Regulation of Phenylalanine Hydroxylase Activity by Phenylalanine in Vivo, in Vitro, and in Perfused Rat Liver*

(Received for publication, July 16, 1982)

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We show that phenylalanine is able to control the extent of activation and, as a result, the catalytic activity of rat liver phenylalanine hydroxylase in vivo, in perfused liver, and in vitro. Both phosphorylated and unphosphorylated enzyme activities are controlled by phenylalanine activation and, overall, this mechanism appears to be a major means of regulating the enzyme's activity in rat liver. At normal phenylalanine levels in vivo, phenylalanine hydroxylase is at most 1–4% activated, and phosphorylated enzyme (glucagon-induced) appears at most 5–7% activated under similar conditions. In both cases, a phenylalanine load increased the percentage of activated enzyme found in vivo to about 40% of maximal. In perfused rat livers, a plasma phenylalanine concentration of only 4 times normal induced a 4-fold increase in the amount of activated enzyme present and a corresponding functional increase in the rate of phenylalanine hydroxylation by the tissue. Under the latter conditions, more than 25% of the amino acid could be hydroxylated in a single pass through the organ.

Purified phosphorylated phenylalanine hydroxylase must be activated to be catalytically active. The activation with phenylalanine, at equilibrium, is a cooperative process, and the phosphorylated enzyme is activated more rapidly at pH 6.8 and 8.0 and at lower phenylalanine concentration than the unphosphorylated species. Overall, phosphorylation appears to allow phenylalanine hydroxylase to be more easily activated at relatively low phenylalanine concentrations.

Several considerations suggest that phenylalanine hydroxylase activity in mammals must be carefully controlled: (a) most mammals possess very high levels of the enzyme (liver has almost all the activity) (1); (b) the hydroxylation reaction is irreversible (2); and (c) phenylalanine is an essential amino acid. The problem is exemplified in rats where, based on the normal plasma level (3), a 100-g animal can have at most 5 μmol of free phenylalanine; whereas, from in vitro measurements, the same animal would have sufficient liver enzyme to hydroxylate at least 10–15 μmol of phenylalanine/min (4).

* This work was supported in part by United States Public Health Service Grants CA14881 and AM21624. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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From what is known of the isolated enzyme, the logical mechanism for controlling phenylalanine utilization in vivo would be by phenylalanine activation. Studies in vitro have shown that both crude and purified enzyme can be reversibly activated by phenylalanine, that the rate of activation is directly proportional to the phenylalanine concentration, and that in the absence of activation the enzyme has little or no catalytic activity (4–6). Furthermore, activation is quite specific and phenylalanine is the only protein amino acid found to have this effect (5). Certain detergents, lyssolecithin being the best studied (5, 7), can also activate phenylalanine hydroxylase to yield an enzyme that is functionally identical to the phenylalanine-activated enzyme (5).

The current investigation was undertaken to provide direct evidence on whether or not phenylalanine activation of phenylalanine hydroxylase is a significant regulatory mechanism in rats in vivo, and to determine what role phosphorylation, induced in situ by glucagon (8, 9), plays in controlling phenylalanine hydroxylase activation in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Perfusion and in Vivo Experiments—Male Lewis rats (Microbiological Associates) were perfused as indicated (Table II) with components described previously (10). At the end of the perfusion, a section of liver was freeze-clamped between aluminum blocks precooled in liquid N₂. The frozen tissue was then powdered and stored under liquid N₂. To make the extract, the frozen sample was added to 10 volumes of cooled (2°C) 0.03 M Tris-HCl buffer, pH 7.3, containing 20 mm phenylalanine and 0.2 mM KCl. The mixture was phospho¬


erized in a Tekmar homogenizer ( precooled to 2°C) for 15 s at ½ speed. The sample was then centrifuged at 45,000 × g for 15 min at −2°C. The clear supernatant was immediately assayed (see Table I and II).

To obtain samples from in vivo experiments, rats were anesthe¬
tized with sodium pentobarbital (8 mg/100 g), the livers exposed, and a 1-g segment freeze-clamped. All other details are as above in Table I.

Measurement of [14C]Tyrosine—To 2.0 ml of perfusate were added 50 μl of 7 N HClO₄. After 5 min at 0°C, the sample was clarified by centrifugation. After warming and degassing, 1.0 ml was applied to a column (0.5 × 4.6 cm) of Q16S resin (Bio-Rad) equilibrated with 0.7 N HCl containing 40% ethanol by volume (6). The column was developed in the same buffer, 0.3-ml fractions were collected every 10 min, tyrosine elutes before phenylalanine. Samples were counted using a Triton-114 scintillation mix (11). [14C]Tyrosine concentration was calculated from the specific radioactivity of the [14C]phenylalanine in the perfusion medium.

Purification of Phosphorylated and Unphosphorylated Phenylalanine Hydroxylase—Animals were injected with 0.3 ml of buffer with or without added glucagon (Table I); 25 min later, they were killed with a blow to the back of the head. The livers were rapidly removed, cooled to 0°C, minced, and immediately homogenized at 0°C for 18 s in a Tekmar homogenizer in 4 volumes of 0.08 M Tris-HCl buffer, pH 7.3, containing 100 mm phenylalanine, 10 mM KF, 5 mM EDTA, and 0.2 mM KCl (total elapsed time was less than 5 min). The homogenates were centrifuged and enzymes purified using pro¬
cedures I.I.C and II.D (4) exactly as described except 5 mM KF was added to all buffers to suppress phosphatase activity and extracts were activated for 7 min at 25°C.

Corrections for Intracellular Dilution and for Assay Conditions—The intracellular release of free phenylalanine from liver protein under our perfusion conditions should be near basal and was calculated to be 133 nmol/min/g of liver (12, 13). The results in line D (Table II) imply that with 310 μmol phenylalanine in the medium, phenylalanine can be transported into liver at a minimum rate of 760 nmol/min/liver. From these numbers, the intracellular [14C]phenylalanine specific radioactivity was calculated to be at least 0.85 of the
extracellular. Assuming a linear decrease in transport rate with amino acid concentration, the intracellular specific radioactivity of [14C]phenylalanine must be at least 0.6 of the extracellular at 84 μM phenylalanine. The corrected values represent the measured [14C]tyrosine divided by its dilution factor.

The intracellular tetrahydrobiopterin concentration in liver in vivo is about 14 μM (14) and the phenylalanine concentration is about 0.1 mM (15). Apparently due to the increased hydroxylation rate, increasing phenylalanine in the medium from 84 to 310 μM had no measurable effect on the average intracellular phenylalanine concentration found in freeze-clamped liver samples. From measurements in control reactions, factors of 0.6 × 0.97 × 2.5 × (to give 1.45 × total) were determined to correct for differences between in vitro and in vivo rates due to tetrahydrobiopterin concentration, phenylalanine concentration, and temperature (5), respectively. Multiplying 1.45 by the unpreincubated enzyme activity, yields the "Corrected PAH Activity" (Table II).

Miscellaneous Methods—Protein was determined (16) using crystalline bovine serum albumin (Sigma) as the standard.

RESULTS

Cooling liver extract rapidly to 0 °C in a phenylalanine containing buffer traps activated phenylalanine hydroxylase by blocking further activation or inactivation of the enzyme (4). In the experiments of Table I, a section of liver of an anaesthetized rat was rapidly frozen by freeze-clamping. The frozen samples were homogenized in buffer containing 20 mM phenylalanine and then centrifuged, care being taken to keep the temperature at 0 °C or less at all times. If the temperature were to rise, enzyme would be activated (4); that this was not a serious problem here is shown by the low level of activated enzyme found in liver extracts of normal rats (Table IA). The amount of activated enzyme in each extract was measured using tetrahydrobiopterin in the assay system; since, even then the hydroxylation rate is a cofactor for activated phenylalanine hydroxylase, it will specifically and almost completely inhibit the unactivated enzyme (5).

As shown in Table IA, at most 1–4% of available phenylalanine hydroxylase in livers of normal well-fed rats, appears to be in an activated state in vivo. In rats given a phenylalanine load, about 35% of the enzyme was found to be activated. In separate experiments, animals given no injection at all or an injection of 70 mg of valine, which will not activate phenylalanine hydroxylase in vitro (5), also showed little (averaging 3%) phenylalanine hydroxylase in an activated state.

Animals were also injected with glucagon to induce complete phosphorylation of phenylalanine hydroxylase (8) and then the state of activation was determined (Table IB). The phosphorylated enzyme in normal well-fed animals, appeared to be 5–7% activated, about 2-fold more than that found in the comparable non-glucagon-treated animals. Extracts of livers of glucagon-treated rats made in a phenylalanine-free buffer to avoid trapping activated enzyme appeared only 1–3% activated, showing that the phosphorylated enzyme was in a partially activated state in the extract containing phenylalanine. When glucagon-treated rats were given a phenylalanine load and the extracts made in the usual way, a large percentage of enzyme was found activated (Table IB).

To establish that the phenylalanine concentration in the

| Injected | PAH specific activity | % Activated |
|----------|-----------------------|------------|
| Extract  | Activated extract     |            |
| A. 1. Saline | 0.2 | 19.4 | 1 |
| 2. Saline | 0.7 | 16.6 | 4 |
| 3. Phe | 12.6 | 35.2 | 36 |
| 4. Phe | 8.1 | 24.5 | 33 |
| B. 1. Glucagon | 1.4 | 20.6 | 7 |
| 2. Glucagon | 1.5 | 29.8 | 5 |
| 3. Phe + Glucagon | 7.8 | 27.6 | 28 |
| 4. Phe + Glucagon | 7.3 | 19.5 | 37 |

plasma was responsible for the enzyme activation in situ, experiments were performed with livers perfused in a single-pass mode. A defined perfusion medium was used, and the only variable component was phenylalanine: its concentration was either 84 μM (normal plasma concentration) or 310 μM. Livers perfused with 84 μM phenylalanine (Table II) had the same low level of activated phenylalanine hydroxylase as found in vivo. Raising the phenylalanine concentration to 310 μM, increased about 4-fold the percentage of activated enzyme seen (Table II). Thus, phenylalanine alone can induce an increase in phenylalanine hydroxylase activation, and the amino acid is effective at physiological concentrations. When all amino acids in the medium were increased to 4 times their normal plasma concentrations, the change in phenylalanine hydroxylase activation was comparable to that shown in Table II (data not shown).

In the experiments of Table II, an estimate of the phenylalanine hydroxylation rate of the perfused livers was obtained by adding [14C]phenylalanine to the perfusion medium and measuring [14C]tyrosine in the perfusate. One mM tyrosine, about 10 times the normal plasma level, was present in the perfusion medium to minimize losses of [14C]tyrosine. Significant [14C]tyrosine was produced at 84 μM [14C]phenylalanine, and increasing the concentration in the medium to 310 μM caused a very large increase in the rate of appearance of [14C]tyrosine. To compare the rate of tyrosine formation in situ with the rate measured in the liver extract, the [14C]tyrosine isolated from the perfusate was corrected for intracellular dilution of the [14C]phenylalanine precursor, and the enzyme activity measured in the extract was corrected to conditions of tetrahydrobiopterin and phenylalanine concentration and of temp.
Phenylalanine Control of Phenylalanine Hydroxylase

TABLE II
Phenylalanine activation in perfused liver

| Medium [\(^{14}C\)Phe] | PAH Activity | Perfusate [\(^{14}C\)Tyr] | Corrected |
|------------------------|--------------|-----------------|-----------|
| \(\mu M\) | \(\mu M/\min/\text{liver}\) | \(\%\) | \(\mu M/\min/\text{liver}\) | \(\mu M/\min/\text{liver}\) |
| A | 84 | 0.11 | 2.3 | 7.6 | 0.073 | 0.16 | 0.12 |
| B | 84 | 0.14 | 1.9 | 9.5 | 0.106 | 0.20 | 0.17 |
| C | 310 | 0.89 | 8.0 | 57 | 0.46 | 1.3 | 0.34 |
| D | 310 | 1.27 | 9.3 | 81 | 0.76 | 1.8 | 0.90 |

These results are essentially the same as those found above using crude extracts and imply that activation of phenylalanine hydroxylase is required for expression of appreciable catalytic activity whether or not the enzyme is phosphorylated. Nonetheless, phosphorylation does affect the activation process. At a given phenylalanine concentration, phosphorylated enzyme is activated more rapidly than unphosphorylated (control) enzyme (Table III). When characterized, the phosphorylated as well as the control enzyme showed first order activation curves that fit Model A:

\[ E_i \rightarrow E_n \]

where \( E_i \) is an inactive species, \( E_n \) is the activated enzyme, and \( k_i \) is an apparent first order rate constant of activation (5). The \( k_i \) was significantly greater for the phosphorylated than for the control species (Table III). The \( k_i \) values for the phosphorylated and unphosphorylated (5) enzymes increase with pH.

When phosphorylated phenylalanine hydroxylase was preincubated at pH 7.4 and 25°C with different known concentrations of phenylalanine and the amount of activated

TABLE III
Rate of activation of phosphorylated and unphosphorylated

| Enzyme | pH 6.8 | pH 8.0 |
|--------|--------|--------|
| \( k_i \) | \( V_i \) | \( k_i \) | \( V_i \) |
| Unphosphorylated | 0.17 | 6.2 | 0.43 | 12.3 |
| Phosphorylated | 0.38 | 10.6 | 1.4 | 14.1 |

Initial velocity (\( V_i \)) for fully activated enzyme and \( k_i \), the apparent first order rate constant of activation defined by Model A, were determined, using the methods described (5), for both the pure phosphorylated and pure unphosphorylated phenylalanine hydroxylase. The reaction mixture contained 60 \( \mu M \) 6-methyl tetrahydropterin, 1 \( \mu M \) phenylalanine, 6 \( \mu M \) dithiothreitol, 64 \( \mu g \) of catalase, and 0.1 M KCl in 0.05 M Tris, 0.05 M phosphoric acid. The reaction pH was 6.8 or 8.0; the temperature was 15°C, the volume was 1.00 ml, and identical aliquots of enzyme were used at pH 6.8 and 8.0.

Fig. 1. Phenylalanine activation of phosphorylated phenylalanine hydroxylase at equilibrium. Identical aliquots of pure phosphorylated phenylalanine hydroxylase (2 \( \mu g \)) were preincubated at 25°C in 0.84 ml of 0.06 M Tris, 0.06 M phosphoric acid, pH 7.4, containing 0.12 M KCl, 64 \( \mu g \) of catalase, and the indicated concentrations of phenylalanine (PHE). After 4 min, dithiothreitol, tetrahydropterin, and phenylalanine were added simultaneously to give final concentrations of 6 \( \mu M \), 30 \( \mu M \), and 1 \( \mu M \), respectively, in a total volume of 1.00 ml. The reaction was monitored at 275 nm and initial velocities (\( V_i \)) determined (5). The initial velocity of enzyme preincubated without phenylalanine was subtracted from all other determinations as the blank reaction. The same set of data is plotted in the inset as in the main figure.

It is clear from these data that one can easily purify phosphorylated phenylalanine hydroxylase using the present procedure when the normal, appropriate precautions are taken to suppress phosphatase activity. This result is in distinction to one published report where only relatively low yields of phosphorylated enzyme were recovered (18) using our purification procedure (4). In this case (18), the authors apparently took no precautions to prevent phosphatase action, which, at least in part, may explain their problems.
enzyme present at equilibrium measured, the activation process appeared cooperative (Fig. 1, inset), just as we had previously found for the unphosphorylated enzyme (5). The activation curve can be fit by an equation of the second degree in phenylalanine (Fig. 1). The phosphorylated enzyme was 50% activated at 0.08 mM phenylalanine compared to 0.06 mM for the unphosphorylated enzyme determined under the same conditions (5). These data indicate that the greater $k_a$ of the phosphorylated enzyme (Table III) is due in part to an increased sensitivity of enzyme activation to phenylalanine concentration. This result appears to explain the increased level of activated enzyme in extracts of rats treated with glucagon as compared with control animals (Table I). Whether all this increase is due to activation in vivo or some to activation that occurred during extract formation is not known.

**DISCUSSION**

We have shown that in vivo phenylalanine can control the amount of catalytically competent (activated) phenylalanine hydroxylase in liver. Furthermore, in perfused liver, and by inference, in vivo, an increase in the amount of activated enzyme correlated with a functional increase in the rate of tyrosine formation by the liver. Our results, combined with the lack of any known effect of tyrosine on the enzyme reaction, suggest that the major physiological role of phenylalanine hydroxylase is to degrade excess phenylalanine.

Substrate activation is an attractive mechanism for regulating the degradation of an essential amino acid, since it allows the amount of available active enzyme to vary in response to the amount of available substrate. Of particular importance is that when, as here (Fig. 1 and Ref. 5), the activation process is cooperative, a relatively large response can be obtained over a narrow concentration range. The need for such a control mechanism was made clear in the perfusion experiments which showed that, once the enzyme is activated, rat liver has an extraordinary capacity for hydroxylyzing phenylalanine. At only 4 times its normal plasma concentration, greater than 25% of the phenylalanine in the perfusion medium was hydroxylated in a single pass through the liver. If the enzyme were not carefully regulated, the enzymatic reaction could rapidly strip all free phenylalanine from the animal.

The perfusion experiments also demonstrated that the measured amount of activated enzyme (2-9% depending upon the case) in the liver could be used to calculate a phenylalanine hydroxylase rate. The calculations assumed that both the substrates and the enzyme are uniformly distributed throughout the cytoplasm. This appears to be true for the enzyme (19), but little is known about the distribution of phenylalanine or tetrahydrobiopterin. In any case, the important point is that, considering the nature of the comparisons, the calculated rates are reasonably close to the actual rates of tyrosine formation by the tissue. If we had assumed 100% of the enzyme to be activated, the calculated hydroxylase rates would have made little sense.

That the percentage of activated enzyme in vivo was less than would be predicted from the known dependence of activation on phenylalanine concentration for either purified phosphorylated (Fig. 1) or unphosphorylated (5) enzyme is to be expected, since the effect of tetrahydrobiopterin on the activation equilibrium has not been taken into account. As mentioned earlier, tetrahydrobiopterin forms an inactive complex with the unactivated enzyme, whether the latter is phosphorylated or not. The complex formation appears to be reversible and in competition with the activation process (5).

If the same reversible inhibition occurs in vivo, at any given phenylalanine concentration, the effect of tetrahydrobiopterin would be to decrease the amount of activated enzyme present. The competition is as yet too poorly characterized to make a quantitative prediction.

In our results in all properties relating to activation, the phosphorylated and unphosphorylated enzyme appear qualitatively alike. The original work characterizing phosphorylated phenylalanine hydroxylase (8, 9) used an assay system in which enzyme activity was proportional to the extent of phosphorylation of the enzyme; the assay did not distinguish activated (in the sense used here) from inactivable enzyme. This was a convenient assay for the original discovery; but it obscured the role of phenylalanine activation. We have specifically chosen assay methods which will separately measure effects on enzyme activity and effects on enzyme activation (4-6). It is now clear that the role of phosphorylation is to increase the sensitivity of phenylalanine hydroxylase activation to phenylalanine concentration; but in the absence of activation, the phosphorylated enzyme has little or no catalytic activity. The prediction would be that in situ only at relatively low phenylalanine levels would differences between phosphorylated and unphosphorylated hydroxylase activity become evident. At very low levels, neither species would be active. This interpretation is in complete accord with recently reported results of the phenylalanine hydroxylating ability of isolated rat hepatocytes in the presence and absence of glucagon (20).

Overall, our findings on the effects of phosphorylation make physiological sense. Given the potential of liver for hydroxylating phenylalanine and the fact that hydroxylation is irreversible, if phosphorylation directly activated the enzyme as has been suggested (9), the effects could be very serious for the animal. From this standpoint, the in vivo role of lysolcithin (7) or any other effector able to circumvent an overall phenylalanine-control of phenylalanine hydroxylase activity seems questionable.

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