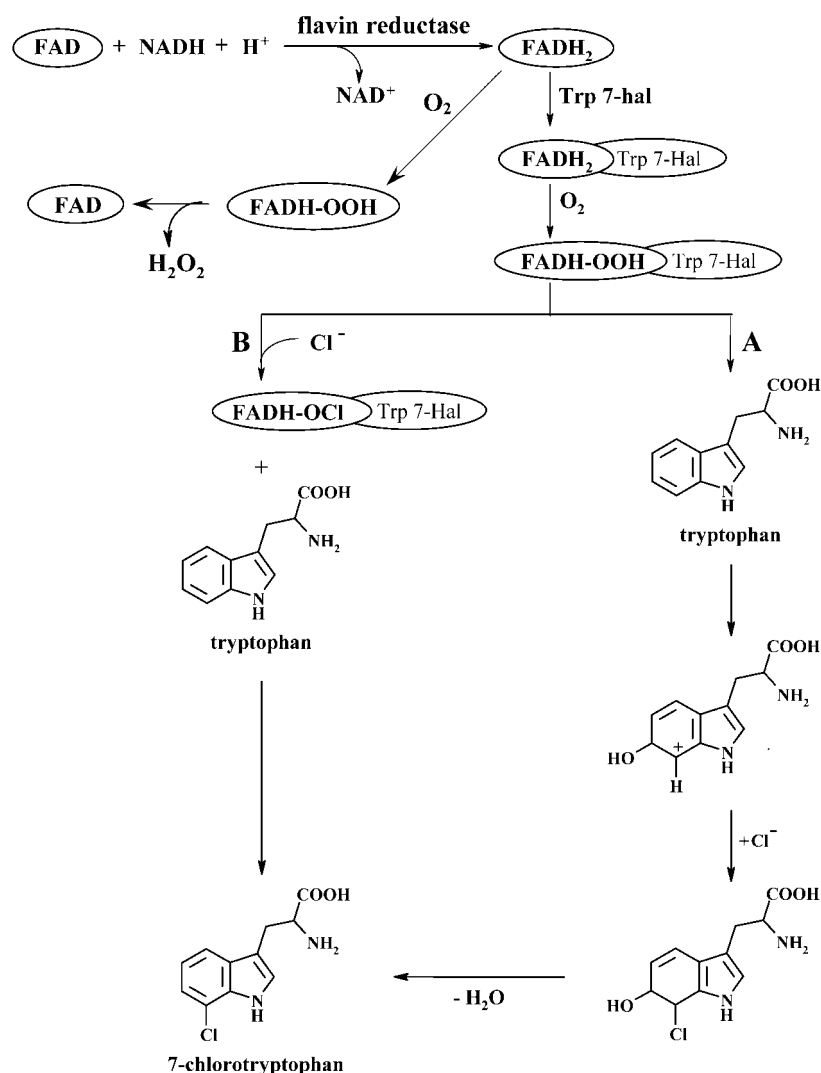


Figure 1. Time-dependent chemoenzymatic formation of 7-chlorotryptophan (♦) and theoretical amount of FADH₂ regenerated by [Cp*Rh(bpy)(H₂O)]²⁺ (▲). General conditions: 10 mM phosphate buffer (pH 7.2), 0.25 mM L-tryptophan, 10 μM FAD, 25 mM Cl⁻, 40 mM formate, 30 °C; (♦): 85 μM Trp 7-hal, 0.2 mM [Cp*Rh(bpy)(H₂O)]²⁺; (▲): 0.2 mM [Cp*Rh(bpy)(H₂O)]²⁺.

NADH. The FADH₂ must be protected from the unproductive spontaneous reaction with molecular oxygen by binding to flavin-free Trp 7-hal. This hypothesis is supported by the fact that all halogenases purified in active form so far, do not contain flavin (unpublished results). Through the reaction of the halogenase-bound FADH₂ with oxygen, enzyme-bound 4α-flavin hydroperoxide would be formed which could attack the organic substrate. This would lead to activation for the attack of chloride as a nucleophile. The resulting halohydrin could give rise to the end product 7-chlorotryptophan *via* dehydration. Hubbard and Walsh^[13] suggested an alternative for the actual halogenation step in which chloride would react with the 4α-flavin hydroperoxide and thus form a flavin-bound, activated electrophilic halide species (Scheme 2). Both hypotheses are based on the initial formation of enzyme-bound 4α-flavin hydroperoxide. Hopefully, further investigations, based on the recently elucidated three-dimensional structure of Trp 7-hal^[14] will help to elucidate the reaction mechanism of this new type of enzymes.

Conclusions

Tryptophan 7-halogenase is the first member of the new type of halogenating enzymes, the flavin-dependent halogenases. The flavin-free enzyme requires FADH₂ for the regioselective chlorination or bromination of tryptophan. It could be shown that FADH₂ can be used by this enzyme in freely diffusible form; direct contact between the flavin reductase providing FADH₂ and the actual halogenase is not absolutely necessary. The flavin reductase and NADH can be substituted by a chemoenzymatic FADH₂ regeneration system consisting of the organometallic catalyst [Cp*Rh(bpy)(H₂O)]²⁺ and for-



Scheme 2. Proposed hypothetical reaction mechanisms of FADH₂-dependent Trp 7-hal with (A) nucleophilic attack of chloride^[3] or (B) via formation of an electropilic, flavin-bound chlorine.^[13]

mate as the electron donor for FAD reduction. These results suggest similarities in the reaction mechanism with a group of monooxygenases which can also use freely diffusible FADH₂ and they are an important step towards the elucidation of the reaction mechanism of the new type of halogenases.

Experimental Section

Purification of Trp 7-hal and Enzyme Assay

Trp 7-hal was purified as described previously.^[4] Chlorinating activity was determined by incubating 55 μ L of Trp 7-hal-containing protein solution with 0.55 mU flavin reductase (Fre) from *E. coli*, 10 μ M FAD, 0.25 mM L-tryptophan, 12.5 mM MgCl₂, and 2.4 mM NADH in a total volume of 200 μ L in 10 mM potassium phosphate buffer (pH 7.2) at 30 °C for 6 h

(standard Trp 7-hal assay). The reaction was stopped and analysed by HPLC as described by Keller et al.^[4]

Reaction with Separated Flavin Reductase and Trp 7-hal

Trp 7-hal and the flavin reductase (Fre) were separated with the help of a Serva dialysis tubing 20/32 membrane with an exclusion limit of 12–14 kDa. The dialysis tubing was filled with 0.55 mU Fre in a total volume of 100 μ L in 10 mM potassium phosphate buffer (pH 7.2). The dialysis tubing was then placed in a solution of 110 μ L Trp 7-hal-containing protein solution (0.3 mg mL⁻¹), 10 μ M FAD, 0.25 mM L-tryptophan, 12.5 mM MgCl₂, and 2.4 mM NADH in a total volume of 700 μ L in 10 mM potassium phosphate buffer (pH 7.2) and incubated at 30 °C for 6 h. As a reference, an assay was used in which the dialysis tubing had been filled with 100 μ L 10 mM potassium phosphate buffer (pH 7.2) and Fre had been added to the Trp 7-hal-containing solution. Detection and quantification

of the formed 7-chlorotryptophan was done by HPLC analysis as described previously.^[4]

Reactions without Flavin Reductase

Chemical reduction of FAD was performed by adding a freshly prepared titanium(III) citrate solution (70 mM) to an FAD solution (100 μ M) that had been flushed with argon.^[16] 160 μ L of this solution were added to a Trp 7-hal (0.3 mg mL⁻¹) assay solution (without Fre, FAD, and NADH) that had also been flushed with argon. Oxygen was allowed to diffuse into the assay solution by incubating the open reaction tubes at 30 °C. After 3 h the reaction was stopped and analysed by HPLC.

In preparation for the investigations of the cofactor regeneration with the organometallic complex [Cp*Rh(bpy)(H₂O)]²⁺ and for the determination of the influence of formate on the Trp 7-hal activity, formate was added to the Trp 7-hal standard assay in concentrations of 5–500 mM.

The organometallic complex [Cp*Rh(bpy)(H₂O)]²⁺ was prepared as described by Kölle and Grätzel.^[15] The influence of the components of the Trp 7-hal assay on the activity of the cofactor regeneration system was analysed using the already described concentrations of the individual components. For the determination of the activity of the catalyst, the amount of H₂O₂ formed during the reaction was measured using a colorimetric assay.^[17] The Trp 7-hal/[Cp*Rh(bpy)(H₂O)]²⁺ assay consisted of 0.2 mM organometallic complex, 10 μ M FAD, 55 μ L Trp 7-hal-containing protein solution (0.3 mg mL⁻¹), 0.25 mM L-tryptophan, 12.5 mM MgCl₂, and 40 mM formate in a total volume of 200 μ L in 10 mM potassium phosphate buffer (pH 7.2). As references, Trp 7-hal standard assays containing the corresponding formate concentration (40 mM) were used.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft (Pe 348/17–1 and the Graduiertenkolleg “Struktur-Eigenschafts-Beziehungen bei Heterocyclen”), by the Sächsische Staatsministerium für Umwelt und Landwirtschaft, and the Fonds der Chemischen Industrie.

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