Molecular Enzymology of Mammalian Δ¹-Pyroline-5-carboxylate Synthase

ALTERNATIVE SPlice DONOR UTILIZATION GENERATES ISOFORMS WITH DIFFERENT SENSITIVITY TO ORNITHINE INHIBITION

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Δ¹-Pyroline-5-carboxylate synthase (P5CS; EC not assigned), a mitochondrial inner membrane, ATP- and NADPH-dependent, bifunctional enzyme, catalyzes the reduction of glutamate to Δ¹-pyroline-5-carboxylate, a critical step in the de novo biosynthesis of proline and ornithine. We utilized published plant P5CS sequence to search the expressed sequence tag data base and cloned two full-length human P5CS cDNAs differing in length by 6 base pairs (bp) in the open reading frame. The short cDNA has a 2379-bp open reading frame encoding a protein of 793 residues; the long cDNA, generated by “exon sliding,” a form of alternative splicing, contains an additional 6-bp insert following bp +711 of the short form resulting in inclusion of two additional amino acids in the region predicted to be the γ-glutamyl kinase active site of P5CS. The long form predominates in all tissues examined except gut. We also isolated the corresponding long and short murine P5CS transcripts. To confirm the identity of the putative P5CS cDNAs, we expressed both human forms in γ-glutamyl kinase- and γ-glutamyl phosphate reductase-deficient strains of Saccharomyces cerevisiae and showed that they conferred the proline prototrophy. Additionally, we found expression of the murine putative P5CS cDNAs conferred proline prototrophy to P5CS-deficient Chinese hamster ovary cells (CHO-K1). We utilized stable CHO-K1 cell transformants to compare the biochemical characteristics of the long and short murine P5CS isoforms. We found that both confer P5CS activity and that the short isoform is inhibited by l-ornithine with a Kᵢ of ~0.25 mM. Surprisingly, the long isoform is insensitive to ornithine inhibition. Thus, the two amino acid insert in the long isoform abolishes feedback inhibition of P5CS activity by l-ornithine.

Proline can be synthesized either from glutamate or ornithine and is nonessential for full-term human infants and adults (1, 2) but conditionally indispensable for premature neonates (3, 4). Δ¹-Pyroline-5-carboxylate (P5C), 1 which is in nonenzymatic equilibrium with glutamic γ-semialdehyde, is a common intermediate in these pathways (5). Formation of P5C from glutamate in humans has been proposed to be catalyzed either by P5C synthase, a bifunctional enzyme with both γ-glutamyl kinase (γ-GK) and γ-glutamyl phosphate reductase (γ-GPR) activities or a complex of separate γ-GK and γ-GPR enzymes (6). Hu et al. (7) showed that plant P5CS is bifunctional, catalyzing the conversion of glutamate to P5C. By contrast, P5CS activity in prokaryotes and lower eukaryotes like Saccharomyces cerevisiae requires the combined functions of separate γ-GK and γ-GPR enzymes. In rats, P5CS activity is highest in small intestine with measurable activity in colon, pancreas, and thymus (8). Since proline may be a neurotransmitter and because the concentration of proline in the extracellular fluid of the central nervous system is low (<5 μM) (9–14), P5CS may be of particular importance in certain regions of the central nervous system. Wakabayashi et al. (8) showed that detectable P5CS activity is present in rat cerebrum and cerebellum; however, the regional distribution of this enzyme in the central nervous system has not been well characterized. P5C can also be synthesized from ornithine in a reaction catalyzed by ornithine δ-aminotransferase (OAT) (15).

The P5CS genes of plants (7, 16–18) and the γ-GK and γ-GPR genes of prokaryotes (e.g. in Escherichia coli; see Ref. 19 and S. cerevisiae (20, 21) have been cloned and sequenced. Mutant strains of S. cerevisiae auxotrophic for proline because of mutations in γ-GK or γ-GPR genes are known and provide a potential system for expression and analysis of human P5CS. Additionally, CHO-K1 cells are proline auxotrophs and lack both P5CS and OAT activities (22–24).

Early studies of Chinese hamster-human somatic cell hybrids (25) and chromosome localization of glutamate oxaloacetate transaminase (26), a marker linked to P5CS, suggested that the human P5CS gene is on chromosome 10. During the course of our work, Aral et al. (27) reported the sequence of a human cDNA that, based on sequence similarity, appeared to encode P5CS. Direct evidence showing that this cDNA encodes

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) U68758 (HsP5CS.short), U76542 (HsP5CS.long), AF036573 (MmP5CS.long), and AF056674 (MmP5CS.short).

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1 The abbreviations used are: P5C, Δ¹-pyroline-5-carboxylate; γ-GK, γ-glutamyl kinase; CHO-K1, Chinese hamster ovary cells; P5CS, Δ¹-pyroline-5-carboxylate synthase; ORF, open reading frame; γ-GPR, γ-glutamyl phosphate reductase; OAT, ornithine δ-aminotransferase; EST, expressed sequence tag; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; bp, base pair(s); kb, kilobase pair(s); nt, nucleotide(s); UTR, untranslated region; RPA, ribonuclease protection assay.
a functional enzyme was not provided. This group also localized the structural gene encoding this cDNA to chromosome 10 in the interval between q24.3 to q24.6 (28).

To better understand the molecular enzymology and regulation of mammalian P5CS, we utilized homology to Vigna P5CS to clone two full-length human P5CS cDNAs and the corresponding murine P5CS cDNAs. Expressing these cDNAs in mutant yeast strains and in CHO-K1 cells, we showed both forms of mammalian P5CS encode bifunctional enzymes and complement growth phenotype of mutant cells. More interestingly, we found that the short but not the long P5CS isoform is sensitive to feedback regulation of ornithine. Our results suggest a mechanism for differential regulation of P5CS activity.

**EXPERIMENTAL PROCEDURES**

**cDNA Libraries and Plasmids**—We obtained a human small intestine 5′-STRETCH cDNA library from CLONTECH and a human kidney cDNA from Dr. G. Bell. pBluescript II KS(+) was used for all cloning manipulations.

**Cloning by Homology Probing and cDNA Library Screening**—We utilized the deduced amino acid sequence of *Vigna aconitifolia* P5CS (7) as a probe to screen the GenBank® (EST) data base (National Center for Biotechnology Information, Bethesda, MD) with the BLAST algorithm (29). We obtained a candidate human EST clone (GenBank® accession no. R13516) from the National Genome Center, Lawrence Livermore National Laboratory (Livermore, CA) and sequenced the cDNA insert. Using this putative P5CS EST clone as a probe, we screened >5×10⁶ plaques from both the human kidney and small intestine cDNA library as described (30, 31). We purified two positive plaques, one (HsP5CS.KS) from kidney cDNA library and the other (HsP5CS.G1) from the small intestine cDNA library, through two additional cycles of screening, subcloned the inserts (HsP5CS.K5, 2.7 kb; HsP5CS.G1, 2.8 kb) into Bluescript II KS (+), and sequenced them. Using the full-length human P5CS cDNA sequence as a probe, we also identified a full-length murine P5CS long form cDNA (GenBank® accession no. AA0142859 and confirmed its identity by sequencing the insert (MmP5CS.long, 1.3 kb).

**Recombinase and Reverse Transcription PCR**—We performed PCR amplification on either human or murine genomic DNA (100 ng) with primers DV2375 (5′-GGATTTCCATGATGGAGCAAGGC) and DV2374 (5′-CAGTGGGCGGACGGCTTACATTAC), corresponding to sequences flanking the 6-bp insert in HsP5CS.long with an initial denaturation step of 6 min at 96 °C, followed by 2 min at 65 °C, and 2 min at 72 °C. The amplified products were electrophoresed in 1% agarose, excised, gel-purified, and sequenced directly with the 5′-nested primer DV1585 (5′-GCCGAACTCCTAAGTGAAGAATGCGGTTGCTG) with the same conditions as utilized for PCR of genomic DNA.

We cloned the amplified DNA fragments in pCR II (Invitrogen) and sequenced the insert. To provide the sequence at the recombination junctions containing the 6-bp insert, we replaced the relevant region of P5CS.short with the counterpart of the long form containing the 6-bp insert. The sequence at the recombination junctions of these recombinant plasmids was verified by sequencing.

**Functional Complementation of Yeast Auxotrophs**—DT1103 (MATα, ura3-D100, trp1-D102, leu2-D110) was used as recipient strain and DT1103 (MATα, ura3-52, trpl-pro2-URA3) were gifts from M. Brandris (34). We transformed yeast by the lithium acetate method as described (30). Strains HV10, HV11, HV13, and HV14 were constructed by introduction of recombinant plasmid pScPR1, p424GAL1, pHsP5CS.short, and pHsP5CS.long 2 into DT1103, respectively. Strains HV12, HV14, HV15, and HV16 were derived from DT1102 transformed with p424GAL1, pHsP5CS.short, and pHsP5CS.long 2, respectively. The transformation reactions were plated on minimal glucose medium containing 0.1% ammonium sulfate without tryptophan (MGA-trp) or minimal glucose medium containing 0.1% ammonium sulfate and 1% proline without tryptophan (MGA-trp ) (30). After growth for 10 days at 30 °C, plasmid DNA was isolated from yeast transformants and used to transform E. coli 294-competent cells (30).

**Construction of Human P5CS Yeast Expression Vectors**—We used the 5′-based p242GAL1 (32) E. coli yeast shuttle vector plasmid containing TRP1- and Ampβ-selectable markers and a multiple cloning site downstream of an *S. cerevisiae* galactokinase (GAL1) promoter. pT282, a plasmid containing the *S. cervisiae* PR0 gene, was a gift from M. Brandris. We subcloned the yeast PR0 gene from pT282 to p424GAL1 to form pScPR0.

To replace the *S. cervisiae* PR0 ORF with those of the long and short forms of human P5CSs, we used a homologous recombinant strategy *in vivo* expressed. Briefly, we designed two oligonucleotide primers to amplify the entire human P5CS ORF flanking with the antisense strand of the 3′-end of the human P5CS ORF (+1 to +27). The 5′-antisense primer contained 60 nt of sequence corresponding to the antisense strand beginning 60 bp downstream of the stop codon of *S. cervisiae* PR0 ORF (+1668 to +1627) followed by 34 nt of sequence identical to the antisense strand of the 3′-end of the human P5CS.short ORF (+2382 to +2349). We used a combination of Pfu and Taq polymerases (Stratagene) to amplify *Hs* P5CS.short. Homologous recombination in yeast was done by cotransferring the BgIII- and Hpa1-linearized pScPR0 and the yeast/human P5CS fragment into yeast strain DT1103 (see below). We synthesized pHsP5CS.long 2 by replacing the relevant region of P5CS.short with the counterpart of the long form containing the 6-bp insert. The sequence at the recombination junctions of these recombinant plasmids was verified by sequencing.
produce pMmP5CS.short.1. Subsequently, we subcloned the insert of pMmP5CS.short.1 into pcDNA3 to form pMmP5CS.short.2. We verified the replaced segment of the recombinant plasmid pMmP5CS.short.2 by sequencing.

**Transfection and Functional Complementation of CHO-K1 Cells**—We transfected pMmP5CS.short.2 or pMmP5CS.long.2 into a subclone (CHO-K1-NC5) of the proline auxotrophic cell line CHO-K1 (ATCC no. CCL61) by electroporation as described by Melkonyan et al. (35). Briefly, we electroporated 7 × 10^6 cells resuspended in the growth medium containing 1.25% MeSO (300 mM) and 30 μg of plasmid DNA with a Gene Pulser (Bio-Rad) using the setting 960 microfarads, 100 ohms, and 350 V. We selected stable transformants in the minimal essential medium with 10% (v/v) dialyzed fetal calf serum lacking L-proline (22).

**P5CS Enzyme Assay and End Product Regulation**—We assayed P5CS activity radioisotopically as described by Wakahayashi et al. (8). In preliminary experiments (not shown) we verified that P5CS activity is dependent on the presence of ATP, MgCl₂, and NADPH and increases linearly with added protein up to a concentration of 1 mg/ml and with time up to 60 min.

**RESULTS**

**Isolation and Characterization of Human and Murine P5CS cDNAs**—We utilized the C-terminal 200-amino acid sequence of *Vigna* (mothbean) P5CS to perform a BLAST search of the GenBank™ EST database and identified a single human candidate clone (GenBank™ accession no. R13516). The deduced amino acid sequence of this clone had 59% identity to the corresponding region of mothbean P5CS (data not shown). A second BLASTP search utilizing the EST as probe retrieved the corresponding region of mothbean P5CS, a *Caenorhabditis elegans* EST sequence (GenBank™ accession no. D35184) and γ-GPR genes for *S. cerevisiae* and several prokaryotes (data not shown). We obtained this human putative P5CS EST clone, sequenced the entire insert for confirmation, and utilized it to probe human kidney and small intestine cDNA libraries. One cDNA clone (P5CS.K5) had an insert size of 2726 bp extending from +472 (where +1 is the A of the initiation methionine codon) to a poly(A) tail. The second clone (P5CS.G1) lacked the 3′-most 503 bp of P5CS.K5, had a 2225-bp overlap with the 5′-end of P5CS.K5, and extended an additional 529 bp 5′ to −57. Using a similar strategy, we also obtained a murine putative P5CS EST clone (GenBank™ accession no. AA1042889) isolated from 13-day-old mouse heart cDNA library had an insert size of 3343 bp extending from −53 (where +1 is the A of the initiation methionine codon) to a poly(A) tail. It has a 53-bp 5′-UTR, 2385-bp ORF, and 805-bp 3′-UTR. The mouse ORF encodes a polypeptide with 95% sequence identity to its human counterpart.

The overall amino acid sequence of the human putative P5CS has 43 and 40% identity to those of *Arabidopsis thaliana* and *V. aconitifolia* P5CSs, respectively. Examination of the human P5CS sequence indicates that the enzyme is bifunctional. The N-terminal half has 36% identity to *S. cerevisiae* γ-GK, while the C-terminal half has 48% identity to *S. cerevisiae* γ-GPR (Fig. 1). The N-terminal half of human P5CS also has strong similarity to lower eukaryotic and prokaryotic γ-GKs, and the C-terminal half is similar to lower eukaryotic and prokaryotic γ-GPRs (data not shown). As expected for a mitochondrial inner membrane protein, the N terminus of both the human and the murine P5CS sequences has features characteristic of a mitochondrial targeting sequence with frequent basic and no acidic residues (36). Based on the consensus for cleavage of mitochondrial targeting sequences (36, 38–39), we identified several cleavage sites following amino acids 56 and 64 (Fig. 1). Using BLASTP and Blocks data base (39) searches, we identified a 32-amino acid motif (between Asp245 and Pro276) with similarity to a sequence thought to be the active site motif in several other kinases in the aspartokinase family (see Figs. 1 and 2). In this motif, two aspartate residues (Asp245 and Asp258) are conserved in a comparison of eight aspartokinases including one from *Vigna*, two from yeast, and five from prokaryotes. Five other residues are identical in at least seven of the examples. Additionally, the human and murine P5CS sequences have three conserved residues (Asn199, Asp193, and Phe194) predicted by homology to *Vigna* P5CS and *E. coli* γ-GK to be involved in proline feedback regulation of P5CS activity (7, 38, 40) (Fig. 1). Also, there are four putative N-glycosylation sites and a putative NADP(H) binding domain (41) (Fig. 1).

Comparing the sequences of the various human putative P5CS cDNAs, we noted that some had a 6-bp insert following bp +711 while others did not. To determine if this insert was the result of some form of alternative splicing, we amplified genomic DNA with primers complementary to cDNA sequences flanking the region of the insert (5′ DV1475; 3′ DV2374). Amplification of cDNA with these primers produces a 74-bp fragment. However, with genomic DNA as template, the product was −500 bp. Sequence analysis showed that this fragment contains a portion of a 5′ exon followed by a 400-bp intron and a portion of a 3′ exon. We sequenced the amplified P5CS structural gene fragment and found that the 6-bp insert derives from the 5′-end of an intron with two potential donor splice sites in tandem (Fig. 3). Alternative utilization of these two donor splice sites generates two different mature transcripts: a short form, utilizing the most 5′ donor site and lacking the insert (Fig. 3), and a long form, utilizing the most 3′ donor site and containing the 6-bp insert following position +711.

To examine the tissue expression of the two P5CS isoforms, we conducted RT-PCR and RPAs with poly(A) RNA isolated from human small intestine, placenta, and cerebellum. The former has the advantage that individual clones can be scored to give a quantitative result; the latter has the advantage that it avoids PCR. In the RT-PCR assay, we cloned the amplified fragments and performed a second PCR amplification with a pair of nested primers surrounding the region containing the insert. In clones containing the insert, the amplified product is 80 bp; in clones without, the amplified product is 74 bp. We found that the ratio of the short to long forms in small intestinal RNA was 0.3 (58/19), whereas the ratio in placental (15/62) and cerebellum (15/59) RNAs was −0.25 (Fig. 4). The RPA data are generally consistent with this result. The antisense riboprobe (cRNA) used for RPA is 391 bp and has 6 bp of vector sequence at each end flanking 379-bp *HsP5CS* long sequence complementary to bp +474 to +852. When the probe hybridizes to the mature *HsP5CS* long transcript, a 379-bp fragment is protected. Alternatively, when the probe hybridizes to a P5CS.short transcript, a single-stranded loop structure will form at the site of the insert yielding two products of 238 and 135 bp following RNase digestion. As seen in Fig. 4, this assay confirms the short form is predominant in the gut, whereas the converse is true in placenta and cerebellum. PhosphorImager analysis of the intensity of these bands gave short to long ratios of 3.75 in gut, 0.83 in placenta, and 0.55 in cerebellum.

**Tissue Distribution and Size of Human P5CS Transcripts**—Northern blots of mRNA from multiple human tissues showed a single predominant P5CS transcript of about 3.6 kb (Fig. 5). Pancreas, ovary, testis, and kidney had the highest expression, followed by colon, small intestine, placenta, heart, and skeletal muscle.

**Functional Complementation of γ-GK- and γ-GPR-deficient Yeasts**—To confirm the identity and function of the two P5CS cDNAs, we constructed composite human P5CS cDNA clones, designated *HsP5CS*.short.1 and *HsP5CS*.long.1, respectively. The *HsP5CS*.short.1 cDNA is 3256 bp in length including 57 bp of 5′-untranslated sequence, a 2379-bp ORF, and 820 bp of 3′-UTR extending to the poly(A) tail. The *HsP5CS*.short.1 ORF
encodes a 793-amino acid protein with a predicted Mr of 90. HsP5CS.long.1 is identical to HsP5CS.short.1 except that it includes the 6-bp insert following position 1711, resulting in a 2385-bp ORF encoding a 795-amino acid protein. To express these in yeast, we utilized PCR and homologous recombination to construct yeast/human P5CS chimeric minigenes (pHsP5CS.short.2 and pHsP5CS.long.2), comprising the S. cerevisiae PRO1 promoter and 5' and 3'-UTR sequences surrounding the intact human P5CS.short and P5CS.long ORFs. Previous work by ourselves and others has shown that substitution of yeast 5' and 3'-UTR sequences may improve the expression of mammalian genes in yeast (30, 42). DT1103, a proline auxotroph with a partially deleted g-GK gene (pro1), is unable to grow on minimal medium without proline. We transfected this strain with either pHsP5CS.short.2 or pHsP5CS.long.2 and selected transformants on medium lacking both proline and tryptophan (MGA-trp2). Both pHsP5CS.short.2 and pHsP5CS.long.2 restored the PRO1 growth phenotype in DT1103 (see strains HV13 and HV14; Fig. 6) although not as efficiently as a construct containing the yeast g-GK gene (pScPRO1; see HV10, Fig. 6).
P5CS cDNAs encode bifunctional enzymes, we performed a second complementation experiment, transfecting pHsP5CS.short.2 and pHsP5CS.long.2 into DT1102, a mutant yeast strain auxotrophic for proline because it has a partial deletion of the pro2 locus. As shown by the DT1102 transformants, HV15 and HV17, pHsP5CS.short.2 and pHsP5CS.long.2 also complement deficiency of both P5CS and OAT activities (22, 23). We sequenced the P5CS mRNA that was expressed by the transformants was murine rather than hamster. To rule out reversion to the hamster P5CS locus, we confirmed that the P5CS mRNA expressed by the transformants was murine rather than hamster. To do so, we performed an RPA performed as described under “Experimental Procedures.” Lane 1, labeled riboprobe only; lane 2, riboprobe digested with RNase mixture; lanes 3–6, riboprobe hybridized with tissue RNA digested with RNase mixture; lane 3, yeast total RNA (10 μg); lane 4, human small intestine poly(A) RNA (1 μg); lane 5, human placenta poly(A) RNA (1 μg); lane 6, human cerebellum poly(A) RNA (1 μg). B, agarose gel electrophoresis of PCR-amplified fragments of cloned RT-PCR products from RNA isolated from the indicated tissue. The long fragment is 80 bp; the short one is 74 bp.

Both Murine P5CS cDNA Isoforms Complement the CHO-K1 Cell Growth Phenotype—Although the human P5CS cDNAs complement the growth phenotype of the γ-GK- and γ-GPR-deficient yeast strains, we were not able to measure P5CS activity in extracts of these yeast transformants. We reasoned that this might reflect the heterologous environment. To do biochemical studies, therefore, we transfected murine P5CS cDNAs into a subclone (NC5) of CHO-K1 (CHO-K1-NC5) cells previously shown to be auxotrophic for proline (43) because of deficiency of both P5CS and OAT activities (22, 23). We selected stable transformants in medium lacking proline and argued that the role of P5CS in ornithine and citrulline synthesis. Previous studies using extracts of rat small intestine showed that P5CS activity is noncompetitively inhibited by...
ornithine (44, 45). To determine if both isoforms of murine P5CS had similar sensitivity to ornithine inhibition, we assayed their activity in the presence of 5 mM ornithine. We found that P5CS.short, the predominant isoform in gut, is inhibited 75% by 5 mM ornithine with a $K_i$ in this assay of 0.25 mM (Fig. 7). In agreement with the earlier work, we obtained similar results using mouse small intestine as the source of P5CS activity (Fig. 7). By contrast, P5CS.long, the predominant isoform in peripheral tissues, is completely insensitive to 5 mM ornithine (Fig. 7).

**DISCUSSION**

Utilizing homology to *Vigna* P5CS, we isolated two splice forms of cDNAs encoding human P5CS: a short form (P5CS.short) encoding a 793-amino acid protein and a long form (P5CS.long), with an additional 6-bp insert following bp +711, encoding a 795-amino acid protein. The human P5CS polypeptides have high sequence identity to P5CS of plants and to $\gamma$-GK and $\gamma$-GPR enzymes of lower eukaryotes and prokaryotes. Expression of human P5CSs restores proline prototrophy in two mutant yeast strains, one deficient in $\gamma$-GK (pro1), the other in $\gamma$-GPR (pro2), confirming the bifunctional nature of the mammalian enzyme. To study the biochemistry and regulation of mammalian P5CS isoforms, we expressed murine P5CS cDNAs in CHO-K1 cells that lack endogenous P5CS activity. Both murine P5CS cDNAs restored proline prototrophy, and stable transformants expressed measurable P5CS activity. Using extracts of these stable transformants, we showed that the two isoforms differ dramatically in their sensitivity to inhibition by ornithine.

During the course of our studies, Aral et al. (27) also reported the sequence for a human P5CS cDNA. As we did, they identified a partial length EST clone containing the 3'9438 bp of the ORF. They used the sequence of this EST to initiate a series of 5'-rapid amplification of cDNA end reactions followed by direct sequencing of the products to extend the cDNA sequence 138 bp into the 5'-UTR. The nucleotide sequence of our P5CS cDNAs has 14 single bp differences with that of Aral et al. (27), all in the ORF and resulting in 13 amino acid changes. Some of these differences may represent polymorphisms; others may reflect sequence errors. Aral et al. (27) utilized direct sequencing of amplified material. Our composite sequence derives from sequencing multiple cDNA clones in both directions. The over-
lapping sequences of our various cDNAs are 100% identical (for example, clones HsP5CS.G1 and HsP5CS.K5 have a 2225-bp overlap in perfect agreement).

In addition to the overall similarity of the N-terminal half of HsP5CS to γ-GKs from a variety of organisms (e.g. 36% identity with S. cerevisiae γ-GK), BLASTP and Blocks data base (39) searches utilizing the γ-GK domain of the human P5CS as a probe revealed a 32-amino acid motif (from Asp245 to Pro276) in the N-terminal half of human P5CS with similarity to a conserved sequence characteristic for members of the aspartokinase family including aspartokinases, uridylate kinases, and carbamate kinases from a variety of species (46–49). Aspartokinases catalyze phosphorylation of substrates with a carboxyl group as the nucleophilic acceptor (50). The conserved sequence overlaps with and extends more 5’ of a region of sequence similarity between E. coli and Serratia marcescens aspartokinases and γ-glutamyl kinases identified by Omori et al. (46), who suggested it is the active site of these related enzymes. Three residues in this motif corresponding to Asp245, Asp260, and Pro276 in HsP5CS are conserved in all γ-GKs and nearly all members of the aspartokinase family (Fig. 2). Another region likely to be important for HsP5CS function is a cluster of three amino acids (Asn191, Asp193, and Phe194) about 50 residues N-terminal of the putative aspartokinase motif. These are conserved with Vigna P5CS, where mutagenesis experiments have shown that they are essential for feedback regulation of P5CS activity by proline (40) (Fig. 1).

We identified two forms of P5CS cDNAs differing only by a 6-bp insertion at position +711. Two lines of evidence indicate that this insert is generated by a variant of alternative splicing called “exon sliding” (51). First, the insert sequence is colinear with the genomic sequence of the human P5CS structural gene and has the consensus sequence of a 5’ splice site. The same colinearity is also present in murine P5CS (data not shown). Thus, the choice between the two tandem splice donor sequences determines whether the mature transcript will contain the 6-bp insert. The fact that we see markedly different ratios of short/long transcripts in various tissues suggests either that there is tissue-specific stability of the two products or that the choice of splice donor is somehow regulated. How this choice might be regulated is unclear. Based on the calculation of the consensus value of the 5’ splice donor site (28, 52–54), both of these tandem donor sites are relatively “weak” with the consensus values of 77.7 and 73.2, respectively, where the value for a typical donor site ranges from 0.7 to 1.0 with the mean of 0.85. This suggests that there might be tissue- or development-specific factor(s) influencing the relative use of the two competing 5’ splice sites. Multiple mechanisms including cis elements and trans-acting factors influence 5’ splice site selection (53–61). Exonic splicing enhancers are positive acting, cis RNA sequences capable of inducing the assembly of the splicosome at weak 5’ splice sites and are generally purine-rich. Interestingly, we note several purine-rich sequences in both HsP5CS and MmP5CS cDNAs 20–500 bp 3’ of the 6-bp insert, for example, a GGAAATGAAAA 47 bp 3’ (bp 1765 to +775), a GGAGGGGAAGAAG 314 bp 3’ (bp 11032 to +1045), and an AGCAGGGAGAAA 376 bp 3’ (bp +1094 to +1105) of the 6-bp insert of the HsP5CS long cDNA. Other examples of generating
short (<20 codona) inserts in mature transcripts by “exon sliding” have been described in the *Drosophila* Ultraembithorax gene (62), porcine urokinase-like plasminogen activator (63), human β-adducin gene (64), chicken growth hormone-releasing hormone gene (65), and human presenilin-1 gene (66).

Interestingly, the 2-amino acid insert (Val^{238} and Asn^{239} in P5CS.long) is immediately N-terminal of the putative aspartokinase motif (amino acids 245–276) and 43 amino acids C-terminal of the cluster of residues (amino acids 191–194) predicted to be involved in the feedback regulation of plant P5CS by proline (Fig. 1). This location in or near the active site and the apparent variation in the ratio of short versus long isoforms in different tissues suggests that the presence or absence of the insert may play a role in the regulation of P5CS activity. Previous work utilizing extracts of rat small intestinal mucosa showed that mammalian P5CS is inhibited by ornithine with a *K*ᵦ of about 0.4 μM (44, 45). We found similar results for the isoform encoded by the predominant gut transcript (P5CS.short). In mice and humans, plasma ornithine is about 75 μM with a distribution ratio of intracellular/extracellular ornithine of about 5, predicting an intracellular ornithine concentration of ~0.37 μM (15). This suggests that physiologic concentrations of ornithine are in the range predicted to regulate P5CS.short activity. By contrast, we found the long P5CS isoform is insensitive to ornithine inhibition. These results correlate functional differences in the protein with the choice of splice sites. As far as we know, this is the first demonstration of functional differences in protein isoforms generated by exon sliding.

The P5CS reaction serves two physiologic functions in mammals: proline biosynthesis and arginine biosynthesis. In tissues with a high protein synthetic requirement for proline (e.g. cartilage and possibly central nervous system), P5CS catalyzes the first committed step in the two-step pathway synthesizing proline from glutamate (5). In prokaryotes and plants, this pathway is regulated by allosteric inhibition of P5CS by the product of the P5CS reaction, isomerizes the first committed step in proline biosynthesis, is sensitive to inhibition by proline (77). Combining our results with this information suggests a tissue-specific model for regulation of the pathway converting glutamate to either arginine or proline. In gut, where the emphasis is on arginine biosynthesis, P5CS.short is the predominant isoform, and ornithine regulates the pathway by its inhibitory effect on P5CS. In peripheral tissues where proline biosynthesis is important, P5CS.long predominates, and the pathway is insensitive to ornithine but is regulated at the level of mammalian P5C reductase by proline (Fig. 8).

Aral et al. (27) and Kamoun et al. (78) describe two siblings with joint hyperlaxity, skin hyperelasticity, cataracts, and mental retardation who are the product of a consanguineous union. Their biochemical phenotype (low plasma proline, citrulline, and ornithine) suggests P5CS deficiency, and both were shown to be homozygous for the P5CS missense mutation, L396S. The functional consequence of this mutation on P5CS activity was not tested. Availability of the human P5CS cDNA sequences and the expression systems provided by P5CS-deficient CHO-K1 cells and yeast strains provides the molecular resources to study the P5CS genes of these and similar patients.

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