Genetic Evidence for a Common Enzyme Catalyzing the Second Step in the Degradation of Proline and Hydroxyproline

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ABSTRACT The initial step in the degradation pathways of proline and hydroxyproline is catalyzed by proline oxidase and hydroxyproline oxidase, yielding Δ^1-pyrroline-5-carboxylate and Δ^1-pyrroline-3-hydroxy-5-carboxylate, respectively. The second step is the oxidation of Δ^1-pyrroline-5-carboxylate to glutamate and Δ^1-pyrroline-3-hydroxy-5-carboxylate to γ-hydroxyglutamate. To determine if this second step in the degradation of proline and hydroxyproline is catalyzed by a common or by separate enzyme(s), we developed a radioisotopic assay for Δ^1-pyrroline-3-hydroxy-5-carboxylate dehydrogenase activity. We then compared Δ^1-pyrroline-3-hydroxy-5-carboxylate dehydrogenase activity with that of Δ^1-pyrroline-5-carboxylate dehydrogenase in fibroblasts and leukocytes from type II hyperprolinemia patients, heterozygotes, and controls. We found that cells from type II hyperprolinemia patients were deficient in both dehydrogenase activities. Furthermore, these activities were highly correlated over the range found in the normals, heterozygotes, and patients.

We conclude from these data that a common Δ^1-pyrroline-5-carboxylate dehydrogenase catalyzes the oxidation of both Δ^1-pyrroline-5-carboxylate and Δ^1-pyrroline-3-hydroxy-5-carboxylate, and that this activity is deficient in type II hyperprolinemia.

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INTRODUCTION

Proline and hydroxyproline are degraded by a series of analogous reactions (1). The biochemical similarity of these two degradative pathways suggests that the corresponding reactions in each pathway could be catalyzed by a common enzyme which can act on either substrate. This common-enzyme hypothesis appears, however, to be incorrect for the first reaction in which proline and hydroxyproline are oxidized to Δ^1-pyrroline-5-carboxylate (PC)[1] and Δ^1-pyrroline-3-hydroxy-5-carboxylate (3-OH-PC), respectively. Genetic and biochemical evidence indicates that separate enzymes specific for either proline or hydroxyproline catalyze this step in man (2–6).

The second reaction in proline and hydroxyproline degradation is the oxidation of PC and 3-OH-PC to glutamate and γ-hydroxyglutamate, respectively (7, 8). Whether this reaction is catalyzed by two distinct enzymes or a common enzyme is uncertain. Adams and Goldstone (7) reported biochemical evidence in factor of a common enzyme, whereas the two-enzyme hypothesis is supported by the fact that plasma hydroxyproline concentrations are only minimally elevated in patients with type II hyperprolinemia (HP2), an inherited deficiency of PC dehydrogenase activity (9).

The recent discovery by Goodman et al. (10) and Applegarth et al. (11) that both PC and 3-OH-PC are present in the urine of individuals with HP2 may be explained by either hypothesis. Abnormal 3-OH-PC oxidation in HP2 would be a direct result of the documented deficiency of PC dehydrogenase if this enzyme is actually a common Δ^1-pyrroline dehydrogenase.

[1] *Abbreviations used in this paper*: HP2, type II hyperprolinemia; 3-OH-PC, Δ^1-pyrroline-3-hydroxy-5-carboxylate; PC, Δ^1-pyrroline-5-carboxylate.

Alternatively, the oxidation of 3-OH-PC may be catalyzed by a separate enzyme. The presence of 3-OH-PC in the urine of HP2 patients might then be explained by an inhibition of the putative 3-OH-PC dehydrogenase by the high concentrations of either proline or PC.

To determine if the dehydrogenation of PC and 3-OH-PC is catalyzed by a common or separate enzyme(s), we developed a sensitive, radioisotopic assay for 3-OH-PC dehydrogenase and compared its activity with that of PC dehydrogenase in extracts of leukocytes and cultured fibroblasts from HP2 patients, heterozygotes, and normals. Our results show a close correlation of PC and 3-OH-PC dehydrogenase activity and support the hypothesis of a common Δ^1-pyrroline dehydrogenase capable of catalyzing the oxidation of either PC or 3-OH-PC.

METHODS

Preparation of substrates

L-[U-^{14}C]PC. We synthesized [U-^{14}C]PC enzymatically using a previously reported method (12). In brief, [U-^{14}C]-ornithine (New England Nuclear, Boston, Mass.) and α-ketoglutarate were incubated with ornithine-ketoacid aminotransferase purified 400- to 500-fold from rat liver. The resultant [U-^{14}C]PC was purified by cation exchange column chromatography.

L-[G-^{3}H]3-OH-PC. Labeled substrate was prepared independently by Dr. Phang and Dr. Goodman by different enzymatic methods, both of which were modified from that described by Adams and Goldstone (13). In Method 1, ≅150 μCi of L-[G-^{3}H]hydroxyproline (New England Nuclear) was incubated with 60 mg of rat liver mitochondria in 25 ml of 0.1 M potassium phosphate buffer, pH 7.4. After 6 h at 37°C, the reaction mixture was deproteinized with the addition of 50% (wt/vol) TCA to a final concentration of 5% (wt/vol) and centrifuged at 25,000 *g* for 30 min. To remove the TCA, the supernate was extracted repeatedly with ether to and end point of pH 4–5. The solution was then applied to a 30-ml Dowex-50 WX8 (Dow Chemical Co., Midland, Mich.), hydrogen form, 100–200 mesh column (Sigma Chemical Co., St. Louis, Mo.) and eluted with 0.2 N HCl. Fractions of 3 ml were collected. A peak of [G-^{3}H]3-OH-PC was found between 190 and 240 ml of eluant and was separated from the peak of precursor hydroxyproline by at least 15 ml. The fractions between 200 and 235 ml were pooled and lyophilized. The residue was dissolved in 1 ml of 0.1 N HCl and 250-μl aliquots were applied to a 1 ml Dowex 1-X8, formate form, 100–200 mesh (Bio-Rad Laboratories, Richmond, Calif.) column to further remove contaminants. [G-^{3}H]3-OH-PC was eluted with water, and the first 2 ml was lyophilized and taken up in 1 ml of 0.1 N HCl and stored at 4°C. Unlabeled 3-OH-PC was prepared in a similar manner with 10 mM L-hydroxyproline was substrate and 10^5 cpm of [G-^{3}H]3-OH-PC prepared by Method 2 (described below) to trace the pattern of 3-OH-PC elution from Dowex-50 and Dowex-1.

In Method 2 of [G-^{3}H]3-OH-PC preparation, ≅200 μCi of L-[G-^{3}H]hydroxyproline and 4.2 mg of unlabeled L-hydroxyproline were incubated with a 16,000-*g* pellet of rabbit kidney homogenate prepared from 4.2 g of tissue. The final incubation volume was 8 ml and contained 0.05 M potassium phosphate buffer (pH 7), 0.12 M potassium chloride, and 2 mM EDTA. The mixture was incubated for 16 h at 37°C, and the reaction was terminated with the addition of 400 mg of dry TCA. After standing at 4°C for 3 h, this mixture was centrifuged at 10,000 *g* for 10 min, and the supernate was extracted 10 times with 10 ml of ether to remove the TCA. Aliquots containing 250 μl of the extracted supernate were than passed through an amino acid analyzer column (Beckman UR-30 resin, Beckman Instruments, Inc., Fullerton, Calif.) and eluted with 0.2 M sodium citrate, pH 2.9. 1-min fractions were collected. The amount of [G-^{3}H]3-OH-PC in the fractions eluted from the column was measured colorimetrically with the *o*-aminobenzaldehyde technique (14), and the radioactivity was determined by liquid scintillation spectrometry. The fractions that contained *o*-aminobenzaldehyde-reactive material were pooled and passed over a column of Dowex-50 to remove citrate. The effluent was lyophilized, taken up in water, and stored at −20°C. This purified substrate was identified as 3-OH-PC by several criteria: It eluted as a single discrete peak from the amino acid analyzer with an elution time and a 570:440 mμ absorbance ratio similar to that described for 3-OH-PC by Heacock et al. (15), and reduction of the material in this peak with sodium borohydride produced only L-hydroxyproline and no allohydroxyproline.

Enzyme preparations

Fibroblasts. Normal human fibroblasts (D550, D551) were obtained from the American Type Culture Collection (Bethesda, Md.) or by skin biopsy (D.V., K.M., and B.R.). The HP2 fibroblasts were from patients K.K. and E.D. who have been previously described (9). Our methods of culturing, harvesting, and sonicating the fibroblasts have also been described in complete detail (9).

Leukocytes. Peripheral leukocytes from normals, HP2 patients (E.D. and G.F.), and obligate HP2 heterozygotes (parents of E.D. and G.F.) were prepared from 10 ml of heparinized (10 U/ml) venous blood by dextran (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) sedimentation and osmotic lysis of contaminating erythrocytes (16). These HP2 kindreds have been described in detail (9). Leukocytes were washed three times with phosphate-buffered saline and sonicated for 40 s at a setting of 2 with the micro-tip of a Branson model W 180 sonicator (Branson Sonic Power Co., Danbury, Conn.).

Enzyme and protein assays

3-OH-PC dehydrogenase. We assayed this activity by measuring the incorporation of radioactivity into product γ-hydroxyglutamate, which was separated from precursor 3-OH-PC by anion exchange chromatography. The elution patterns of these compounds from a 1-ml Dowex-1 formate column are shown in Fig. 1. A mixture of [G-^{3}H]3-OH-PC and L-allo-γ-hydroxyglutamate (Calbiochem, San Diego, Calif.) in 0.2 ml of 0.1 N HCl was applied to the column. The pyrroline compound was eluted with water, and the γ-hydroxyglutamate was subsequently eluted with 1 N formic acid. Ninhydrin was used to detect γ-hydroxyglutamate. We found that 74.8 ±0.5% (mean±1 SEM) of the applied γ-hydroxyglutamate was recovered in the second milliliter of formic acid eluant.

The standard reaction mixture was pH 6.8 and contained 50 mM potassium phosphate, 680 μM NAD^+, 0.11 mM [G-^{3}H]-3-OH-PC with ≅10^5 cpm (a mixture of the substrates was adjusted to pH 6.8 immediately before use) and 10–100 μg of enzyme protein in a final volume of 0.25 ml. After a 30-min incubation at 37°C the reaction mixture was acidified with 0.05 ml of 0.6 N HCl and centrifuged at 10,000 *g* for 10 min.

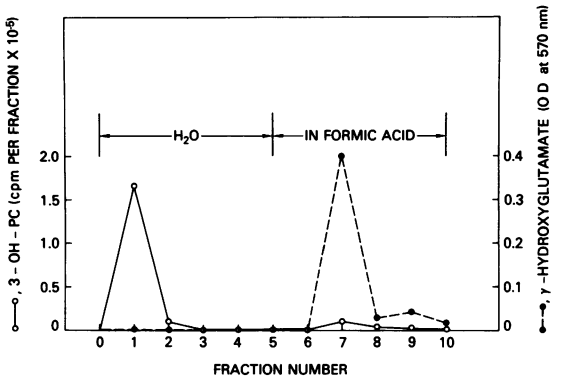

FIGURE 1 Separation of 3-OH-PC and L-allo-γ-OH-glutamate by ion exchange chromatography. A mixture of [G-^{3}H]3-OH-PC (200,000 cpm) and γ-OH-glutamate (0.2 mg) in 0.2 ml of 0.1 N HCl was applied to a 1-ml bed volume column of Dowex-1. The initial eluant was water (10 ml) followed by 1 N formic acid (5 ml). Fractions 1–5 each contained 2 ml of effluent whereas fractions 6–10 each contained 1 ml of effluent. The amount of 3-OH-PC in each fraction was determined by liquid scintillation spectrometry, and the amount of γ-hydroxyglutamate was assayed by reacting 0.2 ml of each fraction with ninhydrin.

The supernate (0.2 ml) was applied to a 1-ml bed volume Dowex-1 formate column. The column was then washed with 10 ml of H_2O, followed by 1 ml of 1 N formic acid. The product then was eluted in the second ml of 1 N formic acid directly into scintillation vials which also contained 12 ml of Aquasol (New England Nuclear).

PC dehydrogenase. We assayed PC dehydrogenase activity as we have previously reported (9).

Protein. The protein content of the various enzyme extracts was determined by the method of Lowry et al. (17).

PC and 3-OH-PC. The concentration of pyrroline compounds used in the assays was determined by the method of Strecker (8).

RESULTS

Assay of 3-OH-PC dehydrogenase activity. We assayed 3-OH-PC dehydrogenase activity in extracts of cultured human fibroblasts and human peripheral leukocytes. The activity in any given extract was comparable using [G-^{3}H]3-OH-PC prepared by either Method 1 or Method 2. Most assays used substrate prepared by Method 1 because this method was simpler and quicker. All results were verified, however, using the substrate prepared by Method 2 where [G-^{3}H]3-OH-PC was more rigorously identified and its purity verified.

The amount of product recovered above blank depended upon the source of the enzyme. Product radioactivity increased linearly with time and protein to ≅40% above blank with fibroblast extracts (Fig. 2). Leukocyte extracts maintained linearity up to 80% above blank (Fig. 2).

Activity of 3-OH-PC dehydrogenase in HP2 fibroblasts. We sought genetic evidence for the one

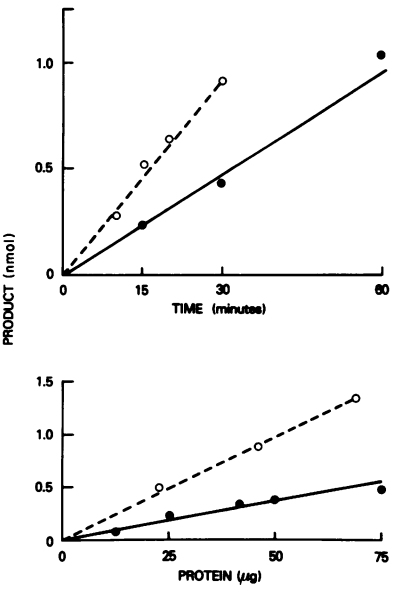

FIGURE 2 Formation of γ-OH-glutamate by fibroblast and leukocyte extracts. The top panel shows the amount of product γ-hydroxyglutamate formed from precursor [G-^{3}H]3-OH-PC when 56 μg of fibroblast protein (● —— ●) or 46 μg of leukocyte protein (O - - - O) was incubated at 37°C for increasing duration. The bottom panel shows γ-hydroxyglutamate formation by increasing amounts of fibroblast (● —— ●) or leukocyte (O - - - O) protein during a 30-min incubation.

enzyme hypothesis by assaying 3-OH-PC dehydrogenase activity in fibroblasts from two HP2 patients. We have previously shown fibroblasts from both patients to be deficient in PC dehydrogenase activity (9). As predicted by the one-enzyme hypothesis, the HP2 fibroblasts had no detectable 3-OH-PC dehydrogenase activity (Table I). This deficiency of 3-OH-PC dehydrogenase activity was not a result of the presence of a soluble inhibitor because the activity assayed in mixtures of HP2 and normal fibroblast extracts was not different from that predicted by the amount of normal fibroblast protein (Table II). Furthermore, dialysis of the HP2 extracts did not restore 3-OH-PC dehydrogenase activity. Our studies strongly suggest that the mutation that causes HP2 results in deficiency of both PC and 3-OH-PC dehydrogenase activities.

Leukocyte 3-OH-PC dehydrogenase. To corroborate the findings in cultured fibroblasts we assayed 3-OH-PC dehydrogenase activity in normal and HP2 leukocytes. In extracts of leukocytes from normals, the 3-OH-PC dehydrogenase was 12.6±1.1 nmol/h per mg (mean±1 SEM) (Fig. 3). In agreement with the results in fibroblasts, the HP2 leukocytes had no detectable 3-OH-PC dehydrogenase activity. Leukocytes from obligate HP2 heterozygotes had 3-OH-PC dehydrogenase levels intermediate between those of control and affected individuals. The activity in cells from four obligate heterozygotes was 4.0±0.7 nmol/h per mg,

TABLE I
Absence of 3-OH-PC Dehydrogenase Activity in HP2 Fibroblasts

Fibroblasts	3-OH-PC dehydrogenase activity
	nmol/h/mg protein
Normals	
D550	16.0
D551	21.6
D.V.	8.0
B.R.	12.7
K.M.	11.6
	14.0±2.3
HP2	
E.D.	Undetectable
K.K.	Undetectable

The activity in cultured fibroblasts was determined as described in Methods. The values in normal fibroblasts are the average of duplicate determinations on at least two separate cell harvests. HP2 fibroblasts were assayed on at least three separate cell harvests.

which is different from normal activity at the 99% confidence level.

Finally, we compared the entire spectrum of leukocyte PC dehydrogenase and 3-OH-PC dehydrogenase activities in patients ($n = 2$), parents ($n = 4$), grandparents ($n = 2$), siblings ($n = 6$), and normals ($n = 11$). Linear regression of 3-OH-PC dehydrogenase activity

TABLE II
Evidence Against Inhibitors of 3-OH-PC Dehydrogenase in HP2 Fibroblast Extracts

Fibroblast extract	3-OH-PC dehydrogenase activity
	nmol/h/mg protein
Dialysis	
Normal (D550)	15.8
HP2 (K.K.)	Undetectable
Mixing	
Normal (D550)	14.5±4.3
HP2 (K.K.)	Undetectable
Normal + HP2	11.2±2.1

Dialysis: Fibroblast extracts were dialyzed for 90 min at 4°C against 100 vol of buffer with two buffer changes. In parallel preparations that monitored the disappearance of labeled proline, we found that 85% of the proline was removed by the dialysis procedure. Mixing: The extract from normal fibroblasts was assayed in the presence and absence of HP2 (K.K.) extract. Amounts of normal and HP2 extract that contained equivalent protein (50 μg) were assayed. Data are expressed on the basis of protein in the normal extract. The values shown are the mean±SEM of three experiments.

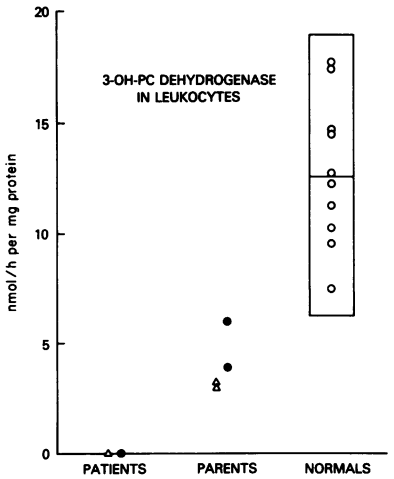

FIGURE 3 3-OH-PC dehydrogenase activity in leukocytes. The levels of enzyme activity in two patients (E.D. △; G.F. ●) and their respective parents are compared with the levels in 10 normals (○). The box represents the mean±2 SD.

on PC dehydrogenase activity in the same leukocyte extracts from each individual showed highly significant correlation ($r = 0.89$), further supporting the hypothesis that a single enzyme functions to catalyze the dehydrogenation of both substrates (Fig. 4).

DISCUSSION

A common Δ^1-pyrroline carboxylate dehydrogenase which can catalyze the conversion of either PC to glutamate or 3-OH-PC to γ-hydroxyglutamate has been suggested by several investigators. Adams and Goldstone (7) showed that PC and 3-OH-PC dehydrogenase activities share many physical, chemical, and catalytic properties. Goodman et al. (10) and Applegarth et al. (11) showed that abnormal amounts of both PC and 3-OH-PC are found in the urine of patients with HP2.

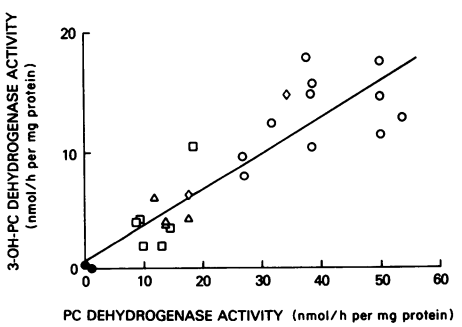

FIGURE 4 Correlation of PC dehydrogenase and 3-OH-PC dehydrogenase activities. The activities for both PC and 3-OH-PC dehydrogenase were concomitantly measured in leukocyte extracts prepared from normal controls (○), HP2 patients (●), parents (△), grandparents (◇), and siblings (□) of HP2 patients.

This result indicates that derangements in the metabolism of both PC and 3-OH-PC are present in patients with deficient PC dehydrogenase. To more directly test the common enzyme hypothesis, we devised specific radioisotopic assays for both PC and 3-OH-PC dehydrogenase activities. By applying these assays in biochemical and genetic studies, we conclude that PC and 3-OH-PC are indeed dehydrogenated by a common enzyme or at least by enzymes which share common subunits.

Our conclusion is based on the finding that cells from individuals known to have mutant genes resulting in a deficiency of PC hydrogenase activity also were deficient in 3-OH-PC dehydrogenase activity. That the deficiency of both dehydrogenase activities occurred as a common primary defect is indicated by the significant reduction in 3-OH-PC dehydrogenase activities in cells from obligate heterozygotes. Indeed, the activities of 3-OH-PC dehydrogenase and PC dehydrogenase are highly correlated over their entire range as would be predicted by the common enzyme hypothesis. Thus, the genetic evidence strongly suggests a common dehydrogenase for both Δ^1-pyrroline compounds.

In light of these findings, it may seem paradoxical that HP2 patients, who have 10-fold elevations in plasma proline, have only a twofold elevation in plasma hydroxyproline (10, 11). This difference in precursor accumulation may be explained by several factors. First, the amounts of proline and hydroxyproline which are degraded are different. Significant amounts of hydroxyproline are found only in collagenous protein (1), whereas proline is present in nearly all proteins. Even in collagen there are greater amounts of proline than hydroxyproline (18). Thus, significantly more proline than hydroxyproline may require degradation.

Secondly, both Goodman et al. (10) and Applegarth et al. (11) have shown that quantitatively the most significant Δ^1-pyrroline compound in the urine of HP2 patients is 3-OH-PC and its derivatives. Therefore, significant amounts of 3-OH-PC are lost in the urine, whereas most of the PC is apparently converted back to proline. Contributing to this preferential loss of 3-OH-PC are the different affinities of the reductase(s), which convert PC and 3-OH-PC back to proline and hydroxyproline, respectively. The K_m for PC reductase is 0.2 mM (19), whereas the K_m for 3-OH-PC reductase is 4.4 mM (20).

We cannot at present assign a physiologic significance to a common dehydrogenase enzyme catalyzing the second step of the proline-hydroxyproline degradation pathway. An analogous situation exists for the degradation of the branch-chain amino acids, leucine, isoleucine, and valine. These three amino acids are initially transaminated to their keto-analogues by two or three distinct transaminases (21). The keto-analogues are then decarboxylated by an enzyme(s), which, if not identical, at least shares common subunits. In maple syrup urine disease, the branch-chain keto-acid decarboxylase activity is deficient for the keto-analogues of all three amino acids.

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