Tetrahydropterin Oxidation without Hydroxylation Catalyzed by Rat Liver Phenylalanine Hydroxylase

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SUMMARY

In the presence of lysolecithin or α-chymotrypsin, rat liver phenylalanine hydroxylase catalyzes the p-tyrosine-dependent oxidation of tetrahydropterin. p-Tyrosine is not hydroxylated during this reaction. Thus, tetrahydropterin oxidation occurs in the absence of hydroxylation of the substrate. For each equivalent of TPNH (or tetrahydropterin) oxidized, 1 equivalent of oxygen is reduced to hydrogen peroxide. Therefore, in the presence of lysolecithin or α-chymotrypsin and p-tyrosine, phenylalanine hydroxylase functions as a tetrahydropterin oxidase.

Phenylalanine hydroxylase is a mixed function oxidase which catalyzes the hydroxylation of phenylalanine to form tyrosine. The enzyme utilizes 2 electrons from a tetrahydropterin cofactor and consumes a molecule of atmospheric oxygen in catalyzing the formation of tyrosine, quinonoid dihydropteridine, and water (Equation 1) (1):

$$XH_4 + \text{phenylalanine} + O_2 \rightarrow XH_2 + \text{tyrosine} + H_2O \quad (1)$$

$$XH_2 + \text{TPNH} + H^+ \rightarrow XH_4 + \text{TPN}^+ \quad (2)$$

In these and following equations, $XH_4$ represents tetrahydropterin and $XH_2$ represents quinonoid dihydropteridine. The quinonoid dihydropteridine is reduced to tetrahydropterin by dihydropteridine reductase and TPNH or DPNH, as shown in Equation 2 (1). Therefore, when phenylalanine is used as the amino acid substrate, and 6,7-dimethyltetrahydropterin, 6-methyltetrahydropterin, or tetrahydrobiopterin (the natural cofactor (2)) is used as cofactor, the ratio of TPNH (or tetrahydropterin) oxidized to tyrosine formed is 1:1 (1, 3). When p-fluorophenylalanine is substituted for phenylalanine, tetrahydropterin oxidation is partially uncoupled from tyrosine formation (3, 4). We have used the term "uncoupled" or "loosely coupled" to describe the hydroxylation reaction when there is more tetrahydropterin oxidized than tyrosine formed, and "tightly coupled" when the ratio of tetrahydropterin oxidized to tyrosine formed is close to unity (5). A ratio of 3 moles of TPNH oxidized to 1 mole of tyrosine formed has been obtained when phenylalanine is used as the amino acid substrate and 7-methyltetrahydropterin or tetrahydropterin is used as cofactor (3). The TPNH to tyrosine ratio for the combination of phenylalanine and 7-methyl-tetrahydropterin was found to be a function of enzyme concentration, temperature, and ionic strength (6). At very dilute enzyme concentrations, a TPNH to tyrosine ratio of nearly 5:1 was obtained. These results suggested that conditions might be found where hydroxylation is completely uncoupled from TPNH oxidation. This report describes those conditions under which the phenylalanine hydroxylase-catalyzed reaction is completely uncoupled.

EXPERIMENTAL PROCEDURE

TPNH, catalase, peroxidase, and sodium pyruvate were obtained from Boehringer Mannheim Corp., West Germany. Lysolecithin and l-p-tyrosine were from Sigma Chemical Co., St. Louis, Mo. Tetrahydropterin was a gift from Dr. Long of Hoffmann-LaRoche, Nutley, N. J. 6,7-Dimethyltetrahydropterin was from Aldrich Chemical Co., Milwaukee, Wis. Dihydropteridine reductase was purified from sheep liver through the calcium phosphate gel step (7). Rat liver phenylalanine hydroxylase was purified through the Sephadex G-200 step (the peak fractions were 90% pure and the side fractions were 70% pure) (8). p-p-Tyrosine was obtained from Mann Research Laboratories, Inc., New York, N. Y. l-p-[14C]Tyrosine (uniformly labeled with a specific activity of 384 mCi per mmole) was from New England Nuclear Corp., Boston, Mass. Purified erythropoietin was a kind gift from Dr. H. Deutsch, University of Wisconsin.

Oxygen uptake was determined with a Gilson Oxygraph (model K, Gilson Medical Electronics, Middleton, Wis.) fitted with a Clark-type oxygen electrode. Determinations were made at 25°C; we assumed that the initial oxygen concentration was 0.255 mm (9). In experiments in which high concentrations of pyruvate were used, it was necessary to determine TPNH concentration by its native fluorescence (excitation 340 nm and emission 460 nm (10)) because pyruvate absorbs too strongly at 340 nm to use ultraviolet absorbance to measure TPNH concentrations.

p-[14C]Tyrosine was identified by its migration on Whatman No. 3MM paper using a solvent system of 2-propanol-NH₄OH-H₂O (8:1:1) (11). In this system p-tyrosine has an $R_f$ of 0.2. p-[14C]Tyrosine was also purified and identified by the use of an amino acid analyzer column which contained a 50-cm column of cation exchange resin AA-15 (Beckman Instruments, Fullerton, Calif.). The column was run at 45°C with a flow rate of 54 ml per hour. Samples were applied to the column in dilute HCl (pH 2.2) and were eluted with 0.2 M sodium citrate, pH 4.25. p-Tyrosine was completely resolved from m-tyrosine.
TABLE I

Requirements for Tyrosine-dependent TPNH Oxidation by Phenylalanine Hydroxylase

Each reaction contained the following components in 1 ml total volume: 0.1 M potassium phosphate buffer, pH 6.9; 150 μg of dihydropteridine reductase; 20 nmoles TPNH; 100 μg of catalase; and 150 μg of 80% pure phenylalanine hydroxylase. D- or L-Tyrosine was present at 1.0 mM where indicated. Tetrahydrobiopterin concentration was 10 μM and 6,7-dimethyltetrahydropterin (DMPH₄) concentration was 60 μM. The lysoseline concentration was 1.0 mM. The autoxidation rate of the tetrahydropterin in the absence of the amino acid substrate or lysoseline was used as the blank rate. This value was the same with or without phenylalanine hydroxylase and was equal to 0.5 n mole and 1.0 n mole of TPNH oxidized per min with tetrahydrobiopterin and DMPH₄, respectively.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Cofactor</th>
<th>Lysoseline</th>
<th>Initial velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tyrosine</td>
<td>Tetrahydrobiopterin</td>
<td>–</td>
<td>0.1</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>Tetrahydrobiopterin</td>
<td>+</td>
<td>7.5</td>
</tr>
<tr>
<td>D-Tyrosine</td>
<td>Tetrahydrobiopterin</td>
<td>–</td>
<td>0.0</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>Tetrahydrobiopterin</td>
<td>+</td>
<td>0.0</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>DMPH₄</td>
<td>–</td>
<td>0.0</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>DMPH₄</td>
<td>+</td>
<td>2.3</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>DMPH₄</td>
<td>+</td>
<td>0.0</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>DMPH₄</td>
<td>–</td>
<td>0.0</td>
</tr>
</tbody>
</table>

under these conditions, as was originally reported by Tong et al. (11). Tyrosine was chemically determined by a fluorometric modification of the nitrosonaphthol method (12).

RESULTS

p-Tyrosine-dependent TPNH Oxidation by Phenylalanine Hydroxylase—We have recently found that lysoseline (13) and α-chymotrypsin1 dramatically stimulate the activity of phenylalanine hydroxylase when phenylalanine is used as the amino acid substrate and tetrahydrobiopterin is used as the cofactor. Both of these treatments alter the structure of the hydroxylase, the lysoseline by changing the conformation of the enzyme, and the protein by decreasing its size by about 30%. These agents increase the rate of hydroxylation of other aromatic amino acids, i.e. tryptophan and m-tyrosine, to an even greater extent than they increase phenylalanine hydroxylation. Thus, it appears that lysoseline and α-chymotrypsin decrease the specificity of phenylalanine hydroxylase toward its amino acid substrate. Accordingly, we tested the efficiency of the product of phenylalanine hydroxylation, p-tyrosine, as a substrate for the hydroxylase in the presence and absence of lysoseline. In the absence of lysoseline with tetrahydrobiopterin as cofactor, there was barely detectable p-tyrosine-dependent TPNH oxidation by phenylalanine hydroxylase (Table I). In the presence of lysoseline, however, there was appreciable p-tyrosine-dependent TPNH oxidation, which was 10% of the rate phenylalanine-dependent TPNH oxidation. p-p-Tyrosine did not stimulate TPNH oxidation in the presence or absence of lysoseline. When 6,7-dimethyltetrahydropterin was used as cofactor, lysoseline also stimulated the p-tyrosine-dependent TPNH oxidation, although to a lesser extent than was observed with tetrahydrobiopterin. To verify that this TPNH oxidation was actually a reflection of tetrahydrobiopterin oxidation, the hydroxylase-catalyzed reaction was run in the absence of the TPNH and dihydropteridine reductase regenerating system, and tetrahydropterin oxidation was followed directly by the increase in absorbance at 330 nm due to the formation of dihydropoterin (1). Under these conditions, the rate of p-tyrosine-dependent tetrahydrobiopterin oxidation was the same as the rate of TPNH oxidation with the regenerating system. Therefore, in the presence of p-tyrosine, phenylalanine hydroxylase catalyzes the oxidation of the tetrahydropterin.

Kinetic analysis of this p-tyrosine-dependent TPNH oxidation showed that the reaction rate increased with tetrahydrobiopterin concentration in a hyperbolic manner with an apparent Kₘ at pH 6.9 of 2.7 × 10⁻⁵ M. This Kₘ is nearly identical with the Kₘ for tetrahydrobiopterin obtained under the same conditions when phenylalanine was the amino acid substrate. The apparent Kₘ for p-tyrosine, based on the oxidation of TPNH, is 0.75 mM. This value is nearly 8 times higher than the apparent Kₘ for phenylalanine under the same conditions (13). From these results it was predicted that in the presence of lysoseline, product inhibition would occur when p-tyrosine concentration is high relative to the phenylalanine concentration. Indeed, when the hydroxylase was assayed at 0.1 mM phenylalanine, 0.8 mM p-tyrosine decreased the rate of TPNH oxidation by 50%, as was predicted from the relative Kₘ values of the two amino acids. This apparent competition of the p-tyrosine for the phenylalanine site on the hydroxylase and the similar Kₘ for tetrahydrobiopterin in the presence of either amino acid suggest that p-tyrosine-dependent tetrahydrobiopterin oxidation by the hydroxylase occurs at the same active site as does the phenylalanine-dependent TPNH oxidation.

p-Tyrosine-dependent TPNH oxidation was stimulated by α-chymotrypsin pretreatment of the hydroxylase to the same extent as it was by lysoseline. This finding further supports our conclusion that α-chymotrypsin and lysoseline activate the hydroxylase in a similar manner.1

Lack of Alteration of p-Tyrosine during p-Tyrosine-dependent TPNH Oxidation by Phenylalanine Hydroxylase—To investigate the fate of the amino acid substrate during the p-tyrosine-dependent TPNH oxidation by the hydroxylase, the reaction was allowed to proceed for 60 min at 37° starting with a low concentration of 14C-labeled tyrosine (0.2 μmole per ml, with a specific activity of 1 μCi per μmole). Since 0.36 μmole of TPNH was oxidized in a reaction mixture containing the hydroxylase (after correcting for the TPNH oxidized in a control reaction mixture containing all components except the hydroxylase), the amount of TPNH oxidized was nearly double the amount of tyrosine originally present (Table II). This result suggested that p-tyrosine might be acting catalytically and that it was not altered during the reaction. To test this model, the amounts of tyrosine remaining in the reaction mixtures run with and without hydroxylase were determined chemically by the nitrosonaphthol reaction (12). Table II shows that the same amount of tyrosine was present in both reaction mixtures after incubation and that the final amount of tyrosine equaled the amount of tyrosine originally added. To characterize further the structure of the tyrosine remaining at the end of the reaction, the percentage of original [14C]tyrosine which remained as p-[14C]tyrosine at the end of the reaction was determined by paper chromatography. In both the reaction mixture containing the hydroxylase and the reaction mixture lacking the hydroxylase, 98.5% of the original [14C]tyrosine co-chromatographed with nonradioactive p-tyrosine (Table II). No [14C]-la-

TPNH oxidation was followed spectrophotometrically by the decrease in absorbance at 340 nm. The complete reaction mixture contained 156 μg of 80% pure phenylalanine hydroxylase with 32 μM tetrahydrobiopterin, 200 μM l-[3H]tyrosine (1 × 10^6 cpm, uniformly labeled), and 1 mM lysolecithin. An identical mixture without hydroxylase served as the control. The reaction was run for 60 min at 37°C and was stopped by the addition of 200 μl of 3 N trichloracetic acid. The acidified reaction mixtures were then analyzed for tyrosine.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Tyrosine-dependent TPNH oxidation</th>
<th>Tyrosine remaining at end of reaction</th>
<th>Rate of oxygen uptake (μoles/min/1 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylnalanine hydroxylase</td>
<td>0.00</td>
<td>0.20</td>
<td>98.5</td>
</tr>
<tr>
<td>Phenylalanine hydroxylase</td>
<td>0.36</td>
<td>0.21</td>
<td>95.0</td>
</tr>
</tbody>
</table>

- Analysis for tyrosine by a fluorometric modification of the nitrosonaphthol method (12).
- Analysis for tyrosine by paper chromatography on Whatman No. 3MM (with a solvent of 2-propanol-H2O-NH4OH, 8:1:1).
- Analysis for tyrosine by chromatography in an amino acid analyzer column according to Tong et al. (11).

beled 3,4-dihydroxyphenylalanine was detectable. Since this paper chromatography system does not resolve m-tyrosine from p-tyrosine, the reaction mixtures were chromatographed on an amino acid analyzer column by a technique which completely separates m- and p-tyrosine (11). By this technique it was shown that after the enzymatic reaction, 95% of the p-[3H]tyrosine originally present in the reaction mixture containing the hydroxylase co-chromatographed with cold p-tyrosine (Table II). Similarly, 94.5% of the p-[3H]tyrosine originally present in the control reaction mixture in which the hydroxylase had been omitted was unchanged during the assay incubation.

These results demonstrate that there is no detectable alteration in p-tyrosine during the p-tyrosine-dependent TPNH oxidation catalyzed by phenylalanine hydroxylase. Therefore, in this reaction there is no hydroxylation of the amino acid substrate and hydroxylation is completely uncoupled from TPNH (tetrahydropterin) oxidation.

Production of Hydrogen Peroxide—Our previous studies have indicated that hydrogen peroxide is formed under conditions in which the phenylalanine hydroxylase reaction is loosely coupled (3). We therefore looked for hydrogen peroxide production under the conditions of complete uncoupling described above, using peroxidase to detect hydrogen peroxide. Peroxidase catalyzes the oxidation of tetrahydropterins by hydrogen peroxide (3), as shown in Equation 3:

\[ \text{H}_2\text{O}_2 + \text{XH}_4 \rightarrow \text{XH}_3 + 2\text{H}_2\text{O} \]  

(3)

When this reaction is combined with the dihydropterin reductase reaction shown in Equation 2, the rate of hydrogen peroxide formation can be determined as an increase in the rate of TPNH oxidation. If the p-tyrosine-dependent TPNH (tetra-

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**Table II**

Effect of p-tyrosine-dependent TPNH oxidation on structure of p-tyrosine substrate

In Experiment 1, 10 μM tetrahydrobiopterin and 0.4 mM tyrosine were incubated with 100 μg of 70% pure phenylalanine hydroxylase. In Experiment 2, 4 μM tetrahydrobiopterin and 0.74 mM tyrosine were incubated with 156 μg of 80% pure phenylalanine hydroxylase. The total volume for each assay was 1 ml. The catalase concentration was 100 μg per ml and the peroxidase concentration was 2 μg per ml.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>Initial velocity (μoles TPNH/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Catalase</td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>Peroxidase</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>Catalase + peroxidase</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Catalase</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td>Peroxidase</td>
<td>13.7</td>
</tr>
<tr>
<td></td>
<td>Catalase + peroxidase</td>
<td>7.0</td>
</tr>
</tbody>
</table>

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**Table III**

Effect of peroxidase on rate of tyrosine-dependent TPNH oxidation

The rate of oxidation of the tetrahydropterin can be measured directly, as mentioned earlier, or indirectly, by coupling Reaction 5 with the dihydropterin reductase-catalyzed reaction (Equation 2) to give Equation 6:

\[ 2\text{XH}_4 + \text{O}_2 \rightarrow 2\text{XH}_3 + 2\text{H}_2\text{O} \]  

(5)

The rate of oxidation of the tetrahydropterin can be measured directly, as mentioned earlier, or indirectly, by coupling Reaction 5 with the dihydropterin reductase-catalyzed reaction (Equation 2) to give Equation 6:

\[ 2\text{TPNH} + 2\text{H}^+ + \text{O}_2 \rightarrow 2\text{TPN}^+ + 2\text{H}_2\text{O} \]  

(6)

As shown in Table III, addition of peroxidase did double the rate of TPNH oxidation in the uncoupled reaction. Further evidence in support of the conclusion that hydrogen peroxide is the product of the reaction was obtained by the observation that an excess of catalase, which catalyzes the following reaction:

\[ 2\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \]  

(7)

eliminated the effect of peroxidase on the rate of TPNH oxidation (Table III). These results support the model that in the p-tyrosine-dependent reaction a molecule of hydrogen peroxide is formed for each molecule of tetrahydropterin oxidized.

The reaction proposed to occur in the presence of p-tyrosine (Equation 4) was investigated further by studying the ratio of TPNH oxidation to oxygen uptake in the presence and absence of catalase. The sum of reactions shown in Equations 2, 4, and 7 is the same as the reaction shown in Equation 6, which predicts that in the presence of catalase the TPNH to oxygen ratio should be 2:1. As shown in Table IV, this prediction was confirmed since the experimentally determined TPNH to oxygen ratio was 2.03:1 in the presence of catalase.

To determine the ratio of TPNH oxidized to oxygen consumed in the absence of catalase, endogenous catalase in our phenylalanine hydroxylase had to be inhibited. (Even our most highly purified preparations of phenylalanine hydroxylase (85% pure) contain 0.5% catalase.) We found that it was possible to inhibit this catalase activity with sodium azide, which, at 10^-4 M, completely inhibited the catalase present in the hy-
Three samples was determined fluorometrically (excitation 350 nm). The TPNH content of each of these samples were diluted in base. The TPNII content of the same enzyme when phenylalanine was the substrate; 100 mm sodium pyruvate where indicated; 0.1 mm sodium azide where indicated; 100 mm per ml of catalase where indicated) were added to the water-jacketed, constant temperature (25°) electrode chamber (Clark electrode attached to a Gilson Oxygraph) in a total volume of 1.8 ml. Fifty microliters were removed and diluted to 2.0 ml with 0.1 N NaOH. Five minutes later, another 50 ml of reaction mixture were similarly diluted and the enzymatic reaction was begun by the addition of the amino acid substrate in a 0.3-ml volume (the final concentration of tyrosine was 1.1 mm and the phenylalanine concentration was 0.2 mm). Five to 8 min later, another 50 ml of reaction mixture were diluted in base. The TPNH content of each of these three samples was determined fluorometrically (excitation 350 nm, emission 450 nm). The decrease in the concentration of TPNH and oxygen during the period before the addition of the amino acid substrate was used as a blank rate. For each set of conditions, the results of two separate experiments are shown.

**Table IV**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions</th>
<th>TPNH consumed</th>
<th>Oxygen consumed</th>
<th>Ratio of TPNH to oxygen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylalanine</td>
<td>Pyruvate</td>
<td>mmoles</td>
<td>mmoles</td>
<td>mmoles</td>
</tr>
<tr>
<td>+ azide</td>
<td>147</td>
<td>165</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>130</td>
<td>147</td>
<td>0.88</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Pyruvate</td>
<td>140</td>
<td>148</td>
<td>0.96</td>
</tr>
<tr>
<td>+ azide</td>
<td>161</td>
<td>190</td>
<td>0.91</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>324</td>
<td>156</td>
<td>2.08</td>
<td></td>
</tr>
<tr>
<td></td>
<td>291</td>
<td>147</td>
<td>1.98</td>
<td></td>
</tr>
</tbody>
</table>

Erythrocuprein was used to test whether the superoxide anion is also involved as an intermediate in the phenylalanine hydroxylase reaction. Purified erythrocuprein (as high as 100 μg per ml) did not inhibit the hydroxylation of phenylalanine or the p-tyrosine-dependent TPNH oxidation by phenylalanine hydroxylase. These results imply that either the superoxide anion is not an intermediate in the phenylalanine hydroxylase-catalyzed reaction or that the superoxide anion is not available to the dismutase. Recent workers have found that two flavin hydroxylases, a dioxygenase (18), a dioxygenase (19), and a microsomal cytochrome P-450 hydroxylase (20) are inhibited by superoxide dismutase, while Massey et al. (21) failed to find any inhibition of flavin hydroxylases by superoxide dismutase.

If the superoxide anion were an intermediate in the hydroxylase reaction, it might appear in solution during the uncoupled reaction. However, under uncoupled conditions phenylalanine hydroxylase did not reduce cytochrome c or oxidize epinephrine. These results suggest that the superoxide anion is not produced during the uncoupled reaction. It is of interest that although superoxide dismutase did not inhibit the hydroxylation, it did inhibit by 50% the autoxidation of the tetrahydropterin. It therefore seems likely that in contrast to the hydroxylase-catalyzed oxidation of tetrahydropterin, the nonenzymatic oxidation involves the superoxide anion as an intermediate.

**Discussion**

The ability of a hydroxylase to catalyze a reaction in which the oxidation of the electron donor is partially uncoupled from hydroxylation was first demonstrated in 1961 with phenylalanine hydroxylase (4). At that time it was shown that in the presence of p-fluorophenylalanine, an analogue of the normal substrate, almost twice as much tetrahydropterin was oxidized as could be accounted for by the sum of the amount of tyrosine formed and organic fluorine reduced to fluoride ions. Later it was shown that analogues of the pterin cofactor, such as 7-methyltetrahydropterin, could also lead to partial uncoupling (3). Subsequently, it was reported that many hydroxylases exhibit substrate-analogue-induced uncoupling (22-24).

We have now defined conditions under which the phenylalanine hydroxylase-catalyzed reaction is completely uncoupled. These conditions require a structural modification of the hydroxylase—either by treatment with lysolecithin or by limited proteolysis—and the presence of an analogue of the substrate, in this case, the normal hydroxylated product of the reaction, tyrosine. As far as we are aware, this is the first example of product-induced uncoupling of a hydroxylation reaction.
Our present results can be related to the previous ones of Okamoto et al. (25), who showed that a structural modification of an oxygenase, i.e. blockage of the enzyme's thiol groups by various reagents, could, in the presence of the normal substrate, lead to uncoupling. It should be emphasized, however, that with phenylalanine hydroxylase, treatment with neither lysolecithin nor chymotrypsin leads to uncoupling with the normal substrate, phenylalanine.

In all cases in which it has been studied, including the present one, it has been shown that hydrogen peroxide is formed under conditions of partial or complete uncoupling. As we have previously discussed (3), the significance of this finding is that it provides the first evidence that oxygen at the reduction level of one, it has been shown that hydrogen peroxide is formed under conditions in which the enzyme is in the structurally altered form (due, for example, to the inhibition. If a portion of phenylalanine hydroxylase in the liver is in the structurally altered form (due, for example, to the inhibition, then under conditions in which the enzyme is in the structurally altered form (due, for example, to the inhibition.

In the case of the pterin-dependent hydroxylases, an attractive candidate for the intermediate is a pterin hydroperoxide. On the basis of evidence that this type of derivative might be involved in the nonenzymatic oxidation of reduced flavins and pteridines, Mager et al. (26, 27) have suggested that pterin and flavin hydroperoxides might also be intermediates in certain enzymatic hydroxylation reactions. Further work is needed to test the validity of this suggestion.

The ability of tyrosine to uncouple the reaction catalyzed by phenylalanine hydroxylase represents a new form of product inhibition. If a portion of phenylalanine hydroxylase in the liver is in the structurally altered form (due, for example, to the effect of endogenous phospholipids), then under conditions in which tyrosine concentrations are high relative to those of phenylalanine (tyrosinemia and tyrosinosis (28)), the rate of tyrosine formation from phenylalanine might be decreased due to the uncoupling phenomenon.

REFERENCES
Tetrahydropterin Oxidation without Hydroxylation Catalyzed by Rat Liver Phenylalanine Hydroxylase
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