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Proline dehydrogenase is a positive regulator of cell death in different kingdoms

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Proline dehydrogenase (ProDH) catalyzes the flavin-dependent oxidation of Pro into Δ1-pyrroline-5-carboxylate (P5C). This is the first of the two enzymatic reactions that convert proline (Pro) into glutamic acid (Glu). The P5C thus produced is non-enzymatically transformed into glutamate semialdehyde (GSA), which acts as a substrate of P5C dehydrogenase (P5CDH) to generate Glu. Activation of ProDH can generate different effects depending on the behavior of other enzymes of this metabolism. Under different conditions it can generate toxic levels of P5C, alter the cellular redox homeostasis and even produce reactive oxygen species (ROS). Recent studies indicate that in Arabidopsis, the enzyme potentiates the oxidative burst and cell death associated to the Hypersensitive Responses (HR). Interestingly, activation of ProDH can also produce harmful effects in other organisms, suggesting that the enzyme may play a conserved role in the control of cell death.

Key words: proline, proline dehydrogenase, cell death, hypersensitive response (HR), reactive oxygen species (ROS)

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ProDH, an Enzyme that Has Maintained the Capacity to Regulate Cell Death in Various Kingdoms?

Although the four-electron oxidation steps that transform Pro into Glu with P5C as intermediate take place in all living systems,[1,2] the ProDH and P5CDH activities are organized in two different ways. In some organisms, both activities are independently displayed by monofunctional enzymes, while in others they are fused into a unique bifunctional enzyme called "Proline utilization A" (PutA).[3] To date, bifunctional enzymes have only been found in bacteria whereas monofunctional ProDHs have been detected in bacteria and eukaryotes. Curiously, in some organisms, PutA also acts as a Pro-sensitive transcriptional regulator and is therefore considered a trifunctional protein.[3,4] In particular circumstances related to stress, the ProDH activity is associated with ROS accumulation and lethality, suggesting that the enzyme may promote cell death by enhancing the ROS levels (**Table 1**).

The PutA enzymes from *Bradyrhizobium japonicum* (bifunctional) and *Escherichia coli* (trifunctional), have mild effect on the tolerance of *E. coli* cells to oxidative burst.[5] In contrast, the bifunctional enzymes from *Helicobacter pylori* and *Helicobacter hepaticus* potentiate the toxicity of oxidative stress in these cells, apparently due to the generation of superoxide anion.[5]

In *Saccharomyces cerevisiae*, exposure to Pro produces growth inhibition and increase in ROS levels in *put2* (P5CDH) deficient cells.[6,7] The cause of this inhibition is unknown but could involve the activity of Put 1 (ProDH), as a similar response is detected in *p5cdh* mutant plants treated with Pro, which show hyperactivation of ProDH with consequent generation of mitochondrial oxidative burst.[8]

In humans, the overexpression of ProDH (proline oxidase; POX), results in Pro-dependent ROS accumulation and apoptosis.[2,9-11] In this case, cell death is signalled through caspase-dependent

Table 1. Examples of ProDH activity associated with ROS accumulation and cell death

Organism	Enzyme	Type	Increased ROS	Cell death	Reference
Arabidopsis thaliana/ Nicotiana tabacum	ProDH	Monofunctional	+	+	8, 24
Homo sapiens	POX	Monofunctional	+	+	9, 12
Saccharomyces cerevisiae	Put 1	Monofunctional	+	+	6, 7
Thermus thermophilus	Put A	Monofunctional	+	nd	31
Helicobacter pilori/ Helicobacter hepaticus	Put A	Bifunctional	+	+	5
Bradyrhizobium japonicum	Put A	Bifunctional	-	-	5
Escherichia coli	Put A	Trifunctional	-	-	5

+, -: presence or absence of the trait; nd: not determined.

pathways and is reduced by the expression of mitochondrial MnSOD, again suggesting that it is mediated by the enhancement of superoxide at mitochondria.[12,13]

Toxic levels of ROS may also result from activation of the plant ProDH. As mentioned above, this was originally reported for Arabidopsis *p5cdh* mutants exposed to high levels of Pro,[8] and more recently for wild-type Arabidopsis plants responding to pathogens.[14] In the latter case, ProDH is required for normal development of the hypersensitive response (HR), a localized reaction against pathogens that is stimulated by ROS to produce the collapse of infected tissues. In pathogen-treated leaves, the activation of ProDH is restricted to cells destined to die and is signalled through the salicylic acid (SA)-sensitive pathway. Interestingly, this activity is necessary for maximum development of both the oxidative burst and cell death,[14] suggesting that the plant ProDH may contribute to disease resistance programs mediated by cell death. As shown in **Table 1**, mono and bifunctional ProDHs potentiate ROS-mediated cell death in several kingdoms. Then, the involvement of ProDH in suicide programs might constitute a still unrecognized ancient function of the enzyme that has been conserved through evolution.

Is the ProDH Activity under the Control of Cell Death Programs?

If ProDH contributes to a death pathway, then the enzyme may be under the control of a cell death regulator. In animals, this is apparently the case, as the *ProDH* gene is a target of the pro-apoptotic and tumor suppressor protein p53.[15] ProDH is induced by p53 under genotoxic stress to initiate apoptosis by both the mitochondrial (intrinsic) and death receptor (extrinsic) pathways. Moreover, ProDH is induced by PPARγ (Peroxisome Proliferator Activated Receptor gamma), with some of the antitumor effects of PPARγ being dependent on this enzyme.[2]

In Arabidopsis, the zinc finger protein LSD1 (Lesion Stimulating Disease 1) has direct implications on ROS homeostasis[16] and negative effect on hypersensitive cell death.[17,18] The runaway cell death phenotype of the *lsd1* mutant is positively regulated by the transcription factor AtbZIP10, with LSD1 and AtbZIP10 having antagonistic effects on cell death.[18] LSD1 controls the transcriptional activity of AtbZIP10 associated to cell death apparently by affecting its intracellular partitioning, since LSD1 is able to retain AtbZIP10 outside the nucleus. Interestingly, AtbZIP10 mediates *ProDH1* gene expression,[19] then it is plausible that the activation of *ProDH1* occurring in HR[14] derives from changes in LSD1-mediated AtbZIP10 partitioning occurring in the infected tissues.

How does ProDH Contribute to the Plant Hypersensitive Cell Death?

Although the mechanisms by which ProDH contributes to hypersensitive cell death are unknown,[14] deleterious effects derived from this enzymatic activity have been described. One of them results from the incomplete oxidation of Pro occurring when ProDH activation is not accompanied by P5CDH activation. Under this condition, accumulation of P5C may result toxic, as this intermediate has high reactivity with several cellular compounds and even acts as a signal molecule[20-22] with harmful effects on plant, animal and yeast cells.[6,7,9,23-25] During HR activation, the infected tissues accumulate *ProDH*, but not *P5CDH*, transcripts suggesting they may sustain an incomplete oxidation of Pro. Nevertheless, these tissues do not accumulate P5C at 6, 8, 12 h post-infection and transient increases of P5C in these tissues remain to be demonstrated.[14]

Another putative contribution of ProDH to hypersensitive cell death involves its capacity to enhance the oxidative burst.[14] To date, the mechanisms underlying ROS accumulation remain unknown for most ProDHs, but this response seems to involve the ability of the enzyme to load electrons into the mETC or directly into O_2. Concerning the first possibility, it is important to note the alterations occurring at the mitochondria during HR. Among them, the loss of membrane potential, release of cytochrome *c*, and inhibition of the mETC complex I,[25-27] which increase the chances of ROS accumulation. In addition, the SA increase may impede the electron flux in the mETC, leading to over-reduction of the ubiquinone pool.[28] Superoxide dismutase, alternative oxidase and other antioxidant enzymes are induced under such circumstances, but may be insufficient to prevent ROS accumulation. In this context, the activation of a mitochondrial enzyme such as ProDH, able to load electrons into the mETC, would substantially increase the ROS content in the organelle.

Regarding the capacity of ProDH to use O_2 as the electron acceptor for proline oxidation, this depends on the ability of

the flavoprotein to expose the FAD cofactor to the solvent.[3] The monofunctional ProDH from *Thermus thermophilus* and the ProDH domain of *E. coli* PutA have been characterized by X-ray crystallography and biochemical analysis.[29-31] These studies reveal that both enzymes are highly similar at the catalytic core but significantly different in the FAD conformation, with the FAD being more exposed to the solvent in the *T. thermophilus* ProDH. Interestingly, the latter enzyme, but not the *E. coli* PutA, reacts with O_2 to generate superoxide anion.[5,31] To date, however, the reactivity with O_2 has not been formally demonstrated for plant or animal ProDHs, nor has been described the crystal structure of these enzymes.

The capacity of ProDH to generate ROS by any of these alternatives can be potentiated if the enzyme participates in the Pro-P5C cycle. This cycle was originally described in animals[2] and more recently in plants,[8] and it involves the constant inter-conversion between Pro and P5C by activation of ProDH and P5C reductase, but not P5CDH. The stimulation of this cycle increases the transference of reducing equivalents to the mitochondria altering the $NADP^+$/NADPH ratio at the cytosol, with putative implications on redox-sensitive pathways, such as the oxidative pentose pathway that generates several defence components.[1]

Therefore, ProDH may contribute to hypersensitive cell death by altering the levels of ROS[14] or P5C,[24] or the ratio $NADP^+$/NADPH,[1] among other effects derived from this enzymatic activity.

Perspectives

The major challenges in the coming years include the elucidation of mechanisms leading to ROS generation by novel ProDHs, including the plant enzymes. Crystallography and biochemical analysis could certainly be an aid to this end. At the physiological level, the relative contribution of different metabolic alterations derived from ProDH activity (P5C, ROS, $NADP^+$/NADPH ratio), remains to be determined. The presence of two ProDH with non-redundant functions seems to be a conserved trait in various kingdoms, with one of these forms in plants being located at the vascular tissues.[32,33] Determine the specific functions of these isoforms will contribute to define the role of the enzyme in responses to stress.

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