ANTIOXIDANTS & REDOX SIGNALING
Volume 19, Number 9, 2013

DOI: 10.1089/ars.2012.5074

Discoveries

FORUM REVIEW ARTICLE

Proline Mechanisms of Stress Survival

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Abstract

***Significance*:** The imino acid proline is utilized by different organisms to offset cellular imbalances caused by environmental stress. The wide use in nature of proline as a stress adaptor molecule indicates that proline has a fundamental biological role in stress response. Understanding the mechanisms by which proline enhances abiotic/biotic stress response will facilitate agricultural crop research and improve human health. ***Recent Advances*:** It is now recognized that proline metabolism propels cellular signaling processes that promote cellular apoptosis or survival. Studies have shown that proline metabolism influences signaling pathways by increasing reactive oxygen species (ROS) formation in the mitochondria *via* the electron transport chain. Enhanced ROS production due to proline metabolism has been implicated in the hypersensitive response in plants, lifespan extension in worms, and apoptosis, tumor suppression, and cell survival in animals. ***Critical Issues*:** The ability of proline to influence disparate cellular outcomes may be governed by ROS levels generated in the mitochondria. Defining the threshold at which proline metabolic enzyme expression switches from inducing survival pathways to cellular apoptosis would provide molecular insights into cellular redox regulation by proline. Are ROS the only mediators of proline metabolic signaling or are other factors involved? ***Future Directions*:** New evidence suggests that proline biosynthesis enzymes interact with redox proteins such as thioredoxin. An important future pursuit will be to identify other interacting partners of proline metabolic enzymes to uncover novel regulatory and signaling networks of cellular stress response. *Antioxid. Redox Signal*. 19, 998–1011.

Introduction

Almost three decades ago, the proline metabolic pathway was proposed to have a regulatory function in oxidation–reduction homeostasis and cell survival (95). Now, numerous laboratories have shown that the imino acid proline impacts a wide range of cellular processes, including bioenergetics, differentiation, growth, lifespan, and apoptosis (30, 70, 74, 75, 84, 95, 97, 99, 153). It is well established that proline metabolism leads to increased mitochondrial reactive oxygen species (ROS) production *via* the electron transport chain (ETC) and that proline metabolism impacts cell survival and cell death in different species (14, 30, 84, 97). In plants, the protective effect of proline during stress is especially well documented (123). Here, we review proline metabolic stress adaption in plants and examine the potential mechanisms of proline stress protection in different organisms.

Proline Metabolic Enzymes

The reactions of the proline metabolic pathway are shown in Figure 1. Proline is synthesized from glutamate by the enzymes Δ^1-pyrroline-5-carboxylate (P5C) synthetase (P5CS) and P5C reductase (P5CR). Alternatively, proline can be formed from ornithine, which is converted into P5C/GSA *via* ornithine-δ-aminotransferase (OAT) (2). The conversion of proline back to glutamate is catalyzed by proline dehydrogenase (PRODH) and P5C dehydrogenase (P5CDH). An overview of the proline metabolic enzymes is provided next.

P5C synthetase

P5CS catalyzes the NADPH-dependent reduction of glutamate to γ-glutamate-semialdehyde (GSA), which then spontaneously cyclizes to P5C (49, 110). The full-length cDNA encoding P5CS in multicellular eukaryotes was first cloned

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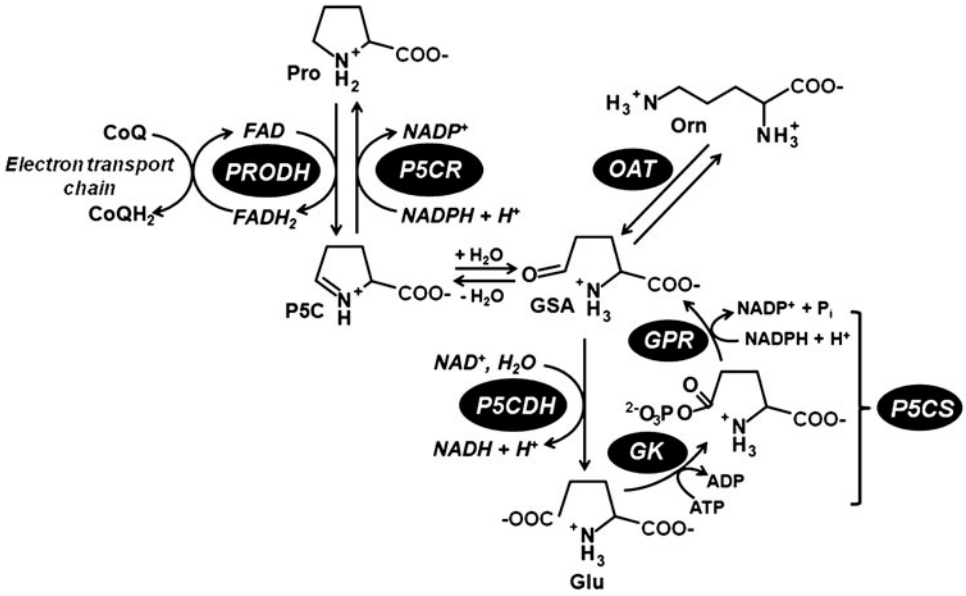

FIG. 1. Reactions of the proline metabolic pathway. Proline (Pro) is synthesized from glutamate (Glu) starting with the enzymes glutamate kinase (GK) and γ-glutamyl phosphate reductase (GPR), which in plants and animals are fused together in the bifunctional enzyme P5C synthetase (P5CS). The intermediate, γ-glutamate-semialdehyde (GSA), spontaneously cyclizes to Δ^1-pyrroline-5-carboxylate (P5C), which is then reduced to proline by P5C reductase (P5CR). Alternatively, GSA/P5C can be generated from ornithine and ornithine-δ-aminotransferase (OAT). Proline is oxidized back to glutamate by proline dehydrogenase (PRODH) and P5C dehydrogenase (P5CDH) in the mitochondrion. PRODH couples proline oxidation to the reduction of ubiquinone (CoQ) in the electron transport chain (ETC). In Gram-negative bacteria, PRODH and P5CDH domains are fused together in the PutA protein.

and characterized in plants (49). P5CS is a bifunctional adenosine triphosphate (ATP) and an NAD(P)H-dependent enzyme in higher eukaryotes that displays glutamate kinase (GK) and γ-glutamyl phosphate reductase (GPR) activities (49, 110). In primitive organisms such as bacteria and yeast, GK and GPR are monofunctional enzymes (6, 94). The structure of bifunctional P5CS has not been reported, but individual structures of GK and GPR are available from bacteria (79, 89). Important residues for glutamate binding in the GK domain are conserved among GK of different species (93, 94). Proline biosynthesis is feedback inhibited by proline binding to the GK domain and interfering with the glutamate binding site (93).

Recently, Engelhard *et al.* identified human P5CS as a potential target of mitochondrial thioredoxin 2 using an *in situ* kinetic trapping assay (32). This interesting finding indicates that P5CS is in the mitochondrial matrix and subject to redox regulation.

P5C reductase

The product of the P5CS reaction, P5C, is reduced to proline by P5CR using NAD(P)H as an electron donor (2). P5CR is conserved among bacteria, plants, insects, and vertebrates (82, 103). X-ray crystal structures of P5CR from different organisms, including humans, have been solved showing a conserved N-terminal Rossmann fold for NADPH binding (6, 82). In plants, P5CR is not only located in the cytosol but has also been shown to be expressed in chloroplasts (123, 131). There are three isoforms of P5CR in humans: PYCR1, PYCR2, and PYCRL. PYCR1 and PYCR2 are localized in mitochondria (24, 103), whereas PYCRL is cytosolic (24). P5CR is not only critical for synthesizing proline, but also has a critical role in cycling proline and P5C between cellular compartments and in maintaining proper $NADP^+$/NAPDH levels in the cytosol to drive the pentose phosphate pathway (84, 95).

Ornithine-δ-aminotransferase

OAT catalyzes the interconversion of ornithine and GSA with the flux direction determined by nutritional needs such as in neonate, where the overall flux from proline to arginine is favored (98, 142). In yeast, OAT is cytoslic (55); whereas in plants and humans, OAT is localized in the mitochondria (65, 116, 123).

Recently, Jortzik *et al.* reported that OAT of the malaria parasite *Plasmodium falciparum* (PfOAT) interacts with thioredoxin *via* Cys154 and Cys163, which are highly conserved residues in *Plasmodium* OAT but absent in other organisms (56). PfOAT also interacts with other cellular redox proteins such as glutaredoxin and plasmoredoxin and is reversibly regulated by S-glutathionylation (56, 60). It is of interest to note that no homolog of *P5CS* or *GK* and *GPR* can be found in the genome of *Plasmodium,* indicating that proline synthesis in *Plasmodium* may only be through the degradation of ornithine (56). These data suggest that proline biosynthesis in *Plasmodium* is redox regulated *via* OAT, and they reveal new molecular linkages between redox homeostasis and proline metabolism.

Proline dehydrogenase/P5C dehydrogenase

PRODH and P5CDH are well conserved in eukarya and bacteria with PRODHs sharing a catalytic core domain of a

distorted $(\alpha\beta)_8$ barrel (117, 126). It should be noted that PRODH enzymes from Archaea have a structural fold that is distinct from eukarya/bacteria PRODHs (59, 126). In eukaryotes, PRODH and P5CDH are localized in the mitochondrial matrix with PRODH associated with the inner membrane of the mitochondria. In Gram-positive bacteria, PRODH binds peripherally to the cytoplasmic membrane, whereas P5CDH is cytosolic (126, 137). Interestingly, in Gram-negative bacteria, PRODH and P5CDH are combined into a single protein known as proline utilization A (PutA) with the PRODH domain linked N-terminal to the P5CDH domain (73, 83, 126). In some Gram-negative bacteria such as *Escherichia coli*, PutA also has an N-terminal DNA-binding domain (ribbon-helix-helix motif), enabling PutA to function as a transcriptional repressor and a proline metabolic enzyme (72, 155).

PRODH contains a noncovalently bound flavin adenine dinucleotide (FAD) and is responsible for catalyzing the first step of L-proline oxidation (6). The reaction catalyzed by PRODH results in the transfer of two electrons from proline to the flavin cofactor to generate P5C and reduced flavin. Next, PRODH transfers two electrons from reduced flavin to an electron acceptor such as ubiquinone in the inner membrane of the mitochondria (or cytoplasmic membrane in prokaryotes) (86, 135). After P5C spontaneously converts into GSA, GSA is oxidized to L-glutamate by P5C dehydrogenase (P5CDH) using nicotinamide adenine dinucleotide as an electron receptor (6, 121). Glutamate generated by proline oxidation enters the tricarboxylic acid cycle after enzymatic conversion to α-ketogluturate. The oxidation of one molecule of L-proline can yield approximately 30 ATP equivalents, thus providing important energy for the cell, particularly under nutrient deplete conditions (44, 96, 98).

Proline Metabolic Adaptation in Plants During Stress

Proline accumulation is a common phenomenon observed in response to environmental stress in bacteria, protozoa, algae, plants, and marine invertebrates (21, 81, 123, 131). In plants, intracellular proline levels have been found to increase by >100-fold during stress (42, 131). Proline accumulation in plants occurs during exposure to various stresses, including salt (150), drought (9, 19), UV radiation (108), heavy metal ions (18), pathogens (33), and oxidative stress (146). Proline accumulation and stress tolerance have been studied in plants by exogenously and endogenously manipulating proline levels (45). Under stress conditions (*e.g.*, drought, cold shock, and biotic challenges), proline accumulation in plants involves reciprocal regulation of *P5CS* and *PRODH* (44, 45, 101, 131). In tobacco, overexpression of *P5CS* results in higher levels of proline, enhanced osmotolerance, root biomass, and flower development (45, 46). Here, we provide a summary of proline metabolic gene expression changes in plants in response to stress. For a more detailed description of metabolic changes in plants, readers are encouraged to see the excellent review by Szabados and Savouré (123).

Proline biosynthesis

Glutamate appears to be the main precursor in stress-induced proline accumulation in plants, as the ornithine pathway mainly facilitates nitrogen recycling from arginine to glutamate (41, 123). The rate-limiting enzyme for proline synthesis is P5CS, the increased expression of which correlates with proline accumulation in *Arabidopsis* (110). Changes in P5CR (*P5CR*; At5g14800) expression levels seem to associate less with proline accumulation, which is consistent with P5CS catalyzing the rate-limiting step of the pathway. However, there are a few reports that *P5CR* transcripts levels are moderately enhanced in the root of soybean and pea, and in the leaves of *Arabidopsis* in response to osmotic stress (26, 132, 138). In addition, Cecchini *et al.* recently showed that *P5CR* was up-regulated by the hypersensitive response (HR) in *Arabidopsis* after infection with an avirulent strain of *Pseudomonas syringae* (13). Thus, P5CR may have an important role in stress response that is not yet fully realized.

There are two isoforms of *P5CS* in plants; in *Arabidopsis*, isoform 1 (*P5CS1*; At2g39800) is localized in the chloroplasts and is required for stress-induced proline accumulation (80, 124). Isoform 2 (*P5CS2*; At3g55610) is localized mainly in the cytosol and is essential for embryo and seedling development (80, 124). Disruption of *P5CS1* by T-DNA insertion in *Arabidopsis* leads to significantly lower proline accumulation in plants during stress, resulting in hypersensitivity to salt stress and high levels of ROS (124). Disruption of *P5CS2* does not significantly impact proline accumulation but impairs development of seedlings and fertile plants (124). Consistent with an important role in proline accumulation, *P5CS1* expression is up-regulated in response to drought and salt stress (1, 122, 150).

Recently, Verslues *et al.* identified a splice variant of *P5CS1* in *Arabidopsis* that resulted in a nonfunctional transcript (62). The alternatively spliced transcript led to reduced P5CS1 protein levels and proline accumulation (62). In a comprehensive study of how proline content and the abundance of the nonfunctional splice variant varied with climate, it was found that the nonfunctional *P5CS1* transcript correlated better with climate variability than with proline content (62). These interesting findings suggest that proline accumulation may not be the sole factor for adaptation to environmental stress, but rather the proline biosynthesis pathway may have an important role in climate adaptation that is not yet fully realized (62).

The signaling mechanisms by which environmental stress induces proline biosynthesis in plants includes several molecules such as abscisic acid (ABA) (109, 122), calcium, and phospholipase C (91, 109, 148). Recently, Sharma *et al.* reported that proline metabolism is required for ABA-mediated growth protection in plants under water deficit (112). Evidence for ROS-mediated regulation of proline biosynthesis has also been found (33, 134, 146). Fabro *et al.* reported that in *Arabidopsis*, HR triggered by incompatible plant pathogen interaction results in proline accumulation *via* up-regulation of *P5CS2* but not *P5CS1* in a salicylic acid and an ROS-dependent manner (33). Later, Verslues *et al.* also reported that hydrogen peroxide (H_2O_2) may cause proline accumulation or promote ABA-induced proline accumulation (134). Recently, Yang *et al.* suggested that H_2O_2 may induce proline accumulation by up-regulation of P5CS and down-regulation of PRODH activity in coleoptiles and radicles of maize seedlings (146).

In addition to transcriptional regulation, plant P5CS is feedback inhibited by proline (154). The feedback inhibition of P5CS is similar to that of bacterial GKs and involves competitive inhibition by proline with regard to glutamate (93,

94). Structural analysis and site-directed mutagenesis of bacterial GKs suggest that proline partially occupies the glutamate binding site when bound to GK, thus interfering with glutamate binding and inhibiting GK activity (93). Incorporating a GK variant that is insensitive to proline inhibition has been used to overproduce proline in bacteria (23, 119) and yeast (125). In plants, expression of a P5CS variant lacking proline inhibition increased proline levels by two fold (46). In a recent review, Pérez-Arellano *et al.* (94) noted that under stress conditions, some bacteria and plants accumulate proline at a concentration (>100 m*M*) that is well above that which is needed to inhibit GK activity with K_I values ranging from ~0.2 to 1 m*M* proline for bacterial GK and plant P5CS enzymes, respectively (10, 27, 61, 93, 94). It has been proposed that under stress conditions, proline inhibition of GK activity is attenuated by the high levels of other solutes such as glutamate (61, 93, 94).

Proline degradation

During stress response, it is generally anticipated that along with up-regulation of proline biosynthesis, a corresponding decrease in the proline degradation pathway occurs that maximizes proline accumulation. Similar to proline biosynthesis, the first step in the pathway of proline degradation (*i.e.*, PRODH) is rate determining. *Arabidopsis* has two functional PRODH isoforms, both of which are localized to the mitochondria: PRODH1 (*PRODH1*; At3g30775), also known as ERD5 gene (Early Responsive to Dehydration) (40, 64) and PRODH2 (*PRODH2*; At5g38710) (40, 123). PRODH1 is widely expressed in plants and is considered the predominant isoform (40). Expression of *PRODH2* is significantly lower than *PRODH1* with *PRODH2* expressed mainly in the vasculature (40). *PRODH1* and *PRODH2* are up-regulated by exogenous proline but surprisingly, they respond differently to drought and salt stress (131, 136). It is well documented that *PRODH1* expression decreases in response to cold, drought, and salt stress (57, 64). Drought-tolerant wheat (*Tritium aestivum*) cultivars were found to contain significantly lower PRODH activity than drought-sensitive plants (147). Moreover, when seedlings were exposed to lead ($Pb(NO_3)_2$), an elevation of PRODH activity was found in drought-sensitive wheat cultivars (Ningchun) but not in drought-tolerant ones (Xihan), which is consistent with down-regulation of proline degradation and providing a benefit to plants during stress (147).

In *Arabidopsis*, salt stress has been shown to induce *PRODH2* expression, while *PRODH1* expression is significantly down-regulated (40). Differential regulation of the two PRODH isoforms has also been reported in tobacco (104). Thus, it appears that PRODH1 and PRODH2 have distinct physiological roles, which will require further investigation to fully understand the benefits of proline in stress tolerance. Funck *et al.* suggest that proline degradation in the vasculature may provide important energy for the plant during stress exposure (40). Indeed, some tissues in plants have been found to maintain proline oxidation under stress. At low water potential (drought), *PRODH1* expression was found to remain high in root apex and shoot meristem in *Arabidopsis*, whereas *PRODH1* expression was reduced in the bulk of shoot tissue (112). A PRODH1 mutant in *Arabidopsis* exhibited significantly lower oxygen consumption in the root apex, indicating that proline catabolism is a important pathway for driving oxidative phosphorylation (112). Furthermore, the apical region of barley roots under NaCl stress had less free proline accumulation even though L-proline transporter and P5CS activities were increased (128). Thus, proline transport and utilization may vary in different tissues depending on the energy demands of different regions of the plant.

The second enzyme of the proline catabolic pathway in *Arabidopsis*, P5CDH (*P5CDH*; At5g62530), is up-regulated by exogenous proline, although the induction of *P5CDH* is much slower than that of *PRODH* (29). For the most part, *P5CDH* expression remains constant during stress. Interest in *P5CDH* has been in whether *P5CDH* expression levels attenuate the toxicity of proline, which is observed in plants at high proline levels (29). The adverse effect of exogenous proline has been postulated to be caused by the build-up of P5C/GSA due to low P5CDH activity (123, 131). P5C/GSA has been reported to increase intracellular ROS (88) and to react with other metabolites (35). Knockout of *P5CDH* in *Arabidopsis* generates mutant plants that are hypersensitive to exogenous proline, whereas *P5CDH*-overexpressing plants are more tolerant to exogenous proline treatment (28). However, *Arabidopsis* with limited PRODH activity still exhibits sensitivity to exogenous proline, suggesting that other mechanisms contribute to proline toxicity such as inhibition of endogenous proline synthesis (13, 44, 78, 123).

During the recovery phase after stress, proline is considered as serving as an important energy source (44, 123). Proline oxidative metabolism in the mitochondria helps drive oxidative phosphorylation and ATP synthesis in recovering tissues (44). Accordingly, *PRODH* and *P5CDH* expression are increased during rehydration (64). Accumulated proline has been shown to be rapidly degraded during stress recovery in cultured tomato cells (*Lycopersicon esculentum* cv VFNT-Cherry) (43).

Mechanisms of Proline Stress Protection

The molecular mechanisms of how proline protects cells during stress are not fully understood but appear to involve its chemical properties and effects on redox systems such as the glutathione (GSH) pool (Fig. 2). The function of proline in stress adaptation is often explained by its property as an osmolyte and its ability to balance water stress (27). However, adverse environmental conditions often perturb intracellular redox homeostasis, necessitating mechanisms that also work to balance oxidative stress. Thus, proline protective mechanisms have also been proposed to involve the stabilization of proteins and antioxidant enzymes, direct scavenging of ROS, balance of intracellular redox homeostasis (*e.g.*, ratio of $NADP^+$/NADPH and GSH/GSSG), and cellular signaling promoted by proline metabolism. The potential mechanisms by which proline provides stress protection are discussed next.

Osmolyte function

Proline is one of the several small molecules classified as an osmolyte or an osmoprotectant (22). Other biologically important osmolytes are glycerol, trehalose, sorbitol, sucrose, taurine, sarcosine, glycine betaine, and trimethylamine *N*-oxide (145). These osmolytes are accumulated in response to conditions of drought, salt, and temperature extremes. Osmolytes help mitigate water stress and balance turgor pressure during stress (22). Osmolytes are also excellent

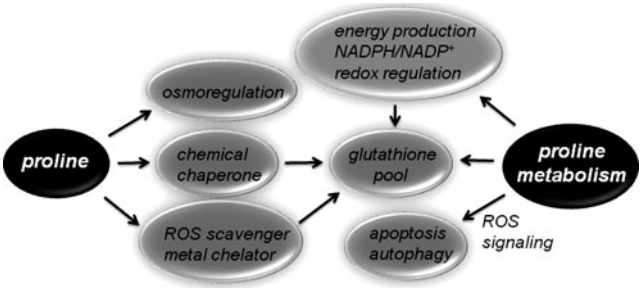

FIG. 2. Potential functions of proline and proline metabolism in stress protection.

cryoprotectants (92). For example, proline has been shown to increase the freeze tolerance of yeast (85, 125) and plants (45, 123, 151), and to be a useful cryoprotector of protein crystals (92), fly larvae (67, 68), plant cells (140), and human stem cells (39). Thus, as an osmolyte, proline is an important molecule that is employed by various organisms to combat stress.

Chemical chaperone

Proline has been shown to act as a chemical protein chaperone and to prevent protein aggregation (71, 107). A thermosensitive *dnaK*-mutant strain of *E. coli* was rescued by increased intracellular proline levels using a GK variant that is insensitive to proline inhibition (15). The higher proline levels reduced protein aggregation and thermodenaturation. In an *in vitro* experiment, proline (1 *M*) protected nitrate reductase under osmotic, metal, and H_2O_2 stress (111). Ignatova *et al.* reported that proline can prevent the aggregation of P39A cellular retinoic acid-binding protein (an aggregation prone protein) under salt stress (51). Proline also diminished the aggregation of a cellular retinoic acid-binding protein that is fused to a pathogenic polyglutamine repeat of the human huntingtin protein (51).

Due to its chaperone properties, proline protection against oxidative stress has been proposed to involve enhancement and stabilization of redox enzymes. Exogenous application of proline to cell cultures has been found to increase the activity of different antioxidant enzymes under salt (47), cadmium (53, 54, 144), and oxidative stress (17), resulting in increased stress tolerance. These enzymes include superoxide dismutase (53, 144), catalase (17, 48, 53, 144), and GSH related or ascorbate (ASC)-GSH cycle-related enzymes (47, 54). All these are important antioxidant enzymes (52, 87) and in plants, the ASC-GSH cycle is especially critical for mitigating ROS (87). In the *P5CS1* knockout of *Arabidopsis*, significantly lower activities of ASC-GSH cycle-related enzymes, including ascorbate peroxidase, GSH peroxidase, and GSH-S-transferase, were observed under NaCl stress conditions (124). Chen *et al.* reported that the addition of proline in the growth medium quenched ROS as efficiently as other ROS scavengers, such as N-acetyl cysteine in the fungal pathogen *Colletotrichum trifolii* (17). The decrease in ROS was shown to be due to an increase in catalase activity by proline treatment (17). Altogether, different groups have reported that increased proline levels enhance antioxidant enzyme activity.

Studies comparing the ability of different biological osmolytes to stabilize proteins have provided insights into the chaperone properties of proline. Proline stabilizes protein structures by driving burial of the peptide backbone and protein folding (7, 130, 143). This is different than protein folding in the absence of osmolytes, which is driven by favorable burying of nonpolar side chains (7, 130, 143). Relative to other osmolytes, proline is categorized as a weak stabilizer of protein folding and ranks lower in ability to induce protein folding (7, 11). Thus, although proline helps stabilize proteins, besides facilitating protein folding, additional mechanisms likely contribute to the protective effect of proline during stress.

Metal chelator

Another mechanism by which proline protects cells against stress has been suggested to involve the chelation of metals. High proline content in metal-tolerant plants is not unusual (113). One of the major toxicities of heavy metals is perturbation of cellular redox balance by ROS production (114). A potent oxidizing agent of biological macromolecules in the cell is the hydroxyl radical ($OH^{\bullet}$), which is formed by the reduction of H_2O_2 by transition metal ions such as Cu^{+} and Fe^{2+} (114). The function of proline as a metal chelator was suggested by Sharma *et al.*, who reported that proline can protect enzymes from zinc- and cadmium-induced inhibition by forming proline-metal complexes (115). A copper–proline complex was also reported in the copper-tolerant *Armeria maritima* (34).

ROS scavenger

The ability of proline to directly react with ROS has been investigated by numerous laboratories (58, 114). Previous studies have shown that free and polypeptide-bound proline can react with H_2O_2 and $OH^{\bullet}$ (pH 7–8) to form stable free radical adducts of proline and hydroxyproline derivatives as shown in Figure 3 (*e.g.*, 4-hydroxyproline and 3-hydroxyproline) (38, 58, 102, 106, 127). Although Floyd and Nagy (38) observed that nitroxyl radicals accumulate during the incubation of proline with H_2O_2, the reaction is very slow relative to that of proline and $OH^{\bullet}$ ($5.4\times10^{8}\ M^{-1}s^{-1}$) (3). Recently, the ability of proline to scavenge H_2O_2 was compared with pyruvate, a well-established scavenger of H_2O_2. At 30 min, H_2O_2 levels were diminished by >90% in cell medium supplemented with 1 m*M* pyruvate, whereas no significant decrease was observed with proline (5 m*M*) (70). This observation further indicates that a direct reaction between H_2O_2 and proline does not significantly contribute to

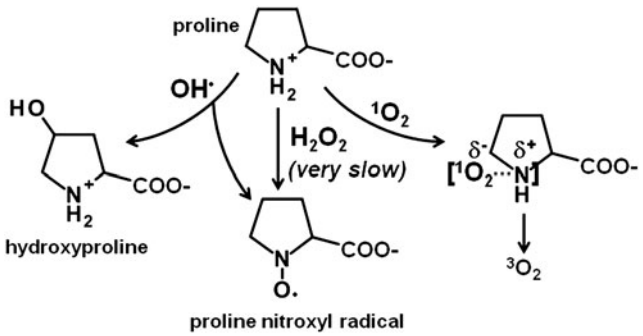

FIG. 3. Potential reactive oxygen species (ROS) scavenging mechanisms of proline.

the scavenging of cellular H_2O_2 (48). Proline has also been shown not to directly scavenge $O_2^{\bullet -}$ (58).

An ROS-scavenging mechanism that is important to proline stress protection is the facile reaction of proline with singlet oxygen ($^1O_2$). In cultured skin fibroblasts, exogenously added proline has been shown to diminish $^1O_2$ levels (141). Proline has been shown to protect human skin cells from photo-induced apoptosis, suggesting that proline suppresses photo-oxidative stress and skin carcinogenesis (141). In plants, Alia *et al.* reported that during strong illumination, the production of $^1O_2$ in the thylakoids from the cotyledons of *Brassica juncea* was dramatically suppressed by proline (5).

The five-membered ring of proline, pyrrolidine, has a low ionization potential that effectively quenches $^1O_2$ most likely through a charge transfer mechanism in which molecular oxygen returns to the ground triplet state ($^3O_2$) (Fig. 3) (20, 81, 152). Alia and coworkers used irradiation of various photosensitizers to produce $^1O_2$, which is detected by measuring the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) by EPR (4). TEMPO formation was completely inhibited by adding 20 m*M* proline to the reaction, indicating that proline directly scavenged or quenched $^1O_2$ (4). Quenching of $^1O_2$ is also documented for other secondary amine compounds as well, such as spermine (63). Due to its action as a $^1O_2$ quencher, proline may help stabilize proteins, DNA, and membranes (81). Prolyl residues in proteins also provide protection against oxidative stress caused by $^1O_2$. For example, the epithelial small proline-rich protein, which is a precursor of the cornified envelope of the epidermal skin, is strongly induced after exposure of the skin to UV radiation (37, 133). Figure 3 summarizes the reactivity of proline with different ROS.

Energy homeostasis and NADP$^+$/NADPH

In addition to the chemical properties of proline discussed earlier, changes in proline metabolic flux can also impact stress tolerance. The effects of proline metabolism on the intracellular redox state have been well studied by Phang and coworkers, who first proposed that the proline-P5C cycle can help maintain proper NADP$^+$/NADPH levels in the cytosol and drive the oxidative pentose phosphate pathway (95). The cycling of proline and P5C *via* PRODH and P5CR results in the transfer of reducing equivalents from the cytosol into the mitochondria (84, 95). Electrons are passed into the mitochondrial ETC directly from PRODH *via* ubiquinone, leading to increases in oxidative phosphorylation and mitochondrial ROS production (84, 95). The proline-P5C cycle is, thus, thought to help maintain a proper NADP$^+$/NADPH ratio (84, 95). The proline-P5C cycle may especially be important when increased PRODH activity is not balanced with P5CDH activity (84, 149). Currently, it is not known how P5C shuttles in/out of the mitochondria. An excellent review on the proline-P5C cycle and the wide ranging effects of proline metabolism was recently provided by Phang (97).

Evidence for proline metabolic flux influencing the NADP$^+$/NADPH ratio in plants has been reported by several groups and summarized in Figure 4 (44, 66, 112). Proline metabolic cycling was found to increase oxidation of NADPH in the soybean nodule, thereby enhancing the oxidative pentose phosphate pathway (66). Increased flux through the oxidative pentose phosphate pathway would support purine nucleotide biosynthesis during stress recovery (Fig. 4) (44, 66). Up-regulation of proline synthesis has also been proposed to maintain the NADP$^+$/NADPH ratio at normal levels during photoinduced stress (44, 123). Significant decreases in the NADP$^+$/NADPH ratio has been reported under different stress conditions due to decreased Calvin cycle activity (44, 123). Without sufficient levels of NADP$^+$ available for electron transfer, photosynthetic cells under stress conditions produce more $^1O_2$ when exposed to high light (16, 123). Light exposure, however, promotes *P5CS* expression, leading to increased proline biosynthesis and NADP$^+$ levels, which ultimately diminishes $^1O_2$ production in the chloroplasts (Fig. 4) (110, 124). These observations suggest a link between enhanced proline synthesis and photoinduced oxidative stress. Hare *et al.* suggest that the redox modulation accompanying proline synthesis may be more important than proline accumulation (44).

Manipulation of proline metabolic enzyme expression has also provided evidence for proline metabolism influencing NADP$^+$ levels in plants. A comparison of sense-orientated and antisense-orientated *P5CR* gene transgenic soybean plants showed that sense plants had higher NADP$^+$ levels and higher stress tolerance relative to antisense plants (25). Antisense knockdown of *P5CR* resulted in lower NADP$^+$ levels and higher sensitivity to stress (25). Recently, Sharma and coworkers reported that under low water stress, plants deficient in P5CS1 or PRODH1 exhibit a lower NADP$^+$/NADPH ratio than wild-type plants (112). In addition, L-proline catabolism was suggested to be important for maintaining the NADP$^+$/NADPH ratio, as the NADP$^+$/NADPH ratio is significantly lower in the *prodh* mutant of *Arabidopsis* than wild-type *Arabidopsis* (112). Although PRODH1 activity apparently declines during stress, a low level of cycling between proline and P5C may be enough to support the maintenance of proper NADP$^+$/NADPH (44).

GSH pool

Different studies have shown that proline addition to the cell medium and up-regulation of endogenous proline biosynthesis leads to increased total GSH and protection of intracellular reduced GSH (47, 118, 144). Guarding reduced GSH is especially important in heavy metal stress, as heavy metal ion toxicity is often associated with depletion of GSH (114). The ability of proline to protect GSH during metal ion stress was tested in *Chlamydomonas reinhardtii* in which transgenic algae expressing the mothbean *P5CS* gene had 80% higher intracellular proline levels relative to wild-type algae (118). After exposing cells to 50 μM Cd^{2+}, the GSH:0.5GSSG ratio was four-fold higher in transgenic algae relative to wild-type cells, indicating that proline prevents GSH depletion during heavy metal stress (118). The higher GSH levels in the *P5CS* transgenic algae were suggested to increase phytochelatin synthesis and the formation of Cd-thiolate complexes in the vacuole, thereby protecting against heavy metal stress (118). The manner in which proline protects the GSH pool is not clear, but it has been proposed that proline directly scavenges $OH^{\bullet}$ and $^1O_2$ generated by heavy metal stress and helps stabilize ROS detoxifying enzymes (47, 118, 144).

The proline and GSH synthesis pathways share the intermediate, γ-glutamyl phosphate, suggesting possible crosstalk

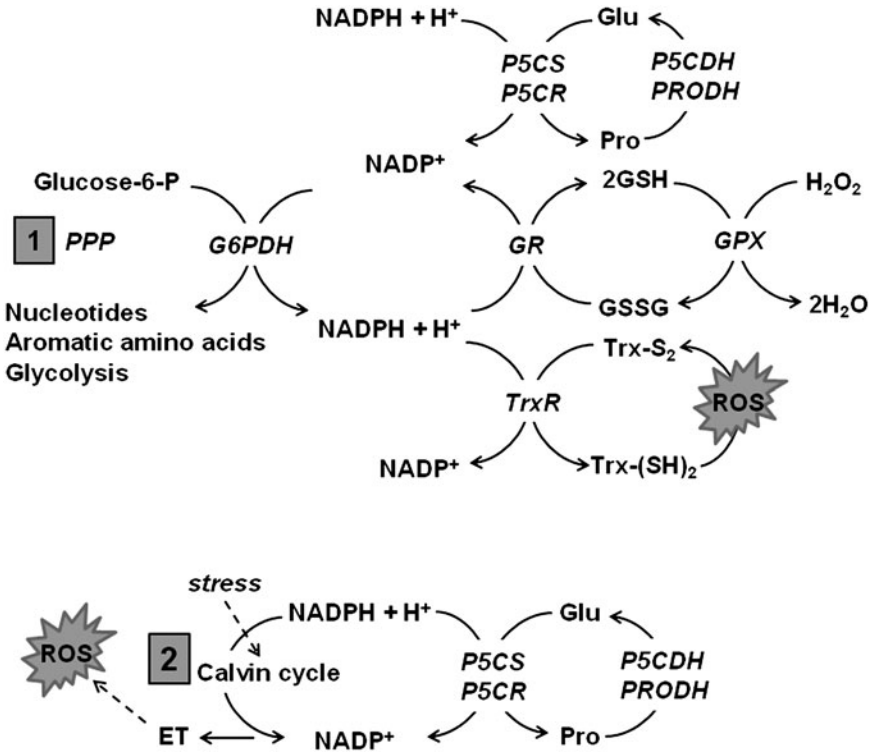

FIG. 4. Proposed mechanisms by which proline metabolism mediates redox homeostasis and energy production *via* NADP$^+$/NADPH. (1) NADP$^+$ produced by proline biosynthesis may stimulate the pentose phosphate pathway (PPP), thereby supporting energy production and the biosynthesis of key molecules such as nucleotides. NADPH is utilized for reductive biosynthesis pathways and is critical for glutathione (GSH) and thioredoxin (Trx) antioxidant systems. (2) In plant chloroplasts, NADP$^+$ produced from proline biosynthesis may replenish depleted NADP$^+$ pools caused by inhibition of the Calvin cycle during stress. Maintaining adequate levels of NADP$^+$ for electrons transfer to the ETC would help minimize ROS generation during stress. Dashed line indicates inhibition. GR, glutathione reductase; GPx, glutathione peroxidase; GSSG, oxidized glutathione; GSH, reduced glutathione; TrxR, thioredoxin reductase; Trx-$(SH)_2$, reduced thioredoxin; Trx-S_2, oxidized thioredoxin; NADP$^+$, nicotinamide adenine dinucleotide phosphate; NADPH, nicotinamide adenine dinucleotide phosphate reduced form; Glucose-6-P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase.

between these pathways. Evidence of proline biosynthesis contributing to GSH synthesis has been recently shown using a GSH-deficient mutant strain of *E. coli* that lacks GshA (glutamate-cysteine ligase), the enzyme which links glutamate and cysteine to form γ-glutamylcysteine and is, thus, auxotrophic for GSH (129). Using a random mutagenesis screen for mutants that rescue GSH auxotrophy, Veeravalli *et al.* discovered that a mutant having both *proB* (GK) and *proA* (GPR) mutations may repress the GSH auxotrophy of *gshA* mutation (129). The mutation in *proB* resulted in a GK mutant that lacks proline feedback inhibition, and the mutation in *proA* generated a GPR mutant which lacks NADPH dehydrogenase activity. The *E. coli* strain with both *proA* and *proB* mutations rescued the GSH auxotropy of the *gshA* mutant by providing an alternative route for generating γ-glutamylcysteine as shown in Figure 5. The mechanism is thought to involve L-cysteine reacting with γ-glutamyl phosphate bound to GPR *via* an S-to-N acyl shift reaction (129).

Intriguingly, bioinformatic analysis of several bacterial genomes showed that in prokaryotes which synthesize GSH, some lack GshA. This suggests that in certain organisms, proline biosynthesis is partly diverted toward GSH production *via* γ-glutamyl phosphate (129). In yeast, it was found that a specific mutation in PRO2 (GPR) is the only suppressor of the GSH auxotrophy of the *gsh1* (homolog of gshA in bacteria) null mutant (120). The rescue of a GSH auxotroph by a *pro2* mutant was due to a trace amount of GSH synthesis by wild-type PRO1 and the PRO2 mutant enzyme (120). Whether proline biosynthesis significantly contributes to GSH pools during stress is not yet known, but these studies demonstrate that γ-glutamyl phosphate derived from the proline biosynthetic pathway is a sufficient precursor for GSH synthesis.

ROS signaling

Overproduction of ROS is well known to cause intracellular damage of biological molecules and to contribute to pathological mechanisms. ROS (*e.g.*, H_2O_2), however, is also an important physiological signaling molecule that triggers adaptive and survival responses by regulating cell death, proliferation, and apoptosis (36, 139). Various studies have shown evidence that proline metabolism leads to increased endogenous ROS (75, 84, 97, 123, 153). Thus, the wide array of effects reported for proline on cellular processes may be due, in part, to the numerous signaling roles of ROS as illustrated in Figure 6.

The first evidence for proline metabolic signaling *via* ROS was provided by Phang's group (30, 97). They have shown that in mammalian cells, PRODH activity increases

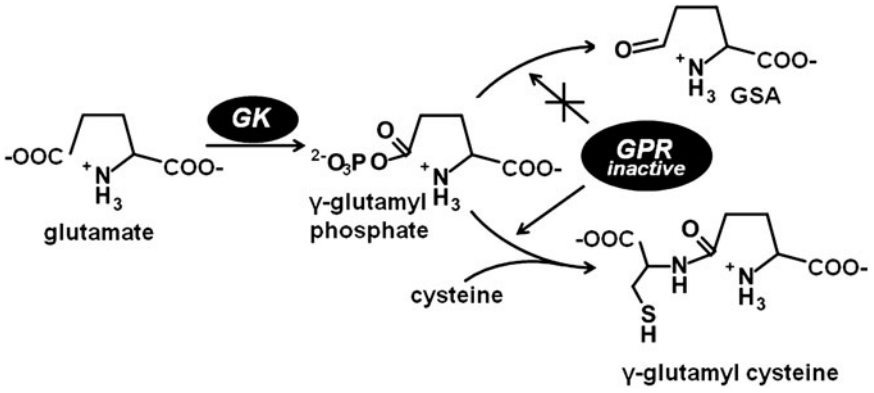

FIG. 5. Formation of γ-glutamylcysteine from the proline biosynthesis pathway. Lack of NADPH dehydrogenase activity in GPR allows cysteine to react with γ-glutamyl phosphate and to generate γ-glutamylcysteine.

mitochondrial ROS production, leading to induction of intrinsic and extrinsic apoptotic cell death pathways (50, 76). The *PRODH1* gene, which encodes PRODH, is a p53-inducible gene (PIG6) (100). Up-regulation of PRODH by p53 increases mitochondrial superoxide ($O_2^{\bullet -}$) production most likely through complex III, leading to cytochrome c release and caspase 9 activation (50, 75). Increased ROS levels due to PRODH have also been implicated in activating apoptotic pathways through the Ca^{2+}/calcineurin-NFAT cascade (76). Phang and colleagues have also shown that PRODH is activated by peroxisome proliferator-activated receptor γ (PPARγ) with PRODH-dependent ROS being an important mediator of apoptosis in cancer cells treated with PPARγ ligands (31, 90, 96, 156). Furthermore, tumor growth is significantly inhibited by overexpression of *PRODH* in mice (77). Recently, Phang *et al.*, showed that the oncogenic transcription factor c-MYC down-regulates *PRODH* expression through miR-23b* and increases the expression of proline biosynthesis enzymes (75). Altogether, the studies by Phang's group have implicated PRODH as an important tumor suppressor protein (97).

ROS signaling stimulated by PRODH has also been implicated in cell proliferation, survival, and autophagy (96–98, 153). Recently, proline and PRODH were found to extend lifespan in *Caenorhabditis elegans* (153). In a *C. elegans daf-2* mutant with impaired insulin and IGF1 signaling, knockdown of *PRODH* significantly decreased lifespan (153). Complementary to the effect of *PRODH* knockdown on lifespan, proline treatment extended the lifespan of wild-type worms expressing *PRODH*. The mechanism by which PRODH increased lifespan was shown to involve transient ROS signals generated by PRODH *via* the mitochondrial ETC (153). Increased mitochondrial ROS production by proline metabolism has been proposed to activate the worm homologues of p38 MAP kinase and Nrf2, leading to increased expression of antioxidant enzymes and lifespan (153). In tumor cells grown under hypoxic conditions, PRODH and proline metabolism generate a protective effect that involves ROS and autophagic signaling (74). Exogenous addition of proline has also been shown to protect mammalian cells against oxidative stress (69). PRODH was recently shown to be essential for proline protection against oxidative stress with the mechanism of protection involving activation of the Akt survival pathway (70). Whether ROS mediates the effects of PRODH on Akt is not yet known.

Increased endogenous ROS formation due to proline metabolism has an important cell signaling role in plants as well (123). In contrast to abiotic stress response described earlier, *PRODH1* expression has been observed to increase in *Arabidopsis* on infection by a nonvirulant strain of *P. syringae* (13). The increase in *PRODH1* levels is dependent on salicylic acid and is considered a part of the initial HR in infected tissues (13). Interestingly, increased PRODH activity correlated with the oxidative burst of the HR (13). Plants in which *PRODH* expression was silenced exhibited increased susceptibility to infection relative to wild-type plants. These results suggest that PRODH may participate in the HR by helping to induce cell death and to prevent pathogen growth in plants (13).

Exogenous proline application has also been shown to lead to increased mitochondrial ROS in *Arabidopsis*, especially in *p5cdh* mutant plants (84). The higher levels of ROS in *p5cdh* plants were suggested to be due to increased proline-P5C cycling, resulting in more flux through the mitochondrial ETC (84). The increased ROS production resulting from exogenous proline addition is proposed to contribute to proline toxicity that is often observed with plants treated with high levels of proline (123). P5CDH was proposed to be an important regulator of ROS production in plants by controlling flow through the proline-P5C cycle to avoid overproduction of mitochondrial ROS. Consistent with this, the expression of *P5CDH* is

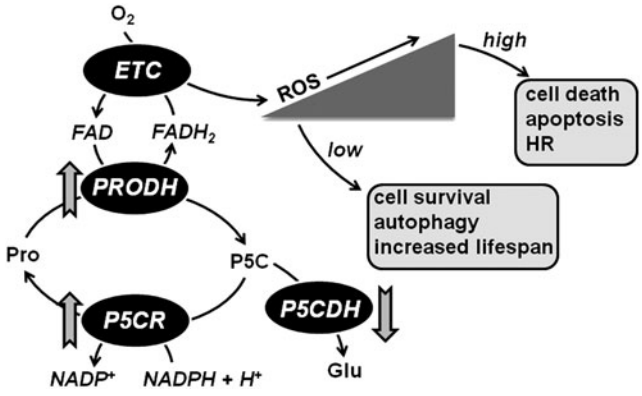

FIG. 6. Proline metabolism and ROS formation. PRODH activity leads to ROS formation in mitochondria by coupling proline oxidation to reduction of the ETC. Increases in PRODH and P5CR activities along with down-regulation of P5CDH are predicted to increase proline-P5C cycling and ROS levels. ROS levels flucuate according to changes in proline metabolism and activate diverse signaling pathways, thereby enabling proline to influence different cellular processes.

down-regulated by 24-nt SRO5-*P5CDH* natural silencing RNAs during salt treatment, leading to an increase in ROS production (12, 131). Furthermore, it was found in flax (*Linum usitatissimum*) that reduced expression of the flax homologue of *Arabidopsis P5CDH*, *FIS1*, resulted in increased sensitivity to exogenous proline and higher levels of H_2O_2 (8, 105).

Conclusion

The ability of proline to protect different organisms during stress involves a plethora of molecular mechanisms, each of which contribute differently according to the physiological and metabolic contexts. Deciphering the mechanisms of how proline influences stress response and redox homeostasis is also complicated by the fact that proline is a proteinogenic amino acid. Thus, the effects of proline metabolism during stress need to be carefully distinguished from potential impacts on protein synthesis that may perturb normal cellular processes and cell survival.

The mechanisms by which proline abates stress can be divided into two general strategies. One strategy is for the organism to accumulate proline *via* up-regulation of proline biosynthesis with proline serving as an osmolyte, a chemical chaperone, and a direct scavenger of $OH^{\bullet}$ or $^1O_2$. A second strategy depends on active proline metabolic flux and linkages to other metabolic pathways. Proline metabolic flux leads to cell protection by helping maintain cellular energy and $NADP^+$/NADPH balance, activating signaling pathways that promote cell survival, and contributing to other pathways such as the tricarboxylic acid cycle and GSH biosynthesis.

Figure 6 shows that the ability of proline metabolism to influence various signaling pathways resulting in either cell survival or cell death may be mediated by ROS. Proline metabolism feeds electrons directly to the ETC *via* PRODH, which leads to superoxide anion formation and H_2O_2. The amount of ROS generated depends on the availability of proline and the level of PRODH activity in the mitochondria. Low ROS generation (*i.e.*, constitutive PRODH expression) would be predicted to lead to protective effects such as activation of Nrf2 and lifespan extension as found in *C. elegans*. High ROS generation due to increased PRODH expression would lead to apoptosis and cell death and contribute to physiological processes such as the HR in plants. Mitochondrial ROS production linked to proline would depend not only on PRODH but also on the activities of P5CR and P5CDH. An increased ratio of PRODH/P5CDH activity, for example, would be predicted to increase proline metabolic cycling with P5C being converted back to proline *via* P5CR and NADPH. Thus, proline metabolic flux determined by the activities of PRODH, P5CR, and P5CDH will have a profound impact on ROS-mediated signaling and ultimately, cell fate. In the future, it will be important to understand not only the regulation of proline metabolism, but also how the activities of these key enzymes are correlated with cell survival and cell death. Potentially, this cycle could be exploited to further improve plant stress behavior and as Phang has already suggested, as a novel target of cancer therapy (97).

Acknowledgments

The work was supported in part by grants GM079393, P20 RR-017675, and P30GM103335 from National Institutes of Health.

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Date of first submission to ARS Central, November 6, 2012; date of final revised submission, March 22, 2013; date of acceptance, April 14, 2013.

Abbreviations Used

ABA = abscisic acid
ASC = ascorbate
ATP = adenosine triphosphate
ETC = electron transport chain
FAD = flavin adenine dinucleotide
GK = glutamate kinase
GPR = γ-glutamyl phosphate reductase
GPx = glutathione peroxidase;
GSA = γ-glutamate semialdehyde
GSH = glutathione
GSSG = oxidized glutathione
H_2O_2 = hydrogen peroxide
HR = hypersensitive response
$NADP^+$ = nicotinamide adenine dinucleotide phosphate
OAT = ornithine-δ-aminotransferase
P5C = Δ^1-pyrroline-5-carboxylate
P5CDH = P5C dehydrogenase
P5CR and PYCR = P5C reductase
P5CS = P5C synthetase
PPARγ = peroxisome proliferator-activated receptor γ
PRODH = proline dehydrogenase
put = proline utilization
PutA = proline utilization A
ROS = reactive oxygen species
TEMPO = 2,2,6,6-tetramethylpiperidine-1-oxyl
Trx = thioredoxin
TrxR = thioredoxin reductase
Trx-S_2 = oxidized thioredoxin
Trx-$(SH)_2$ = reduced thioredoxin