Studies on the Phenylalanine Hydroxylase System in Vivo

AN IN VIVO ASSAY BASED ON THE LIBERATION OF DEUTERIUM OR TRITIUM INTO THE BODY WATER FROM RING-LABELED L-PHENYLALANINE

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The rate of release of deuterons into the body water from 2,3,4,5,6-pentadeutero-L-phenylalanine has been shown to be a valid measure of the activity of the phenylalanine hydroxylase system in vivo. At a dose of 0.5 g/kg, the rate of release of deuterons is linear for 60 to 90 min. Male rats, which had previously been shown to have 22 to 25% more phenylalanine hydroxylase activity in liver extracts than female rats, produced deuterons from deuterated phenylalanine at a rate 20 to 30% greater than female rats.

p-Chlorophenylalanine, which irreversibly inhibits phenylalanine hydroxylase in vivo, caused a similar degree of inhibition of the rate of deuteron formation as was found when phenylalanine hydroxylase was measured in extracts from the same group of animals. Methotrexate, which inhibits the phenylalanine hydroxylase system by preventing regeneration of the tetrahydropteridine cofactor, caused parallel inhibition of the in vivo assay as well as when the conversion of phenylalanine to tyrosine was measured in liver slices.

Randomly ring-tritiated phenylalanine can be used interchangeably with ring-deuterated phenylalanine if greater sensitivity is needed in the in vivo assay for phenylalanine hydroxylase. However, a dose of 20 to 30 μCi/kg is required.

The in vivo deuterium release assay described in this paper should be useful in studying the physiological control of the phenylalanine hydroxylating system. It also may be of value in differentiating between individuals who are heterozygotes for phenylketonuria and those who are homozygotes for hyperphenylalaninemia.

Phenylalanine hydroxylase (L-phenylalanine, tetrahydropteridine: oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1) in mammalian liver catalyzes the tetrahydropterin-dependent hydroxylation of phenylalanine to tyrosine (1). A deficiency of this enzyme leads to the disease known as phenylketonuria.

Although it had been thought that there is a total lack of hepatic phenylalanine hydroxylase activity in phenylketonuria patients, it recently has been shown that a patient with the classical form of the disease has about 0.27% of the normal enzyme activity (2). This finding raises the possibility that measures that could enhance this low level of activity might be of therapeutic value in phenylketonuria and has provided an impetus for the study of the in vivo regulation of phenylalanine hydroxylase.

In order to evaluate the effectiveness in vivo of procedures that are known to be able to markedly activate the purified hydroxylase, e.g. exposure of the enzyme to phospholipids or to limited proteolysis (3), as well as to aid in the exploration of other possible modes for regulating the activity of this enzyme, it was necessary to develop a method which could be used to measure accurately the enzyme activity without resorting to a liver biopsy. Currently, the best method available for assessing the amount of phenylalanine activity in vivo is the phenylalanine tolerance test (4). This method, however, is relatively insensitive and cannot be used to distinguish between a heterozygote for phenylketonuria and an individual with hyperphenylalaninemia.

A possible method for the in vivo measurement of phenylalanine hydroxylase would be one based on the formation of tritiated water from p-tritophenylalanine upon hydroxylation. However, since nearly all of the isotope in the para position migrates to the meta position in the reaction (5), this assay would require the use of fully ring-labelled phenylalanine. Although this method would be useful in animal studies, it might necessitate the use of unduly large doses of radioactive activity in humans. An alternative approach would be the use of fully ring-deuterated phenylalanine as the substrate. Hydroxylation then would lead to the release of deuterium, a nontoxic, nonradioactive substance, which can be measured by a number of physical methods (6).

This paper describes the preparation of L-phenylalanine (ring-deuterated) and its use in an in vivo assay for phenylalanine hydroxylase. Measurements of the rate of release of deuterons into the body water, after administration of this compound to rats, have shown that this assay is a valid one for
the measurement of phenylalanine hydroxylase activity in whole animals.

We also have prepared phenylalanine that is randomly labeled on the phenyl ring with tritium. Preliminary experiments analogous to those carried out with the deuterated compound indicate that the tritiated phenylalanine also can be used for the in vivo assay of the hydroxylase. The latter assay should be a useful adjunct to the deuterium release assay, especially in animal studies on the physiological regulation of the phenylalanine hydroxylation system.

MATERIALS AND METHODS

All rats used weighed 100 to 150 g and were of the Sprague-Dawley strain. They were obtained from local sources. L-Phenylalanine was obtained from Sigma Chemical Co, N-sulfuric acid-D and D$_2$O were from Merck and Co., Rahway, N. J. Benzene-D$_4$ was from Aldrich Chemical Co. All other chemicals were reagent grade.

Preparation of L-Phenylalanine-D$_6$—Phenylalanine was deuterated by a modification of the acid-catalyzed exchange procedure described by Moss and Schoenheimer (7). To 10 g of L-phenylalanine were added 10 ml of D$_2$O. The mixture was cooled in an ice bath while 100 g of sulfuric acid-D$_2$ were added slowly. The mixture then was flushed with nitrogen, sealed, and stirred for 7 days at room temperature. The yellow solution then was slowly added to 500 g of ice water, keeping the temperature below 20°. A solution of 0.25 g of barium acetate in 500 ml of water was added, and the precipitate of BaSO$_4$ was removed by centrifugation. The supernatant fraction was passed through a Dowex 50 (H+, 100 to 200 mesh) column (5 x 20 cm) to remove any remaining sulfate and sulfonated phenylalanine. The column was washed with 5 column volumes of water and the deuterated phenylalanine was eluted with 1 column volume of 2 M H$_2$SO$_4$. Ethanol was added to precipitate the product, which then recrystallized from a water-ethanol mixture. The yield was generally between 4 to 5 g of greater than 90% ring-deuterated phenylalanine.

The ultraviolet spectrum of the deuterated phenylalanine was identical with that of authentic phenylalanine. Upon direct introduction into the probe of a chemical ionization mass spectrometer, a parent ion at m/e = 170 (H$_2$O, 100 to 200 mesh) column (5 x 20 cm) to remove any remaining sulfate and sulfonated phenylalanine. The column was washed with 5 column volumes of water and the deuterated phenylalanine was eluted with 1 column volume of 2 M H$_2$SO$_4$. Ethanol was added to precipitate the product, which then recrystallized from a water-ethanol mixture. The yield was generally between 4 to 5 g of greater than 90% ring-deuterated phenylalanine.

Preparation of [ring-H]Phenylalanine—L-Phenylalanine (25 mg) was added to 0.2 g of H$_2$SO$_4$, containing 25 mCi of H$_2$O (specific activity, 1 mCi/ml) and incubated at 50° for 7 days. The solution was worked up and the phenylalanine was isolated as described for the deuterated phenylalanine with care to avoid contamination with any volatile H$_2$O. The yield was 13.4 mg containing 300 μCi of tritium (specific activity, 3.7 mCi/mmol). Very little tritium was released on hydroxylation (<2%) with purified phenylalanine hydroxylase as expected for random tracer labeling and retention of 90% of para-H upon hydroxylation (5).

Preparation of Ring-deuterated Tyrosine—2,3,5,6-Tetradecutero-p-tyrosine was synthesized de novo from benzene-D$_6$ in about 5% yield according to published procedures (9, 10). The following sequence was used: benzene → nitrobenzene → aniline → phenol → anisole → p-methoxybenzyl chloride → ethyl-p-methoxybenzylacetamidomalonate → n-p-tyrosine.

Previous reports from this laboratory on the in vitro properties of liver phenylalanine hydroxylase have been published in previous issues of this journal (9, 10).

RESULTS

Fig. 1 shows the appearance of deuterium in the body water of rats after different doses of L-phenylalanine-D$_6$. The rate of deuterium release is linear for 60 to 90 min and is maximal at a dose of about 0.5 g/kg. It should be noted that the dose usually used for an oral phenylalanine tolerance test is 0.1 g/kg.

It has previously been found that liver extracts from male rats have 22 to 25% more phenylalanine hydroxylase activity than those from female rats (14). As shown in Fig. 1, male rats release deuterium from L-phenylalanine-D$_6$ at a rate that is 70 to 30% faster than female rats. These results not only show that the in vivo assay is capable of detecting a small quantitative difference in hydroxylase activity, but also that the sex difference in hydroxylase activity that was previously observed in liver extracts, can also be seen in whole animals.

p-Chlorophenylalanine in vivo irreversibly inhibits phenylalanine hydroxylase (15). A group of rats were injected intraperitoneally with 300 mg/kg of DL-p-chlorophenylalanine methyl ester hydrochloride. After 96 hours, half of the animals were killed and their livers were removed and assayed for phenylalanine hydroxylase activity in liver slices (12) and in extracts of the same livers. The activity in the remainder of the animals was measured by the deuterium release assay. The results are given in Table I. As can be seen, there is a good correlation in the trend of inhibition by the p-chlorophenylalanine treat-

In Vivo Assay—The in vivo assay of phenylalanine hydroxylase was carried out as follows: Animals were injected intraperitoneally with solutions of phenylalanine-D$_6$ or [ring-^3H]phenylalanine in 1 ml of 0.9% NaCl solution (saline) containing 1 equivalent of NaOH. At various times after injection, the animals were decapitated and whole blood was collected in a beaker containing 50 mg of disodium EDTA to prevent clotting. The whole blood then was lyophilized as described by Stansell and Mojica (11). The deuterated water content was determined in the trapped water by infrared spectrophotometry with the use of a temperature-regulated 0.2-mm CaF$_2$ cell and Beckman Acculab 4 infrared spectrophotometer with an accessory recorder for scale expansion (11). Tritiated water was determined by counting an aliquot of the trapped water in a liquid scintillation counter (Beckman LS-250).

Phenylalanine hydroxylase activity in liver slices was determined by measuring the conversion of [^14C]phenylalanine to [^14C]tyrosine (12). Phenylalanine hydroxylase activity in liver extracts was measured as described by Kaufman (13).

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![Fig. 1. The rate of appearance of deuterium in the body water of rats following intraperitoneal injections of L-phenylalanine-D$_6$.](http://www.jbc.org/)
TABLE I

Inhibition of phenylalanine hydroxylase by p-chlorophenylalanine and methotrexate

Rats were injected intraperitoneally with 300 mg/kg of n-p-chlorophenylalanine methyl ester 96 hours prior to measurement of their phenylalanine hydroxylase activity as described in the text. Eight rats for the in vivo assay to obtain a time course similar to that in Fig. 1. Methotrexate-treated rats received 2.5 mg/kg intraperitoneally 1 hour prior to the phenylalanine hydroxylase assay. The release of deuterium was linear with respect to time for 60 min, the slope of the line being used to calculate inhibition. Two control rats and two drug-treated rats were killed at the same time and their liver phenylalanine hydroxylase activities measured as described in the text.

| Treatment             | In vivo | Slice | Extract |
|-----------------------|---------|-------|---------|
|                       | mM D2O/min | % Inhibition | nmol tyrosine/min/g liver | % Inhibition | nmol tyrosine/30 min/mg protein | % Inhibition |
| Control ............. | 0.085 | 18.9 | 483     |
| p-Chlorophenylalanine | 0.05   | 41.2 | 10.8 | 42.8 | 50.0 | 282 | 41.4 |
| Methotrexate .......... | 0.038 | 55.0 | 9.5 | 50.0 | 282 | 41.4 |

We have shown that methotrexate inhibits the phenylalanine hydroxylase system by inhibiting dihydropteridine reductase (16), thereby preventing the regeneration of the tetrahydropteroxin. This inhibition is usually not evident in assays done on extracts, where excess regenerating system is added routinely. It has been shown, however, that methotrexate inhibits the conversion of phenylalanine to tyrosine in liver slices (12). To see if the in vivo assay can detect the lowered hydroxylase activity caused by inhibition of the system by methotrexate, two groups of rats were given 2.5 mg/kg of methotrexate intraperitoneally. After 1 hour, the animals were killed and the conversion of phenylalanine to tyrosine in liver slices was determined. There was 50% inhibition compared to saline-injected controls. The second group of rats was given a load of L-phenylalanine-D3, 1 hour after the methotrexate injection and the rate of formation of deuterium was measured. There was 55% inhibition compared to controls. Here again, the results obtained with the in vivo assay are in excellent agreement with those of the in vitro assay.

In Fig. 2 are shown the results of giving rats a load of tritiated phenylalanine (3 μCi/rat, 0.5 g of L-phenylalanine/kg). The appearance of 4H in the body water is linear with time and is inhibited by methotrexate (2.8 mg/kg) to a similar extent as in the deuterium release assay as shown in Table I.

To test whether phenylalanine hydroxylation or a subsequent step in the pathway for conversion of tyrosine to CO2 and H2O is rate-limiting, the rate of release of deuterons from 2,3,4,5,6-pentadeuterio-L-phenylalanine and from 2,3,5,6-tetradeterio-L-tyrosine was compared. Deuterium was released from tyrosine-D4 in the rat at a rate at least 2 times greater than from phenylalanine D3.

The first enzyme in the metabolic pathway for the conversion of tyrosine to CO2 and H2O is tyrosine transaminase. As a further test of the hypothesis that phenylalanine hydroxylation is the rate-limiting enzyme in catalysis of phenylalanine, a group of rats was treated with triamcinolone (100 mg/kg, intraperitoneal in 1 ml of 0.9% NaCl) to induce tyrosine transaminase. After fasting for 14 hours, two animals were killed and their liver tyrosine transaminase levels were measured (17). The remainder were given a load of tritiated phenylalanine as described above and the rate of appearance of tritiated water in the body water was measured. Liver tyrosine transaminase levels were increased 6-fold over the untreated controls and the rate of appearance of 4H in the body water was identical to that shown in Fig. 2. These results strongly indicate that phenylalanine hydroxylation is the rate-limiting enzyme in the pathway for the catabolism of phenylalanine.

Besides its conversion to tyrosine, incorporation into proteins is one of the important reactions in the metabolism of phenylalanine. It was of interest to know, therefore, how sensitive the rate of phenylalanine hydroxylation might be to competition from this alternate metabolic pathway. If variations in the rate of protein synthesis could alter the rate of phenylalanine hydroxylation (by altering the concentration of phenylalanine available to the hydroxylase), this sensitivity to competition from other metabolic pathways might limit the usefulness of the deuterium release assay. To evaluate this possibility, protein synthesis in rats was inhibited by administration of cycloheximide (3.5 mg/kg). The rate of release of deuterium from L-phenylalanine-D3 was unaffected by this treatment, an indication that agents that can lead to changes in the endogeneous pool size of amino acids have no effect on the in vivo hydroxylase assay. These results also indicate that in rats the rate of protein synthesis is low compared to the rate of conversion of phenylalanine to tyrosine.

DISCUSSION

The phenylalanine hydroxylation system in mammalian liver catalyzes the following reactions (1):

\[
\text{Tetrahydropteroxin} + \text{phenylalanine} + O_2 \rightarrow \text{quinonoid dihydropteroxin} + \text{tyrosine} + \text{H}_2\text{O} \tag{1}
\]

\[
\text{Quinonoid-dihydropteroxin} + \text{DPNH (TPNH) + H}^+ \rightarrow \text{tetrahydropteroxin} + \text{DPN}^+ (\text{TPN}^+) \tag{2}
\]

Reaction 1 is catalyzed by phenylalanine hydroxylase and Reaction 2 by dihydropteridine reductase. The major pathway of catabolism of tyrosine proceeds via transamination to p-hydroxyphenylpyruvate, followed by a series of reactions that lead to fumarate and acetoacetate; these last two products are ultimately oxidized to CO2 and H2O. In mammals, there is no known pathway in which the benzene ring of phenylalanine can be oxidized to CO2 and H2O without the phenylalanine first being converted to tyrosine.

**Fig. 2.** The liberation of 4H into the body water of female rats following intraperitoneal injections of ring-tritiated phenylalanine (3 μCi/rat, 0.5 g/kg). The methotrexate-treated rats received 2.8 mg/kg 15 min prior to receiving the tritiated phenylalanine. Each point represents the value found from a single rat.
If the rate-limiting reaction in the pathway leading to the complete oxidation of phenylalanine is the conversion of phenylalanine to tyrosine, the rate of appearance of deuterium in the body water after the administration of 2,3,4,5,6-pentadeutero-L-phenylalanine would be a quantitative measure of the in vivo activity of the phenylalanine hydroxylase system. If a reaction subsequent to the phenylalanine hydroxylating step was rate-limiting, however, the rate of appearance of only one out of five of the deuteriums (i.e. the one released during the hydroxylation reaction per se) would be a measure of the hydroxylation step, whereas the rate of appearance of the major part of the deuterium would be a measure of other reactions in the metabolic sequence. This latter circumstance might complicate the interpretation of deuterium release data.

Because of these considerations, it was necessary not only to delineate the general characteristics of the in vivo deuterium release assay, but also to try to determine whether or not phenylalanine hydroxylation is the rate-limiting step in the catabolic pathway. The finding that deuterium is released from ring-deuterated tyrosine faster than it is released from ring-deuterated phenylalanine indicates that the conversion of phenylalanine to tyrosine is the rate-limiting step in the catabolic pathway.

The results obtained with the two inhibitors of the phenylalanine hydroxylating system, p-chlorophenylalanine and methotrexate, provide independent evidence that the hydroxylase-catalyzed step is the rate-limiting one in the sequence that leads to deuterium release.

From the data in Fig. 1, based on the deuterium release assay, the rate of tyrosine formation in the whole animal can be calculated and is equal to 0.15 μmol of tyrosine/min/animal. From the data in Table I for liver slices, and from a separate assay on liver extracts carried out in the presence of tetrahydrobiopterin (the rate shown in Table I for extracto was determined in the presence of 6,7-dimethyltetrahydropterin), it can be calculated that the rate of conversion of phenylalanine to tyrosine in liver slices and extracts is 0.13 and 0.11 μmol of tyrosine/min/animal, respectively. It should be noted that these three values, which are in reasonable agreement, were not corrected for the temperature difference between the in vivo assay, carried out at the body temperature of the rat, and the two in vitro assays, which were carried out at 37°.

In summary, a simple, stable isotope, in vivo assay for phenylalanine hydroxylase activity has been developed. That the rate of release of deuterium after a single dose of L-phenylalanine-D₉ is a valid measure of the phenylalanine hydroxylase system in vivo is shown by the parallel results obtained with this assay and with the more direct assays for hydroxylase activity in liver slices and extracts. Since the assay, as it would be applied to humans, should be as innocuous as the commonly employed phenylalanine tolerance test, requiring only a few small samples of blood (1 ml/time point), and equipment found in most laboratories, it may find widespread use. It should be especially useful in differentiating between individuals who are heterozygotes for phenylketonuria, with about 15% of normal phenylalanine hydroxylase levels, and those who are homozygotes for hyperphenylalaninemia, with about 5% of normal phenylalanine hydroxylase activity (18). The standard phenylalanine tolerance test cannot with certainty discriminate between these two conditions. The formation of tritiated water from ring-tritiated phenylalanine can also be used for an in vivo assay for phenylalanine hydroxylase. The greater sensitivity of this assay could outweigh the potential hazards of the radioactive label when attempting to differentiate between a homozygote for phenylketonuria with a small amount of residual phenylalanine hydroxylase activity and the hyperphenylalaninemic.

Further experiments are also in progress on the use of this assay in evaluating alternate methods of therapy for phenylketonuria which are based on increasing the level of activity of the phenylalanine hydroxylase system in vivo.

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