$FADH_2$-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 $FADH_2$ 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 $FADH_2$ 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 $FADH_2$, the organometallic complex (pentamethylcyclopentadienyl)rhodium-bipyridine $\{[Cp^*Rh(bpy)(H_2O)]^{2+}\}$ 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; $FADH_2$ 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 organohalogens, 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 compounds.[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 pyrrolnitrin-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 ($FADH_2$) 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

 DOI: 10.1002/adsc.200505029 © 2005 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

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 $FADH_2$ or an additionally required NADH.

For the elucidation of the reaction mechanism, the interplay of the two protein components with respect to the $FADH_2$ transfer and the NADH requirement was investigated in detail.

Results and Discussions

Direct Interaction of the Flavin Reductase and the Halogenase Component

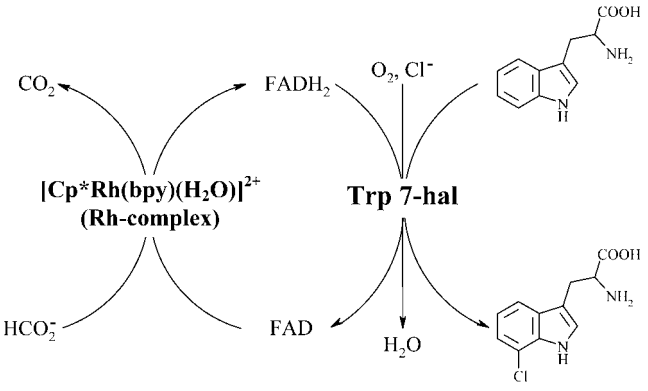
To investigate whether a direct contact between the flavin reductase and the halogenase is necessary, the two protein components were separated by a dialysis membrane. Even under these conditions, chlorination of tryptophan occurred, although only with about 60% (130 μU compared to 220 μU of halogenating 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 $FADH_2$. 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 μM) 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 μM) occurred. Since $FADH_2$ is consumed through the very rapid, spontaneous reaction with molecular oxygen to 4α-flavin hydroperoxide which then decomposes into FAD and H_2O_2[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 $FADH_2$ clearly prove that the halogenase can use freely diffusible $FADH_2$ for the catalysis of the chlorination of tryptophan. When FAD was substituted by FMN, no reaction occurred, showing that the halogenase is specific for $FADH_2$.

Halogenation using an $FADH_2$ 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 $FADH_2$ regeneration. For this purpose an enzyme-independent *in situ* regeneration system consisting of the organometallic (pentamethylcyclopentadienyl)rhodium-bipyridine complex $\{[Cp^*Rh(bpy)(H_2O)]^{2+}\}$ 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, $FADH_2$ 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 $FADH_2$ 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 μM catalyst concentration, indicating that $FADH_2$ 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 $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

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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 $FADH_2$ 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 $FADH_2$ 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 $FADH_2$ 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 $FADH_2$-producing enzyme, the flavin reductase, to the $FADH_2$-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 $FADH_2$-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 $FADH_2$ transfer.[8] Only in the luciferase/flavin reductase system from *Vibrio harveyi*, could an $FMNH_2$-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 $FADH_2$-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 $FADH_2$ dependence a monooxygenase-like mechanism can be postulated for Trp 7-hal (Scheme 2). In a first reaction, preceding the actual halogenation reaction, $FADH_2$ is produced by a flavin reductase with oxidation of

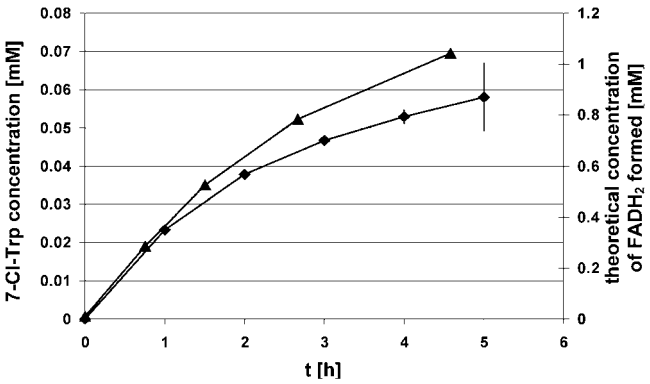

Figure 1. Time-dependent chemoenzymatic formation of 7-chlorotryptophan (◆) and theoretical amount of $FADH_2$ regenerated by $[Cp^*Rh(bpy)(H_2O)]^{2+}$ (▲). General conditions: 10 mM phosphate buffer (pH 7.2), 0.25 mM L-tryptophan, 10 μM FAD, 25 mM Cl^-, 40 mM formate, 30 °C; (◆): 85 μM Trp 7-hal, 0.2 mM $[Cp^*Rh(bpy)(H_2O)]^{2+}$; (▲): 0.2 mM $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

NADH. The $FADH_2$ 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 $FADH_2$ 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 $FADH_2$ for the regioselective chlorination or bromination of tryptophan. It could be shown that $FADH_2$ can be used by this enzyme in freely diffusible form; direct contact between the flavin reductase providing $FADH_2$ and the actual halogenase is not absolutely necessary. The flavin reductase and NADH can be substituted by a chemoenzymatic $FADH_2$ regeneration system consisting of the organometallic catalyst $[Cp^*Rh(bpy)(H_2O)]^{2+}$ and for-

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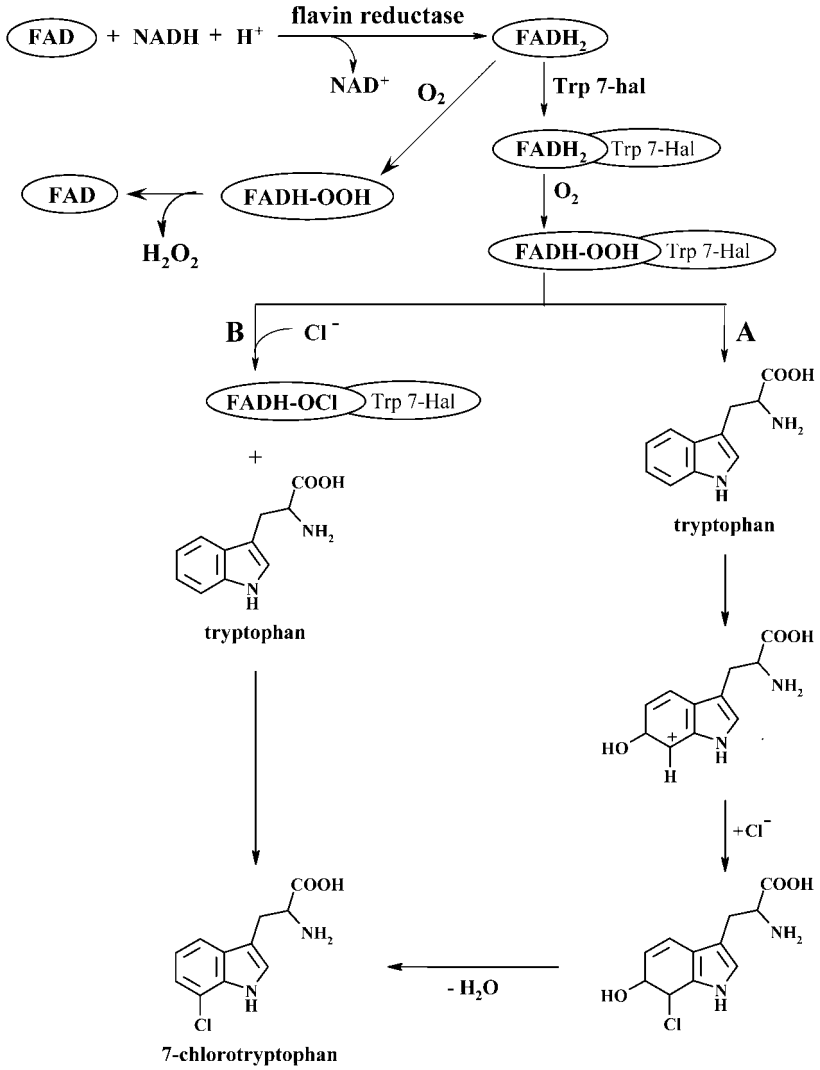

Scheme 2. Proposed hypothetical reaction mechanisms of $FADH_2$-dependent Trp 7-hal with (**A**) nucleophilic attack of chloride[3] or (**B**) *via* formation of an electropilic, flavin-bound chlorine.[13]

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 $FADH_2$ 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 μL of Trp 7-hal-containing protein solution with 0.55 mU flavin reductase (Fre) from *E. coli*, 10 μM FAD, 0.25 mM L-tryptophan, 12.5 mM $MgCl_2$, and 2.4 mM NADH in a total volume of 200 μL 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 μL in 10 mM potassium phosphate buffer (pH 7.2). The dialysis tubing was then placed in a solution of 110 μL Trp 7-hal-containing protein solution (0.3 mg mL^{-1}), 10 μM FAD, 0.25 mM L-tryptophan, 12.5 mM $MgCl_2$, and 2.4 mM NADH in a total volume of 700 μL 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 μL 10 mM potassium phosphate buffer (pH 7.2) and Fre had been added to the Trp 7-hal-containing solution. Detection and quantification

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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 μM) that had been flushed with argon.[16] 160 μL 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 $[Cp^*Rh(bpy)(H_2O)]^{2+}$ 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 $[Cp^*Rh(bpy)(H_2O)]^{2+}$ 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 H_2O_2 formed during the reaction was measured using a colorimetric assay.[17] The Trp 7-hal/$[Cp^*Rh(bpy)(H_2O)]^{2+}$ assay consisted of 0.2 mM organometallic complex, 10 μM FAD, 55 μL Trp 7-hal-containing protein solution (0.3 mg mL^{-1}), 0.25 mM L-tryptophan, 12.5 mM $MgCl_2$, and 40 mM formate in a total volume of 200 μL 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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