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Proline Biosynthesis and Degradation in Mammalian Cells and Tissue

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ABSTRACT

Radioisotopic assays have been developed for the enzymes of proline metabolism. These assays are specific and sensitive enough to measure enzyme activities in cultured cells and biopsy specimens. Measurements of these enzymes in tissues and cultured cells suggest that endogenous biosynthesis of proline may be an important source of this amino acid.

Proline and its hydroxylated derivative, hydroxyproline, are important constituents of collagen. Together, these two amino acids make up 22 percent of this structural protein.[3] Although previous workers have shown that glutamate and ornithine can be converted to proline, little is known of the proline biosynthetic enzymes in bone or cartilage cells.

The biochemical pathways and relevant enzymes for proline metabolism are shown in figure 1. The immediate precursor of proline, Δ^1-pyrroline-5-carboxylate (PC) is converted to proline by PC reductase.[7,13] PC, the central intermediate of the pathway is derived from either ornithine by ketoacid transamination[8,14] or from glutamate.[1] Ornithine-δ-transaminase catalyzes the conversion of ornithine to PC, but the enzyme(s) catalyzing the conversion of glutamate to PC are undefined in higher organisms. The degradation of proline is catalyzed by proline oxidase converting proline to PC[4] and PC can be recycled back to glutamate by PC dehydrogenase.[15]

In order to study these enzymes in bone, it was necessary to develop specific assays sensitive enough to measure these enzymes in cultured cells and in hypocellular tissues, e.g., bone.

Materials

Materials include ornithine-U-^{14}C (260 mC per mMole),* proline-U-^{14}C (240 mC per mMole),* *o*-aminobenzaldehyde,† nicotinamide adenine dinucleotide (reduced form),† cytochrome C (equine heart)† and Dowex-50W (H^+).† Established cell lines were from the American Type Culture Collection.

Methods

ENZYME PREPARATION

Cells were grown in Minimum Essential Eagle's (MEM) supplemented with 10 percent fetal calf serum, glutamine (2mM) and nonessential amino acids. All cells were harvested in late log phase, washed three

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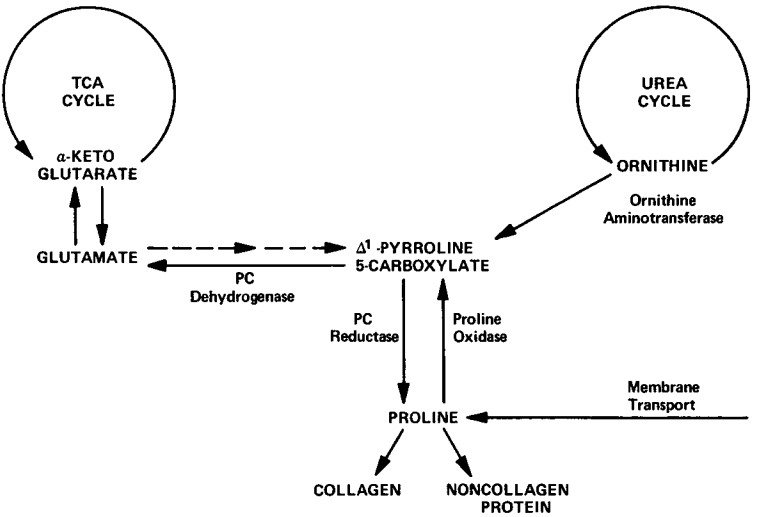

Figure 1. Schematic diagram of the pathways for proline synthesis and degradation. The dotted arrows indicate the enzymatic conversion of glutamate to Δ^1-pyrroline-5-carboxylate. These enzymes have been demonstrated in microorganisms but not in animal cells.

times in phosphate-buffered saline, resuspended in the appropriate buffer and sonicated for 40 secs. Tissues were minced and homogenized in the appropriate buffer with a Teflon pestle in a Potter-Elvehjem homogenizer and centrifuged at 25000 × *g* for 30 minutes. Calvaria were isolated from term rat fetuses, frozen with liquid nitrogen and pulverized with a mortar and pestle. The powder was taken up in the appropriate buffer, sonicated for 40 sec, centrifuged at 25,000 × *g* for 30 minutes and the supernatant dialyzed for 18 hours.

ENZYME ASSAYS

These assays have been described in detail.[9,10,11] A brief summary of the methods follows.

Ornithine-δ-transaminase. Uniformly labeled ornithine-^{14} C (0.7 mM) is incubated with α-ketoglutarate (0.7 mM) and enzyme in 0.1 M PO_4 pH 8.0 buffer. The reaction mixture is acidified with HCl to a final concentration of 1 N and applied to a Dowex 50 column. Product Δ^1-pyrroline-5-carboxylate is eluted with 1 N HCl.

PC reductase. PC-^{14}C is enzymatically prepared from precursor ornithine-U-^{14}C and then isolated by Dowex 50 column chromatography. To assay conversion of PC to proline cell or tissue, extracts are incubated with PC-^{14}C, organically synthesized PC (0.045 mM) and NADH (0.68 mM) in 0.1 M phosphate pH 6.8 buffer. Following incubation, the reaction mixture is acidified to 1 N with HCl and a portion is reacted with an equal volume of *o*-aminobenzaldehyde (10 mg per ml in 10 percent ethanol and 1 N HCl). PC combines with OAB, forming a dihydroquinazolinium compound which binds tightly to Dowex 50. Product proline-^{14}C is then eluted with 1 N HCl.

Proline oxidase. Proline-^{14}C (0.1 mM) is incubated with cell or tissue extracts and cytochrome C (0.1 μM) in 0.1 M Tris pH 7.2. Product PC is reacted with OAB and the mixture applied to a 1 ml bed volume Dowex 50 column. The column is washed with 16 ml of 2 N HCl to elute unreacted proline. PC-^{14}C (as the dihydroquinazolinium complex) is then eluted with 2 N NaOH.

Proteins in the extracts were determined by the method of Lowry.[6]

Results

ENZYME ACTIVITIES IN CULTURED CELLS

Using the assays described, the authors were able to quantitate the activities of the

TABLE I

Enzymes of Proline Synthesis and Degradation: Activities in Cultured Mammalian Cells

Cell Line	Enzyme Activity (nMoles per hr - mg protein) Ornithine-δ-transaminase	PC Reductase	Proline Oxidase
Normal human fibroblast (D550)	156 ± 19	746 ± 75	Undetectable
Chinese hamster lung (CHL)	180 ± 41	461 ± 27	Undetectable
Rat liver (BRL)	234 ± 17	542 ± 24	Undetectable
Rabbit kidney (LLC)	3600 ± 200	310 ± 20	2200 ± 300

proline metabolic enzymes in 10^6 to 10^7 cultured cells. Cell lines studied include normal human fibroblasts (D550), rabbit kidney cells (LLC), Chinese hamster lung cells (CHL) and normal rat liver cells (BRL). The activities of ornithine-δ-transaminase, PC reductase and proline oxidase in these cells are shown in table I.

TABLE II

Pathways for Δ^1-pyrroline-5-carboxylate Formation: Comparison Between a Proline Auxotroph and Proline Prototroph

Cell Line	Characteristics	Relative OTA Activity	Relative Incorporation of Glutamate-^{14}C into Cell Protein: Recovered as Glutamate	Recovered as Proline
CHO-K1	Proline Auxotroph	8	92	0
CHL	Proline Prototroph	100	100	100

ENZYME DEFECT(S) IN A PROLINE AUXOTROPH

Previous workers have shown that Chinese hamster ovary cells (CHO-K1) require proline in the media for growth.[5] PC, substituted for proline in the media could also support growth. These findings suggest that the defect in these cells was in the biosynthesis of PC rather than in the conversion of PC to proline. Since PC can be derived from either ornithine or glutamate, the defect could be in either or both pathways for PC formation. Therefore, ornithine-δ-transaminase was measured by the radioisotopic method described. In addition, the conversion of glutamate to PC by *in vivo* incorporation studies was estimated using glutamate-^{14}C and quantitating proline-^{14}C in protein. As shown in table II, CHO-K1 cells were deficient in both biochemical pathways for synthesizing PC.[16]

PROLINE ENZYMES IN FETAL BONE

Since rapidly proliferating bone requires proline for collagen synthesis, it was of interest whether or not bone possessed the capacity for synthesizing this amino acid. The levels of ornithine-δ-transaminase and PC reductase found in fetal calvaria were much higher than activities found in either fetal liver or kidney. In fact, enzyme activities in calvaria were comparable to those found in cultured cells (table III). These findings indicate that developing bone has a high capacity for synthesizing its own proline for protein and collagen synthesis. In contrast,

TABLE III

Ornithine-δ-transaminase and PC Reductase Activities in Rat Tissue*

	Enzyme Activity (nMoles per hr - mg protein) Ornithine-δ-transaminase	PC Reductase
Adult rat liver	176 ± 70	26 ± 3
Fetal rat liver	26 ± 1	71 ± 6
Fetal rat kidney	48 ± 7	263 ± 34
Fetal rat bone (calvaria)	132 ± 29	713 ± 71

*Values are mean ± SEM

the activities of the degradative enzyme, proline oxidase, were relatively low.

Discussion

The capability of mammalian cells to synthesize proline from a number of precursors is well known. Little attention, however, has been given to the tissue specificity or the regulation of these biosynthetic processes. Although investigators have speculated that derangements of proline metabolism may be involved in the pathogenesis of human disease,[12] little direct experimental evidence is available to support these hypotheses. The lack of sensitive enzyme assays has been a major obstacle in pursuing these studies.

Specific sensitive radioisotopic assays have been developed for measuring the enzymes of proline synthesis and degradation. Methodologic innovations include (1) the production and isolation of high specific activity Δ^1-pyrroline-5-carboxylate-U-^{14}C and (2) the separation of the reaction products on cation exchange chromatography. Treatment of the product with *o*-aminobenzaldehyde, which combines with PC, was especially helpful in a number of the assays. With these methods, a number of questions relevant to the regulation of proline metabolism can be considered.

Since most cultured cells are prototrophic for proline, it is expected that the proline biosynthetic enzymes would be present in these cells. Indeed, all prototrophic cell lines had ample activities of these enzymes. It is interesting that a proline-requiring cell line (CHO-Kl) was deficient in the enzymes of both pathways for PC formation. Regulating interactions between these two pathways is an intriguing possibility.

Since fetal bone and cartilage cells actively synthesize collagen, it was thought that these cells would have an abundance of proline biosynthetic enzymes. This, indeed, was the case. The activity of PC reductase in fetal calvaria was three times that in fetal kidney and 10 times that found in fetal liver. The pattern of enzyme activities suggests that bone cells can readily produce their own proline for protein and collagen synthesis.

It is hoped that these initial studies will stimulate investigation of proline metabolism and collagen synthesis in osseous tissue. Since collagen synthesis is routinely measured using exogenous labeled proline, the interaction between proline from endogenous synthesis and proline accumulated by membrane transport will be of special interest.

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