

# Studies on the Possible Mechanism of Inactivation of Phenylalanine Hydroxylase by Destructive Oxygen Species

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The enzymic hydroxylation of phenylalanine by phenylalanine hydroxylase (E.C. 1.14.16.1.) *in vitro* is dependent on the presence of hydrogen peroxide removing processes.

The loss of phenylalanine hydroxylase activity can be prevented to the same extent by catalase as well as the presence of optimized amounts of both peroxidase and superoxide dismutase.

Peroxidase alone exhibited only two third of the maximal protective effect of catalase whereas superoxide dismutase alone was not able to exert any protective influence on phenylalanine hydroxylase. These findings suggest that the termination of phenylalanine hydroxylation in the absence of hydrogen peroxide removing reactions is probably due to destructive oxygen species generated at the active site iron of phenylalanine hydroxylase in the presence of  $H_2O_2$  and the tetrahydropterin cofactor.

## Introduction

Besides the substrates phenylalanine,  $O_2$  and the tetrahydropterin cofactor, rat liver phenylalanine hydroxylase also requires catalase for maintaining its activities *in vitro* as shown by Kaufman and Fisher and other groups [1–7].

In the absence of catalase the enzymic conversion of phenylalanine into tyrosine ceased approximately 3 min after starting the reaction [8]. A presumptive explanation of the protective effect of catalase for the phenylalanine hydroxylase activity has been given by Kaufman and Fisher [1] who assumed that during the enzyme catalysis oxidized, inhibitory products could be generated or, alternatively  $H_2O_2$  could oxidise tetrahydropterin to the inactive 7,8-dihydropterin where the active quinoid dihydropterin is no longer involved as an intermediate.

Since the protective effect of catalase is still not well understood the purpose of this study was to

investigate the mechanism of inactivation of rat liver phenylalanine hydroxylase with respect to the function of destructive oxygen species generated during enzyme catalysis *in vitro*.

## Experimental procedures

### Materials

Dithiothreitol, 6,7-dimethyltetrahydropterin, superoxide dismutase and bovine serum albumin were purchased from Sigma; catalase and peroxidase from Boehringer, Mannheim; phenyl-Sepharose CL-4B from Pharmacia; Chelex 100 from BioRad; all other chemicals (analytical grade) were obtained from Merck, Darmstadt.

### Methods

The preparation of rat liver phenylalanine hydroxylase (RL-PAH) was performed by hydrophobic affinity chromatography on phenyl-Sepharose CL-4B according to Shiman [2] as modified by Gottschall *et al.* [9].

### Protein determination

The protein content was determined by a modified method of Lowry using Folin-Ciocalteus reagent [10].

**Abbreviations:** PAH, phenylalanine hydroxylase; E.C. 1.14.16.1.; RL-PAH, rat liver phenylalanine hydroxylase; CAT, catalase; E.C. 1.11.1.6.; POD, peroxidase; E.C. 1.11.1.7.; SOD, superoxide dismutase; E.C. 1.15.1.1.; DTT, dithiothreitol; BSA, bovine serum albumin.

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### Determination of enzyme activity

Rat liver phenylalanine hydroxylase activity was assayed by indirect spectrophotometric tyrosine determination according to the method of Shiman [2].

1.0 ml assay mixture contained:

0.1 M potassium buffer pH 6.8 (passed through a Chelex 100 column), 1 mM phenylalanine, variable amounts of catalase, peroxidase, superoxide dismutase or formate, respectively, RL-PAH sample, 6 mM dithiothreitol and 60  $\mu$ M 6,7-dimethyltetrahydropterin (instead of 6-methyltetrahydropterin in the original "Shiman" test system [2]).

For activation of phenylalanine hydroxylase, the reaction mixture was incubated for 3 min at 25 °C prior to the addition of dithiothreitol and tetrahydropterin cofactor.

The rates of tyrosine formation were monitored at  $\lambda = 275$  nm ( $\Delta A/\text{min}$ ,  $\epsilon = 1700 \text{ mol}^{-1} \cdot \text{cm}^{-1}$ ). The reference cuvette contained all components of the assay mixture except the RL-PAH sample.

## Results

### 1. Photometric determination of rat liver phenylalanine hydroxylase activity in dependence of catalase

Varying amounts of catalase in the assay mixture showed that the protective effect of catalase exhibits three phases (Fig. 1). Maximal tyrosine formation rate was maintained by catalase in the range higher than 10  $\mu$ g CAT/ml assay mixture.

Lower catalase concentrations resulted in a marked decrease in PAH activity. Of interest in this context is that already very low catalase concentrations (approximately 300 ng/ml assay mixture = 20 units) were sufficient to support half maximal rate of spectrophotometric detectable tyrosine formation.

### 2. Effect of peroxidase on rat liver phenylalanine hydroxylase activity

In order to examine the possibility that catalase as protective agent could be replaced by other hydroperoxide "consuming" enzymes, we tested the effect of peroxidase in catalase free test systems.

As shown in Fig. 2, peroxidase also exhibited a protective influence but not to the same extent as catalase. Peroxidase concentrations between

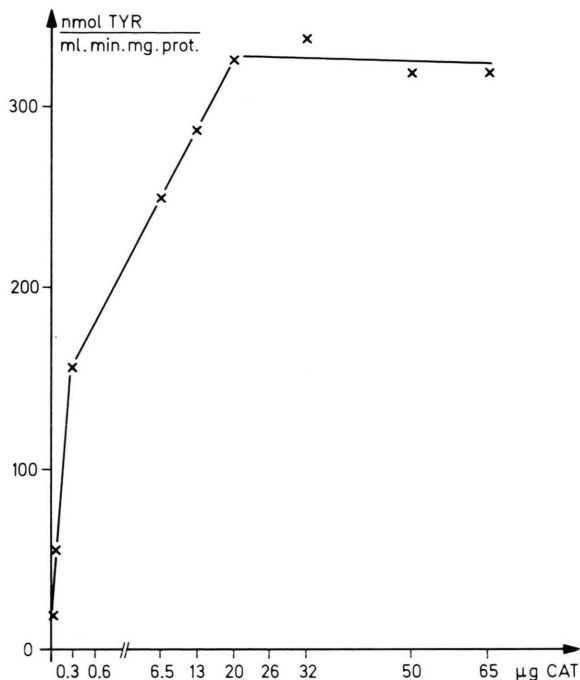


Fig. 1. Spectrophotometrical determination of tyrosine formation: Influence of increasing catalase concentration on phenylalanine hydroxylase activity. Reaction conditions were as described under Methods. The reaction mixtures contained 16  $\mu$ g rat liver phenylalanine hydroxylase.

20–150  $\mu$ g per ml assay mixture provided 60–70% of the control rate of tyrosine formation in the presence of 65  $\mu$ g catalase/ml assay mixture.

Peroxidase concentrations under 10  $\mu$ g POD/ml also yielded in a conspicuous decrease of PAH activity.

### 3. Optimization of peroxidase effects by superoxide dismutase

As shown in Fig. 2, the rate of tyrosine formation in catalase free test systems can be increased by peroxidase and superoxide dismutase up to the level of maximal tyrosine production by phenylalanine hydroxylase as measured in presence of 65  $\mu$ g catalase [11]. The protective effects of 100  $\mu$ g POD/ml assay mixture which resulted in maintaining approximately 70% of maximal tyrosine formation were elevated to 99% of the maximal rate by the addition of 50  $\mu$ g superoxide dismutase (Table I).

Fig. 2 also demonstrates that the sole presence of SOD in test systems without catalase or peroxidase,

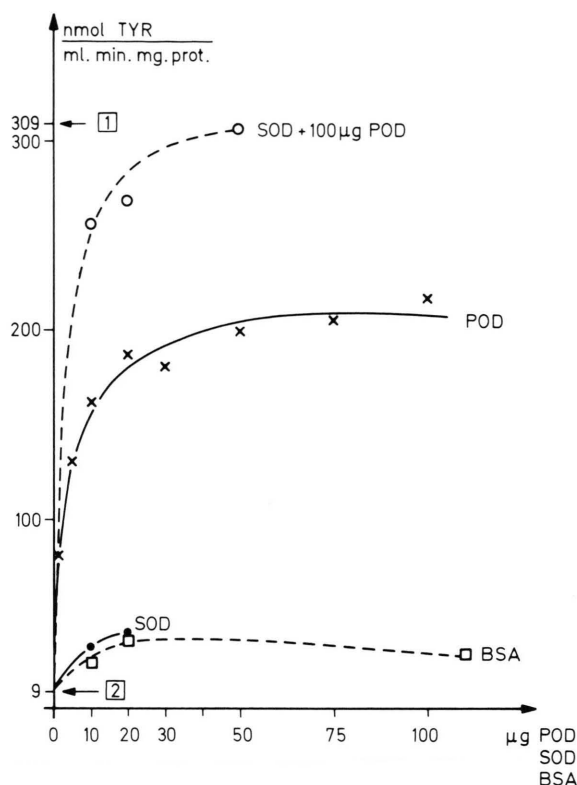


Fig. 2. Comparison of photometrically determined activities of rat liver phenylalanine hydroxylase: Protective effects of peroxidase (POD), superoxide dismutase (SOD), bovine serum albumin (BSA) and the combination of POD and SOD. Reaction conditions were as described under Figure 1. Symbols:  $\square$  65  $\mu$ g catalase were added instead of POD;  $\square$  the assay mixture contained neither catalase nor peroxidase.

respectively, is not able to protect phenylalanine hydroxylase against inactivation. SOD (10  $\mu$ g and 20  $\mu$ g) yields only very low protective effect, comparable with that of unspecific proteinaceous effects of equivalent amounts of bovine serum albumin (Table I).

#### 4. Influence of formate on the photometric detectable rate of tyrosine formation

The addition of 0.5–10  $\mu$ mol Na-formate to the test system in exchange for catalase as well as peroxidase resulted in an obvious decrease in the rate of tyrosine formation compared with the catalase and peroxidase free test system.

Therefore, the  $\text{OH}^\cdot$ -radical scavenger formate did not exhibit any protective effect against the inactivation of phenylalanine hydroxylase.

Table I. Photometric determination of phenylalanine hydroxylase activities in dependence of catalase, peroxidases and superoxide dismutase.

| Additions                    | nmol TYR    |                | % TYR formed |
|------------------------------|-------------|----------------|--------------|
|                              | ml          | min mg protein |              |
|                              | (3.–4. min) |                |              |
| + CAT                        | 309         |                | 100          |
| – CAT                        | 9           |                | 3            |
| – CAT + POD                  | 219         |                | 71           |
| – CAT + POD + 50 $\mu$ g SOD | 306         |                | 99           |
| – CAT + POD + 20 $\mu$ g SOD | 269         |                | 87           |
| – CAT + POD + 10 $\mu$ g SOD | 256         |                | 83           |
| – CAT – POD + 20 $\mu$ g SOD | 40          |                | 13           |
| – CAT – POD + 10 $\mu$ g SOD | 33          |                | 11           |
| – CAT + 110 $\mu$ g BSA      | 29          |                | 9            |
| – CAT + 20 $\mu$ g BSA       | 37          |                | 12           |
| – CAT + 10 $\mu$ g BSA       | 24          |                | 8            |

Assay mixtures were as described under Methods. The test systems contained 16  $\mu$ g rat liver phenylalanine hydroxylase and where indicated, 65  $\mu$ g catalase (CAT), 100  $\mu$ g peroxidase (POD), superoxide dismutase (SOD) or bovine serum albumin (BSA), respectively. TYR = tyrosine.

#### Discussion

The results confirm that the spectrophotometric monitoring of tyrosine formation in the applied test system is dependent on removal of hydrogen peroxide from the test system as also known for other determination methods of phenylalanine hydroxylase activity. PAH inactivation was prevented to the same extent by catalase as well as by peroxidase, optimized in combination with superoxide dismutase.

Peroxidase alone only achieved up to 70% of the maximal protective effect brought about by catalase alone.

The additional protection caused by the simultaneous presence of superoxide dismutase may be attained by preventing peroxidase from the destructive influence of  $\text{O}_2^{\cdot -}$  which is known to affect the activities of certain heme proteins. Since SOD alone was not able to maintain phenylalanine hydroxylase activity this observation indicates that  $\text{O}_2^{\cdot -}$  is not responsible for damaging phenylalanine hydroxylase and its loss of activity.

$\text{H}_2\text{O}_2$  is generated by autoxidation of the tetrahydropterin cofactor [1, 12] and thus appears to be the main intriguing by-product during the enzymic turnover since the free radical scavenger Na-for-

mate was also unable to provide a protective effect for RL-PAH.

As shown by earlier investigations [11], a minimal system consisting of tetrahydropterin and Fe-III-/EDTA (0.1 mM) causes methionine fragmentation (yielding ethylene as indicator) probably due to a cooperation between a "Fenton-type" oxidant or ferryl species (Fe-III-OH<sup>•</sup>) or a highly reactive agent called "crypto-OH-radical", generated by the interaction of tetrahydropterin radical (H<sub>3</sub>Pt<sup>•</sup>) and H<sub>2</sub>O<sub>2</sub>.

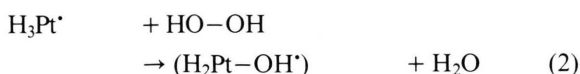
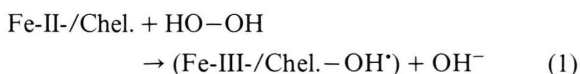
This type of methionine fragmentation can also be completely inhibited by catalase, indicating the essential role of H<sub>2</sub>O<sub>2</sub> as reacting component [11].

It is known that phenylalanine hydroxylase contains iron in its active site which can be removed by certain chelators [9], resulting in a loss of activity. Extensive ESR studies by Kaufman [1] and also by Gottschall [13] indicate that purified phenylalanine hydroxylase shows an ESR signal typical for Fe-III- in its high spin state which is very sensitive to changes of its ligand environment.

As demonstrated by Gottschall [13], the addition of peroxide (H<sub>2</sub>O<sub>2</sub>) to the pure PAH sample did not influence the signal. Therefore it seems not likely that H<sub>2</sub>O<sub>2</sub> alone would inactivate phenylalanine hydroxylase by interacting with the active site iron, (Fe-III-).

In presence of all components, necessary for the hydroxylation reaction (PAH, tetrahydropterin, O<sub>2</sub>

and phenylalanine), however, we speculate the generation of highly reactive oxygen species by reaction of the reduced active site iron (Fe-II-) with H<sub>2</sub>O<sub>2</sub> yielding a Fenton-type oxidant (see equation (1)) and/or by reaction of tetrahydropterin radical with H<sub>2</sub>O<sub>2</sub> resulting in the production of a "crypto-OH-radical" like specie (see equation (2)).



Both types of destructive oxygen species will react non specific and may be able to cause local destructions of the active site, probably irreversibly changing the ligand environment of the active site iron of phenylalanine hydroxylase. Therefore, in absence of H<sub>2</sub>O<sub>2</sub> removing reactions (most effectively represented by catalase) phenylalanine hydroxylase likely seems to be subjected to a suicidal side effect during its catalysis *in vitro*.

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