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The proline biosynthesis in living organisms

Review Article

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Summary. In this article we review recent work on the physiology of proline and Δ^1-pyrroline-5-carboxylate (P5C) in living organisms and consider recent progress in our understanding of the role of P5C synthetase in collagen metabolism and the regulation of urea cycle in vertebrates. Much of this recent progress has been made possible by advances in our knowledge of the enzymes and genes involved in proline biosynthesis in man. The availability of well characterized P5C synthetase deficiency in man has been an impetus for the cloning of the cDNA encoding for this enzyme from man and facilitated the establishment of the phenotype-genotype relationships in P5C synthetase deficiency in higher vertebrates.

Keywords: Proline – Δ^1-pyrroline-5-carboxylate synthetase – cDNA – Collagen – Urea cycle

Abbreviations: GK: γ-glutamyl kinase; GPR: γ-glutamyl phosphate reductase; P5CR: Δ^1-pyrroline-5-carboxylate reductase; GSA: glutamic-γ-semialdehyde; P5C: Δ^1-pyrroline-5-carboxylate; P_i: Inorganic phosphate; AMP, ADP, ATP: Adenosine 5′-mono-, di-, triphosphate; NAD^+, NADH: nicotinamide adenine dinucleotide, and its reduced form; $NADP^+$, NADPH: nicotinamide adenine dinucleotide phosphate, and its reduced form; DEAE: diethylaminoethyle; OAT: ornithine amino transferase; CHO: Chinese hamster ovary; IGF-1: insulin-like growth factor-1; P5CDH: pyrroline 5-carboxylate dehydrogenase; IMP: inosine 5′-monophosphate.

Introduction

Proline represents a unique class of molecules among amino acids; besides being classified as a non-essential amino acid it belongs to the class of "glutamate family" of amino acids whose biosynthesis depends on the ability of the carbon skeleton of glutamic acid to give rise to glutamine, proline,

arginine, ornithine (Conn et al., 1987). In fact, a specific system of enzymes with special properties has evolved to mediate the metabolism of proline. These metabolic interconversions among proline, ornithine, and glutamate have pyrroline-5-carboxylic acid (P5C) as the sole common intermediate. As pointed out by Adams, the inclusion of the α-amino group within a ring structure bestows a number of special properties (Adams, 1970). With its peptide bond within the pyrrolidone ring, proline confers rigidity and three-dimensional stability to proteins. The high content of proline and hydroxyproline in collagen contributes to the unique physical properties of this structural protein. Moreover, in several unicellular species, the intracellular concentration of proline serves as a defense against osmotic challenge (Csonka, 1989; Csonka and Hanson, 1991; Strom et al., 1983; Yancey, 1994). Also as it has been reviewed extensively elsewhere, the accumulation of proline is a common metabolic response of higher plants to water deficits, salinity stress and cold stress (Csonka and Baich, 1983; Delauney and Verma, 1993; Dorffling et al., 1993; Hanson and Hitz, 1982; Stewart, 1981; Stewart and Larher, 1980; Thomson, 1980). In this article we review recent work on the metabolic functions of proline and P5C unravaled by the results of molecular studies from their deficiencies in humans.

I. Pathways of synthesis of P5C and proline

Nutritional and other studies with isotopic carbon have provided strong evidence for the interconversion in vivo of glutamic acid and proline in both animal tissues and microorganisms (Stetten, 1951). The metabolic pathways with P5C as the central intermediate (Fig. 1) were initially defined as a system for proline biosynthesis (Adams, 1970; Adams and Frank, 1980; Baich, 1969) and this physiologic endpoint has been emphasized by a number of investigators (Mezl and Knox, 1977; Smith and Phang, 1978; Yip and Knox, 1972). In both prokaryotes (Baich, 1969) and eukaryotes (Baich, 1977; Smith and Phang, 1979; Valle et al., 1973) the inability to synthesize P5C results in proline auxotrophy. On the other hand if one of the pathways for P5C synthesis is present, i.e. from either ornithine or glutamate, normal growth occurs in proline-free medium (Smith and Phang, 1979).

a. In bacteria

Enzyme systems catalyzing the reduction of this intermediate to proline have been described in microorganisms (Meister et al., 1957). In bacteria such as E. coli, Pseudomanas aeruginosa and Salmonella typhimurium, proline synthesis from glutamate is catalyzed by three enzymes: γ-glutamyl kinase (GK), γ-glutamyl phosphate reductase (GPR) (also called glutamic semialdehyde dehydrogenase) and Δ^1-pyrroline-5-carboxylate reductase (P5CR) (Fig. 2), encoded by genes proB, proA and proC, respectively (Adams and Frank, 1980; Baich, 1969, 1971; Csonka and Baich, 1983; Deutch et al., 1982, 1984; Gamper and Moses, 1974; Hayzer and Leisinger, 1980, 1981, 1982; Hayzer and

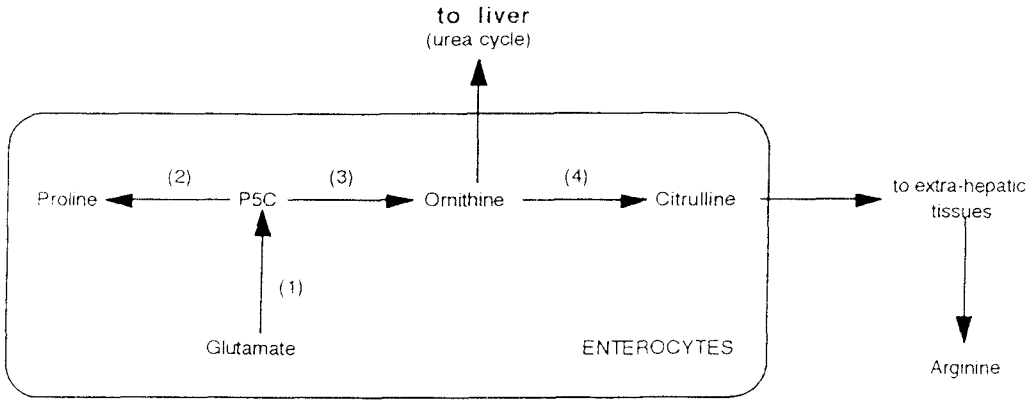

Fig. 1. Metabolic pathways illustrating the relantionships between proline, ornithine and citrulline biosynthesis and the urea cycle substrates. *(1)* Δ^1-pyrroline-5-carboxylate synthetase (P5CS); *(2)* Δ^1-pyrroline-5-carboxylate reductase; EC 1.5.1.2; *(3)* Δ ornithine amino-transferase (OAT); EC 2.6.1.13; *(4)* ornithine trans-carbamoylase (OTC); EC 2.1.3.3; *P5C* Δ^1-pyrroline-5-carboxylate

Moses, 1978a,b; Krishna and Leisinger, 1979; Krishna et al., 1979; Smith et al., 1984). The step from glutamic-γ-semialdehyde (GSA) to Δ^1-pyrroline-5-carboxylate (P5C) is reversible and spontaneous. The reaction catalysed by GK is irreversible. The product of the reaction, γ-glutamyl phosphate, is extremely labile. It can be chemically trapped by reaction with hydroxylamine to yield the more stable γ-glutamyl hydroxamate which can be quantified with a ferric chloride reagent (Baich, 1969; Hayzer and Leisinger, 1980). In the absence of hydroxylamine, the γ-glutamyl phosphate product spontaneously degrades to pyroglutamate (5-oxoproline) and inorganic phosphate (P_i) (Seddon et al., 1989). By using two proline-requiring mutants of E. coli, Strecker has shown that the addition of pyruvate, lactate, glucose, or formate increased the formation of P5C in washed cells in the presence of either L-glutamate or L-glutamine; further stimulation was obtained on the addition of either AMP or ADP but not ATP. The production of P5C was inhibited by the endogenous proline (Strecker, 1960b) Δ1-pyrroline-5-carboxylic acid hydrochloride has been synthesized and isolated and assayed for its conversion to proline in 80% yield by chemical reduction. Solutions of the compound are unstable both at room temperature and in the frozen state but are relatively stable at 3°C; depending on the pH of the reaction, reaction with ninhydrin results in the formation of two products; the 2,4-dinitrophenylhydrazone derivative and the addition product with o-aminobenzaldehyde have been prepared (Strecker, 1960a). The GK of E. coli is inhibited by proline (Baich, 1969; Rushlow et al., 1984; Smith, 1985) and has a relatively low affinity for glutamate (K_m = 7–10mM) (Baich, 1969). Marked substrate activation of the enzyme is observed when the concentration of glutamate exceeds 50mM (Baich, 1969). Proline increases the apparent K_m for glutamate (Baich, 1969). Proline overproducing mutants have a GK with markedly altered feedback inhibition (Csonka, 1981; Rushlow et al., 1984; Smith, 1985). The reaction

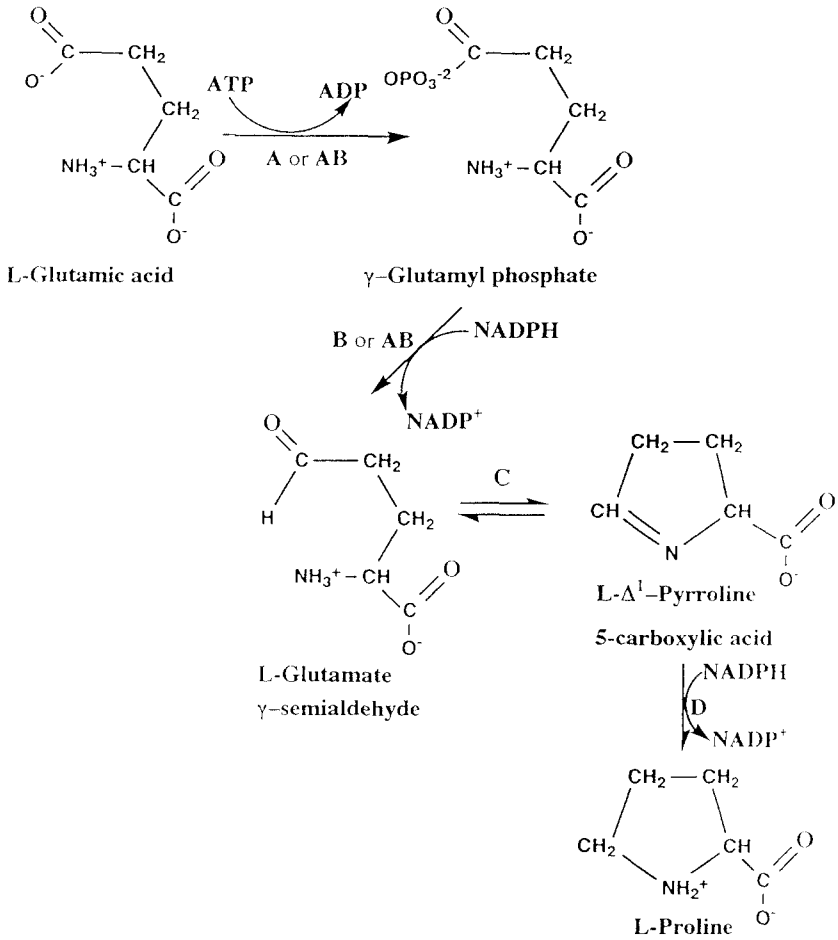

Fig. 2. Pathways of proline biosynthesis in different species. The enzymes are: *A* γ-glutamyl kinase (γ-GK) (EC 2.7.2.11); *B* γ-glutamyl phosphate reductase (γ-GPR) (EC 1.2.1.41); *AB* PC5 synthetase (P5CS); *C* non-enzymatic step; *D* P5C reductase (EC 1.5.1.2)

catalysed by the second enzyme is reversible and this reversibility forms the basis of a selective assay for GPR in which P5C-GSA-dependent, and P_i-dependent reduction of $NADP^+$ is measured. The γ-glutamyl phosphate generated in this reverse reaction spontaneously degrades to pyroglutamate and P_i (Hayzer and Leisinger, 1981). Pyroglutamate has the following distinctive attributes: (i) unlike other amino acids it does not bind to Dowex-50-H^+ (Hayzer and Leisinger, 1981); (ii) it is ninhydrin negative (Hazyer and Leisinger, 1981); and (iii) it can be converted to glutamate by boiling in aqueous acid (Hayzer and Leisinger, 1981; Mazelis, 1979). The assay for GPR (P5C- ⇔ GSA- and P_i-dependent reduction of $NADP^+$) is subject to interference by an active P5CR in crude extracts (Hayzer and Leisinger, 1980). Thus, as NADPH is formed, this can be oxidized back to $NADP^+$ by the catalytic activity of P5CR. This interference by P5CR can lead to markedly non-linear $NADP^+$ reduction kinetics in bacterial crude extracts. However, GPR and

P5CR can be separated easily on a diethylaminoethyl (DEAE)-cellulose column (Hayzer and Leisinger, 1980).

b. In yeast

The yeast Saccharomyces cerevisiae synthesizes proline from glutamate via the intermediates γ-glutamyl phosphate, γ-glutamyl semialdehyde, and P5C (Brandriss, 1979; Brandriss and Falvey, 1992; Orser et al., 1988; Tomenchok and Brandriss, 1987). The isolation and preliminary characterization of mutations leading to proline auxotrophy in the yeast S. cerevisae have been reported (Brandriss, 1979). Three complementation groups (pro1, pro2, and pro3) were identified. The requirement for proline in the pro1 and pro2 strains could be satisfied by arginine or ornithine, since these compounds can be converted to glutamate semialdehyde by enzymes in the arginine degradation pathway, bypassing the early biosynthetic steps. pro3 mutants can grow only when proline is added to the medium and are defective in P5C reductase enzyme activity. The three enzymes involved in this pathway are γ-glutamyl kinase (the product of the PRO1 gene), γ-glutamyl phosphate reductase (the product of the PRO2 gene), and P5C reductase (the product of the PRO3 gene). The gene-enzyme relationships were demonstrated by interspecies complementation of proB, proA, and proC mutants of E. coli and Salmonella typhimurium by the yeast PRO1, PRO2, and PRO3 genes, respectively (Tomenchok and Brandriss, 1987), and of a yeast pro1 mutant by the E. coli proB gene (Orser et al., 1988). The first two enzyme activities were never measured in the studies of S. cerevisae. In S. cerevisae, the enzyme P5C reductase also functions in the pathway for arginine degradation (Brandriss and Magasanik, 1980). The ornithine that is formed from arginine is converted to glutamate semialdehyde and P5C in the cytoplasm. P5C is then converted to proline by P5C reductase, and the proline is transported into the mitochondria, where it is converted to glutamate (Brandriss and Magasanik, 1981). Therefore, P5C reductase functions in both the proline biosynthetic and the arginine degradative pathways.

c. In higher plants

Enzyme systems catalyzing the reduction of the glutamate to proline have been described in plants. (Bogges et al., 1976; Morris et al., 1969; Heyser et al., 1989a; Heyser et al., 1989b; Rhodes and Handa, 1989; Rhodes et al., 1986). A number of in vivo labelling studies with ^{14}C-labelled precursors (Boggess et al., 1976; Morris et al., 1969) or [^{13}C]glutamate (Heyser et al., 1989a,b) or $^{15}NH4^{+}/NO_3^{-}$ (Rhodes and Handa, 1989; Rhodes et al., 1986) strongly suggest that glutamate is a major precursor of osmotic stress-induced proline accumulation in plants and that osmotic stress results in an increase of proline biosynthesis rate (Boggess et al., 1976; Rhodes and Handa, 1989; Rhodes et al., 1986; Stewart, 1981). The prevailing evidence [reviewed extensively by others (Delauney and Verma, 1993; Hanson and Hitz, 1982; Stewart, 1981;

Thompson, 1980)] is that the increase in proline synthesis rate may, in part, involve induction or activation of enzymes of proline biosynthesis, possibly coupled with a relaxation of proline feedback inhibition control of the pathway (Boggess et al., 1976; Delauney and Verma, 1993; Stewart, 1981). Interestingly, this feedback control of the proline pathway may also be modulated during development (Oaks, 1992; Oaks et al., 1970). Decreased proline oxidation to glutamate (Elthon and Stewart, 1982; Huang and Cavalieri, 1979; Stewart et al., 1977), decreased utilization of proline in protein synthesis (Boggess and Stewart, 1980; Stewart, 1981) and enhanced protein turnover (Fukutoku and Yamada, 1984) may all contribute to net proline accumulation. There are indications that de novo transcription and translation are required for stress-induced proline biosynthesis in higher plants. For example, stress-induced proline accumulation is inhibited by both cycloheximide and cordycepin in Arabidopsis (Verbruggen et al., 1993). In contrast, because cycloheximide is inhibitory to 200 mM NaCl-induced proline accumulation in D. spicata cells, whereas actinomycin D is not inhibitory, Ketchum et al. (1991) suggest that translation, but not transcription, is necessary for production of proline in stressed cells.

In addition to the pathway of synthesis of proline from glutamate, there is also the possibility of synthesis of proline from ornithine (derived from the N-acetyl-glutamate pathway) in higher plants. This can occur by two routes, depending upon whether the α- or δ-NH_2 moiety of ornithine is transaminated (Adams and Frank, 1980; Csonka and Baich, 1983; Delauney and Verma, 1993; Mestichelli et al., 1979; Thompson, 1980). In the case of δ-NH_2 transamination, the product is GSA which, in spontaneous equilibrium with P5C, can be converted readily to proline by P5CR. Ornithine δ-transaminase has been partially purified from several plant sources, including pumpkin cotyledons (Splittstoesser and Fowden, 1973). The alternative pathway involves α-NH_2 transamination of ornithine to α-keto-δ-aminovalerate $\Leftrightarrow$ Δ^1-pyrroline-2-carboxylate (P2C). This product can be reduced to proline by a P2C reductase. In vivo tracer studies with labelled ornithine strongly suggest that the P2C pathway of ornithine metabolism is the main route of conversion of ornithine to proline in several plant species (Mestichelli et al., 1979). There is a paucity of in vitro enzymological data to support the occurrence of a glutamate pathway of proline synthesis in higher plants similar to that establislied for bacteria. Although P5CR has been identified and characterized in several plant species (e.g. Krueger et al., 1986; LaRosa et al., 1991; Treichel, 1986), there have been no reports of GK or GPR activities in higher plants since the first report of the occurrence of in vitro synthesis of ^{14}C GSA from ^{14}C glutamate, Mg^{2+}, ATP and NADPH in cell-free extracts of suger beet (Morris et al., 1969). The enzyme activity described by Morris et al. (1969) is similar to that reported for the P5C synthetase activity of mammalian cells (Lodato et al., 1981; Wakabayashi and Jones, 1983). Efforts devoted to identifying a GK activity in plant extracts by the γ-glutamyl hydroxamate assay procedure have been hindered by a large background of γ-glutamyl hydroxamate formation activity catalysed by glutamine synthetase in plant tissues (Miflin and Lea, 1977). There does not appear to have been a sys-

tematic search for GPR in higher plants. In addition to the problem of interference from P5CR in detecting GPR activity in crude extracts there is also an interference from an enzyme capable oxidizing P5C to glutamate; P5C:NAD(P)' oxidoreductase. This enzyme is the second enzyme of the two-step pathway of proline oxidation in higher plants (Elthon and Stewart, 1982; Huang and Cavalieri, 1979; Rayapati and Stewart, 1991; Stewart, 1981; Stewart and Boggess, 1978; Thompson, 1980). These problems associated with measurement of GPR are alleviated by monitoring P_i-dependent pyroglutamate production from P5C $\Leftrightarrow$ GSA and $NADP^+$ instead of monitoring $NADP^+$ reduction to NADPH.

d. In animals

Enzyme systems catalyzing the reduction of the P5C to proline have been described in rat and other animal tissues (Meister et al., 1957). Workers showed that an enzyme activity converting glutamate to P5C is present in homogenates of intestinal mucosa incubated with [U-^{14}C] glutamate (Ross et al., 1978). The conversion of glutamate to P5C is most likely catalyzed by two enzymes or a multifonctional enzyme complex. The γ carbon of glutamate is activated by ATP followed by the NADPH-dependent reduction of γ-glutamyl phosphate. As a trivial designation, "P5C synthetase" refers to the cell-free activity in which glutamate is converted to P5C in an ATP and NADPH-dependent reaction. The activity was measured in cultured cells and its presence or absence correlated with proline prototrophy or auxotrophy, respectively (Smith et al., 1980). Using solubilized mitochondrial preparation from rat intestinal mucosa, Wakabayashi et al. characterized this activity in greater detail (Wakabayashi et al., 1983). The activity is sensitive to inhibition by ornithine (Lodato et al., 1981). And unlike the bacterial enzyme, the conversion of glutamate to P5C in mammalian tissues is not inhibited by proline (Kramer et al., 1985). Interestingly, in rat tissues the enzyme has been detected only in intestine and thymus (Wakabayashi et al., 1983). However, the presence of the pathway in a variety of cultured cells is supported by metabolic studies (Lorans et al., 1978; Smith and Phang, 1979; Valle et al., 1973) as well as by direct enzyme assay in cell-free extracts (Smith et al., 1980). Derepression or activation of the enzyme accompanying mitogenesis is an intriguing possibility. Mutants of Chinese hamster fibroblasts which are proline hyperproducers and thereby resistant to the toxic effects of azetidine carboxylic acid have either an extremely high level of P5C synthetase or an enzyme which is insensitive to inhibition by ornithine (Smith et al., 1981; Lodato et al., 1981). The extent and the rate of inactivation depend upon the time and the temperature of prior incubation of the enzyme with ornithine in the absence of substrates, which is suggestive evidence for an allosteric regulation of the enzyme; the inactivation was prevented by 0.2 mM ATP or ADP (Wakabayashi et al., 1985).

Although demonstrable in vitro (McGivan et al., 1977), the physiological significance of the carbon transfers between the urea cycle and glutamatic acid-P5C-proline metabolism was in doubth. Widely distributed in mam-

malian tissues and localized to the mitochondrial matrix, ornithine aminotransferase catalyzes the ω-transamination of ornithine to α-ketoacid acceptors thereby forming P5C $\Leftrightarrow$ GSA. The K_{eq} of hepatic OAT greatly favors P5C formation. The properties of the purified enzyme have been recently reviewed (Valle and Simell, 1995). Animals fed a high protein diet have markedly elevated levels of OAT activity in liver (Volpe et al., 1969). Moreover, recent studies in patients with gyrate atrophy of the choroid and retina, an inherited disorder with deficiency of OAT (Valle et al., 1977; Valle and Simell, 1995) strongly supports this idea (Valle et al., 1980). The deficiency of OAT results in markedly elevated plasma ornithine, low ammonia, and low glutamate and glutamine, findings which are consistent with the interpretation that OAT controls the level of ornithine available to the urea cycle. In order to prove the importance of the ornithine pathway, Smith and Phang have selected mutant Chinese hamster ovary (CHO) cells with specific defects in the proline metabolic pathways and have found that ornithine can be converted to proline at a rate adequate to support maximal cell growth in the absence of any other sources of proline; although the importance and net direction of the OAT reaction in cultured CHO fibroblasts was in the direction ornithine $\Rightarrow$ P5C $\Rightarrow$ glutamate, the OAT-catalyzed reaction is reversible and net flow from glutamate or proline to ornithine may occur in other cell types (Smith and Phang, 1979). Glucagon which induces the hepatic enzyme may be responsible, in part, for the elevated levels of OAT in liver in response to high protein diet. Liver OAT would be inducible not by glucagon but estrogens (Lyons and Pitot, 1976). In fact, transcript quantification experiments showed a largely transcriptional-independent mechanism for the increase in enzyme synthesis with increased dietary protein (Mueckler et al., 1983). The combination of theses two activities may supply urea cycle intermediates for the liver. The back reaction by ornithine aminotransferase, i.e. the synthesis of ornithine from P5C, clearly can occur in intestine. Ross et al. obtained ornithine synthesis from glutamate in rat intestinal mucosa homogenates: in their studies, ^{14}C-labeled ornithine was separated from other radioactive chemicals by ion-exchange chromatography, and was specifically identified by its capacity to serve as a substrate for OTC. The synthesis of ornithine required ATP, $Mg^{2}+$ and NADPH in addition to glutamate (Ross et al., 1978). Windmuellar and Spaeth showed citrulline production from glutamine in isolated perfused rat intestine (Windmueller and Spaeth, 1975). The formation of [^{14}C]ornithine from [^{14}C]glutamate was found in a subcellular fraction enriched in mitochondria of rat small intestinal mucosa; synthesis of ornithine from glutamate was not observed with liver or kidney homogenates; pyridoxal 5′-phosphate stimulated both ornithine synthesis and endogenous OAT activity; partially purified rat liver OAT stimulated ornithine synthesis (Henslee and Jones, 1982). In order to establish the interorgan dependency of arginine synthesis, Wakabayashi et al. quantified P5C synthetase activity in many rat tissues and found that the activity was concentrated only in the upper small intestine. Minor activity was found in pancreas, thymus, lymph node, and some other tissues: this was confirmed by the dependency on the specific substrates, the loss of activity in the pres-

ence of an inhibitor, and identifying the reduced product as proline (Wakabayashi et al., 1991). Riby et al. established the developmental profile of the two intestinal mitochondrial enzymes, P5C synthetase and OAT, responsable for the conversion of glutamate to ornithine. Both enzymatic activities were found to be significantly elevated throughout the suckling period with a peak of activity during the second week of life; in contrast, glutamate dehydrogenase activity in the intestine did not appear to be developmentally regulated during the suckling and weaning periods and ornithine decarboxylase activity was undetectable in the intestine of the mouse during the suckling period and was detected briefly at weaning, indicating that ornithine synthesized in the intestinal mitochondria is not diverted actively into the polyamine pathway and is available for synthesis of arginine by the enzymes of the urea cycle (Riby et al., 1990). Wu et al. showed no evidence of P5C synthetase activity in chick entorocytes therefore resulting in the lack of synthesis of ornithine and citrulline from glutamine, in contrast to cells from post-weaning pigs (Wu et al., 1995ab; Wu, et al., 1994). In the lens, glutamate is necessary for the synthesis of products including glutamine, glutathione, and proline, as well as proteins such as the glutamate-rich crystallins. The transamination reactions in human lenses appeared to preferentially involve amino groups derived from glutamate; but the glutamate used for human lens proline biosynthesis is derived from glutamine and glutaminase, which converts glutamine to glutamate, is located in mitochondria in all tissues examined as well as in rat lens, suggesting that the glutamate at the site where proline synthesis begins (i.e. in mitochondria) may arise primarily from deamidation of glutamine by glutaminase (Jernigan, 1990).

L-Pyrroline-5-Carboxylic Acid (P5C) has been obtainable by chemical synthesis only as a mixture of the D- and L-stereoisomers. Smith et al. reported a method for the enzymatic synthesis of P5C on a preparative scale. The P5C is formed from L-ornithine by purified OAT and then isolated by Dowex-50W cation-exchange resin chromatography. With [^{14}C]ornithine as precursor, high specific activity uniformly labeled [^{14}C]P5C can be obtained (Smith et al., 1977). Using an assay based on a preparation of P5C reductase purified from genetically engineered E. coli, Fleming et al. measured P5C in normal human plasma, urine, and saliva. In addition, they have found pyrroline-5-carboxylate levels 10× to 20× normal in two patients with type II hyperprolinemia. P5C concentrations in normal human plasma were 0.38 ± 0.11 nmol/mL; those of saliva were approximately 50% of plasma levels. Urinary excretion of P5C was up to 1.2μmol/24h in normal subjects (Fleming et al., 1984).

Mixson et al. developed a very sensitive and specific new method of assay based on the interaction of P5C with cysteine. [^{35}S] Cysteine at a defined specific activity is reacted with P5C and the P5C-Cys adduct can be separated from the reactants by cation exchange column chromatography (Mixson, 1991).

P5C reductase, a cytosolic enzyme which catalyzes the conversion of P5C to proline with reduced pyridine nucleotide as cofactor, is ubiquitous in ani-

mal cells. The enzyme has been purified from E. coli (Deutch et al., 1982; Rossi et al., 1977) and yeast (Matsuzawa and Ishiguro, 1980) but not from mammalian cells. Nevertheless, characterization of the activity from animal tissues reveals a number of interesting features. Heretofore, investigators have considered that NADH is the preferred cofactor for P5C reductase. But studies in intact cells argue that it is NADPH which is oxidized by the enzyme in situ (Yeh and Phang, 1980; Yeh et al., 1981). The explanation may lie in the kinetic characteristics of the enzyme. Although V_{max} activities are higher with NADH, preference for NADPH may be due not only to a markedly higher affinity for NADPH but also a higher affinity for P5C with NADPH as cofactor (Yeh et al., 1981). Thus, at least in certain cells, NADPH is oxidized concomitantly with the conversion of P5C to proline. Also of interest P5C reductase from various tissue or cellular sources are differentially sensitive to inhibition by proline (Valle et al., 1973; Yeh et al., 1981), $NADP^+$ (Smith and Greenberg, 1957; Yeh and Phang, 1981), and adenine nucleotides (Smith and Greenberg, 1957). The enzyme from cultured fibroblasts is sensitive to inhibition by proline and its inhibition of the NADH-mediated reaction ($K_i = 2 \times 10^{-4}$M) is much greater than of the NADPH-mediated reaction ($K_i = 2 \times 10^{-3}$M). By contrast, the hepatic and erythrocyte enzymes are insensitive to proline but very sensitive to inhibition by $NADP^+$ (Yeh and Phang, 1981). Adenine nucleotides also inhibit the hepatic enzyme (Smith and Greenberg, 1957) but not the erythrocyte enzyme. These differential patterns of inhibition suggest that there are isozymes of P5C reductase found in various tissues, an interpretation further supported by the relative deficiency of the NADH-mediated activity in a leukemia cell line (Lorans and Phang, 1981). Whether these putative isozymes are products of different genes or are interconvertible forms is an intriguing question which awaits definitive studies on enzyme prepared from different sources. In addition to the kinetic characteristics and allosteric regulation, the amount of P5C reductase in cells appears to be regulated. In contrast to enzyme systems for amino acid synthesis in prokaryotes P5C reductase in cultured cells is not repressed by high concentrations of proline in the growth medium. However, marked increases in enzyme activity are observed in human peripheral lymphocytes activated with mitogenic lectins (Valle et al., 1975). In mammary gland tissues stimulated to undergo lactogenesis, coordinated increases in arginase, ornithine aminotralisferase, and P5C reductase are observed (Yip and Knox, 1972). An enzyme has been purified approximately 160-fold from a calf liver extract catalyzing the reduction of P5C to proline and gave preliminary kinetic data. Most of the P5C reductase activity were associated with the microsomal and supernatant fractions whereas both proline oxidase and P5C dehydrogenase activities are associated with the mitochondrial fraction (Johnson and Strecker, 1962). The reductive reaction cannot be reversed, lending support to the hypothesis that the reduction of glutamate to proline proceeds by a pathway which differs from that for the oxidation of proline to glutamate. Phang et al. developed a new radioisotopic assay in which they measured the radioactivity incorporated into the product, L-proline, from precursor

Δ^1-pyrroline-5-carboxylate [U-^{14}C] (Phang et al., 1973). A high P5C reductase activity was also shown in epidermal and neutral ectoderm such as cornea, lens, retina and retinal pigment epithelium (Matsuzawa et al., 1982b). Bovine retinal P5C reductase (L-proline: NAD(P)$^+$ 5-oxidoreductase, EC 1.5.1.2) was purified 180-fold from crude extract, using affinity chromatography with 2′,5′-ADP Sepharose 4B. The purified enzyme utilized either NADH or NADPH as the coenzyme. The enzyme activity was inhibited with ATP, and activated with K^+ and phosphate ions, suggesting that it may be regulated by the energy level and the redox state in the retina. The distribution of the enzyme activity in bovine cornea and lens tissues have been provided (Shiono et al., 1986; Matsuzawa, 1982b).

In brain for example, the correlation between OAT and P5C reductase was significant ($\gamma = 0.75$). This is because the brain uptake index of proline is as low as those of other putative neurotransmitters (glutamate, aspartate and glycine), the existence of an endogenous proline synthetic pathway favouring the ornithine $\Rightarrow$ P5C $\Rightarrow$ proline pathway in the CNS is suggested (Matsuzawa and Obara, 1987). Yeh et al. (1984) reported a P5C reductase activity in human erythrocytes. Morover, P5C reductase activity was the only proline metabolic enzyme activity present in these cells; The kinetic properties and regulation of the erythrocyte enzyme were distinctly different from those for human fibroblast P5C reductase (First, the enzyme has a preferential affinity for NADPH; second, it lacked of sensitivity to feedback inhibition by proline and instead this enzyme is sensitive to feedback inhibition by product $NADP^+$), suggesting that the function of the enzyme in human erythrocytes may be to generate oxidizing potential in the form of $NADP^+$ (Yeh and Phang, 1981). Generally, P5C reductase capacity correlates with that of OAT in the same tissue; this indicates that there is a widespread potential among tissues for proline synthesis from ornithine. In contrast, the significant correlations of the activities of the proline oxidase, P5C dehydrogenase with one another indicate a real but less widespread potential for proline oxidation in rat tissues. In fact, a flow to form ornithine would appear to be distinctly atypical, because proline oxidase activities are limited and are not significantly associated with OAT activities in the same tissues, and because there is no detectable conversion of P5C into glutamate by the reversal of the P5C dehydrogenase reaction in the same tissues (Herzfeld et al., 1977).

Proline oxidase catalyzes the conversion of proline to P5C and transfers electrons into the mitochondrial electron transport with an intervening flavoprotein (Meyer, 1977). Thus, the pair of electrons donated by proline can effect the phosphorylation of two ADP molecules. The enzyme is tighly bound to mitochondrial inner membranes and is limited in its tissue location to liver, kidney, brain and heart (Herzfeld et al., 1977). Treatment of mitochondria with Triton X-100 yields a solubilized preparation but enzyme activity then requires the presence of an electron acceptor such as iodonitrotetrazolium (Kowaloff et al., 1977). The enzyme can be induced by glucocorticoids both in animals (Kowaloff et al., 1976) and in cultured cells

(Kowaloff et al., 1978). The activity is sensitive to inhibition by lactate (Kowaloff et al., 1977): the K_m for proline increases as a function of increasing lactate concentration.

P5C dehydrogenase catalyzes the conversion of P5C to glutamic acid (Adams and Goldstone, 1960). The enzyme is ubiquitous but shows marked differences in specific activity among tissues. Although the activity of P5C dehydrogenase is generally high in tissues with proline oxidase, e.g., liver and kidney, suggesting that for these tissues proline serves as a source of glutamate, the activities of these two enzymes are not always parallel. For example, rat brain has P5C dehydrogenase/proline oxidase ratios which are one-tenth of those for liver or kidney (Yeh and Phang, unpublished observations). Considered to be localized primarily in mitochondria, P5C dehydrogenase is also found in the cytosol. Differential utilization of NAD^+ versus $NADP^+$ may help distinguish enzymes from the two subcellular sources. P5C dehydrogenase isolated from both yeast and animal tissues appears to be sensitive to inhibition by amino acids. In yeast cells the sensitivity of the enzyme to specific amino acids in vitro correlates with the sensitivity to growth inhibition of a mutant strain auxotrophic for glutamate (Lundgren and Ogur, 1973). Similarly, the enzyme isolated from kidney is sensitive to inhibition by a variety of amino acids. At saturating concentrations, these amino acids inhibit in an additive fashion suggesting distinct regulatory sites for each amino acid (Lundgren and Ogur, 1972). Small et al. reported an 800-fold purification P5C dehydrogenase from rat liver mitochondria to yield an essentially homogenous protein with a M_r = 59,000. The substrate P5C and NAD^+ have apparent dissociation constant of 0.16 and 1.0 mM, respectively. Studies established that the reverse reaction, the conversion of glutamate and NADH to P5C and NAD^+, is not catalyzed by this enzyme. Survey of various rat tissues for P5C dehydrogenase activity used [^{3}H]P5C as substrate, revealed higher activities in liver, kidney and heart, intestinal mucosa, retina and xiphoid cartilage had lowest activities (Small and Jones, 1990). The deficiency of P5C dehydrogenase has been identified as the basis for the inborn error of metabolism, Type II hyperprolinemia (Valle et al., 1976). In these patients, plasma proline concentrations are elevated markedly and P5C is excreted in the urine. Due to the insensitivity of previously available assays for P5C, this molecule was not measurable in the plasma of these patients. But recent studies using a sensitive enzyme-coupled radioisotopic assay has quantitated P5C levels in the plasma of patients with hyperprolinemia II as well as in normal subjects. Plasma P5C in this disease is 15 to 20 times elevated over that of normals (Fleming et al., 1984). An assay which measures radiolabeled glutamate production by P5C dehydrogenase in the presence of NAD^+ from radiolabelled P5C has been developed. Separation of substrate from product is achieved by column chromatography using Dowex-50 cation-exchange resin. The product isolated by this procedure was identified as glutamate (Small and Jones, 1987). P5C dehydrogenase has been purified to homogeneity from the human liver by the use of ion exchange chromatography on CM-Sephadex and affinity chromatography on Blue Sepharose CL-6B and 5′-AMP Sepharose 4B. The reaction catalyzed appears to be irreversible.

Although NADP can be used, NAD is the preferred coenzyme. The enzyme also exhibits an unusual property of being subject to substrate inhibition by NAD (Forte-McRobbie and Pietruszko, 1986).

II. Regulation of proline biosynthesis in development, ageing and in other physiological conditions

The physiologic relevance of P5C as an extracellular effector molecule besides being an intracellular intermediate in amino acid metabolism, was supported by the identification of P5C in normal human plasma (Fleming et al., 1989; Fleming et al., 1984). Strikingly, plasma P5C levels showed marked diurnal fluctuations, which correlated with feeding and which were markedly attenuated by fasting. Although the rise in plasma P5C seemed to be nutrionally dependent, it did not relate simply to ingestion of specific precursor amino acids, e.g., large oral bolus of proline did not increase plasma P5C. Presumably, the release of P5C into plasma is dependent on a number of metabolic and perhaps hormonal factors. Semon et al. reported that cultured human fibroblasts not only release P5C into the medium but also respond with increased release when stimulated by either insulin or insulin-like growth factor 1 (IGF-1) (Semon and Phang, 1991).

Isotopic evidence for the differential regulation of arginine and proline synthesis shown in man: In one study the subjects ingested a single dose of a mixture of uniformly (U)-^{13}C-labeled amino acids and carbohydrate; the appearance of U-^{13}C-amino acid in the plasma reflects entry of the dietary amino acids, and the appearance of ^{13}C in lower mass isotopomers demonstrates synthesis of the respective amino acids by the subject. The [M+5] isotopomer of plasma arginine was labeled as rapidly as [M+6] (i.e., tracer) arginine, which reflects the synthesis of citrulline from dietary [M+5]-glutamate in the first pass. The [M+1] to [M+3] isotopomers of arginine were also labeled for a prolonged period of time, suggesting that systemic glutamate was also a precursor for arginine synthesis. In fed subjects, only the [M+5] isotopomer of proline was significantly labeled. Fasting was associated with increased labeling of the [M+1] to [M+3] isotopomers of both arginine and proline, suggesting an increase in the contribution of de novo synthesis to their plasma flux (Berthold et al., 1995).

P5C concentration in the small intestine of fetuses was 1/5 that of adults and reached an adult level as early as postnatal Day 1. The highest peak was observed at Day 14, and then activity decreased to the adult level. However, P5C in the brain was highest at birth and quickly inactivated in a few days. Pregnancy and lactation reduced intestinal P5C considerably up to day 14 after parturition. P5C concentration in the small intestine of senescent rats was almost halved compared to young controls on a whole tissue basis. These results indicate that P5C synthetase in the small intestine is highly activated in suckling and weaning and amino acids derived from dietary ornithine are not fully dispensable in pregnancy, lactation, and senescence (Yamada and Wakabayashi, 1991).

III. Pathological states affecting the metabolism of P5C and glutamate family of amino acids

Stewart et al. (1981) showed that cats that develop hyperammonemia after an arginine-free meal have low hepatic ornithine levels. This group concluded that the sensitivity of the cat to an arginine free meal was the result of low levels of hepatic ornithine. With arginine deprivation, the net synthesis of ornithine becomes a critical source of urea cycle intermediates for the removal of excess ammonia. As a possible explanation for the unusual response of the cat to arginine deprivation Rogers and Phang (1985) have focused on the activity of P5C synthetase in intestinal mucosa of the cat. They found that P5C synthetase activity of the intestinal mucosa of cats, when compared to that of rats, was only 18% as high per gram of mucosa and only 5% as high per kilogram body weigh. This severe limitation in the first step in the de novo synthesis of ornithine may be the metabolic basis for the severe hyperammonemia found in cats fed an arginine-deficient diet. This lack of ornithine synthesis makes the cat completely dependent on dietary arginine for the ornithine required for the removal of ammonia via urea synthesis in the liver.

Normally, in bovine ocular tissues, cornea and retinal outer layers provide the intracellular proline synthetic pathway from ornithine, but not from glutamate. In C3H retinal degeneration mice, P5C reductase activity in the retinal outer layers was decreased to about one third lesser than that of CRJ control mice in the specific activity, throughout suckling and weaning periods. The activity of an incorporation of ^{14}C-ornithine into proline in the retinal outer layers (including the choroid) which presumably participates in the biosynthesis of proline-rich proteins such as collagen and glycoproteins, was also decreased to the same extend at the weaning period, in the retinal degeneration mice (Matsuzawa et al., 1982a; Matsuzawa, 1982a).

In man, disorders of proline and hydroxyproline metabolism result in hyperprolinemia. Two distinct conditions are known, each due to a mutation in a different genetic locus; both are apparently autosomal recessive. Type I hyperprolinemia is due to a deficiency of proline oxidase, and type II hyperprolinemia is due to a deficiency of P5C dehydrogenase. Proline levels are elevated only three- to fivefold in the former condition but ten- to fifteenfold in the latter. The distinguishing biochemical findings in type II hyperprolinemia are high plasma P5C levels and urinary excretion of P5C. Although the metabolic disorders in the hyperprolinemias are consistent with normal adult life, recent findings strongly suggest that there is a casual relationship between type II hyperprolinemia and neurologic manifestations in childhood (Phang et al., 1995).

Rabier et al. (1992) described a clinical case where two children born to consanguineous parents presented with a joint hyperlaxicity, skin hyperelasticity, bilateral cataract and mental retardation with hyperammonemia and low citrulline, ornithine and proline in plasma. They suggested that these patients affected with Δ1-pyrroline-5-carboxylate synthetase (P5CS) deficiency. Molecular studies confirmed this hypothesis (Aral et al., 1997).

Gyrate atrophy of the choroid and retina is a progressive chorioretinal degeneration which is inherited as an autosomal recessive trait. It is caused by a deficiency of OAT. Plasma ornithine values range from 400 to 1400μM, and 0.5 to 10mmol of ornithine is excreted daily. Plasma glutamate, glutamine, lysine, creatine and creatinine concentrations are modestly reduced. OAT activity in the cells and tissues of patients is from 0 to 6% that in control patients, and obligate heterozygotes have intermediate values (Phang et al., 1995).

IV. Gene-enzyme relationships in the proline biosynthetic pathway

In contrast to the poor progress in measuring enzymes of proline synthesis in higher plant extracts, there has been rapid progress in recent years in the cloning of cDNAs encoding these enzymes from higher plants by complementation of proline-requiring mutants of E. coli. A cDNA encoding P5CR was cloned by direct complementation of an E. coli proC proline auxotroph with a soybean nodule cDNA expression library (Delauney and Verma, 1990). This cDNA then facilitated the isolation of an homologous P5CR gene from Pisum sativum (Williamson and Slocum, 1992). The sequence of the cDNA encoding P5CR of soybean nodules also enabled the selection of suitable primers for the isolation of a P5CR gene from Arabidopsis thaliana (Verbruggen et al., 1993). In soybean, pea and Arabidopsis, P5CR transcripts increase in abundance in response to osmotic stress, indicating that P5CR gene transcription is under osmotic stress control (Delauney and Verma, 1990; Verbruggen et al., 1993; Williamson and Slocum, 1992). It is still not certain, however, whether this induction of P5CR mRNA contributes to enhanced proline accumulation under stress. The P5CR cDNA from soybean has been over-expressed in tobacco (Szoke et al., 1992). The transgenic plants exhibit 100-fold greater P5CR activity than wild-type. However, proline concentrations were not increased significantly in these transgenic plants (Szoke et al., 1992). Thus, P5CR may not be the rate-limiting step in proline accumulation (Delauney and Verma, 1993; LaRosa et al., 1991).

A cDNA encoding an ornithine-δ-aminotransferase was isolated from a mothbean (Vigna aconitifolia) cDNA expression library employing a proBA mutant of E. coli (CSH26) grown on nitrogen-free medium supplemented with 10mM ornithine. This mutant cannot utilize ornithine as a proline source. Introduction of the ornithine-δ-aminotransferase cDNA from Vigna allowed conversion of ornithine to GSA $\Leftrightarrow$ P5C which was then readily converted to proline by P5CR encoded by the endogenous E. coli proC gene, facilitating growth on ornithine in the absence of exogenous proline (Delauney and Verma, 1993; Delauney et al., 1993; Verma et al., 1992). In striking contrast to P5CR, ornithine-δ-aminotransferase appears to be markedly down-regulated under salinity stress (Delauney et al., 1993).

A cDNA encoding both GK and GPR similarly was isolated from a mothbean cDNA expression library employing E. coli proA, proB and proBA

proline auxotrophs, and screening for cDNAs which permit growth in the absence of proline. This cDNA was found to encode a bifunctional enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS) (Hu et al., 1992). The single major open reading frame of this cDNA encodes a polypeptide of 73.2 kDa which has two distinct domains exhibiting 55.3% overall similarity to E. coli GK and 57.9% similarity to 168 E. coli GPR, respectively (Hu et al., 1992). The GK activity (as measured by the γ-glutamyl hydroxamate assay (Hayzer and Leisinger, 1980) of this bifunctional enzyme is inhibited by proline (50% inhibition with 6 mM proline) (Hu et al., 1992). This enzyme appears to be much less sensitive to feedback inhibition than the wild-type GK of E. coli (50% inhibition by 0.2 mM (Hu et al., 1992) to 0.1 mM proline (Smith, 1985). Northern analyses indicate that the P5CS gene is induced (particularly in leaves) by treatment of Vigna plants with 200 mM NaCl (Hu et al., 1992). A single substitution of an alanine for a phenylalanine at amino acid residue 129 of the P5CS resulted in a significant reduction of proline feedback inhibition. The 50% inhibition values of γ-GK activity of the wild type and the mutant P5CS were observed at 5 mM and 960 mM proline, respectively (Zhange et al., 1995). Garcia-Rios et al. (1991, 1994) have also recently cloned a cDNA encoding both GK and GPR, from a tomato fruit cDNA expression library by complementation of prob E. coli mutants. It was subsequently found that this cDNA (designated PR01) complemented both proA and proBA mutants of E. coli (Garcia-Rios et al., 1991, 1994). The nucleotide sequence of the PRO1 cDNA revealed two protein domains; a GK domain consisting of 283 amino acids at the 5′ end, and a GPR domain consisting of 429 amino acids at the 3′ end (Garcia-Rios et al., 1994). The GK domain has a 66–69% amino acid sequence similarity of the GKs of E. coli and yeast, and a 69% similarity to the GK domain of mothbean P5CS. The GPR domain has a 69 and 66% similarity to the corresponding enzyme from E. coli and the corresponding domain of mothbean P5CS, respectively (Garcia-Rios et al., 1994). In marked contrast to mothbean P5CS, the tomato PRO1 cDNA has an inframe TAA (translation termination) codon between the two segments that specify GK and GPR (Garcia-Rios et al., 1994).This codon is followed after 5 bp (i.e. at a -1 or $+2$ frameshift with respect to GK) by a potential translation start ATG codon (Garcia-Rios et al., 1994). Consistent with this, when expressed in E. coli maxi cells, PRO1 directs the synthesis of two separate polypeptides (30 and 45 kDa) of the approximate mass predicted from the sequence analysis (Garcia-Rios et al., 1994).

The conversion of L-glutamate to proline in E. coli is achieved in a four-step reaction catalysed by GK, GPR and P5CR which are encoded by proB, proA, and proC, respectively. The E. coli proB, proA (Deutch et al., 1984) and proC (Deutch et al., 1984) loci have been cloned and sequenced. In E. coli and Serratia marcescens, the proB gene is located directly upstream of the proA gene and they appear to share a common promoter (Deutch et al., 1984). Similarly, the proB and proA genes of Salmonella typhimurium and of C. jejuni (Mahan and Csonka 1993; Lee et al., 1985) have been reported to form a single operon with proB located proximally to the promoter. The gene-enzyme relationships were demonstrated by interspecies complemen-

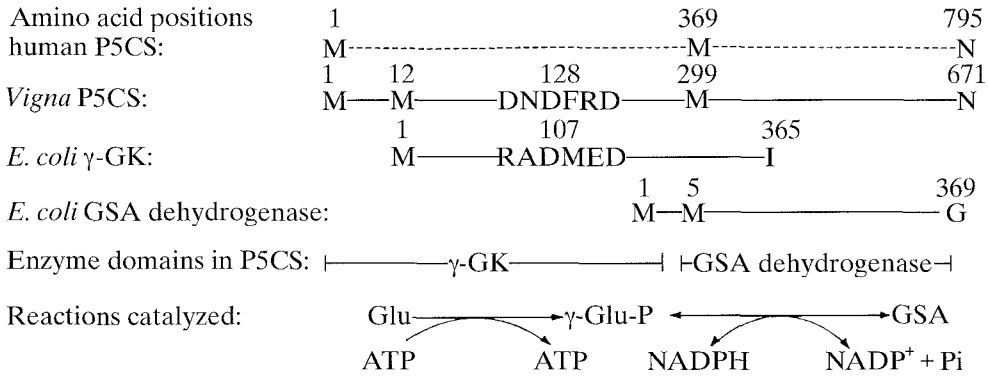

Fig. 3. Gene-enzyme relationship of the P5CS in different organisms (adapted from Hu et al., 1992)

tation of proB, proA, and proC mutants of E. coli and S. typhimurium by the yeast PRO1, PRO2, and PRO3 genes, respectively (Tomenchok and Brandriss, 1987) and of a yeast pro1 mutant by the E. coli proB gene (Orser et al., 1988). There is substantial deduced amino acid sequence similarity between the E. coli (Deutch et al., 1984) and S. cerevisiae (Li and Brandriss, 1992) GK, between the E. coli (Deutch et al., 1984) and S. cerevisiae (Brandriss, unpublished data) GPR, and among the P5C reductases of bacteria (Deutch et al., 1982; Hamilton and Reeve, 1985; Savioz et al., 1990), S. cerevisiae (Brandriss and Falvey, 1992), plants (Delauney and Verma, 1990) and mammals (Dougherty et al., 1992).

A human P5CS cDNA has been cloned by "database cloning" strategy and 2,907 bp sequenced from this cDNA which has a closed open reading frame (ORF) of 2,385 bp coding for a polypeptide of 795 amino acid residues (Aral et al., 1996). This cDNA, as its plant counterpart, encodes a bifunctional enzyme (Fig. 3), with both γ-glutamyl kinase GK and γ-glutamyl phosphate reductase GPR activities that catalyzes the first two steps in proline biosynthesis and it hybridizes to a 4.5 kb mRNA from various tissues, including spleen, thymus, prostate, testis, ovary, small intestine, heart and skeletal muscle, pancreas, among others. In humans, the expression levels found in heart, skeletal muscle and pancreas were relatively high compared to other tissues (Aral et al., 1996). In rats, assay for P5CS activities showed values 10–1,000 times higher in small intestine in comparison to other tissues (Wakabayashi et al., 1991). However, our results showed that in humans the mRNA levels detected in other tissues equals, or even higher, those in small intestine, contrasting with the tissue distribution of P5CS activity in rats given by Wakabayashi (Wakabayashi et al., 1991). Northern blot analyses showed that the P5CS gene is induced (particularly in chondrocytes) by treatment of rabbit cartilage cells in culture with IGF-1 (Aral, unpublished data; Smith et al., 1979), indicating that P5CS gene transcription is under hormonal control. The human gene encoding P5CS has been assigned to 10q24.3 by in situ hybridization (Liu et al., 1996). Dougherty et al. (1992) cloned a human P5C reductase

cDNA by complementation of proline auxotrophy in a S. cerevisiae mutant strain (DT1100). The 1,810-bp P5C reductase cDNA hybridizes to a 1.85-kb mRNA in samples from human cell lines and predicts a 319-amino acid, 33.4-kDa protein. The derived amino acid sequence is 32% identical with that of S. cerevisiae. By genomic DNA hybridization analysis, the human P5C reductase appears to be encoded by a single copy gene which maps to chromosome 17. The tissue expression profile of P5C reductase mRNA is ubiquitary. The P5C dehydrogenase, the gene defective in type II hyperprolinemia has also been cloned by using published peptide sequence data and degenerate primer PCR. Hu et al. (1996) cloned two full length human P5CDH cDNAs, differing in length by 1 kb. Both have a 1,689 bp ORF encoding a protein of 563 residues with a predicted molecular mass of 60 kDa. The predicted amino acid sequence has 100% identify to human P5CDH peptide sequence and 43% identity to S. cerevisiae P5CDH. The human P5CDH mRNA transcript has 3.2 kb length. The human OAT cDNA and structural gene have been cloned and characterized and the OAT locus mapped to 10q26 (Mitchell et al., 1988; Inana et al., 1986; Ramesh et al., 1986, 1987; Barrett et al., 1987).

V. The physiological roles of P5C and proline

A specific system of enzymes with special properties has evolved to mediate the metabolism of proline. These metabolic interconversions among proline, ornithine, and glutamate have P5C as the sole common intermediate. There are three poles of production, namely the intestinal mucosa which produces ornithine and citrulline, the liver which produces urea and the kidney which produces arginine. The intestinal mucosa, produces and exports both proline and ornithine. Only in the light of human genetic pathology and animal models related to the deficiencies of enzymes surrounding P5C-proline metabolism, we can truly speak about tissue specificity for each metabolic function.

The P5CS deficiency has shed some light on the importance of the ability to synthesize P5C and convert it to ornithine in enterocytes. The mutation these patients show is in the structural gene for P5C synthetase (Aral et al., 1997). As a result, these patients have a paradoxical hyperammonemia associated to hypocitrullinemia, hypoornithinemia and hypoprolinemia. This situation, coupled with the impaired production of urea, indicates that the conversion of P5C to proline and to ornithine is important for the maintenance of normal blood (and tissue) glutamate family amino acids and for the normal turnover of urea cycle and blood ammonemia levels. Whereas hyperammonemia is worsened after a protein load in most inborn errors of metabolism (such as urea cycle disorders and organic acidemias), in these patients hyperammonemia was more severe preprandially. It has been suggested that the cause of the paradoxical nature of hyperammonemia is that food intake provides the necessary ornithine to urea cycle whereas in fasting the ornithine is provided from the conversion of the P5C to ornithine in enterocytes via OAT reaction. Besides, in OAT $-/-$ mice, during the neonatal periods, there is a marked reduction in plasma ornithine,

arginine and citrulline (Wang et al., 1995). These animals also exhibit hyperammonemia and profound orotic aciduria which suggests impaired ammonium detoxification due to deficiency of urea cycle intermediates and the availability of intramitochondrial ornithine is inadequate for citrulline synthesis (Hauser et al., 1990). These data indicate that the flux in the OAT reaction in the neonatal period is in the direction of ornithine synthesis rather than degradation and that the survival of the rapidly growing neonatal mouse depends on an intact P5CS and OAT reaction to synthesize ornithine and arginine. The other animal model which support the usefulness of glutamate $\Rightarrow$ P5C $\Rightarrow$ ornithine pathway is the deficiency of P5CS in the intestinal mucosa of the cat, which makes the cat completely dependent on dietary arginine for the ornithine required for the removal of ammonia via urea synthesis in the liver (Rogers and Phang, 1985).

On the other hand the lack of P5CS in the liver, and the very high level of P5CDH which forms glutamate from P5C, indicates that glutamate synthesis may be important in liver. The high level of OAT in liver may indicate that OAT play a central role in regulating ornithine levels available to urea cycle; There is a human genetic disease, gyrate atrophy (GA), where patients suffering from it present themselves with limitations in peripheral vision due to atrophy of the retina, which starts at the periphery and then gyrates toward the center of the retina. The mutation these patients show is in the structural gene for OAT. As a result, these patients have high levels of blood ornithine. This situation, coupled with the normal production of urea, indicates that the conversion of ornithine to P5C (ornithine $\Rightarrow$ P5C) is important for the maintenance of normal blood (and tissue) ornithine levels. It has been suggested that the cause of the retinal atrophy is that cellular conversion of ornithine to P5C and conversion of P5C to proline (ornithine $\Rightarrow$ P5C $\Rightarrow$ proline pathway) is essential. Knockout adult mice (OAT $-/-$) for OAT on a standart diet exhibit profound hyperornithinemia to an extend similar to that of humans with GA (Wang et al., 1995).

In some initial studies, it was shown that P5C markedly activated the metabolism of glucose through the pentose phosphate pathway (Phang et al., 1979, 1982). The effect was a function of P5C concentrations and was observed at all concentrations of glucose (Phang, 1985). The unaltered response to P5C in cells deficient in either OAT or P5CDH, suggested that P5C reductase is the enzyme mechanism mediating the effect. In human erythrocytes, P5C reductase is the only enzyme of the proline metabolic system (Yeh and Phang, 1981), and in incubations of human red cells P5C stimulated the metabolism of glucose through the pentose phosphate pathway and there was a good correlation between two molecules of P5C converted to proline for every glucose 6-phosphate metabolized to ribose 5-phosphate. These initial studies support the general hypothesis that the interconversions of proline, ornithine, and glutamate can stimulate a metabolic pathway dependent on the $NADP^+$/NADPH redox ratio. There was also an effect of P5C on PP-ribose and purine nucleotides. Indeed, as PP-ribose-P levels in incubated human erythrocytes markedly increased when cells were treated with P5C (Phang, 1985; Yeh and Phang, 1981; Yeh and Phang, 1983). Although circulating

human erythrocytes lack the pathways for de novo purine synthesis they readily incorporate preformed purines into nucleotides by the salvage pathway in a PP-ribose-P-dependent process (Pritchard et al., 1975). P5C added to red cells markedly increased the incorporations of labeled purines into their respective nucleotides (Phang, 1985; Yeh and Phang, 1981), P5C also stimulated the IMP synthesis from hypoxanthine. A possible mechanism for the P5C effect on nucleotides via PP-ribose-P synthetase has been evoked (Phang, 1985).

Proline belongs to a class of compounds known as "compatible" or "counteracting solutes" which includes several amino acids (e.g. proline, alanine, β-alanine and taurine). These compounds tend to be uncharged at neutral pH, and are of high solubility in water (Ballantyne and Chamberline, 1994). In fact, exogenously supplied proline is osmoprotective for bacteria, facilitating growth in highly saline environments (Csonka, 1989; Csonka and Hanson, 1991; Strom et al., 1983; Yancey, 1994). Overproduction of proline in bacteria caused by altered feedback inhibition of the proline biosynthesis pathway can result in increased osmotolerance (Csonka, 1981; Smith, 1985). In higher plants, the accumulation of proline is a common metabolic response of higher plants to water deficits, salinity stress and cold stress (Csonka and Baich, 1983; Delauney and Verma, 1993; Dorffling et al., 1993; Hanson and Hitz, 1982; Steward, 1981; Steward and Larher, 1980; Thompson, 1980). Fasciola hepatica manipulates the metabolism of the host animal using a mechanism involving proline. While still in the host liver, the immature fluke has high proline concentrations and has a large proline biosynthetic capacity (Isseroff, 1979). The fluke releases proline into the host's bile increasing the concentration of biliary proline more than 1,000-fold, and inducing ipso facto, proliferation and enlargement of the host's bile duct so that it becomes a suitable habitat.

Conclusions

Plants, microorganisms and animal species have all three necessary enzymes which produce (the P5C-producing enzymes) and utilize (the P5C-utilizing enzymes) the P5C. But they use the P5C for different purposes. Certainly, the glutamate $\Rightarrow$ P5C $\Rightarrow$ proline pathway is the most studied in three organisms. Proline biosynthesis as a substrate to be incorporated into proteins must exist in plants, microorganisms and animal species. The tissues with high collagen content use this pathway in order to supply proline during tissue development. In microorganisms and plants the biosynthesis of proline is a necessary biological response to water deficits, salinity stress and cold stress. It serves as "compatible" or "counteracting solutes". In animal species and in man, the pathway glutamate $\Rightarrow$ P5C $\Rightarrow$ ornithine is also used in enterocytes in order to produce ornithine, in the absence of exogenous ornithine, for a correct urea cycle homeostasis (and also citrulline and arginine biosynthesis). The cloning of cDNAs encoding key enzymes of proline biosynthesis, such as P5CS from animal species and man, may prove to be as useful as the specific probes for these genes and their products will clarify our understanding of

urea cycle homeostasis and collagen metabolism. Hepatocytes and retina cells use the ornithine ⇒ P5C ⇒ proline pathway in order to adjuste the ornithine levels available to urea cycle and to supply proline to neuronal cells.

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