Peptidyl Proline Hydroxylase in Adult, Developing, and Neoplastic Rat Tissues

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SUMMARY

A sensitive assay system, optimally supplemented with tritiated protocollagen substrate and cofactors, is described which is suitable for determining the peptidyl proline hydroxylase (PPH) content of a wide spectrum of rat tissues. In most tissues, less than 50% of the total activity was soluble; the particulate portion of the activity (concentrated in the mitochondrial and microsomal fractions) was doubled by pretreatment with Triton X-100.

Among normal adult tissues, lung had the highest total PPH activity (2.4 times that of liver) and small intestine had the lowest (25% that of liver). In brain and lactating mammary gland, the activity was similar to that in skin (60% of that in liver). Fetal tissues contained 3 to 8 times more PPH than the corresponding adult tissues, and a much lower portion of the total activity was soluble.

In four tissues studied in detail (lung, liver, kidney, and brain), the total PPH declined rapidly during the last few days of gestation; brain attained its low adult value before term, whereas the other three tissues continued to decrease in the course of postnatal development. An injection of cortisol to fetal rats enhanced the decline of PPH in lung, liver, and skull. These experiments suggest that during normal differentiation the decline in collagen synthesis is initiated by fetal glucocorticoid secretion which is maximal on the 19th gestational day.

PPH activity appears to be a sensitive indicator of neoplastic growth. In renal, mammary, muscle, and hepatic tumors, the PPH activities were 4 to 10 times higher than in the cognate adult tissue. Even in well-differentiated, slow-growing tumors, the activity was considerably higher than in any normal, mature, or immature tissue, with the exception of the skull and lung of the 19-day-old fetus.

INTRODUCTION

The essential role of PPH in collagen biosynthesis is well documented by studies of its catalytic function (1, 16) and by demonstration of high activities in tissues particularly active in collagen production (24). One might expect that many growing tissues would contain higher amounts of this enzyme than the corresponding mature tissues. Indeed, it has been reported that the PPH activity decreases in chick shortly before hatching (11), in the mouse embryo after the 18th day of gestation (13), and in rat tissues after birth (24), but systematic quantitative comparisons between the PPH activity of fetal and adult organs are not available for any species. Neoplasms may also be expected to possess the equipment for collagen synthesis. Accordingly, primary mammary tumors (4) and sarcomas (26) in the mouse, as well as a hepatoma and a chondrosarcoma in the rat (26), were reported to have high PPH activities. However, the activities of tumors relative to the cognate adult and fetal tissues, or as a function of their growth rate, have not been studied. Furthermore, recent reports of the presence of considerable PPH activity in the particulate fraction of mouse mammary tumors (4) and rat tissues (10) suggest that previous assays restricted to the supernatant fraction of centrifuged homogenates may have given an incomplete picture of the relative tissue concentrations of this enzyme. The present study explores this question, describes the conditions that provide a sensitive assay of PPH in crude tissue preparations, and presents the results of a systematic comparison of the levels of PPH in a series of developing, mature, and neoplastic rat tissues. The time course of change in some selected tissues during normal development, and in response to cortisol treatment, is also described and related to previous observations on the biochemical differentiation of these tissues.

MATERIALS AND METHODS

Animals. Normal tissues were obtained from inbred Kx rats. The age of fetal rats was read off a body weight-age curve (6); adult rats were 60- to 90-day-old males. Mammary glands were obtained from 86- to 96-day-old female rats on the 14th to 18th day of gestation. Tumors were serially transplanted in Kx (Walker carcinoma 256), Fischer (mammary tumors 1C, 5A and 3230AC), and Buffalo (hepatoma 7777 and renal carcinoma MK1) rats. Their exponential rate constants of growth in volume, \(b_{vol}\), calculated from the linear part of the relation, \(\log_{10} V = A + b_{vol} \times \text{days}\) (log 2/\(b_{vol}\) equals doubling time in days) (20), have been previously published (20, 23).

Substrate Preparation. The protocollagen substrate was
prepared by a method similar to that of Fleckman et al. (5). Femur and tibia (0.21 g) from four 11-day-old chick embryos were incubated for 3 hr at 37° with continuous shaking in an atmosphere of 95% O₂-5% CO₂ in 5 ml Krebs-Ringer buffer (28) containing 1 mm of α,α′-dipryridyl and 300 μCi of L-[3,4,14C]proline (38 Ci/m mole, New England Nuclear, Boston, Mass.). After centrifugation, the pellet was suspended in 4 ml of 50 mM Tris-HCl buffer, pH 7.5, 1.0 mM NaCl, and 20 mM KCl and homogenized in a ground-glass homogenizer. After shaking for 10 hr at 4°, it was centrifuged at 105,000 × g for 60 min, and the supernatant was dialyzed against 4 changes of water for 25 hr. The supernatant substrate used in most of the experiments to be described contained 0.87 mg protein per ml with a specific radioactivity of 7 × 10⁴ dpm/mg. It was stored at −20° and was stable for at least 6 months.

**Assay of PPH.** After the rats were killed by decapitation, tissues were excised and immediately homogenized in 9 volumes of 0.25 m sucrose containing 0.1 mM DTT, using a glass-Teflon homogenizer. For routine purposes, an aliquot of this homogenate was centrifuged at 105,000 × g for 60 min, and the enzyme was assayed (in duplicate) in both the supernatant fraction and in the uncentrifuged whole homogenate. The latter was allowed to stand before assay for 30 min on ice in the presence of Triton X-100 (0.5% final concentration). The same Triton treatment was applied when assaying various particulate fractions sedimented by centrifugation.

The assay mixture (final volume, 0.255 ml) contained 1 to 100 μl of tissue preparation, 99 to 0 μl of the homogenizing medium, 100 μl of the protocollagen substrate, 5 μl of a catalase solution of 2.5 mg/ml (bovine liver catalase, twice crystallized, C-100; Sigma Chemical Co., St. Louis, Mo.), and 50 μl of a "cofactor solution," so that the final concentrations in the assay mixture were 0.15 mM Fe(NH₄)₂(SO₄)₂, 12.5 mM ascorbate, 0.3 mM α-ketoglutarate, 0.1 mM DTT, and 0.1 mM Tris-HCl buffer (pH 7.0). After incubation with shaking in air for 20 min at 37°, the reaction was stopped by placing the tubes on ice and by adding 2 ml of 0.04 M EDTA. The mixture was then vacuum distilled (12). A 2.00-ml aliquot of the distillate was added to 15 ml of Aquasol (New England Nuclear, Boston, Mass.) and its radioactivity was determined at 55% gain with 50 to 1000 windows in a Model 3375 Packard liquid scintillation counter. Efficiency, determined with a standard ¹⁴H₂O sample (New England Nuclear), was 29%. Storing of the mixture for 48 hr at 4° before distillation did not alter the results. Enzyme-free blanks, run with each determination, gave 30 to 100 dpm; similarly low values were obtained with the complete mixture incubated for 0 min.

The enzyme activity units (dpm/min) obtained have no absolute meaning since the specific radioactivity of the available proline in the substrate (which varies among preparations) is unknown. Therefore, most of the results (units/g fresh tissue weight) were converted to reference units (L.U.), relative to the activity found with the same substrate in normal, adult rat liver (taken as 1.0 L.U.) and included in each series of measurements. The arbitrary choice of rat liver as the reference tissue is justified by its ready availability, highly reproducible PPH activity, and by the fact that, in this tissue, the largest numbers of enzymes and other chemical constituents have been quantified (18).

**RESULTS**

The method of preparing the protocollagen substrate differed from that described by Fleckman et al. (5) in only 1 respect: preincubation of chick bones prior to the addition of ²H)proline was avoided because it decreased the yield by about 25%. Additional tests showed that extraction with neutral salt solution, recommended by Fleckman et al. (5), was very advantageous; extraction with 0.5 N acetic acid, as described by Hutton et al. (12), resulted in only 18% relative yield.

Adult rat lung homogenates were used to establish optimal conditions for the assay of PPH. As seen in Chart 1, maximal activity was obtained in the presence of 100 μl substrate. With another substrate preparation, 250 μl were required for saturation, and the resulting activity (dpm/min/g lung) was 1.8 times higher. Product formation was proportional to the amount of tissue extract added (Chart 2, A) and increased linearly during the 1st 30 min of incubation (Chart 2, B). To obtain maximal activity, we increased the amounts of ascorbate and Fe, recommended by Fleckman et al. (5), to 12.5 and 0.15 mM, respectively (Chart 3). Under these conditions, DTT did not cause a further increase, and catalase caused a 27% rise. Using 10 times more catalase and twice the standard amount of α-keto glutarate did not augment the activity. At pH 6.8 and 7.2, the activity was about 90% but, at pH 7.5, the activity was only 15% of that at pH 7.0. The blank values obtained with the complete assay mixture at 0 time, or without enzyme after 20 min of incubation, were below 300 dpm so that the counts obtained with even the least active tissue (using 100 μl of a 10% intestinal homogenate) were highly significant (8650 dpm).

In agreement with Guzman and Cutroneo (10), we found that less than one-half of the total PPH activity of adult rat liver was soluble. This was also true for lung and brain (Table 1; for additional tissues, see Table 2). Triton treatment more than doubled the activity of the particulate fraction and had no effect on that of the soluble fraction. Most of the particulate activity in liver and brain appeared...
to be evenly divided between the mitochondrial (P₂) and the microsomal (P₃) fractions. Since the activity of whole homogenates (Line 1) was the sum of the soluble (Line 3) and particulate fractions (Line 4), 2 measurements, those in whole homogenates and in the soluble fraction, were routinely made. However, since the stability of the 2 fractions differs significantly, this approach is applicable only if assays are done on fresh tissue preparations (i.e., within 60 to 90 min after killing the animal). After storage of lung preparations at 0° for 5 hr, there was no change in soluble activity, but 37% of that of the particulate fraction and some 25% of that of the whole homogenate was lost.

Table 2 summarizes the tissue distribution of PPH activity expressed in reference units (see explanation under “Materials and Methods”). The results given in Table 2 (and in all other tables and charts) were obtained with the same substrate preparation. With alternate substrate preparations, the absolute activities were different, but activities of various tissues relative to liver were similar. For example, another substrate yielded 290,000 and 609,000 dpm/min/g liver and lung, respectively, so that, in reference units, the value for lung (2.12 L.U.) was similar to that in Table 2 (2.37 L.U.). The 1st column of Table 2 shows that, among 9 adult tissues, only lung had a higher total PPH activity than liver. The activity of brain was surprisingly high, 59% of that of liver, and similar to that of skin. All fetal tissues had much higher activities than their cognate adult tissues. Table 2 also shows that tumors contain 4 to 10 times more PPH than the cognate adult tissues and more than any normal tissue, except for the lung and skull of the fetus. The volume-doubling time of these tumors, ranging from 1 day (Walker) to 14 days (MK1), did not correlate with their PPH content.

The soluble portions of the total PPH activity, listed for each tissue in Table 2, reveal a remarkable change with
The total and soluble PPH activities of adult, fetal, and neoplastic rat tissues

Activities of whole homogenates (with Triton) are given in reference units (1 L.U. = 173,000 dpm/min/g adult liver); the activity of the soluble fraction as percentages of those of the whole homogenate are listed under %S (100 - %S = % particulate activity). Mammary gland was excised from female rats on the 16th day of lactation; all other normal adult tissues were from male rats. Values for adult or neoplastic tissues refer to single animals or to mean ± S.D. of results obtained with 3 to 6 animals. Fetal values (19th day of gestation) refer to pools of tissues from 3 to 8 fetuses (for additional assays of immature tissues, see Chart 4 and Table 3).

Table 2

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Adult L.U.</th>
<th>Fetal L.U.</th>
<th>Neoplastic Tumor L.U.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>2.37 ± 0.38</td>
<td>7.29 ± 17</td>
<td>7777 ± 0.188</td>
</tr>
<tr>
<td>Heart</td>
<td>1.02</td>
<td>38</td>
<td>3.62 ± 24</td>
</tr>
<tr>
<td>Liver</td>
<td>1.00 ± 0.21</td>
<td>3.12 ± 15</td>
<td>2376 ± 0.21</td>
</tr>
<tr>
<td>Lactating</td>
<td>0.66, 0.37</td>
<td>38, 50</td>
<td>5A (0.287)</td>
</tr>
<tr>
<td>mammary gland</td>
<td></td>
<td></td>
<td>3230AC (0.104)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.64 ± 0.14</td>
<td>2.85 ± 21</td>
<td>Walker (0.351)</td>
</tr>
<tr>
<td>Skin</td>
<td>0.62</td>
<td>4.49 ± 19</td>
<td>1C (0.057)</td>
</tr>
<tr>
<td>Brain</td>
<td>0.59 ± 0.08</td>
<td>1.49 ± 13</td>
<td>MK1 (0.021)</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.52</td>
<td>4.19</td>
<td>NS104 (0.181)</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.25, 0.29</td>
<td>11.5</td>
<td>5.49, 3.16</td>
</tr>
<tr>
<td>Skull</td>
<td></td>
<td>40</td>
<td>30</td>
</tr>
</tbody>
</table>

* Numbers in parentheses, growth rate of the tumors as kobs, described in "Materials and Methods."

In fetal tissues (with the exception of skull), less than 21% of the activity was soluble whereas, in all adult tissues, this percentage [except in brain (see “Discussion”)] ranged from 38 to 64.

The change of total PPH content throughout late gestation and postnatal life has been determined in lung, liver, kidney, and brain (Chart 4) (values obtained with homogenates of whole, 13-day-old fetuses are also shown for comparison). In each tissue a rapid decline began 2 days before term. Neonatal (brain and lung) tissues had activities that were similar to or (liver and kidney) about twice as high as the analogous adult tissues.

The decrease in PPH content that occurs during normal development can be enhanced by the administration of cortisol to fetal rats. Table 3 shows that in the lung, liver, and skull of the experimental fetuses, the activity was lower than in their control littermates. Since the latter (presumably because of some “leakage” of cortisol) (14) had lower activities than those found in completely untreated fetuses, intralitter comparisons may somewhat underestimate the effect of cortisol. Cortisol treatment did not decrease significantly the total weight of lung and liver.

In newborn rats, a dose of cortisol (similar, per g body weight, to that which was effective in fetuses) did not alter the PPH activity of liver, lung, and kidney. Estradiol treatment of immature female rats has been previously shown to increase the amount of uterine PPH (27). In 9-day-old rats, given injections of estradiol benzoate (0.01 mg/g, on Days 5 and 8), we found the rise in PPH to be very small (10 to 20%) per g uterus but, per whole uterus, the changes in PPH were less significant.

Chart 4. PPH in rat tissues as a function of age. The PPH of whole homogenates is expressed in reference units, as in Table 2. The adult values are taken from Table 2; all other points are means of closely agreeing duplicate measurements on a pool of tissues from 4 to 6 rats of the indicated prenatal or postnatal ages. Whole embryos were measured on the 13th day of gestation.
4, the pregnant rats were laparotomized under anesthesia, and fetuses in 1
closely agreeing duplicates obtained by assaying pools of tissues from 4 to 6
in Triton-treated whole homogenates of fetal tissues. Each value (expressed
relative to that of a simultaneously assayed normal adult liver) is a mean of
completely agreeing duplicates obtained by assaying pools of tissues from 4 to 6
fetuses of the same litter.

Table 3
The effect of cortisol on PPH in fetal tissue
Litters 1 and 3 received no injections. In experiments with Litters 2 and
4, the pregnant rats were laparotomized under anesthesia, and fetuses in 1
uterine horn received injections of 0.05 ml 0.9% NaCl solution and, in the
other horn, injections of 0.05 mg cortisol (hydrocortisone acetate) in 0.05
ml 0.9% NaCl solution. The maternal abdomen was then sutured, and
restoration was allowed to continue for 24 (Litter 2) or 48 hr (Litter 3), at
which time the animals were killed. The PPH activity was then determined
in Triton-treated whole homogenates of fetal tissues. Each value (expressed
relative to that of a simultaneously assayed normal adult liver) is a mean of
consistently agreeing duplicates obtained by assaying pools of tissues from 4 to 6
fetuses of the same litter.

<table>
<thead>
<tr>
<th>Litter</th>
<th>Gestational age when assayed (days)</th>
<th>Treatment</th>
<th>Lung</th>
<th>Liver</th>
<th>Skull</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.7</td>
<td>None</td>
<td>5.5</td>
<td>3.4</td>
<td>11.5</td>
</tr>
<tr>
<td>2</td>
<td>17.7</td>
<td>0.9% NaCl solution</td>
<td>3.77</td>
<td>3.10</td>
<td>7.86</td>
</tr>
<tr>
<td>3</td>
<td>19.6</td>
<td>Cortisol</td>
<td>3.34</td>
<td>1.14</td>
<td>4.58</td>
</tr>
<tr>
<td>4</td>
<td>19.6</td>
<td>None</td>
<td>6.00</td>
<td>3.1</td>
<td>11.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.9% NaCl solution</td>
<td>4.09</td>
<td>2.55</td>
<td>9.56</td>
</tr>
</tbody>
</table>

weight of which increased 3- to 4-fold, the rise was
considerable. The same estradiol treatment did not alter the
PPH activity or weight of liver, lung, and kidney.

DISCUSSION

The most sensitive method so far described for assaying
PPH in crude tissue homogenates was designed by Fleck-
man et al. (5) for the study of human skin biopsy samples.
This assay, with minor modifications, appears to be highly
suitable for the determination of PPH in crude extracts of
rat tissues. Even with the least active of the rat tissues
studied (small intestine), only 1 mg of tissue is required to
obtain values 200% above the blank. The quality of the
substrate may be an important determinant of sensitivity.
As a result of incubating chick bones and extracting with a
neutral salt solution (5), protocollagen may represent a
higher percentage of the total protein than it does in the
substrate prepared by Hutton et al. (12) from whole chick
minces and with acid extraction. It is probably due to this
difference that saturation of PPH with substrate was
attained with the present method (Chart 1) but not with that
of Hutton et al. (12). Both the use of excess ascorbate and
Fe^{2+} and restrictions of the reaction to the linear portions
with incubation time and enzyme concentrations were
practiced in this work to assure that the measured activities of
different tissues were proportional to their relative PPH
concentrations. Of course, one cannot assign absolute
meaning (e.g., pmoles proline hydroxylated) to the results
obtained, because the amount and specific activity of the
available proline in the protocollagen is not known and may
vary from preparation to preparation. However, the relative
activities of different tissues (or of the same tissue before
and after hormone treatment) remain constant with different
substrate preparations. It is thus particularly important

in the case of PPH to assure that observations have
Cumulative value by including a convenient standard tissue
with each study (18).

The present survey shows that, in most normal and
neoplastic tissues of the adult rat, less than 50% of the
activity is soluble. To obtain the total activity, whole-tissue
homogenates have to be treated with Triton X-100. This
more than doubles the activity in the particulate fraction.
Upon storage at 0°C for 5 hr, the soluble activity is stable
while the particulate activity shows a 37% loss. This alone is
insufficient to suggest the existence of 2 molecularly differ-
ent entities. In the 2 tissues investigated (liver and brain),
the particulate PPH was evenly distributed between the
microsomal and mitochondrial fractions. The presence of
this enzyme in the microsomal fraction accords with the fact
that proline may become hydroxylated while the protocolla-
orgen peptide is still attached to ribosomes (7, 17). The PPH
content of the mitochondrial fraction may in part be due to
ccontamination with the Golgi apparatus, which has been
shown to be actively involved in collagen synthesis and
export (29). PPH, bound to nascent protocollagen chains
associated with ribosomes, may travel with the completed
protocollagen chain through the Golgi system and become
soluble again after the completion of the hydroxylation
process. The completely hydroxylated collagen would then
be available for export to the extracellular matrix, and PPH
would be free to attach to another nascent protocollagen.

According to the only previously reported distribution of
PPH among rat tissues (24) (7 adult tissues were assayed as
supernatants obtained by centrifugation at 15,000 x g,
without Triton treatment), skin had higher activity than
liver, kidney, and muscle. The present more complete
measurements of total activities raised liver significantly
above the lower and similar values for skin, kidney, and
muscle. The soluble activities of these 4 tissues were about
the same. More importantly, only by assaying the whole
homogenate after Triton treatment was it possible to show
that brain (not included in the previous study) contained
very little soluble activity but as much total activity as did
skin or kidney. High activities in the particulate fraction of
brain homogenates, which includes the synaptosomes, is not
unique for PPH. Thirty to 50% of the total activity of
several glycolytic enzymes in brain is also sedimentable by
centrifugation (22).

Since there is, unfortunately, no quantitative information
available about the rates of collagen synthesis in different
tissues, we cannot ascertain whether they correlate with the
levels of PPH. The uniquely high activity in lung may be
due to the synthesis of both basement membrane and interstitial
collagen; however, intestine, which also produces both types
of collagen, had the lowest PPH activity of all tissues tested.
Lung was also rich (relative to kidney, muscle, brain, and
heart) in glutamine hexosephosphate aminotransferase (25),
necessary for glycoprotein synthesis. Both enzymes tend to
be at high levels in growing tissues. The coprominence of
PPH and glutamine hexosephosphate aminotransferase is
most striking in the skull bone, where both enzymes are
more concentrated than in any other fetal or neoplastic
tissue. This probably reflects the rapid synthesis of both

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collagen and glycoproteins during osteogenesis.

The high PPH activities of tumors (from 4 different tissues of origin) encompass a relatively small (2-fold) range and do not overlap with the low but wider (10-fold) range in the normal adult tissues. This enzyme is one of many which fits Knox's (18) generalization about the "fetalism" of neoplasms, i.e., that they tend to be rich in enzymes highly concentrated in fetal tissues and poor in enzymes that characterize the mature tissue of origin. However, unlike the amounts of other such fetal enzymes (19, 20), that of PPH did not correlate with the growth rates of this series of tumors, suggesting that even the very slow growth of a well-differentiated renal carcinoma (see Table 2, legend) is associated with a maximal potential for procollagen-proline hydroxylation.

Several examples indicate that, in different tissues of the developing organism, the upsurge or diminution of the same enzyme may occur at different times (9). Such asynchrony is to some extent also exhibited by PPH. While in brain it attained the low adult value 1 day before birth, in liver, kidney, and to a lesser extent in lung, there was a slow decrease throughout postnatal life. However, a sudden prenatal decrease in PPH activity is common to all 4 tissues examined and may be attributable simply to a decline in growth rate. It is important to note that the same late fetal period is also characterized by the emergence of new biochemical functions in some or all of these tissues. There is evidence that the emergence at this time of some enzymes in liver (9) is triggered by glucocorticoid secretion, which in fetal rats begins on the 16th day of gestation (15) and becomes maximal on the 19th day (3). In rabbits, cortisol promotes pulmonary epithelial maturation [e.g., the capacity for surfactant synthesis (21)] and at the same time promotes pulmonary epithelial maturation [e.g., the capacity for surfactant synthesis (21)] and at the same time

REFERENCES

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