A Protein That Stimulates Rat Liver Phenylalanine Hydroxylase

(Received for publication, April 13, 1970)

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SUMMARY

A protein has been found in partially purified preparations of sepiapterin reductase that stimulates highly purified rat liver phenylalanine hydroxylase. The stimulation is dependent on the structure of the pterin cofactor used; a large stimulation is observed with the naturally occurring cofactor, tetrahydrobiopterin, a slight one with 6-methyltetrahydropterin, and none with 6,7-dimethyltetrahydropterin. The extent of the stimulation is minimal at low and maximal at high hydroxylase concentrations. Kinetic data suggest that the stimulating protein influences a reversible association-dissociation of the hydroxylase.

The enzyme system from liver that catalyzes the conversion of phenylalanine to tyrosine is complex. One of the essential components of the system is an unconjugated pteridine cofactor which has been isolated from rat liver. It has been identified as 7,8-dihydrobiopterin (7,8-dihydro-2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-erythro)l]-pteridine) (1). Certain synthetic unconjugated pterins, such as B-methyl and 6,7-dimethylpterin, have high cofactor activity (2). In the presence of an active tetrahydropterin, phenylalanine hydroxylase ("rat liver enzyme") catalyzes the reaction shown in Equation 1 (3).

\[ \text{Tetrahydropterin + phenylalanine + O}_2 \rightarrow \text{quinonoid dihydropterin + tyrosine + H}_2\text{O} \] (1)

Another component, dihydropteridine reductase ("sheep liver enzyme"), catalyzes the \( \text{TPNH-mediated reduction of the quinonoid dihydropterin back to the tetrahydro level according to Equation 2 (3). This reaction allows the pterin to function catalytically.} \)

\[ \text{Quinonoid dihydropterin + TPNH + H}^+ \rightarrow \text{tетrahydropterin + TPN}^+ \] (2)

When 7,8-dihydrobiopterin, the form in which the cofactor has been isolated from rat liver, is used, phenylalanine hydroxylation exhibits an additional requirement for dihydrofolate reductase. This enzyme catalyses the \( \text{TPNH-mediated reduction of the 7,8-dihydrobiopterin to the active 5,6,7,8-tetrahydro form (3, 4). Once this reduction occurs, dihydrofolate reductase plays no further role in the hydroxylase system, since during the hydroxylation reaction, the pterin cycles between the tetrahydro and quinonoid dihydro forms (1).} \)

Under certain assay conditions, catalase also stimulates phenylalanine hydroxylation, but this effect is indirect; catalase protects the tetrahydropterin (5), and perhaps other components of the hydroxylase system, against inactivation by \( \text{H}_2\text{O}_2 \) that is formed during the auto-oxidation of the tetrahydropterin.

The present communication describes the properties of another protein, which stimulates phenylalanine hydroxylase.

The stimulating protein was originally detected (5, 6) as a contaminant in bovine liver glucose dehydrogenase preparations (7). It has now been found in purified preparations of sepiapterin reductase from rat liver (8). The properties of the protein from the latter source have been studied in detail and are the subject of the present report.

A marked stimulation by the protein is observed only when the naturally occurring cofactor, reduced biopterin, is used. This protein is distinct from any of the other known components of the system. Kinetic evidence suggests that it stimulates the hydroxylase by influencing the equilibrium between associated and dissociated forms of the enzyme. Of the known proteins that have been assayed for PHS activity, bovine growth hormone (somatotropin) is the only one that shows relatively high activity.

EXPERIMENTAL PROCEDURE

Materials

The source of the PHS used in these studies was a preparation of sepiapterin reductase purified from rat liver according to Method A of a published procedure (8). Phenylalanine hydroxylase that is about 90% pure was prepared from rat liver as described (9). Glucose dehydrogenase (7) and dihydropteridine reductase (2) were prepared as previously described. Highly purified dihydrofolate reductase from chicken liver was a gift from Dr. B. T. Kaufman. Biotperin samples were generously provided by Dr. H. Rembold. Tetrahydrobiopterin and 7,8-dihydrobiopterin were prepared as previously described (4), 6-methyltetrahydropterin was prepared as previously described (6), and 6,7-dimethyltetrahydropterin was purchased from Aldrich Chemical Company. Three times recrystallized \( \alpha-\)

1 Pterin refers to derivatives of 2-amino-4-pteridone.
chymotrypsin was obtained from Worthington Biochemical Corporation. Catalase and TPNH were products of Boehringer Mannheim Corporation. Sephadex G-25 and G-200 were obtained from Pharmacia Fine Chemicals. All of the studies with bovine growth hormone were carried out with an immunologically-homogeneous preparation kindly supplied by Dr. P. Condliffe. Its preparation and physical characterization have been described (10, 11). Human growth hormone was also kindly supplied by Dr. Condliffe. Other pituitary hormones were gifts from the Hormone Distribution Officer, National Institute of Arthritis and Metabolic Diseases. The ovine and rat growth hormone preparations had a biological potency of 1.6 and 1.0 i.u. per mg, respectively. Insulin and bovine serum albumin were purchased from Sigma Chemical Company.

Methods

Protein concentrations were determined by the method of Warburg and Christian (12).

Standard Assay for Phenylalanine Hydroxylase Stimulator—PHS is quantitatively assayed by its ability to stimulate the phenylalanine hydroxylase system. Either tyrosine formation (13) or the phenylalanine-dependent oxidation of TPNH (14) can be followed. The assay conditions are the same as those used for the determination of phenylalanine hydroxylase activity except that the reaction is performed at pH 8.0 and tetrahydrobiopterin is used in place of the 6,7-dimethyltetrahydropterin. The complete reaction mixture for the spectrophotometric assay contained the following components (in micromoles) in a final volume of 1.0 ml: potassium phosphate, pH 8.0, 30; L-phenylalanine, 2.0; TPNH, 0.15; tetrahydrobiopterin, 0.01; dihydropteridine reductase and catalase (7800 units) in excess; phenylalanine hydroxylase (an amount that will catalyze the formation of 0.005 to 0.01 μmoles of tyrosine per min under standard assay conditions); and PHS. A blank cuvette containing the complete reaction mixture but without PHS served as a control. When crude extracts were assayed for PHS activity, the hydroxylase activity in the extract was measured and the appropriate correction was made. A complete reaction mixture without phenylalanine can also be included as a control for non-phenylalanine-dependent TPNH oxidation. Since the rate of TPNH oxidation in the absence of phenylalanine is only 2 or 3% of that in the complete mixture, there is no need to include this control in every assay. The reaction was carried out in a spectrophotometer equipped with a thermostated cell chamber kept at 25° with a circulating water bath. The rate of TPNH oxidation, calculated from the decrease in absorbance at 340 μm, was followed for several minutes and was equal to the rate of tyrosine formation under these conditions (14). The rate obtained in the presence of PHS minus that in its absence is a measure of PHS activity. 7,8-Dihydrobiopterin can be used in place of the tetrahydro compound. In this case, dihydrofolate reductase must be added (4).

The stimulation of the hydroxylase reaction by increasing amounts of purified PHS is shown in Fig. 1.

Incubation of Phenylalanine Hydroxylase Stimulator with Tetrahydrobiopterin and Separation of Pterin from Phenylalanine Hydroxylase Stimulator—L-Tetrahydrobiopterin was prepared from 7,8-dihydrobiopterin (4) by incubation with dihydrofolate reductase. Two tubes were prepared, each containing the following components (in micromoles): potassium phosphate pH 8.0, 50; TPNH, 0.15; dihydrobiopterin (0.008); dihydrofolate reductase and catalase, in excess, in a final volume of 0.5 ml. After a 70-min incubation at 25° in the dark, 0.01 ml of PHS (containing 0.054 mg of protein) was added to one mixture and the tubes were incubated for another 15 min. The tubes were cooled to 4°, and 0.5 ml of 0.07 M mercaptoethanol was added to each tube. The contents of each tube were applied at 4° to columns (1.1 x 4.2 cm) of Sephadex G-25 that had been previously equilibrated with 0.01 M KCl-0.034 M 2-mercaptoethanol. The same mixture was used to elute the tetrahydrobiopterin. The first 4.0 ml contained less than 10% of the pterin (determined spectrophotometrically on a control mixture not containing TPNH) and were discarded. The next 6.0 ml contained 85 to 90% of the tetrahydrobiopterin. The eluates were lyophilized, and the residues were dissolved in 0.5 ml of 0.005 N HCl and assayed immediately. Control experiments showed that there was no detectable PHS activity in the 6-ml eluate from the Sephadex columns. It has not been possible to recover from the Sephadex G-25 PHS in amounts as small as were used in this experiment.

Results

General Properties of Phenylalanine Hydroxylase Stimulator and Its Relationship to Sepiapterin Reductase—As already mentioned, PHS was originally detected as a contaminant in glucose dehydrogenase preparations. Subsequently it was found that glucose dehydrogenase was also contaminated with sepiapterin reductase, the enzyme that catalyzes the TPNH-mediated reduction of sepiapterin (6-lactyl-7,8-dihydropterin) to 7,8-dihydrobiopterin (8). These results indicated a possible functional relationship between PHS and sepiapterin reductase and led to the current finding that purified sepiapterin reductase had PHS activity. The possibility that the reductase activity is responsible for PHS activity was therefore considered. This possibility was eliminated by a study of the heat stability of the two activities. As can be seen in Table I, when fractions that contain both activities were heated for 6 min at 52°, 42% of

![Fig. 1. Stimulation of hydroxylase by increasing amounts of PHS. Standard conditions are as described under “Methods” with 0.005 μmoles of 7,8-dihydrobiopterin and 5.4 μg of hydroxylase.](http://www.jbc.org/content/245/18/4732/F1.large.jpg)
Table I

Sensitivity of sepiapterin reductase and phenylalanine hydroxylase stimulator to heat inactivation and acetate inhibition

The PHS fraction from the alkaline ammonium sulfate (8) stage and containing 3.1 mg of protein per ml was heated under conditions described. Any insoluble protein was removed by centrifugation and the supernatant fraction was assayed for PHS activity as described under "Methods." Sepiapterin reductase was assayed spectrophotometrically (Method A) as previously described (8). The same inhibition by acetate was observed when the reductase was assayed in the reverse direction, starting with 7,8-dihydrobiopterin (4).

<table>
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<tr>
<th>Heating conditions</th>
<th>Activity lost</th>
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<tr>
<td></td>
<td>Sepiapterin reductase</td>
</tr>
<tr>
<td>6 min, 52°</td>
<td>42%</td>
</tr>
<tr>
<td>14 min, 52°</td>
<td>65%</td>
</tr>
<tr>
<td>1 min, 100°</td>
<td>98%</td>
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<table>
<thead>
<tr>
<th>Acetate concentration</th>
<th>Inhibition</th>
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<tbody>
<tr>
<td>M</td>
<td>%</td>
</tr>
<tr>
<td>0.02</td>
<td>61</td>
</tr>
<tr>
<td>0.04</td>
<td>75</td>
</tr>
<tr>
<td>0.10</td>
<td>98</td>
</tr>
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</table>

* +, an apparent increase in activity.

Fig. 2. Optimum pH for hydroxylase in the presence and absence of PHS. Standard conditions with 0.010 μmole of tetrahydrobiopterin and 40 μg of hydroxylase, 108 μg of PHS. The pH values refer to those of the potassium phosphate buffer measured at 0.1 m at 25° with a glass electrode.

**Effect of Cofactor Structure on Phenylalanine Hydroxylase Stimulator Activity**—The stimulation of phenylalanine hydroxylation by PHS is markedly dependent on the structure of the pterin cofactor used in the assay. No stimulation of the rate of hydroxylation has been detected with either 1 x 10⁻⁵ M or 4 x 10⁻⁶ M 6,7-dimethyltetrahydropterin, and only a 20 to 25% stimulation with the 6-methyl compound at a concentration of 8 x 10⁻⁵ M. Only with the naturally occurring cofactor, tetrahydrobiopterin, does PHS show a pronounced (200 to 300%) stimulation. The usual, pronounced PHS stimulation was observed when the assay was carried out with a mixture of tetrahydrobiopterin and the tetrahydrodimethylpterin, each at 1 x 10⁻⁴ M. This result indicates that the failure to observe a PHS stimulation with the dimethylpterin is not due to an inhibition of the PHS response by the dimethyl compound.

Crude hydroxylase fractions from rat liver are contaminated with PHS. It is not until the DEAE-cellulose chromatography step in the hydroxylase purification (9) that the two activities are at least partially separated. Fractions eluted from the column prior to the peak of hydroxylase activity show the maximum stimulation by PHS, an indication that PHS is bound to the DEAE-cellulose more tightly than the hydroxylase.

**Optimum Conditions for Phenylalanine Hydroxylase Stimulator Response**

Under the usual conditions for the assay of phenylalanine hydroxylase in vitro there is only a small stimulation by added PHS even with tetrahydrobiopterin used as the cofactor. The following variables increase the dependence of phenylalanine hydroxylase on PHS: high pH and low phosphate concentration.

**Optimum pH of Hydroxylase with and without Phenylalanine Hydroxylase Stimulator**—In the absence of added PHS, the hydroxylase exhibits a broad optimum between pH 6 and pH 7 (Fig. 2). In the presence of PHS, the optimum is shifted to a more alkaline range (peak between pH 7.6 and pH 8.4), with a plateau of activity between pH 6.3 and pH 7.4. It is clear that the stimulation of the hydroxylase by added PHS is more pronounced at pH 8 than at the lower pH values that have been examined.

**Effect of Phosphate Concentration on Hydroxylase Activity**—It was observed that high concentrations of phosphate reduced the stimulation by added PHS (Fig. 3). The effect of high...
phosphate concentrations under these conditions is 2-fold: it stimulates the hydroxylase in the absence of PHS and inhibits slightly the activity in the presence of PHS. It is evident that a low phosphate concentration is necessary to observe a significant stimulation by PHS. The stimulation of the hydroxylation reaction by phosphate shows some specificity, neither 0.15 M KCl, 0.1 M potassium acetate, nor 0.15 M K$_2$SO$_4$ can substitute for phosphate under these conditions.

**Relationship of Phenylalanine Hydroxylase Stimulator to Other Components of Phenylalanine-hydroxylating System**

*Incubation of Phenylalanine Hydroxylase Stimulator with Tetrahydrobiopterin*—Once assay conditions had been established for demonstrating a large stimulation by PHS, attempts were made to gain some insight into the mechanism of the stimulatory effect.

Since a large PHS effect has so far only been seen when tetrahydrobiopterin is used as the cofactor, it was of interest to determine if PHS was exerting its effect directly on the pterin, *e.g.* by converting the reduced biopterin to a compound with higher cofactor activity. To test this possibility, PHS and tetrahydrobiopterin were previously incubated for 15 min at 25°C in the presence of TPNH, dihydrofolate reductase, and catalase. (A mixture without added PHS served as a control.) The tetrahydrobiopterin in each tube was separated from the enzymes by gel filtration on Sephadex G-25 (as described under “Methods”) and tested in the standard PHS assay system. The two tetrahydrobiopterin samples were equally active in the absence of PHS and inhibits slightly the activity in the presence of PHS. It is evident that a low phosphate concentration is necessary to observe a significant stimulation by PHS. The stimulation of the hydroxylation reaction by phosphate shows some specificity, neither 0.15 M KCl, 0.1 M potassium acetate, nor 0.15 M K$_2$SO$_4$ can substitute for phosphate under these conditions.

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**Effect of Phenylalanine Hydroxylase Stimulator in Absence of Tetrahydropterin-reducing System**—The failure of PHS to directly affect the cofactor activity of tetrahydrobiopterin indicated that it functioned by stimulating one of the enzymes in the hydroxylase system.

The usual assay system for PHS contains ancillary enzymes whose only function is to keep the pterin cofactor in the active, reduced form (5). To determine whether PHS was stimulating the hydroxylation reaction by acting on the hydroxylase, or on one of the secondary enzymes, an assay was performed with stoichiometric, rather than the usual catalytic, amounts of tetrahydrobiopterin. Under these conditions, the need for TPNH, a TPNH-generating system, and dihydropteridine reductase is obviated. The hydroxylation reaction was followed by a spectral determination of the phenylalanine-dependent oxidation of tetrahydrobiopterin to the dihydro compound. (The dihydro compound has an absorption maximum at 330 to 340 nm at pH 7 to 8, whereas the tetrahydro derivative has its maximum close to 300 nm (4).) As can be seen in Fig. 4, a 1.7-fold stimulation by PHS of the initial rate of the reaction is detectable under these conditions. This result eliminates the possibility that the stimulation by PHS is due to its effect on one of the regenerating enzymes, and strongly supports the conclusion that it is due to an interaction of PHS and the hydroxylase.

The results of the last experiment also demonstrate that both isomers of Dl-tetrahydrobiopterin are utilized in tyrosine formation. Based on the reported extinction coefficient for dihydrobiopterin (16), it can be calculated that the observed increase in absorbance in the presence and absence of PHS is 82% and 78%, respectively, of the theoretical value expected if both isomers are active in the hydroxylation reaction. When larger amounts of tetrahydrobiopterin were used, this value was 88% of the theoretical one. A separate experiment of this type in which 0.0125 μmole of tetrahydrobiopterin was used, it was found that 0.0131 μmole of tyrosine was formed, again proving that both isomers of the Dl-tetrahydrobiopterin are utilized for tyrosine formation.
that had not been previously incubated (see Fig. 2). These
adjustment of pH is also close to that found with hydroxylase
the PHS effect is due to protection of the hydroxylase from in-
0.021). The third curve (Curve A) is a control without added
PHS (rate = 0.011). There is some indication that on more
prolonged incubation of the hydroxylase under these conditions,
PHS had been added at the start of the incubation (rate =
0.020) to the rate observed in the cuvette (Curve C) where
hydroxylation reaction (-A absorbance per min for the 6- to
4-min incubation in its absence (Curve B). The rate of the
results show that incubation of the hydroxylase at pH 8.40 in
the presence and absence of PHS. The conditions
were the same as those described in the legend to Fig. 2. The
initial concentration of potassium phosphate was 0.03 M, pH 8.40.
After 12 min, 0.04 ml of 1 M phosphate, pH 5.40, was added. This
addition lowered the pH to 6.94.

Failure to Detect Protective Effect of Phenylalanine Hydroxylase
Stimulator—A mechanism for the PHS effect that would probably
be without physiological significance, and therefore of less
interest, would be one in which PHS protects the hydroxylase
from inactivation under the assay conditions that have been
selected. The fact that the PHS effect is maximal at a somewhat
alkaline pH might be regarded as support for this idea.

Two experiments were performed to examine this model. To
exaggerate any inactivation by alkali, in the first experiment the
hydroxylase was assayed in the presence and absence of PHS
at pH 8.40, an even more alkaline pH than the usual one. After
12 min, the pH was lowered to 6.94 by the addition of the 0.04
ml of phosphate buffer, pH 5.40. As can be seen in Fig. 5,
lowering the pH of the hydroxylase that had been incubated
without PHS led to a 76% increase in activity (Δ absorbance
per min increased from 0.0132 to 0.0232). This increase in
activity on going from pH 8.40 to pH 6.94 is similar to that
observed with hydroxylase that had not been previously incu-
bated (see Fig. 2). Furthermore, the difference in rates ob-
served in the presence and absence of PHS after the downward
adjustment of pH is also close to that found with hydroxylase
that had not been previously incubated (see Fig. 2). These
results show that incubation of the hydroxylase at pH 8.40 in
the absence of PHS does not inactivate the hydroxylase.

In the second experiment, shown in Fig. 6, PHS was added
to the hydroxylase under the standard assay conditions after a
4-min incubation in its absence (Curve B). The rate of the
hydroxylation reaction (Δ absorbance per min for the 6- to
12-min period) after this delayed addition of PHS is close (rate =
0.020) to the rate observed in the cuvette (Curve C) where
PHS had been added at the start of the incubation (rate =
0.021). The third curve (Curve A) is a control without added
PHS (rate = 0.011). There is some indication that on more
prolonged incubation of the hydroxylase under these conditions,
it does lose its responsiveness to PHS.

Results of both of these experiments make it unlikely that
the PHS effect is due to protection of the hydroxylase from in-
activation. The inactivation hypothesis is also made improbable
by the observation that PHS stimulates the initial rate of the
hydroxylation reaction (Figs. 4, 5, and 6).

Effect of Phenylalanine Hydroxylase Stimulator on Apparent
\( K_m \) Values for Tetrahydrobiopterin and Phenylalanine—The
possibility was examined that PHS stimulates the rate of
hydroxylation by decreasing the \( K_m \) of the hydroxylase for its
substrate or coenzyme. It was found that PHS had no signifi-
cant effect on the apparent \( K_m \) for chemically reduced tetra-
hydrobiopterin. At pH 8 in 0.03 M phosphate, the \( K_m \) in the
presence of PHS is \( 7.85 \times 10^{-4} \text{ M} \), whereas, in the absence of
added PHS, it is \( 7.30 \times 10^{-4} \text{ M} \).

The effect of phenylalanine concentration on the rate of the
hydroxylation reaction in the presence and absence of PHS is
shown in Fig. 7. Although PHS has only a slight effect on the
apparent \( K_m \) for phenylalanine, it does markedly increase the
inhibition by excess phenylalanine. By contrast, no inhibition
by excess (i.e. 0.008 M) phenylalanine can be detected in the
presence of the 6,7-dimethyltetrahydropterin, whether or not
PHS is added. When the data in Fig. 7 (excluding those values
above 0.002 M phenylalanine obtained with tetrahydrobipterin)
are replotted according to the method of Lineweaver and Burk
((\( S/\rho \)) against (\( S \))), the following apparent \( K_m \) values for phenyl-
alanine are obtained: in the presence of dimethyltetrahydro-
pterin, 1.1 µmole per ml; in the presence of tetrahydrobipterin
with PHS added, 0.2 µmole per ml; in the presence of tetra-

4 It may be noted that in the experiment described in Fig. 5,
the time course of the reaction is characterized by a high initial
rate that declines during the first 5 to 6 min and then becomes
essentially constant. The initial burst of activity is seen only
when phenylalanine is added to the hydroxylase prior to tetra-
hydrobipterin and not when the order of addition is reversed.
This kinetic phenomenon, which has already been reported (4),
and will be dealt with in detail in a future communication, does
not appear to be related to PHS activity. It has been found
that PHS stimulates the initial rate only slightly more than it
does the final rate. In the experiment shown in Fig. 5, for ex-
ample, the PHS stimulation for the 0- to 6-min interval is 100%,
whereas for the 6- to 12-min interval it is 95%.

Fig. 5. The effect on hydroxylase activity of incubation at pH 8.40 in the presence and absence of PHS. The conditions
were the same as those described in the legend to Fig. 2. The
initial concentration of potassium phosphate was 0.03 M, pH 8.40.

Fig. 6. PHS stimulation after a 4-min incubation of the hydroxylase in its absence. The conditions were the same as
those described in the legend to Fig. 2. The concentration of potassium phosphate, pH 8.11, was 0.02 M. For details, see text.
The effect of phenylalanine concentration on the rate of the hydroxylation reaction in the presence of either tetrahydrobiopterin with PHS (○) or without PHS (▲), or the dimethyltetrahydropterin (□). Identical values were obtained with the dimethyl compound in the presence and absence of PHS. The reaction mixture contained the following components (in micromoles) in a final volume of 1.0 ml: potassium phosphate, pH 7.98, 100; TPNH, 0.15; 7,8-dihydrobiopterin, 0.0038; dihydrofolate reductase sufficient to catalyse the reduction of the dihydrobiopterin in 10 min at 25°C. After a 10-min incubation, the following components were added: L-phenylalanine, 2.0; catalase, 7800 units; dihydropteridine reductase in excess; phenylalanine hydroxylase, 33 µg; and PHS, when added, 54 µg. When the 6,7-dimethyltetrahydropterin was used, its final concentration was 0.040 pmoles per ml and dihydrofolate reductase was omitted.

Table II

<table>
<thead>
<tr>
<th>Hydroxylase</th>
<th>PHS µg/ml</th>
<th>TPNH oxidized µmoles</th>
<th>Tyrosine formed µmoles</th>
<th>Ratio of TPNH oxidized/tyrosine formed</th>
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<tr>
<td>15</td>
<td>0</td>
<td>0.0270</td>
<td>0.0265</td>
<td>1.02</td>
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<td>15</td>
<td>50</td>
<td>0.0848</td>
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<td>30</td>
<td>100</td>
<td>0.1373</td>
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</table>

Fig. 8. The rate of the hydroxylation reaction as a function of hydroxylase concentration in the presence and absence of PHS. The conditions were the same as those described in the legend to Fig. 7 except that the reaction was carried out in 0.05 M potassium phosphate, pH 7.98. PHS, 27 µg, was added where indicated. Phenylalanine concentration, 0.002 M.

hydrobiopterin without added PHS, 0.1 µmole per ml. It is clear that the apparent Kₐₗₚₜ for phenylalanine varies significantly with the pterin cofactor that is used in the assay and that in the presence of tetrahydrobiopterin, PHS increases slightly the apparent Kₐₗₚₜ.

Lack of Effect of Phenylalanine Hydroxylase Stimulator on TPNH to Tyrosine Ratio—Since it has been found that many factors can affect the ratio of 7-methyltetrahydropterin (or TPNH) oxidized to tyrosine formed (19, 20), it was of interest to see if PHS could alter this ratio under conditions where it stimulates the hydroxylation reaction. It was found (Table II) that PHS stimulates tyrosine formation and TPNH oxidation to the same extent, and that the ratio of TPNH oxidized to tyrosine formed is close to 1.0 in the presence and absence of PHS.

Stimulation by Phenylalanine Hydroxylase Stimulator as a Function of Hydroxylase Concentration—A clue to the mechanism by which PHS stimulates the hydroxylation reaction would be to determine if the dimethyltetrahydropterin is present in the same ratio as the tetrahydropterin. This ratio would be determined by the nitrosoponnaphthol method (19).

Although not designed for this purpose, the experiment also shows that under these conditions the maximum rate with the dimethyltetrahydropterin is higher than with tetrahydrobiopterin. Since the dimethylpterin concentration used is closer to saturating than is the tetrahydrobiopterin concentration, this difference would presumably be less at saturating concentrations of both pterins. It should be noted, however, that at 0.1 to 0.2 µmole per ml phenylalanine, which is probably close to the normal tissue concentration (17), and at a dimethylpterin concentration equal to the biopterin concentration used here, which is probably also in the physiological range (18), the rate of the hydroxylation reaction with the dimethyl compound would probably be only 1 or 2% that with tetrahydrobiopterin.
TABLE III
Effect of hydroxylase concentration on inhibition by phenylalanine

The conditions were the same as those described in the legend to Fig. 7. The concentration of 7,8-dihydrobiopterin was 0.0038 μmoles per ml.

<table>
<thead>
<tr>
<th>Hydroxylase concentration µg/ml</th>
<th>Phenylalanine concentration μM</th>
<th>Hydroxylase activity μmoles/14 min</th>
<th>Inhibition %</th>
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<tr>
<td>14</td>
<td>1.2</td>
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<td>14</td>
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<tr>
<td>56</td>
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</table>

* Phenylalanine-dependent oxidation of TPNH.

which PHS stimulates phenylalanine hydroxylation was provided by the observation that at pH 8, the stimulation is extremely dependent on the concentration of hydroxylase. As can be seen in Fig. 8, in the absence of PHS, the specific activity of the hydroxylase decreases with increasing concentrations of hydroxylase from a specific activity of 0.048 at the lowest to a specific activity of 0.014, at the highest hydroxylase concentration tested. By contrast, in the presence of PHS, the specific activity is more independent of hydroxylase concentration.

In contrast, the specific activity of the hydroxylase at pH 6.8 to 6.9 in the absence of PHS decreases much less with increasing enzyme concentration than it does at pH 8. At pH 6.8 the specific activity at 0.055 mg of protein per ml is 24% less than it is at 0.014 mg of protein per ml.

These results suggest that at pH 8 and in low phosphate concentrations the hydroxylase can undergo a concentration-dependent association to give a form of the enzyme that has low catalytic activity with tetrahydrobiopterin. According to this idea, PHS would stimulate the hydroxylation reaction by preventing the association.

Effect of Hydroxylase Concentration on Inhibition by Phenylalanine—If the association-dissociation model is correct, the greater inhibition by excess phenylalanine in the presence of PHS (Fig. 7) would suggest that the dissociated form of the enzyme is more sensitive to inhibition than the associated form. The model predicts that inhibition by phenylalanine should be greater at low than at high hydroxylase concentrations. The data in Table III show that the inhibition by excess phenylalanine is a function of enzyme concentration.*

Phenylalanine Hydroxylase Stimulator-like Activity of Growth Hormone—The original finding that PHS is present as a contaminant in glucose dehydrogenase preparations from bovine liver, together with the present finding that the activity is present in preparations of sepiapterin reductase, raised the question of whether many proteins might not have PHS activity. Although the answer to that question is negative, a random survey of proteins led to the finding that bovine growth hormone does have PHS activity.

It should be noted that the inhibition data in Fig. 7 could also be interpreted as an indication that high phenylalanine concentrations prevent the stimulation of the hydroxylation reaction by PHS. Although this interpretation has not been rigorously excluded, the results in Table III do not support it.

Fig. 9 (above). Stimulation of the hydroxylation reaction by bovine and ovine growth hormones. Standard assay conditions as described under “Methods,” except that 0.5 μmoles of TPNH were added. The hydroxylase concentration was 66 μg per ml. The tubes were incubated for 30 min at 25°. The amount of tyrosine formed in the absence of the hormones was 0.028 μmoles and this blank value has been subtracted from the amount formed in the presence of the hormones.

Fig. 10 (below). Effect of chymotrypsin on PHS activity of bovine growth hormone. A mixture containing 5 mg of growth hormone and 0.25 mg of chymotrypsin in 0.5 ml of 0.01 m potassium phosphate, pH 8.11, was incubated at 25°. A mixture without chymotrypsin served as a control. At the designated times, a 0.1-ml aliquot was removed from both mixtures and tested for PHS activity under standard assay conditions as described under “Methods.” The addition of the amount of chymotrypsin contained in the aliquot (i.e. 25 μg) to the assay reaction mixture did not inhibit. The control in which the growth hormone was incubated without chymotrypsin had full PHS activity even after the 18-hour incubation.
The conditions were the same as those described in the legend to Fig. 9. The amount of PHS added was 70 μg. The amount of growth hormone added was 1.0 mg. The concentration of 7,8-dihydrobiopterin was 0.0076 pmol per ml; the concentration of 6,7-dimethyltetrahydropterin was 0.040 pmol per ml. Tyrosine formation was measured after a 30-min incubation.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Tetrahydropterin used</th>
<th>Tyrosine formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Bioppterin</td>
<td>0.028 pmol</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>Bioppterin</td>
<td>0.128 pmol</td>
</tr>
<tr>
<td>PHS</td>
<td>Bioppterin</td>
<td>0.149 pmol</td>
</tr>
<tr>
<td>Growth hormone + PHS</td>
<td>Bioppterin</td>
<td>0.154 pmol</td>
</tr>
<tr>
<td>None</td>
<td>6,7-Dimethyl</td>
<td>0.186 pmol</td>
</tr>
<tr>
<td>PHS</td>
<td>6,7-Dimethyl</td>
<td>0.186 pmol</td>
</tr>
<tr>
<td>Growth hormone</td>
<td>6,7-Dimethyl</td>
<td>0.184 pmol</td>
</tr>
</tbody>
</table>

As can be seen in Fig. 9, the effect of the hormones shows species specificity; the bovine hormone is about 3 times more active (on a weight basis) than the ovine hormone. Human growth hormone, at a level of 0.5 mg per ml, has barely detectable activity, and rat growth hormone at the same concentration is inactive. The significance of the inactivity of the latter hormone is uncertain because the preparation used was not inhibitory. The still impure PHS fractions from rat liver used in the present study have a specific activity that is 20 times greater than that of the bovine hormone. In spite of this low specific activity, present evidence indicates that the bovine hormone and PHS stimulate the hydroxylation reaction by a similar mechanism. Thus, neither compound stimulates the reaction when the 6,7-dimethyltetrahydropterin is used in place of tetrahydrobiopterin (Table IV). Furthermore, when both the hormone and PHS are added together, the stimulation observed is only slightly greater than that with just PHS.

The PHS activity of bovine growth hormone is not due to a nonprotein contaminant. As can be seen in Fig. 10, the activity is destroyed by treatment with chymotrypsin. In addition, the PHS activity of the hormone is completely lost on boiling for 5 min.

The following proteins have been tested for PHS activity at a level of 0.5 to 1.0 mg per ml in the standard assay for PHS and found to be inactive: insulin, bovine serum albumin, bovine liver catalase, ovine and bovine prolaetin, lysine vasopressin, ovine-lyleneinizing hormone, bovine thyrotropin, and porcine follicle-stimulating hormone.

**DISCUSSION**

The present studies show that the phenylalanine-hydroxylating system, already a complex one, is even more complicated than had been suspected. PHS, a naturally occurring protein, appears to play a role in the regulation of the activity of the hydroxylase. Little is known about the physiological significance of this new component of the system, but the fact that it markedly stimulates the hydroxylase only in the presence of the naturally occurring pterin cofactor is at least suggestive of a physiological role.

Indirect evidence has been presented that PHS stimulates the hydroxylation reaction by interacting with the hydroxylase and that the mechanism of stimulation does not involve protection of the hydroxylase from inactivation. The stimulation can be explained by the model, outlined in Equation 3, in which it is assumed that the hydroxylase can exist in at least two forms that have high (E) and low (EX) catalytic activity in the presence of tetrahydrobiopterin, and that these two forms are in equilibrium.

\[ E + X \rightleftharpoons EX \]  

It should be noted that according to Equation 3, as the concentrations of both X and E are raised, the fraction of the enzyme in the EX form will increase. According to this hypothesis, PHS would stimulate the hydroxylation reaction by shifting the equilibrium toward the left. The equation has been written in general terms in which X may be an inhibitor. Since there is physical evidence, however, that the enzyme can exist in at least three forms that are probably monomer (mol wt 61,000 to 55,000), dimer (mol wt 110,000), and tetramer (mol wt 210,000) (9), it seems more likely that E and X in Equation 3 are identical and that E is the monomer and EX (or EE) is the dimer. It is also possible that the equilibrium being affected by PHS is that between dimer and tetramer.

The present data are consistent with this model. According to it, high pH values would shift the equilibrium toward the right, whereas phosphate would shift it toward the left.

From the kinetic studies one can draw tentative conclusions about the relative properties of the associated and dissociated forms of the enzyme. Based on the equilibrium model, the monomer and the dimer display different relative activities toward various pterin cofactors, the order of discrimination being tetrahydrobiopterin (lowest activity with the dimer relative to the monomer), 6-methyl-tetrahydropterin and 6,7-dimethyltetrahydropterin (equal activity with the monomer and the dimer). An additional property of the dissociated form of the enzyme that can be inferred from the data is that it is more sensitive to inhibition by excess phenylalanine.

Alternatively, one can explain the above kinetic data by assuming that pterins such as the dimethyl compound, but not tetrahydrobiopterin, can shift the equilibrium to the left and that excess phenylalanine can shift it toward the right. It should be possible to distinguish between these alternatives by a direct study of the effect of phenylalanine and various tetrahydropterins on the state of association of the hydroxylase.

Additional indirect evidence that is consistent with the existence of multiple forms of the hydroxylase has been obtained from studies with the 7-methyltetrahydropterin used as the cofactor. It has been found with this pterin, that the ratio of tetrahydropterin (TPNH) oxidized to tyrosine formed varies with hydroxylase concentration; the ratio is greater than 4:1 at low protein concentration, and approaches 1:1 at high protein concentration (20). Furthermore, at high protein concentrations, the addition of PHS increases this ratio. These results indicate that the dissociated form of the enzyme catalyzes a reaction in which the oxidation of the 7-methyltetrahydropterin is only loosely coupled to hydroxylation of phenylalanine (20).

An alternate explanation is that PHS does not affect the equilibrium but increases the catalytic activity of the EX form of the enzyme.

The demonstration that excess phenylalanine inhibits the hydroxylase in the presence of PHS and tetrahydrobiopterin, but not in the presence of the 6,7-dimethyltetrahydropterin, clarifies a point that has been the source of considerable confusion in the literature. Thus, it has recently been stated that in the presence of the dimethylpterin "no evidence of substrate inhibition was obtained even for concentrations (of phenylalanine) as high as \(8 \times 10^{-3}\) M, although other investigators had reported such inhibition at concentrations of approximately \(2 \times 10^{-3}\) M" (21). This confusion was caused by the assumption that inhibition by excess substrate would be manifest with any pterin cofactor.

The finding that substrate inhibition is dependent on the structure of the pterin cofactor may have clinical significance. In a disease such as hyperphenylalaninemia, characterized by low, but detectable, levels of hepatic phenylalanine hydroxylase \textit{in vivo} (22, 23) and high blood phenylalanine levels, the low activity of the enzyme \textit{in vivo} is due to a genetically-determined decrease in the amount of fully-active hydroxylase. It may be, however, that the potential activity of the enzyme is not fully expressed \textit{in vivo} because of inhibition by excess substrate. Since it has been shown in the present study that excess substrate does not inhibit when tetrahydrobiopterin is replaced by the 6,7-dimethyl compound, it is possible that the dimethyltetrahydropterin would relieve the inhibition by excess phenylalanine \textit{in vivo} and may therefore be of therapeutic value in this disease.

The PHS-like effect of bovine growth hormone on phenylalanine hydroxylase is, as far as we are aware, the first example of a stimulation of a purified enzyme by this hormone. The effect shows species specificity and is sensitive to proteolysis. Although the relatively large concentrations of the hormone required for this effect \textit{in vitro} make it unlikely that the effect is of physiological significance, the possible relationship between PHS and growth hormone would appear to merit further study.

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A Protein That Stimulates Rat Liver Phenylalanine Hydroxylase
Seymour Kaufman


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