Intracellular Enzymes of Collagen Biosynthesis in Rat Liver as a Function of Age and in Hepatic Injury Induced by Dimethylnitrosamine

PURIFICATION OF RAT PROLYL HYDROXYLASE AND COMPARISON OF CHANGES IN PROLYL HYDROXYLASE ACTIVITY WITH CHANGES IN IMMUNOREACTIVE PROLYL HYDROXYLASE

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Prolyl hydroxylase was purified from newborn rats by affinity chromatography using poly(L-proline), and antiserum to the enzyme was prepared in rabbits. The rat prolyl hydroxylase was similar to the chick and human enzymes with respect to specific activity, molecular weight and molecular weights of the polypeptide chains. The activity of prolyl hydroxylase and the content of immunoreactive enzyme were measured in rat liver as a function of age and in experimental hepatic injury. Active prolyl hydroxylase comprised about 13.2% of the total immunoreactive protein in the liver of newborn rats and the value decreased to about 3.6% at the age of 420 days. This decrease was due to a decrease in the enzyme activity, whereas only minor changes were found in the content of the immunoreactive protein. In hepatic injury, a significant increase was found in the ratio of active enzyme to total immunoreactive protein, owing to an increase in the enzyme activity. The data indicate that prolyl hydroxylase activity in rat liver is controlled in part by a mechanism which does not involve changes in the content of the total immunoreactive protein.

Prolyl hydroxylase catalyses the synthesis of hydroxyproline in collagen by hydroxylation of prolyl residues in peptide linkages (for reviews, see Cardinale & Udenfriend, 1974; Bornstein, 1974; Kivirikko & Risteli, 1976; Prockop *et al.*, 1976). The enzyme is present in cultured L-929 and 3T6 fibroblasts partly as an inactive proenzyme that can be activated by the addition of micromolar concentrations of ascorbate to the medium (McGee *et al.*, 1971; McGee & Udenfriend, 1972a; Stassen *et al.*, 1974; Levene *et al.*, 1974). The molecular weight of the proenzyme was reported to correspond to that of the subunit monomers of the enzyme, and it was suggested that the enzyme activity may be controlled by subunit association (McGee & Udenfriend, 1972a). The activation of prolyl hydroxylase was also demonstrated *in vitro* in sonicates of early exponential-phase L-929 fibroblasts, but the activation *in vitro* required, in addition to ascorbate, ferrous iron and α -oxoglutarate (Kuttan *et al.*, 1975). The activation *in vitro* did not involve subunit association of the enzyme, and the activatable form of prolyl hydroxylase was as large or larger than the active enzyme (Kuttan *et al.*, 1975). The presence of inactive prolyl hydroxylase protein was demonstrated in other fibroblasts too (Kao *et al.*, 1975),

but the enzyme activity in cultured fibroblasts from chick embryo frontal bones (Blanck & Peterkofsky, 1975) or leg tendons (Kao *et al.*, 1975) is not controlled by an ascorbate-requiring activation mechanism. It has further been demonstrated that all mammalian tissues contain, in addition to active prolyl hydroxylase, large amounts of an inactive, immunologically cross-reacting protein (Stassen *et al.*, 1974; Tuderman *et al.*, 1975b), suggesting that the activation of prolyl hydroxylase may be of physiological significance.

The amount of prolyl hydroxylase activity compared with that of immunoreactive enzyme protein is low in liver compared with that in several other tissues (Stassen *et al.*, 1974). Relatively large changes in liver prolyl hydroxylase activity take place with age [the preceding paper, Risteli & Kivirikko (1976)] and in hepatic injury leading to fibrosis (Takeuchi *et al.*, 1967; Takeuchi & Prockop, 1969; Feinman & Lieber, 1972; Risteli & Kivirikko, 1974, 1976). In the present study the activity of prolyl hydroxylase and the content of immunoreactive enzyme were measured in liver as a function of age and in experimental injury to find out whether changes in the enzyme activity are accompanied by similar changes in the amount of the immunoreactive protein. To

carry out such a study, purification of rat prolyl hydroxylase and preparation of specific antiserum were required.

Experimental

Purification of rat prolyl hydroxylase and preparation of antiserum

Prolyl hydroxylase was purified from newborn rats by affinity chromatography using poly(L-proline) (Tuderman *et al.*, 1975a; Kuutti *et al.*, 1975). Newborn rats were killed 0–12 h after birth and stored frozen. A total of 100 rats (about 600 g) were homogenized in a solution consisting of 0.1 M-NaCl, 0.1 M-glycine, 0.1 % Triton X-100 and 0.01 M-Tris/HCl buffer adjusted to pH 7.8 at 4°C (4 ml/g body wt. of rat), and an $(\text{NH}_4)_2\text{SO}_4$ (25–65 % satn.)-precipitated enzyme was prepared as described previously for chick embryos and foetal human tissues (Tuderman *et al.*, 1975a; Kuutti *et al.*, 1975).

The $(\text{NH}_4)_2\text{SO}_4$ -precipitated enzyme was passed through an affinity column containing poly(L-proline), mol.wt. 30000, linked to agarose and having a bed volume of 50 ml. The column was washed until the E_{230} of the effluent was about 0.05, and the enzyme was then eluted with 20 ml of the equilibrium solution with an addition of 3 mg of poly(L-proline)/ml (mol.wt. 5700). The fractions were pooled, concentrated and centrifuged, and the enzyme was separated from poly(L-proline) by gel filtration. The details of these procedures were identical with those previously reported for purification of chick and human prolyl hydroxylase (Tuderman *et al.*, 1975a; Kuutti *et al.*, 1975).

Antiserum to rat prolyl hydroxylase was prepared in rabbits as described previously (Kuutti *et al.*, 1975).

³H labelling of prolyl hydroxylase

Part of one prolyl hydroxylase preparation was labelled with ³H by using a technique of reductive alkylation with formaldehyde and NaB³H₄ (Rice & Means, 1971; Margolis, 1972). This reaction was carried out as reported for chick and human prolyl hydroxylase (Tuderman *et al.*, 1975b).

Preparation of [¹⁴C]proline-labelled procollagen

Isolated cells obtained from leg tendons of 100 17-day chick embryos were incubated with 60 μCi of [¹⁴C]proline in the presence of 0.3 mM- $\alpha\alpha$ -bipyridyl for 4 h (Dehm & Prockop, 1972), and the procollagen was extracted with 0.1 M-acetic acid (Berg & Prockop, 1973a; Harwood *et al.*, 1974). After centrifugation at 20000g for 30 min, the supernatant was dialysed against a solution containing 0.2 M-NaCl and 0.05 M-Tris/HCl buffer, adjusted to pH 7.8 at 4°C, with several changes. The preparation was then heated to 100°C for 10 min, centrifuged at 1000g

for 10 min to remove the precipitate formed during heating, and the supernatant was stored frozen in batches containing 60000 d.p.m. of radioactivity.

Animals and preparation of liver samples for assays

The experimental animals were female Long-Evans rats. The same rats were used as described in the preceding paper (Risteli & Kivirikko, 1976). The livers were homogenized and the 15000g supernatant of the liver homogenate was prepared as previously reported (Risteli & Kivirikko, 1974, 1976).

Assays for immunoreactive prolyl hydroxylase

Two different assays were used for the measurement of immunoreactive prolyl hydroxylase. The first was a direct radioimmunoassay based on the displacement of radioactively labelled prolyl hydroxylase from its antibody by the non-labelled enzyme, and on the subsequent precipitation of the enzyme-antibody complex by a cellulose-bound second antibody (Tuderman *et al.*, 1975b). The second assay was based on the displacement of the active enzyme from its antibody by inactive forms of the enzyme and on the measurement of the activity of the unbound enzyme (McGee & Udenfriend, 1972b; Stassen *et al.*, 1974). This assay was modified by substituting the [¹⁴C]proline-labelled procollagen substrate described above for the [³H]proline-labelled substrate prepared in whole chick embryos in the original procedure. Consequently the assay of the original procedure by the ³H release method was replaced by the assay with a specific chemical procedure (Juva & Prockop, 1966).

The results of both assays were processed and converted into ng of immunoreactive prolyl hydroxylase on a Honeywell 1644 time-sharing system by a program modified from that of Burger *et al.* (1972).

Assays of prolyl hydroxylase activity

Three different methods were used for the assay of prolyl hydroxylase activity. In experiments on the purification of rat prolyl hydroxylase, most assays were carried out by using a procedure based on the stoichiometric decarboxylation of 2-oxo-[1-¹⁴C]-glutarate (Calatomic, Los Angeles, CA, U.S.A.) (Rhoads & Udenfriend, 1968) with (Pro-Pro-Gly)₅-4H₂O (Protein Research Foundation, Minoh, Osaka, Japan) as a substrate, whereas in other experiments the synthesis of hydroxyproline was studied by quantitative assay (Kivirikko *et al.*, 1967), also with (Pro-Pro-Gly)₅-4H₂O as substrate. The details of these two methods are described in connection with purification of chick prolyl hydroxylase (Tuderman *et al.*, 1975a).

The third assay was based on the synthesis of hydroxyl[¹⁴C]proline with [¹⁴C]proline-labelled procollagen as a substrate. This method was used for the assay of prolyl hydroxylase activity in the super-

Table 1. Purification of prolyl hydroxylase by an affinity chromatography procedure from an $(\text{NH}_4)_2\text{SO}_4$ -precipitated enzyme of newborn rats

For definition of units of enzyme activity, see the Experimental section.

Enzyme fraction	Total protein (mg)	Total activity (units)	Recovery (%)	Specific activity (units/mg)	Purification (fold)
$(\text{NH}_4)_2\text{SO}_4$ (25–65% satn.)	6806	68.1	100	0.010	1
After gel filtration	0.35	27.2	40	77.7	7770

Fig. 1. Inhibition of rat prolyl hydroxylase activity by antiserum to the enzyme

The enzyme reaction was carried out as described in the Experimental section with [^{14}C]proline-labelled proto-collagen substrate and with 80ng of pure prolyl hydroxylase in the presence of increasing amounts of the antiserum.

nant samples of the liver homogenates and in the assay of immunoreactive prolyl hydroxylase by the modified method of McGee & Udenfriend (1972b) and Stassen *et al.* (1974). This assay was carried out as described previously for rat liver samples (Risteli & Kivirikko, 1974), except that the procollagen substrate was prepared in a different manner (see above), the incubation temperature was decreased to 30°C (as used by McGee & Udenfriend, 1972b) and no dithiothreitol was added to the incubation mixture, as this addition had no stimulatory effect in experiments with crude liver samples.

Definition of units of prolyl hydroxylase activity

The unit of prolyl hydroxylase activity was defined as the amount of enzyme required to synthesize 1 μmol of hydroxyproline/h at 37°C under conditions in which the concentrations of cofactors and co-substrates are those used in experiments involving assay of the quantitative synthesis of hydroxyproline, and in which a saturating concentration of (Pro-Pro-Gly) $_n$ ($n = 5, 10$ or 20) synthesized by the solid-state method, was used as a substrate (see Tuderman *et al.*, 1975a).

Other assays

The protein content of the 15000g supernatants of rat liver homogenates was assayed as in our previous work in this series (Risteli & Kivirikko, 1976).

Disc electrophoresis of native enzyme was carried out by using either a two-gel system or only one gel, with 7–7.5% polyacrylamide gels (see Tuderman *et al.*, 1975a). Disc electrophoresis of denatured polypeptide chains was performed in the presence of sodium dodecyl sulphate (Weber & Osborn, 1969). All gels were stained with Coomassie Brilliant Blue.

Double immunodiffusion was carried out by standard procedures on microscope slides covered with 1% agar in 0.02M-barbital buffer, pH8.6 (see Clausen, 1970).

The protein content of the pure enzyme samples was assayed by E_{230} , by using an absorption coefficient $E_{230}^{1\text{mg/ml}} = 7.73$, with a 1cm light-path found for chick prolyl hydroxylase (R. A. Berg, Y. Kishida, S. Sakakibara & D. J. Prockop, unpublished work).

All radioactivity counting was performed in a Wallac liquid-scintillation spectrometer with an efficiency of 85% and a background of 25c.p.m. for ^{14}C radioactivity, or 35% and 10c.p.m. for ^3H radioactivity, by using the scintillants reported for various assay procedures (see Juva & Prockop, 1966; Tuderman *et al.*, 1975a, b).

Results

Purification of rat prolyl hydroxylase

The procedure used here for the purification of rat prolyl hydroxylase is similar to that reported for purifying chick and human prolyl hydroxylases (Tuderman *et al.*, 1975a; Kuutti *et al.*, 1975). Six enzyme preparations were purified from rat sources. The purification of one typical enzyme preparation is shown in Table 1. The specific activity observed for the purified enzyme (77.7 units/mg) is similar to that of 59.3–91.5 units/mg reported for chick prolyl hydroxylase (Tuderman *et al.*, 1975a) or 61.0–82.7 units/mg for human prolyl hydroxylase (Kuutti *et al.*, 1975). The recovery of the enzyme activity was also similar to that found for purification of prolyl hydroxylase from the other two sources.

The mobility of the rat enzyme in gel filtration in an 8% agarose gel column, which was the last step

in the purification procedure, was identical with that of the chick and human enzymes, suggesting that the rat enzyme has a similar molecular weight. Examination of the purity of the native enzyme by polyacrylamide-disc-gel electrophoresis showed the presence of only one band and examination of the dissociated enzyme by sodium dodecyl sulphate polyacrylamide-gel electrophoresis showed the presence of two bands. Calibration of the polyacrylamide gels with standard proteins (bovine serum albumin, two subunits of chick prolyl hydroxylase, pepsin, trypsin and cytochrome *c*) indicated that the mol.wts. of the polypeptide chains were about 60000 and 64000.

Antiserum to rat prolyl hydroxylase, and comments on assays

Antiserum to rat prolyl hydroxylase gave a single precipitin line when examined with the rat enzyme by double immunodiffusion, whereas no line was found between non-immune serum and the enzyme. The antiserum inhibited the enzyme activity, about 5 μ l of antiserum giving a 50% inhibition of the activity of 1 μ g of rat prolyl hydroxylase (Fig. 1).

In initial experiments, the content of immuno-reactive prolyl hydroxylase was measured with the immunoassay based on the displacement of the active enzyme from the antibody by inactivated enzyme (McGee & Udenfriend, 1972*b*; Stassen *et al.*, 1974). The standard curves obtained with pure prolyl hydroxylase could be used for determining the content of immunoreactive prolyl hydroxylase (Fig. 2*a*, closed symbols). However, when similar standard curves were prepared in the presence of supernatant of liver homogenate, the shape of the curve clearly differed from that obtained with pure samples (Fig. 2*a*, open symbols). In further experiments with the other immunoassay (see below), it was found that heat-inactivation, such as that required in this immunoassay, decreased the displacement by about 35%. However, this observation cannot in itself explain the difference between the two curves in Fig. 2(*a*), as the standard curve was also prepared with heat-inactivated enzyme. When the heat-inactivation was carried out in the presence of liver supernatant, a precipitate was always formed, and it seems possible that this phenomenon explains at least part of the problems noted with the liver samples. In spite of a number of experiments, we were not able to prepare satisfactory and reproducible standard curves in the presence of liver supernatant with this method.

In subsequent experiments a direct radioimmunoassay with ^3H -labelled prolyl hydroxylase was used (Tuderman *et al.*, 1975*b*). The standard curves obtained with pure rat enzyme (Fig. 2*b*, closed symbols) were similar to those reported previously for chick and human enzymes (Tuderman *et al.*, 1975*b*). When similar standard curves were prepared

Fig. 2. Standard curves for the two immunoassays in the absence or presence of liver supernatant

The displacement curves were prepared with pure standard enzyme in the presence of bovine serum albumin (2.5 mg/ml) in the absence of liver supernatant (●) or in the presence of the same amount of liver supernatant in each sample of assay (○). The latter values were plotted, corrected for the presence of prolyl hydroxylase in the liver supernatant samples not containing standard enzyme. (*a*) Assay based on displacement of active prolyl hydroxylase; (*b*) direct radioimmunoassay based on displacement of the ^3H -labelled enzyme.

in the presence of supernatant of liver homogenate, the shape of the curve was identical with that obtained for pure enzyme, as shown in Fig. 2*b* (open symbols), for a liver sample containing 13 ng of immunoreactive prolyl hydroxylase.

To compare the enzyme activity with the amount of immunoreactive protein, it was necessary to convert the synthesis of hydroxyproline into units of enzyme activity. Because the units of prolyl hydroxylase activity have been defined with a saturating concentration of a synthetic peptide substrate (see the Experimental section), and because the assays of prolyl hydroxylase activity in liver supernatant samples were carried out with [^{14}C]procollagen

as the substrate, it was not possible to convert directly the synthesis of [^{14}C]hydroxyproline into units of enzyme activity. Therefore a conversion factor had to be experimentally determined for each new [^{14}C]protocollagen substrate preparation by incubation with purified enzyme of known specific activity. The units of enzyme activity were further converted into ng of active enzyme protein by assuming a specific activity of 77.7 units/mg for the pure enzyme, as shown in Table 1. It should be noted that values obtained for the same enzyme standard in various hydroxylation experiments, even with the same protocollagen substrate preparation, vary by about $\pm 10\%$, and that the specific activities of various pure enzyme preparations also vary, probably owing

to inactivation during the purification procedure, the lowest value found in this study being 48 units/mg. Thus the absolute values for amount of active enzyme as a percentage of total immunoreactive protein are subject to some error. However, all samples were analysed together with control samples in the same incubations, and thus the magnitudes of the changes are not subject to the errors described above.

Effect of age

Over an age range of 0–420 days the prolyl hydroxylase activity expressed per mg of protein in supernatant of liver homogenates decreased to about 25% (Fig. 3a). By contrast, no statistically significant changes were observed in the content of the immunoreactive protein measured with the direct radioimmunoassay, the lowest mean value being about 84% of the highest value (Fig. 3a). Active prolyl hydroxylase comprised about 13.2% of the total immunoreactive protein at day 0 (Fig. 3b). This value decreased to about 3.6% at 420 days.

Effect of hepatic injury

The changes in prolyl hydroxylase activity and in the immunoreactive prolyl hydroxylase, measured with the direct radioimmunoassay, compared with values in the control rats on each of the time-points are shown in Fig. 4. Both values are expressed in relation to protein content in the supernatant of the liver homogenates. A rapid increase was found in the prolyl hydroxylase activity, whereas no significant changes were observed in the content of the immunoreactive protein (Fig. 4a). The mean value for this protein on day 3 was about 86% of that in the control and increased to a value of 120% of the control on day 21. The ratio of active enzyme to the total immunoreactive protein increased to about 195% on day 3, and a statistically significant decrease ($P < 0.001$) to a value of 147% compared with controls on day 7 was observed (Fig. 4b). No changes were found in this ratio between day 7 and day 21, the ratio being continuously significantly higher than that in the controls.

When the content of the immunoreactive protein was measured with the immunoassay based on the displacement of the active enzyme (McGee & Udenfriend, 1972b; Stassen *et al.*, 1974), the values were similar to those described above, in that no significant change was found on day 3. However, because of difficulties encountered in preparing standard curves with this assay (Fig. 2a), it was not possible to relate the values obtained to ng of immunoreactive prolyl hydroxylase.

Further comparison of the content of immunoreactive protein was carried out by examining the inhibition of prolyl hydroxylase activity by various amounts of antiserum in one control sample and in

Fig. 3. Age-related changes in prolyl hydroxylase activity and the immunoreactive protein in the liver

(a) The enzyme activity (●) is expressed as munits/mg of protein in the 15000g supernatant of the liver homogenate, and the immunoreactive protein (○) as μg/mg of protein. (b) The ratio of active prolyl hydroxylase to the total immunoreactive protein was calculated as described in the text. Each group contained three rats, and the values are means $\pm$ S.D.

Fig. 4. Changes in prolyl hydroxylase activity and the immunoreactive protein in the liver in hepatic injury

The values are expressed as percentages (means $\pm$ S.D.) compared with the mean values for the control rats on each of the time-points. Each group contained six rats. The injections of dimethylnitrosamine are indicated by arrows. (a) Prolyl hydroxylase activity (●); immunoreactive protein (○). (b) Ratio of active prolyl hydroxylase to total immunoreactive protein.

one sample from a fibrotic liver on day 21, the enzyme activity being in this sample 237% of that in the control sample (Fig. 5a). When the percentages of inhibition observed with both samples were plotted, it was found that the same amounts of antiserum gave identical inhibitions with both samples (Fig. 5b). These data suggest that the amount of antigen was the same in both samples, in spite of the difference in the enzyme activities.

Discussion

Prolyl hydroxylase was initially purified to near homogeneity by conventional protein purification procedures from chick embryos (Halme *et al.*, 1970; Pänkäläinen *et al.*, 1970) and newborn-rat skin (Rhoads & Udenfriend, 1970). Since then, two affinity-column procedures were developed for purifying prolyl hydroxylase, and the enzyme was

Fig. 5. Inhibition of prolyl hydroxylase activity in a sample of normal liver (○) or fibrotic liver (●) with increasing amounts of the antiserum to rat prolyl hydroxylase

(a) Enzyme activities observed with the two samples, expressed as radioactivity (d.p.m.) of $[^{14}\text{C}]\text{hydroxyproline}$ formed/mg of liver supernatant protein; (b) percentage inhibition observed with the two samples. The same amount of liver supernatant protein was used for assaying both samples, but, as shown in (a), the enzyme activity in the sample from the fibrotic liver was 237% of that in the sample from normal liver.

isolated as a pure protein from chick embryos (Berg & Prockop, 1973b; Tuderman *et al.*, 1975a) and foetal human tissues (Kuutti *et al.*, 1975). In the present study prolyl hydroxylase was isolated as a pure protein from newborn rats. The molecular weight of the rat enzyme by gel filtration was identical with that of the chick and human enzymes, and the molecular weights of the subunits were also similar to those of the two other prolyl hydroxylases. The specific activity of the pure rat enzyme was likewise similar to that of the chick and human enzymes.

Comparison of the prolyl hydroxylase activity with the content of the immunoreactive protein in rat liver samples indicated that the enzyme activity decreased to about 25% with age from 0 to 420 days,

whereas only a statistically non-significant decreasing tendency to about 85% was found in the content of the immunoreactive protein. After liver injury, the enzyme activity rapidly increased to about 160% of control on day 3 and 180% on day 21, whereas no change or even a slight tendency to decrease was noted in the content of the immunoreactive protein on day 3 and a slight tendency to increase thereafter. When the ratio of the content of active prolyl hydroxylase to that of the immunoreactive protein was calculated, a significant decrease was found with age and a significant increase after the liver injury.

The present data indicate that prolyl hydroxylase activity in rat liver is controlled in part by a mechanism which does not involve changes in the content of the total immunoreactive protein. Previous studies have indicated the presence of an activation mechanism for prolyl hydroxylase in cultured L-929 and 3T6 fibroblasts (see the introduction). Kao *et al.* (1975) showed that 40–50% of prolyl hydroxylase protein was active in freshly isolated chick-embryo leg-tendon cells, but when these cells were cultured, this value decreased to 15–20%. Studies in animal tissue have indicated that the amount of prolyl hydroxylase activity compared with the content of immunoreactive protein varies greatly between different tissues (Stassen *et al.*, 1974; L. Tuderman, unpublished work). In developing chick embryos, the highest ratios of active enzyme to the total immunoreactive protein, up to 60–65%, were found in cartilage and skin, and the lowest ratios, below 10%, in spleen and the large vessels (L. Tuderman, unpublished work). It thus seems that the amount of prolyl hydroxylase activity may be controlled in several instances by a mechanism, or mechanisms, not involving changes in the content of the immunoreactive enzyme protein.

The present data do not indicate the mechanism of the changes in the ratio of active enzyme to the total amount of immunoreactive protein. Studies in cultured fibroblasts have suggested control of prolyl hydroxylase activity by subunit association in certain situations, whereas in other situations the proenzyme which could be activated was as large or larger than the active enzyme (see the introduction). It is also possible that the specific activity of the enzyme tetramer can vary, as has been reported in studies with pure prolyl hydroxylase preparations (see Tuderman *et al.*, 1975a), and regulation of the enzyme activity by specific inhibitors may also exist. Finally, it is possible that the immunoreactive protein partly represents a pool, or pools, of degradation products of prolyl hydroxylase which may not bear any direct relationship to the activation mechanism of the enzyme.

It is noteworthy that the changes in the content of immunoreactive prolyl hydroxylase with age and in liver injury observed in the present study are very

similar to those occurring in the collagen galactosyl-transferase and collagen glucosyltransferase activities in the same experiments (Risteli & Kivirikko, 1976). This similarity may suggest that the contents of all these three enzyme proteins were regulated similarly, but that an additional mechanism existed for regulating the amount of prolyl hydroxylase activity.

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