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Substrate channeling in proline metabolism

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Abstract

Proline metabolism is an important pathway that has relevance in several cellular functions such as redox balance, apoptosis, and cell survival. Results from different groups have indicated that substrate channeling of proline metabolic intermediates may be a critical mechanism. One intermediate is pyrroline-5-carboxylate (P5C), which upon hydrolysis opens to glutamic semialdehyde (GSA). Recent structural and kinetic evidence indicate substrate channeling of P5C/GSA occurs in the proline catabolic pathway between the proline dehydrogenase and P5C dehydrogenase active sites of bifunctional proline utilization A (PutA). Substrate channeling in PutA is proposed to facilitate the hydrolysis of P5C to GSA which is unfavorable at physiological pH. The second intermediate, gamma-glutamyl phosphate, is part of the proline biosynthetic pathway and is extremely labile. Substrate channeling of gamma-glutamyl phosphate is thought to be necessary to protect it from bulk solvent. Because of the unfavorable equilibrium of P5C/GSA and the reactivity of gamma-glutamyl phosphate, substrate channeling likely improves the efficiency of proline metabolism. Here, we outline general strategies for testing substrate channeling and review the evidence for channeling in proline metabolism.

Keywords

Substrate Channeling; Proline Metabolism; Proline Dehydrogenase; PRODH; Pyrroline-5-carboxylate Dehydrogenase; P5CDH; Pyrroline-5-Carboxylate; P5C; Glutamic semialdehyde; GSA; Gamma-Glutamyl Kinase; Gamma-Glutamyl Phosphate Reductase; Pyrroline-5-Carboxylate Synthase; P5CS; Gamma-Glutamyl Phosphate; Review

2. INTRODUCTION

It is well known that proline metabolism has important roles in carbon and nitrogen flux and protein synthesis. Proline metabolism has also emerged as a relevant pathway in other processes such as cell signaling, cellular redox balance, and apoptosis (1–3). Proline homeostasis is important in human disease, where inborn errors in proline metabolism are thought to lead to neurological dysfunctions such as schizophrenia and febrile seizures, as well as errors in systemic ammonia detoxification and developmental disorders such as skin hyperelasticity (4–7). Recently it was shown that mutations that disrupt proline biosynthesis are linked with progeroid features and osteopenia that are part of the autosomal recessive cutis laxa syndrome (8). In bacteria and plants, proline metabolism is responsive to various environmental stresses such as drought, osmotic pressure, or ultraviolent irradiation leading to proline accumulation as a survival mechanism (9–11). Overall proline has become a very important metabolite that is thought to be involved in many cellular processes.

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Fundamental to understanding the roles of proline metabolism in various processes is knowledge of the relevant enzymes and mechanisms used to maintain proper proline homeostasis. In this review, the unique aspect of substrate channeling in proline metabolism will be explored. Insights into the channeling mechanisms of enzymes responsible for the catabolism and biosynthesis of proline are helping to reveal the many roles of proline within the cell. Here we review the structural and kinetic data that support substrate channeling of P5C/GSA and gamma–glutamyl phosphate in the proline catabolic and biosynthetic pathways, respectively. The data indicate that both intermediates are channeled, which increases the efficiency of proline metabolic flux.

3. PROLINE METABOLIC ENZYMES

3.1. Proline catabolism

The catabolic and anabolic reactions of proline metabolism are shown in Figure 1. The catabolic pathway generates glutamate from the four electron oxidation of proline, which occurs in two catalytic steps (12). In the first step, proline dehydrogenase (PRODH; EC 1.5.99.8) uses a flavin adenine dinucleotide (FAD) cofactor as an electron acceptor to remove two electrons from proline, rendering the intermediate 1-delta-pyrroline-5-carboxylate (P5C). P5C then undergoes a non-enzymatic hydrolysis, which opens the ring structure and generates gamma-glutamate semialdehyde (GSA). Pyrroline-5-carboxylate dehydrogenase (P5CDH; EC 1.5.1.12) next pulls off two additional electrons from GSA using nicotinamide adenine dinucleotide (NAD^+) to complete the conversion of proline to glutamate (12).

The PRODH and P5CDH enzymes involved in the oxidation of proline are highly conserved in both eukaryotes and prokaryotes, but differ in whether they are fused into a bifunctional enzyme called proline utilization A (PutA). As reviewed by Tanner, PRODH enzymes can be divided into three branches (13). One branch consists of monofunctional enzymes, where the PRODH and P5CDH domains are found as separate enzymes. The other two branches have the PRODH and P5CDH domains on a single PutA polypeptide (13). Originally it was thought that all prokaryotes contain bifunctional PutAs, and that all eukaryotes contain monofunctional enzymes. However, it is now known that Gram-positive bacteria contain monofunctional enzymes, thus limiting PutAs to Gram-negative bacteria (14).

Figure 2 summarizes the domain organization of PRODH and P5CDH enzymes. Monofunctional PRODHs typically are 200–540 amino acid residues in length, while monofunctional P5CDHs are composed of 400–600 residues. *Thermus thermophilus* PRODH and P5CDH are currently the only structures of monofunctional enzymes that have been solved (PDB ID 2G37, 2EKG, 2BHP, 2BJA) (14–16). PutAs consist of 1000–1350 residues with the P5CDH domain linked to the C-terminal end of the PRODH domain (13). The two branches of PutA enzymes are distinguished by whether or not PutA also contains an N-terminal ribbon-helix-helix (RHH) DNA binding domain. PutAs that contain a DNA binding domain are trifunctional and are generally longer polypeptides than PutAs that lack a DNA binding domain (17–19). Trifunctional PutAs act as transcriptional repressors—when cellular proline is scarce, PutA binds DNA and represses expression of the *putA* and *putP* (Na^+/proline transporter) genes (20, 21). Regulation of PutA is achieved through a functional switching mechanism, where the redox state of flavin determines whether PutA is bound to the DNA and acts as a transcriptional repressor or is peripherally bound to the membrane where it efficiently catabolizes proline (22).

Recently, the first crystal structure of a complete PutA protein (*Bradyrhizobium japonicum*) was solved by Tanner's group (PDB ID 3HAZ). Previously, the only structures available for PutA were of the isolated PRODH and DNA binding domains. The PRODH domain

structure was solved for PutA from *Escherichia coli* (PDB ID 1K87, 1TJ2, ITIW, 1TJ0, 3ITG) (23–26), and the DNA binding domain of PutA was solved by solution NMR (*Pseudomonas putida*) and X-ray diffraction (*E. coli*) (PDB ID 2JXG, 2GPE, 2RBF) (27–29). These structures show that the PRODH domain is a conserved $beta_8alpha_8$-barrel, while the P5CDH domain contains a well conserved Rossmann fold domain. The evolutionary divergence from bifunctional PutA to monofunctional PRODH and P5CDH is of interest due to substrate channeling between the active sites in bifunctional PutA. Substrate channeling between monofunctional enzymes would necessitate functional PRODH-P5CDH interactions, which have not yet been explored.

3.2. Proline biosynthesis

Proline biosynthesis from glutamate involves three enzymatic steps (Figure 1). The initial two steps are catalyzed by gamma-glutamyl kinase (GK; EC 2.7.2.11) and gamma-glutamyl phosphate reductase (GPR; EC 1.2.1.41). GK uses adenosine-5′-triphosphate (ATP) to generate gamma-glutamyl phosphate, which is subsequently reduced by GPR using nicotinamide adenine dinucleotide phosphate (NADPH) to produce GSA (12). GSA next cyclizes to P5C, which is a crossroads intermediate that, in principle, can be converted not only to proline, but also to ornithine or back to glutamate via P5CDH (12). The reduction of P5C to proline is catalyzed by P5C reductase (P5CR; EC 1.5.1.2), while the production of ornithine from P5C requires ornithine aminotransferase (OAT; EC 2.6.1.13), an enzyme that is important for balancing cellular nitrogen levels (12).

In bacteria and lower eukaryotes such as yeast, GK and GPR are discrete monofunctional enzymes. In animals and plants, the GK and GPR domains are fused together into the bifunctional enzyme P5C synthase (P5CS) (Figure 3). The GK and GPR domains are well conserved in lower eukaryotes and bacteria. The GK domain is normally 250–450 residues in length with an N-terminal amino acid kinase (AAK) domain. In bacteria, GK contains a C-terminal pseudo uridine synthase and archaeosine-specific transglycosylase (PUA) domain, which has no known function (30). It has been suggested, however, that the PUA domain may enable bacterial GK to have a gene regulatory role (31). The structures of GK enzymes from *E. coli* and *Campylobacter jejuni* have been solved (PDB ID 2J5T, 2AKO) (32). *E. coli* GK is composed of an N-terminal catalytic domain made up of eight nearly parallel beta-sheets sandwiched by two layers of three and four alpha-helices. It is connected by a linker region to the PUA domain, which contains a distinctive beta sandwich (32).

GPR typically contains 400–500 residues and consists of an N-terminal Rossmann fold domain for NADPH binding, a catalytic domain, and an oligomerization domain at the C-terminus (33). The X-ray crystal structure of GPR from *Thermotoga maritima* reveals that the catalytic domain has an alpha/beta architecture with a five-stranded parallel beta-sheet (PDB ID 1O20) (33). To date, no complete structure of bifunctional P5CS has been reported. However, the structure of the isolated GPR domain (PDB ID 2H5G; unpublished) from human P5CS is available. The last enzyme of the proline biosynthetic pathway, P5CR, ranges from 400–500 residues in length and has a conserved N-terminal Rossmann fold for NADPH binding. Several crystal structures of P5CR have been determined, including the human form (PDB ID 2GRA) (34). Human P5CR has an active site cleft made of an 8-stranded beta-sheet sandwiched by alpha-helices on either side and oligomerizes to form a decameric structure of dimers (34).

4. INTERMEDIATES OF PROLINE METABOLISM

The P5C/GSA and gamma–glutamyl phosphate intermediates of proline metabolism are appreciably labile and reactive. Figure 4 shows examples of undesirable fates that can occur with these intermediates. The instability of the intermediates implies substrate channeling

may be important for maintaining efficient proline metabolic flux. The intermediate shared by the catabolic and biosynthetic pathways, P5C/GSA, has been shown to inhibit other enzymes, react with metabolites, and act as a signaling molecule. GSA has been reported to inhibit glucosamine-6-phosphate synthase from *E. coli*, cytidine 5′-triphosphate synthase, and the amidotransferase domain of carbamoyl phosphate synthetase (35–37). Additionally, P5C forms adducts with other metabolites such as pyruvic acid, oxaloacetic acid, and acetoacetic acid (38). P5C can also react with pyridoxal phosphate in patients with type II hyperprolinemia. Type II hyperprolinemia is characterized by elevated plasma levels of P5C/GSA due to deficient P5CDH activity (39). The high levels of P5C/GSA generate inactive adducts with pyridoxal phosphate, leading to lower amounts of functional vitamin B6 in patients (Figure 4) (38). P5C also acts as a signaling molecule in eukaryotes and is thought to induce apoptosis by increasing intracellular reactive oxygen species (40, 41). Altogether, it seems that controlling levels of free P5C/GSA would be beneficial.

The reactive intermediate in proline biosynthesis is gamma–glutamyl phosphate. The carbonyl phosphate group is susceptible to nucleophilic attack, resulting in the spontaneous cyclization of gamma–glutamyl phosphate into 5-oxoproline as shown in Figure 4 (42, 43). It has been suggested that 5-oxoproline is a neurotoxin. Interstitial injection of 5-oxoproline into rats produces behavioral and neuropathological effects that resemble Huntington's disease (44, 45). The instability of gamma–glutamyl phosphate seems to necessitate its channeling between GK and GPR during proline biosynthesis.

5. OVERVIEW OF SUBSTRATE CHANNELING

5.1. Rationale for substrate channeling

Substrate channeling is a phenomenon where the product of one reaction is transported to a second active site without equilibrating into bulk solvent (46). Three mechanisms of substrate channeling have been defined, two of which are reviewed by Miles *et al.* (47). The most common form of substrate channeling occurs when a cavity exists within a protein that sequesters the intermediate from solvent, allowing for a means of travel between active sites (47). To date, several enzymes are known to utilize these intramolecular tunnels, with the classic example being tryptophan synthase (48). The second form of channeling does not use intramolecular cavities; rather, electrostatic residues on the surface of the enzyme guide the intermediate from the first active site to the second active site (47). Dihydrofolate reductase-thymidylate synthase complex stands as the common example for this form of channeling (49). A third form of channeling exists in protein complexes such as pyruvate dehydrogenase, which uses cofactor lipoic acid to transfer substrate to multiple active sites without contacting solvent (50).

Substrate channeling has been proposed to be advantageous in the cellular environment for several reasons, as outlined by Ovadi and others (46, 51). First and foremost it increases the efficiency of coupled reactions both by preventing the loss of intermediates to diffusion and by decreasing transit time between active sites. This allows the steady-state flux through the coupled steps to be attained more rapidly (46). Secondly, it prevents labile intermediates from decaying and reacting with other metabolites or enzymes within the cell (46). Third, channeling segregates intermediates that may require a specific environment (e.g., pH) to retain structure or reactivity. Channels can provide an environment that facilitates an equilibrium step that normally would be unfavorable in the bulk solution. Finally, channeling limits intermediates from being siphoned out into competing reactions or pathways (46).

All of the benefits listed above are not necessarily relevant for every channeling system. In the proline catabolic pathway, channeling of P5C/GSA may be most critical for making the

hydrolysis of P5C to GSA more favorable at physiological pH values. The P5C/GSA equilibrium is highly pH dependent (35). GSA is favored only below pH 6.5 due to protonation of the pyrrolinium ring, which facilitates the hydrolysis of P5C to GSA. Thus, one benefit of channeling between PRODH and P5CDH would be to increase the pK_a of the pyrrolinium species above pH 6.5, making the hydrolysis of P5C to GSA more favorable at physiological pH conditions. If we only consider the P5C/GSA hydrolysis step, substrate channeling is likely more critical for the proline catabolic pathway than for proline biosynthesis, since P5C formation is favored at physiological pH. In the proline biosynthetic pathway, protecting the highly labile gamma–glutamyl phosphate would be a clear benefit of substrate channeling between GK and GPR.

5.2. Kinetic approaches to test for substrate channeling

Different strategies have been devised to examine whether channeling occurs between enzymes. Before reviewing the evidence for substrate channeling in proline metabolism, a short description of various experimental methods is described here.

5.2.1. Transient time estimation—A common strategy to test for channeling is to evaluate whether there is a lag time in reaching steady-state formation of the final product in a coupled assay. Figure 5 shows substrate (S) being converted to the final product (P) via the coupled action of two enzymes (E1 and E2). The lag time or transient time, Tau, is the time preceding the build-up to steady-state formation of the final product using the substrate of the first enzyme (52). If no channeling occurs, Tau should be equal to the ratio of K_m/V_{max} of the second enzyme. If the observed lag time is shorter than the K_m/V_{max} ratio, then it infers that the intermediate is transferred between the enzymes, E1 and E2. The extent of the observed lag time may vary among different channeling systems with a shorter transient time being interpreted as more efficient transfer or channeling (53). Along with steady-state assays, pre-steady state measurements can also be made to evaluate the lag time prior to product formation.

5.2.2. Trapping the intermediate—The effect of a reagent that specifically traps the intermediate species (I, Figure 5) on the kinetics of product formation can also be used to evaluate channeling. For example, *o*-aminobenzaldehyde (*o*-AB) which reacts with P5C to form a yellow complex can be used as a trapping agent for the PRODH and P5CDH coupled reaction. *o*-AB would be anticipated to decrease the overall rate of glutamate formation if no channeling occurs, while in a channeling system *o*-AB would have a negligible effect on the reaction kinetics. Using a third enzyme that competes with E2 for the intermediate can also be an effective strategy to test for substrate channeling.

5.2.3. Inactive mutants—Another useful tool is to generate active site mutants of the two enzymes being studied (Figure 5B). In the case of suspected channeling partners, an active site mutant (e.g., E2) would be expected to compete with its native counterpart for interaction with the cognate enzyme (E1). If channeling occurs, adding the inactive E2 mutant in amounts excess to that of native E2 would decrease product formation. If no channeling occurs, adding the inactive E2 mutant to the coupled enzyme assay would have no effect on the rate of product formation. This strategy was effectively used to rule out channeling between aspartate aminotransferase (AAT) and malate dehydrogenase (MDH) (54).

If the channeling involves two enzyme active sites that are covalently linked, active site mutants can be used to generate a non-channeling control. Figure 5C illustrates that combining active site mutants of E1 and E2 creates a mixture of monofunctional enzyme variants that can only generate product via a diffusion mechanism. The transient times of the

native enzyme and the mixed enzyme variants can then be compared to distinguish between channeling and non-channeling mechanisms. This strategy was used recently to demonstrate channeling in bifunctional PutA.

5.2.4. Designing fusion proteins—Two active sites that are in close proximity can sometimes exhibit kinetic behavior that resembles direct channeling (55). One strategy for distinguishing between active channeling and proximity effects is to change the relative orientation of two active sites, which is important for interacting enzymes (56). A polypeptide linker can be engineered to covalently link the two enzymes with various degrees of flexibility and in different orientations (55). If the enzymes are truly channeling, changes in the orientation of the active sites will have a dramatic effect on the kinetics of product formation.

6. CHANNELING OF P5C/GSA

The oxidation of proline to glutamate is catalyzed in consecutive reactions by PRODH and P5CDH (Figure 1). Avoiding release of P5C/GSA into bulk solvent during proline oxidation may be beneficial due to the chemical properties of P5C/GSA as discussed in the previous section. Evidence for channeling P5C/GSA has recently been shown for bifunctional PutA from *B. japonicum* (BjPutA). Srivastava *et al* reported a 2.1 Å resolution crystal structure of BjPutA (999-residue polypeptide) that reveals an interior channel connecting the PRODH and P5CDH active sites (PDB ID 3HAZ) (23). BjPutA purifies as a dimer-of-dimers tetramer. The dimer is the relevant species for discussing channeling and is shown in Figure 6. Both PRODH and P5CDH domains contribute to the formation of the channel, with the two active sites separated by a distance of 41 Å. The connecting channel appears to start at the *si* face of the isoalloxazine ring of FAD and end at the catalytic cysteine (Cys792) of the P5CDH domain (Figure 6). Within the channel, the central cavity is lined by fifteen basic residues (Lys and Arg) and seventeen acidic residues (Glu and Asp), imparting a hydrophilic nature to the channel. Each of the PutA protomers has an individual channel connecting PRODH and P5CDH active sites.

Interestingly, the dimeric structure of BjPutA seems to be critical for sealing the channel and minimizing access to bulk solvent. A beta-flap protrudes from the P5CDH domain (beta strands, residues 628–646, 977–989) from one protomer and forms intermolecular interactions with the P5CDH domain of the second protomer (Figure 6). This beta-flap is structurally conserved in *Thermus thermophilus* P5CDH (PDB ID 1UZB, residues 163–174,506–516) as well as a class I aldehyde dehydrogenase isolated from sheep liver (PDB ID 1BXS, residues 147–159, 486–498) (57) (16) and is sometimes referred to as the oligomerization domain. Thus, in BjPutA, the beta-flap not only helps stabilize dimer formation but also is important for sealing the central cavity.

Along with these structural features of channeling, kinetic evidence for channeling was also reported for BjPutA by Srivastava *et al.* Different experiments have provided strong evidence for channeling. First, the amount of P5C released into bulk solvent was quantitated by using *o*-aminobenzaldehyde (*o*-AB) as a trapping agent. P5C and *o*-AB react to form a yellow complex that can be monitored at 443 nm (58). In the absence of NAD^+, the P5CDH domain is inactive and leads to significant release of P5C into the bulk solvent, as detected by the yellow complex formation. In the presence of NAD^+, however, the P5CDH domain is active, resulting in significantly lower *o*-AB-P5C complex formation, as the majority of P5C is converted into glutamate (23). The apparent fraction of P5C that is channeled in BjPutA from PRODH to P5CDH was estimated to be 0.7 by these measurements.

Substrate channeling in BjPutA was also examined by estimating the transient time to reach steady-state turnover of the second enzyme, P5CDH, using proline as a substrate (23, 59, 60). With native BjPutA, steady-state formation of NADH (product of the P5CDH reaction) occurred without any apparent lag time (23). The absence of a lag time in the approach to steady-state indicates substrate channeling. A non-channeling control was also analyzed using active site mutants of BjPutA that lack PRODH (R456M) and P5CDH (C792A) activity (23). The R456M mutation inactivates PRODH but does not impair P5CDH activity, whereas the C792A mutation inactivates P5CDH but does not impair PRODH activity. The mixture of these monofunctional variants was used as a non-channeling control as described above. In this non-channeling control, P5C formed by the C792A variant must diffuse out into bulk solvent and bind to the R456M variant before NADH is formed. In the assays with the non-channeling variants, a lag time of about seven minutes for NADH formation was observed (23). The observed lag time was similar to the theoretical Tau value calculated from the independent PRODH activity and P5CDH kinetic parameters. An example of these steady-state assays is shown in Figure 7. Figure 7 illustrates the clear difference in the kinetic behavior of native BjPutA and the non-channeling control. With native BjPutA, NADH formation is observed without a lag time, while with the mixed variants a lag time of around 6.5 minutes is observed. The results from these assays are consistent with a substrate channeling mechanism in BjPutA. Kinetic profiles of native BjPutA and the mixed variants were also compared by rapid-reaction kinetics under anaerobic, single-turnover conditions. Rapid mixing of native BjPutA and proline generated NADH with no apparent lag time. For the non-channeling variants, a 10 s lag time for NADH formation was observed after mixing the enzymes with proline. These results show native BjPutA efficiently channels P5C/GSA.

Evidence for channeling in PutA has also been reported from *Salmonella typhimurium* PutA (StPutA). Similar to EcPutA, StPutA contains the N-terminal DNA binding domain and is thus trifunctional. Maloy *et al.* demonstrated that the P5CDH domain shows a 14-fold greater steady-state production of NADH using P5C generated endogenously from proline by PRODH, as compared to exogenously added P5C (61). In addition, they showed exogenous P5C was unable to compete against endogenous P5C. Due to a lack of structural information on trifunctional PutAs, it is not clear whether a channel similar to that characterized in BjPutA exists. Future structural and kinetic experiments will need to be performed to fully address the channeling mechanism in trifunctional PutAs.

7. CHANNELING OF GAMMA-GLUTAMYL PHOSPHATE

As mentioned previously, channeling of the intermediate gamma-glutamyl phosphate would be beneficial because of its instability. Channeling of gamma-glutamyl phosphate is also implicated by the fusion of GK and GPR in bifunctional P5CS. Kinetic data have existed for over forty years suggesting that a complex forms between bacterial GK and GPR in order to conceal gamma-glutamyl phosphate from solvent (62, 63). A typical assay to measure GK activity is to add hydroxylamine along with the substrates, glutamate and ATP. Hydroxylamine reacts with the product gamma-glutamyl phosphate to make gamma-glutamyl hydroxamate, which can be measured at 535 nm (62). Multiple groups have documented that GK activity is dependent on the presence of GPR. GK is inactive or exhibits very low activity in the absence of GPR, suggesting GPR is required for GK activity (42, 43, 62, 64, 65). It was found that a 10:1 GPR:GK ratio was necessary to obtain maximal GK activity, indicating that a GK/GPR complex forms with excess GPR (64). To test for a complex, Smith *et al.* tried incubating different ratios of bacterial GK and GPR, then looked for co-elution of the enzymes by chromatography (64). Both proteins eluted separately meaning either a complex does not form or complex formation is transient and is dependent on substrate binding. Other work suggesting a GK-GPR complex includes assays which contained GK and GPR, but lacked NADPH, the cofactor necessary for GPR activity

(43). In this case the GPR enzyme was inactive, but it still activated GK. GK has also been shown to be activated by incubation with GPR mutants, further demonstrating that GK activation by GPR does not require GPR activity (66). Other experiments that have explored GK/GPR interactions include Chen *et al.*, who created a mutant GK/GPR fusion protein that was able to over-produce proline, making the host *E. coli* strain more resistant to osmotic stress (67). While this work does not support channeling directly, it does show that enhancing the proximity of two active sites can significantly increase the efficiency of a metabolic pathway (67).

Structural data supporting channeling is not directly available for GK and GPR enzymes (68). Figure 8 shows the individual structures of *E. coli* GK (EcGK) and *T. maritima* GPR (TtGPR). Marco-Marin *et al.* modeled a possible interaction between monofunctional GK and GPR, showing GPR in both an open and closed conformation, depending on the binding status of the substrate (32). The model shows a tetrameric form of GK from *E. coli* complexed with a dimer of GPR from *T. maritima* (32). In solving the crystal structure EcGK, Marco-Marin *et al.* noted that GK was well suited for channeling (32). They suggested that channeling is possible if the GK and GPR active sites are positioned so that the active site cysteine of GPR is able to react with gamma-glutamyl phosphate while still bound at the GK active site. A complex as described would allow for a favorable environment and timely transfer of gamma-glutamyl phosphate to the second active site, thereby preventing cyclization to 5-oxoproline (32). Additionally it has been suggested that leucine zipper motifs found in the GK and GPR domains of plants, as well as the GK and GPR enzymes of some bacteria are evidence for a functional complex (69). The leucine zipper of plant P5CS may help with oligomerization or it may be an artifact of evolution. Several domain swapping experiments have shown that leucine zippers mediate protein-protein dimerization in eukaryotic and prokaryotic enzymes. Thus, the leucine zippers in bacterial GK and GPR enzymes and plant P5CS may support a possible channeling complex (70).

8. SUMMARY

Proline metabolism has become a very important area of study due to its involvement in many different cellular processes from maintaining redox balance to countering environmental stress. As described in this review, substrate channeling is a relevant mechanism in proline metabolism for translocating important intermediates between active sites. Rationale for substrate channeling in proline metabolism is two-fold. First, the equilibrium for the hydrolysis of P5C to GSA is unfavorable at physiological pH, indicating channeling may be necessary to increase the overall conversion efficiency of proline into glutamate. Second, P5C/GSA and gamma-glutamyl phosphate are reactive and labile intermediates. Channeling of these intermediates would protect against the formation of unwanted products, such as 5-oxoproline from gamma-glutamyl phosphate.

Future kinetic and structural studies are important for understanding the mechanisms of substrate channeling in proline metabolism. In proline catabolism, BjPutA provides structural and kinetic data supporting channeling, but more work needs to be done on trifunctional and monofunctional enzymes. Substrate channeling in PutAs suggests that bacteria have evolved a strategy to limit the availability of P5C to other competing pathways. P5C is at the crossroads of important metabolic pathways, which include proline oxidation, urea cycle, TCA cycle via glutamate, and the proline biosynthetic pathway (71). Substrate channeling by PutA may help maintain flux through the proline oxidative pathway, which would be especially important under poor nutrient conditions in cells starved for nitrogen and glutamate and other downstream products such as alpha–ketoglutarate. Whether P5C/GSA is channeled in Gram-positive bacteria and eukaryotes is

not yet known. Studies of PRODH-P5CDH coupled kinetics and potential PRODH-P5CDH interactions are needed to address channeling between monofunctional PRODH and P5CDH enzymes. In proline biosynthesis, evidence for interactions between monofunctional GPR and GK has been reported, but structural evidence for channeling in bifunctional P5CS is currently not available. Although channeling interactions between GK and GPR seem likely and are supported by several studies, additional work is required to define the channeling pathway and mechanisms in proline biosynthesis. To date, a full-length structure of P5CS has not been reported. Solving a crystal structure of P5CS would be a significant step toward understanding channeling of gamma-glutamyl phosphate. Not only would a complete structure of P5CS act as a template for modeling the interaction between monofunctional GK and GPR, but it could also be used to identify cavities within the protein that may act as channels for transporting gamma-glutamyl phosphate between active sites.

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Abbreviations

PRODH	proline dehydrogenase
FAD	flavin adenine dinucleotide
P5C	pyrroline-5-carboxylate
GSA	glutamic semialdehyde
P5CDH	pyrroline-5-carboxylate dehydrogenase
NAD^+	nicotinamide adenine dinucleotide
PutA	proline utilization A
P5CS	pyrroline-5-carboxylate synthase
NADPH	nicotinamide adenine dinucleotide phosphate
GK	gamma-glutamyl kinase
ATP	adenosine-5′-triphosphate
GPR	gamma-glutamyl phosphate reductase
gamma-GP	gamma-glutamyl phosphate
P5CR	pyrroline-5-carboxylate reductase
AAK	amino acid kinase

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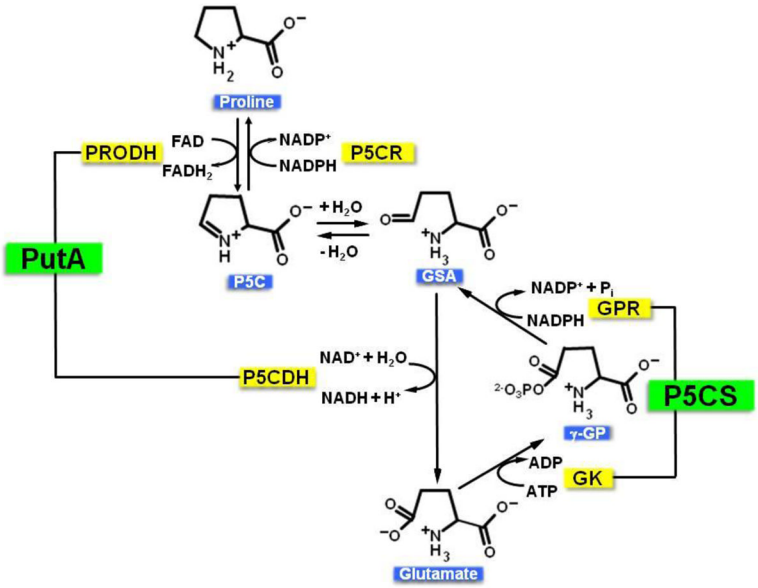

Figure 1.

Reactions of the proline metabolic pathway. In the catabolic pathway, proline is converted to glutamate via a four electron oxidation process. Proline dehydrogenase (PRODH) performs the first oxidative step, resulting in the intermediate pyrroline-5-carboxylate (P5C). P5C is subsequently hydrolyzed to glutamic semialdehyde (GSA), which is then further oxidized by P5C dehydrogenase (P5CDH) to generate glutamate. In Gram-negative bacteria, PRODH and P5CDH are fused together on a bifunctional enzyme called proline utilization A (PutA). Proline anabolism begins with phosphorylation of glutamate by gamma-glutamyl kinase (GK) to generate gamma-glutamyl phosphate (gamma-GP). gamma-GP is reduced by gamma-glutamyl phosphate reductase (GPR) to GSA, which cyclizes to form P5C. P5C is then reduced to proline via pyrroline-5-carboxylate reductase (P5CR). In higher eukaryotes such as plants and animals, GPR and GK are fused together in the bifunctional enzyme pyrroline-5-carboxylate synthase (P5CS).

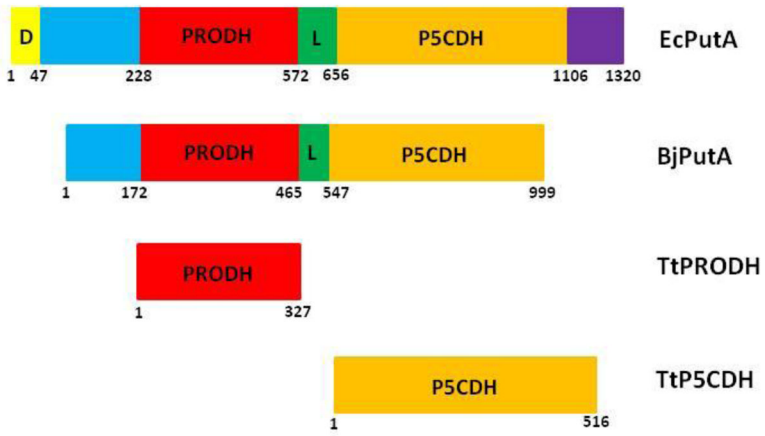

Figure 2.

Domain mapping of PRODH and P5CDH from *E. coli* (EcPutA), *B. japonicum* (BjPutA), and *T. thermophilus.* In PutAs, the PRODH and P5CDH domains are connected by a linker region (L). Trifunctional PutAs such as EcPutA also have a DNA binding domain (D). TtPRODH and TtP5CDH are separate enzymes (monofunctional) in the Gram-positive bacteria, *T. thermophilus*.

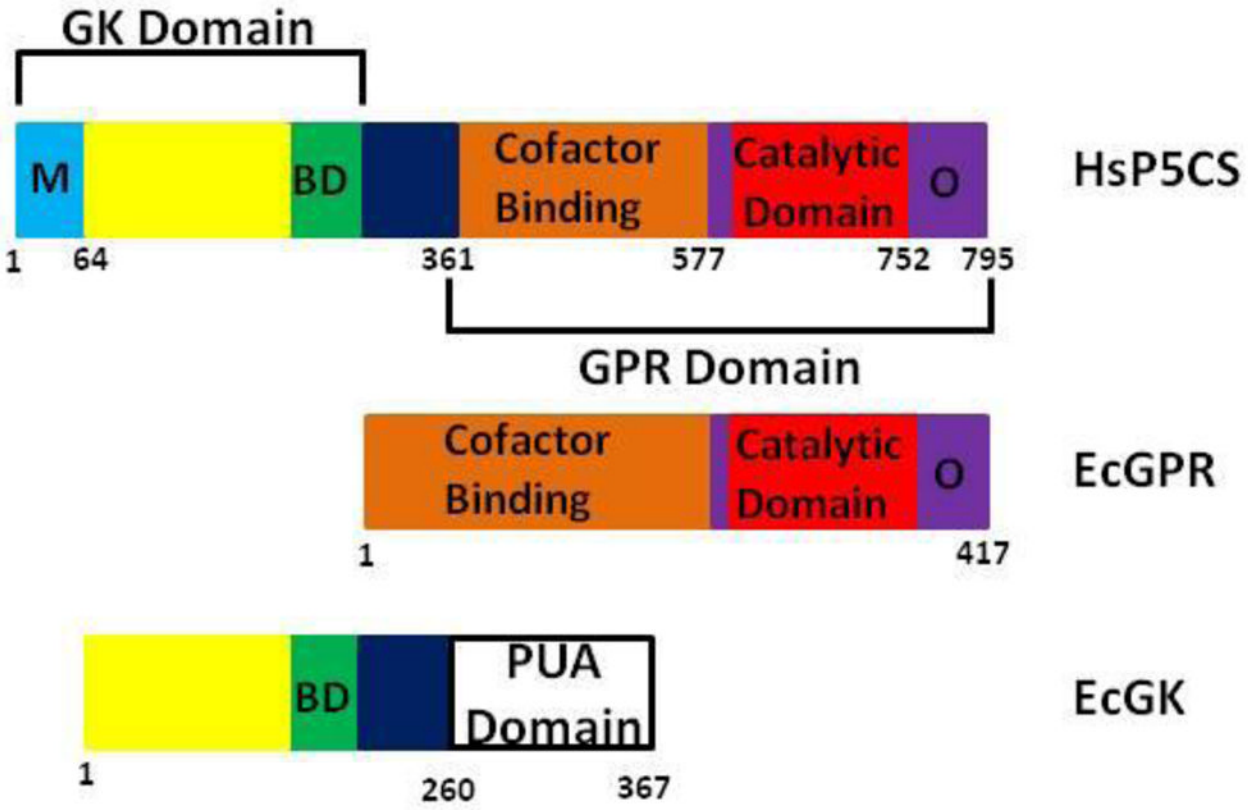

Figure 3.

Domain mapping of monofunctional gamma-glutamyl phosphate reductase (EcGPR) and gamma-glutamyl kinase (EcGK) enzymes from *E. coli* and bifunctional pyrroline-5-carboxylate synthase (P5CS) from *Homo sapiens.* M, putative mitochondrial signaling peptide, BD, binding domain for glutamate and ATP, O, oligomerization domain, and PUA, pseudo uridine synthase and archaeosine-specific transglycosylase domain with no known function in EcGK.

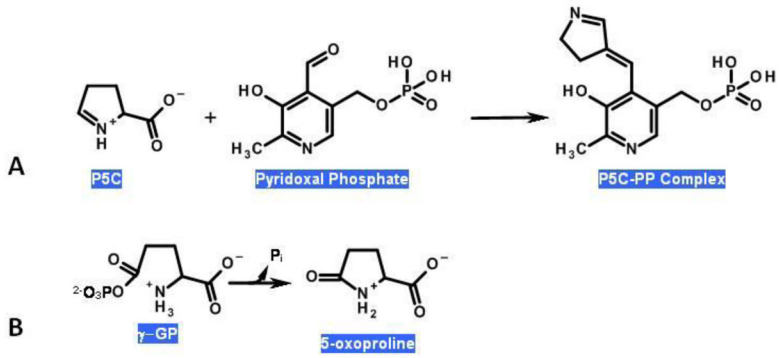

Figure 4.

Side reactions of intermediates pyrroline-5-carboxylate (P5C) and gamma-glutamyl phosphate (gamma-GP). (A) P5C can deactivate pyridoxal phosphate by forming an adduct, resulting in vitamin B6 deficiency in individuals with hyperprolinemia type II. The P5C-pyridoxal phosphate adduct structure is from reference 38. (B) gamma-GP can cyclize and dephosphorylate to form 5-oxoproline, which is suggested to be a neurotoxin in rats.

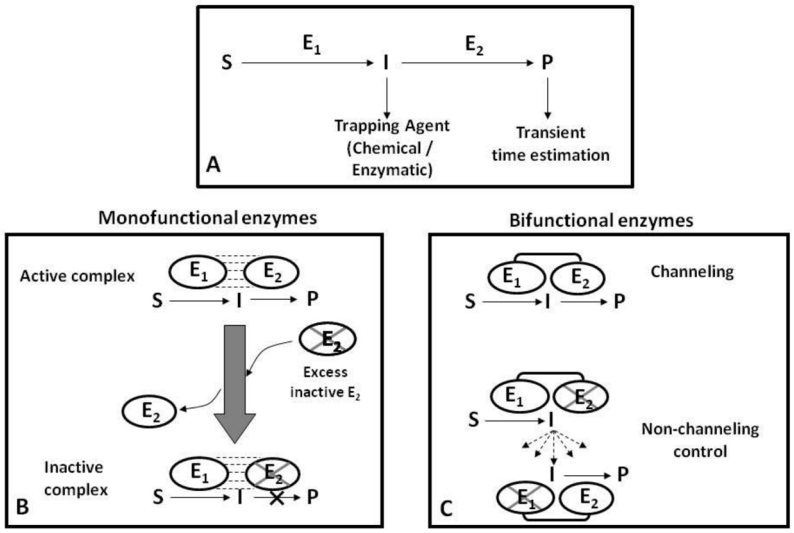

Figure 5.

Strategies for examining substrate channeling. (A) Transient time analysis of a coupled reaction involving two enzymes, E1 and E2, which convert substrate A into product C. A trapping agent can also be used to test whether intermediate B is released into bulk solvent during the reaction. (B) Inactivation of one of the enzyme pairs by site-directed mutagenesis. If channeling occurs, adding inactive E2 would disrupt the active E1–E2 complex resulting in lower steady-state activity. (C) Testing channeling in bifunctional enzymes. Inactivation of the individual domains results in monofunctional variants that can only catalyze the coupled reaction via a diffusion mechanism. The mixture of monofunctional variants is thus a non-channeling control.

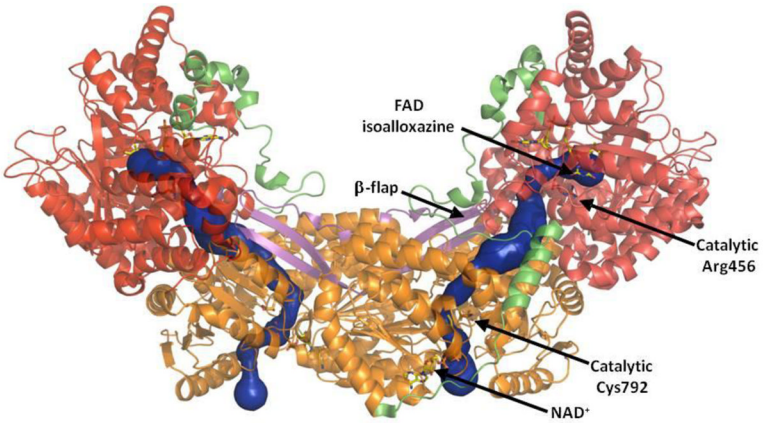

Figure 6.

Structure of dimeric BjPutA shown in ribbon representation. The PRODH domain (red) and the P5CDH domain (orange) of each protomer are connected by a linker region (green). Active site residues (Arg456, Cys792), FAD and NAD^+ are displayed as sticks. β-flap of each protomer is colored as magenta. The substrate channel of each BjPutA protomer is shown as blue surface. This model was made using PyMol, CAVER and PDB 3HAZ.

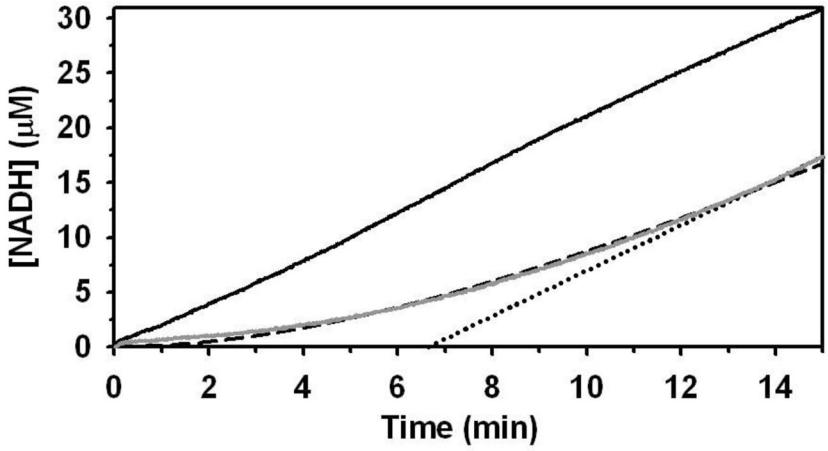

Figure 7.

Example of transient time analysis of BjPutA. Steady-state formation of NADH using proline as a substrate by native BjPutA (solid black curve) and an equimolar mixture of monofunctional variants R456M and C792A (solid grey curve). The mixture of the monofunctional variants serves as a non-channeling control. The dotted line represents the extrapolation used for estimating the lag-time. Native BjPutA shows no apparent lag in NADH formation, while a lag time of about 6.5 min is observed for the non-channeling control. The dashed line overlaying the grey curve of the non-channeling control reaction was simulated using the kinetic parameters of PRODH and P5CDH as described previously and the following equation: $[NADH] = v_1 t + (v_1/v_2)K_m(e^{-v2t/Km} - 1)$ (21, 72). Assays were performed at pH 7.5.

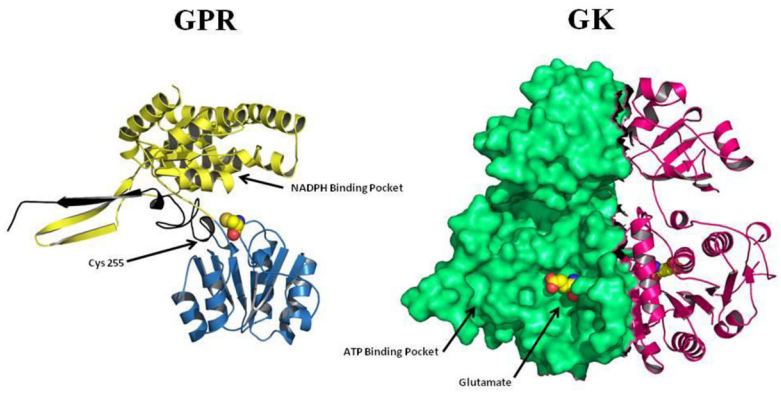

Figure 8.

Structures of GPR from *T. maritima* (TmGPR) and GK from *E. coli* (EcGK). EcGK is shown as a dimer with one monomer shown in surface representation and the other monomer as a ribbon cartoon illustration. Glutamate is shown as spheres in the substrate binding pocket, which is solvent accessible. Only one monomer of GPR (open conformation) is shown, which contains three domains: NADPH binding domain (yellow), catalytic domain (blue) with the catalytic cysteine shown in spheres, and the oligomerization domain (black). The solvent-exposed glutamate binding pocket of GK suggests that the gamma-glutamyl phosphate intermediate would be accessible to GPR in a potential GK-GPR complex. A GK-GPR complex in which the catalytic domain of GPR is aligned with the glutamate binding pocket of GK has been proposed and modeled by Marco-Marin *et al.* (32). Models shown here were made using PyMol and PDBs 2J5T (EcGK) and 1O20 (TmGPR).