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Recent Progress on the Characterization of Aldonolactone Oxidoreductases

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Abstract

L-Ascorbic acid (ascorbate, AsA, vitamin C) is essential for animal and plant health. Despite our dependence on fruits and vegetables to fulfill our requirement for this vitamin, the metabolic network leading to its formation in plants is just being fully elucidated. There is evidence supporting the operation of at least four biosynthetic pathways leading to AsA formation in plants. These routes use D-mannose/L-Galactose, L-gulose, D-galacturonate, and *myo*-inositol as the main precursors. This review focuses on aldonolactone oxidoreductases, a subgroup of the vanillyl alcohol oxidase (VAO; EC 1.1.3.38) superfamily, enzymes that catalyze the terminal step in AsA biosynthesis in bacteria, protozoa, animals, and plants. In this report, we review the properties of well characterized aldonolactone oxidoreductases to date. A shared feature in these proteins is the presence of a flavin cofactor as well as a thiol group. The flavin cofactor in many cases is bound to the N terminus of the enzymes or to a recently discovered HWXK motif in the C terminus. The binding between the flavin moiety and the protein can be either covalent or non-covalent. Substrate specificity and subcellular localization differ among the isozymes of each kingdom. All oxidases among these enzymes possess dehydrogenase activity, however, exclusive dehydrogenases are also found. We also discuss recent evidence indicating that plants have both L-gulonono-1,4-lactone oxidases and L-Galactono-1,4-lactone dehydrogenases involved in AsA biosynthesis.

Keywords

Aldonolactone oxidoreductase; ascorbate; galactonolactone dehydrogenase; gulonolactone oxidase; vanillyl alcohol oxidase family

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1. Introduction

L-Ascorbic acid (AsA, ascorbate, vitamin C) is a key antioxidant for human and plant health. Humans, non-human primates and guinea pigs cannot produce vitamin C due to the absence of L-gulonolactone oxidase (GulLO; EC 1.1.3.8) activity (Burns, 1957). Therefore, we need plant sources to obtain this vitamin. Many approaches have been undertaken to increase vitamin C content in plants yet challenges still remain (reviewed in Gallie, 2013; Locato et al., 2013). In order to successfully engineer elevated AsA content in plants, we need to have a clear understanding about the pathways and enzymes that catalyze the reactions involved in AsA metabolism. In animals, vitamin C biosynthesis proceeds in a single route from D-glucose via D-Glucuronic acid and L-gulonolactone (L-GulL), as discovered from the early works of Isherwood and others (1954). In contrast, plants possess an intertwined biochemical network to make this molecule. This network involves *myo*-inositol, L-gulose, D-mannose/L-Galactose (Man/Gal), and D-galacturonate as entry points. The Man/Gal pathway a.k.a Smirnoff-Wheeler pathway is the best characterized of all and the enzymes involved in this process have been cloned and characterized (Wheeler et al., 1998; Conklin et al., 1999; Laing et al., 2004; Conklin et al., 2006; Laing et al., 2007; Qian et al., 2007; Maruta et al., 2008). Only a few of the enzymes involved in the other three pathways have been studied to date (Agius et al., 2003; Wolucka and van Montagu, 2003; Lorence et al., 2004).

Aldonolactone oxidoreductases catalyze the terminal step in the conversion of an aldonolactone to AsA in plants and animals or its analogues in fungi (Figure 1). Aldonolactone oxidoreductases of different organisms (isoenzymes) are named after the substrate they use, the physiological relevance of substrate and the use of electron acceptors. These two subtopics (substrate specificity and electron acceptors) are discussed in sections 4 and 5 of this paper. There are two immediate precursors to AsA in plants: L-Galactono-1,4-lactone (L-GalL) and L-gulonolactone (L-GulL). L-Galactono-1,4-lactone dehydrogenase (GLDH; EC 1.3.2.3) catalyzes the conversion of L-GalL to AsA, while GulLO catalyzes the transformation of L-GulL into AsA. A detailed study about the existence of aldonolactone oxidoreductases in different prokaryotic and eukaryotic lineages has been recently published (Wheeler et al., 2015). Isoenzymes of GLDH have been characterized from animals, plants, and other organisms. GLDH was suggested to be a good candidate for the control of AsA biosynthesis (Bartoli et al., 2005; Tamaoki et al., 2003; Tokunaga et al., 2005). However, no clear relationship between AsA content and GLDH activity has been found to date (Bartoli et al., 2005). An attempt to increase AsA by overexpressing GLDH in tobacco did not yield positive results (Imai et al., 2009). In contrast one of the earlier successes to increase AsA content in plants, consisted in overexpression of a rat GulLO in lettuce and tobacco that resulted in up to 7-fold increase in foliar AsA content (Jain and Nessler, 2000).

Studies have also been conducted to restore the vitamin C biosynthetic capacity in animal cells by transgenic expression of the rat or mouse *GulLO* gene in guinea pig cells (Krasnov et al., 1998), human cells (Ha et al., 2004), monkey cells (Yagi et al., 1991), and in fish cells such as the rainbow trout and the Far Eastern catfish (Krasnov et al., 1998; Nam et al., 2002). GulLO is also being studied as a potential therapeutic target in protozoans such as *Trypanosoma cruzi* (Logan et al., 2007; Kudryashova et al., 2011), *T. brucei* (Wilkinson et

al., 2005), and *Leishmania donovani* (Biyani and Madhubala, 2011) as well as in the pathogenic yeast *Candida albicans* (Huh et al., 2001). D-Arabinono-1,4-lactone oxidase (ALO; EC 1.1.3.37), the isoenzyme in yeasts, and D-gluconolactone oxidase (GUO; EC 1.1.3.-) have been also studied for their industrial application to synthesize AsA or its analogues using *E. coli* or yeasts (Lee et al., 1999; Hancock and Viola, 2001; Salusjärvi et al., 2004; Sauer et al., 2004). In addition, plant GLDH has been studied for its use as a biocatalyst in commercial AsA production (Leferink, 2009a). Isoenzymes from other species in mammals, fungi, and algae have been reported (Takahashi et al., 1976; Nishikimi et al., 1978; Shigeoka et al., 1979; Blegg and Christensen, 1982; Kiuchi et al., 1982; Okamura, 2001).

A review on recently characterized enzymes from the vanillyl alcohol oxidase (VAO family) including aldonolactone oxidoreductases was published seven years ago (Leferink et al., 2008a). A focused review on the characterization of aldonolactone oxidoreductases was published recently (Leferink and van Berkel, 2014). However, this work does not include any of the plant GulLOs. Therefore, the current review was prepared to expand on the knowledge about the aldonolactone oxidoreductases characterized to date. This paper discusses the history of characterization, cofactor attachment, substrate specificity, electron acceptor, inhibitors, functional residues, and properties of recombinantly expressed GulLO isoenzymes.

2. History of aldonolactone oxidoreductases

L-gulonolactone oxidase (GulLO) activity was first identified in rat liver microsomes by Burns et al. (1956). Eliceiri et al. (1969) partially purified the enzyme from rat liver microsomes. A purification protocol resulting in 8 to 15-fold purification was developed and some properties of the enzyme were studied including the finding of a prosthetic group (Nakagawa and Asano, 1970). Later on, Nishikimi et al. (1976) purified the protein to apparent homogeneity; this aided in antisera generation, which further led to the identification of the cDNA sequence (Koshizaka et al., 1988). These tools allowed the study of the molecular mechanism for lack of vitamin C production in scurvy prone animals including humans (Nishikimi and Udenfriend, 1976; Nishikimi and Yagi, 1991). From there on, research moved in the direction of identifying the molecular mechanism(s) for the lack of vitamin C synthesis in scurvy prone animals. Aberrant gene sequences were identified in the human and the guinea pig genomes for GulLO (Nishikimi et al., 1988; Nishikimi et al., 1992; Inai et al., 2003).

In plants, however, a similar activity, L-Galactone-1,4-lactone dehydrogenase (GLDH), was first identified in mitochondria of pea and mung bean seeds (Mapson et al., 1954). This activity was partially purified and characterized from cauliflower floret mitochondria (Mapson and Breslow, 1958). Since then isoforms from spinach (Mutsuda et al., 1995) and white potato tubers (Ôba et al., 1994) have been reported. cDNA sequences encoding *GLDH* from cauliflower (Østergaard et al., 1997) and sweet potato (Imai et al., 1998) were identified. GLDH and GulLO are both enzymes involved in AsA biosynthesis in plants.

3. Flavin attachment to aldonolactone oxidoreductases

Aldonolactone oxidoreductases are part of the VAO family of flavoenzymes. The flavin group can be either covalently or non-covalently attached to the proteins. Covalent attachment increases the redox power of the enzyme, saturation of the active site with cofactor, protein stability, and prevents flavin modification (Leferink et al., 2008a). Flavin adenine dinucleotide (FAD) is the flavin cofactor associated with all aldonolactone oxidoreductases identified to date, except for the isoform in cauliflower (Mapson and Breslow, 1958), which has flavin mononucleotide (FMN; Table 1).

The flavin attachment is determined from signature spectral analysis of the purified protein, whereas a more detailed analysis usually involves purifying the flavin peptide followed by releasing the cofactor and determining its identity. A review about the methods employed for the identification of flavin linkage is found in Mewies et al (1998). In the spectral analysis, the purified protein is tested for a hypsochromic shift than FAD or FMN at the second maximum (at about 350 nm) indicating covalent flavin attachment to the protein (Nishikimi et al., 1978; Huh et al., 1994). Plant GLDHs with non-covalently bound flavin show the same spectrum as in FAD or FMN or riboflavin (at about 375 nm) without any hypsochromic shift (Table 1). Covalently bound flavin should not be released with trichloroacetic acid, SDS, boiling or acid ammonium sulfate treatment (Nakagawa et al., 1975; Nishikimi et al., 1978; Huh et al., 1994). Trichloroacetic acid treatment may hydrolyze the pyrophosphate bond in FAD to FMN (Nakagawa et al., 1975; Kudryashova et al., 2011) and this should be considered while determining the identity of the flavin. The identity of the flavin cofactor can be determined by TLC, pyrophosphatase treatment (Kiuchi et al., 1982), paper chromatography (Mapson and Breslow, 1958) or HPLC analysis (Logan et al., 2007). SDS and β -mercaptoethanol treated proteins with a covalently bound flavin, when soaked in 7% acetic acid solution, fluoresce under UV illumination (Nishikimi et al., 1977).

A flavin group is covalently attached to all the identified isoforms from animals, fungi, and bacteria while it is non-covalently attached to the isoforms in protozoa and plants (Table 1). GalLOs of mouse, pig, and *Grifola frondosa*, a basidiomycete's fungus may have a covalent flavin due to the presence of a histidine residue in the N terminus FAD domain or a weak fluorescence in SDS-PAGE (Table 1). The activity of *Euglena gracilis* GalLDH was slightly inhibited by riboflavin suggesting flavin involvement in this organism (Shigeoka et al., 1979).

The GalLOs from *T. cruzi* and *T. brucei* were shown to have a non-covalently bound FMN (Logan et al., 2007). However, the cofactor in the *T. cruzi* enzyme was found to be FAD when the factor was extracted with butanol/acetic acid/water system (Kudryashova et al., 2011). The authors point out that the trichloroacetic acid procedure, used to extract the cofactor from the *T. cruzi* GalLO (Logan et al., 2007), may have hydrolyzed the FAD to FMN giving a false result. It is possible that the true cofactor of the GalLO enzyme in *T. brucei* may also be FAD (Wilkinson et al., 2005). The isoenzyme from *Leishmania donovani* seems to have a non-covalently bound FAD (Biyani and Madhubala, 2011).

The GULLDH of *Gluconobacter oxydans* is reported to contain a cytochrome C component in addition to the flavin group (Sugisawa et al., 1995). The recombinant GULLDH of *M. tuberculosis* does not have a flavin cofactor, as no flavin spectrum was seen; in addition the enzyme was insensitive to exogenous FAD or riboflavin addition (Wolucka and Communi, 2006).

GLDHs from plants contain a non-covalent flavin prosthetic group as found in cauliflower, sweet potato, tobacco, and *Arabidopsis* (Table 1). Another GLDH preparation from cauliflower was reported not to have a flavin prosthetic group (Østergaard et al., 1997) and this discrepancy remains unclear. The lack of a shift in the absorption spectrum in the sweet potato GLDH was suggested due to non-covalent flavin attachment (Imai et al., 1998), which was later confirmed in AtGLDH as well (Leferink et al., 2008b). Amino acid sequence analysis of the GLDHs revealed the presence of leucine instead of histidine in the N terminus FAD-binding domain (Imai et al., 1998; Yabuta et al., 2000; Leferink et al., 2008b) and a lysine in the protozoan GalOs (Biyani and Madhubala, 2011). The replacement of leucine instead of histidine in the FAD-binding domain was proposed as the reason for non-covalent flavin attachment (Imai et al., 1998). Leferink et al (2008b) replaced the leucine with a histidine in AtGLDH but did not observe any covalent FAD attachment. Rather a partial release of the FAD factor, something that did not happen when the wild type enzyme was studied.

4. Substrate specificity of aldonolactone oxidoreductases

4.1. Preferred substrates

The preferred substrate for animal GULLOs is L-GuLL and they can also oxidize L-GalL at a rate >70% compared to L-GuLL (Table 2). D-Arabinono-1,4-lactone (D-AL) and L-GalL are used at an equally significant rate by the ALOs from ascomycete fungi. However, L-GuLL was oxidized only at about one-third rate of D-AL (Table 2).

A GUO partially purified from *P. griseoroseum* is specific for D-glucono-1,4 and D-glucono-1,5-lactones among other hexono acid lactones tested (Takahashi et al., 1976) and that of *P. notatum* can use D-glucono-1,4-lactone (Takahashi, 1969; Table 2). L-GuLL was not a substrate for *P. notatum* and GUO of *P. griseoroseum* (Takahashi et al., 1976). L-GalL did not serve as a substrate for the cells of *P. notatum* (Takahashi et al., 1976) and it may not be a substrate for GUO of *P. griseoroseum*. GULLO of *G. frondosa*, a basidiomycetes fungi, is highly specific for L-GuLL and it can also use D-mannono-1,4-lactone at 25% efficiency as L-GuLL. It appears that this enzyme has substrate specificity for the hydroxyl group at C2 and C3 to be in the “D” form and also only six carbon sugar acid lactones served as substrates (Figure 1; Okamura, 2001). The physiological substrate of this enzyme was thought to be 6-deoxy-GuLL because 6-deoxy-5-O-(α -D-glucofuranosyl)-AsA is the naturally occurring AsA analogue in *G. frondosa* (Okamura, 1994). 6-Deoxy-GalL (L-fucono-1,4-lactone) did not serve as a substrate as expected. Similar results were obtained for crude enzyme preparation from *Lentinus edodes* (Okamura, 2001). Bacterial GULLDHs from *M. tuberculosis* and *G. oxydans* were specific for L-GuLL and L-GalL did not serve as a substrate (Sugisawa et al., 1995; Wolucka and Communi, 2006). These enzymes are different from animal, plant, and fungal GULLO isoenzymes in their substrate specificity.

D-Arabinono-1,4-lactone and L-GalL are the preferred substrates for protozoan GalLOs (Table 2). None of the three protozoan enzymes used L-GulL as a substrate (Table 2). The physiologically relevant substrate for *T. cruzi* was found to be L-GalL (Logan et al., 2007).

Plant GLDHs are highly specific to L-GalL as seen in cauliflower, sweet potato, spinach, tobacco, strawberry, and *Arabidopsis* (Table 2). L-GalL was found to be the physiological substrate for this enzyme (Østergaard et al., 1997), while L-GulL was not a substrate for these enzymes (Table 2). Recombinant tobacco GLDH used L-GulL as a substrate at 7% as L-GalL but not the native enzyme (Yabuta et al., 2000). Sweet potato GLDH was only 1% efficient towards L-GulL. Recombinant AtGLDH catalyzed L-GulL at a significant rate with a K_m of 19.1 mM (Leferink et al., 2008b).

4.2 Stereoisomers

Different stereoisomers among hexono and pentono lactones have been studied for their function as substrates for aldonolactone oxidoreductases. A comprehensive list of substrates was studied for enzymes from *G. frondosa*, *L. edodes* (Okamura, 2001) and rat GulLO (Ashwell et al., 1961; Okamura, 2001). Rat GulLO can use substrates, although at low rate, from hexono, pentono and tetrono sugar acid lactones as long as the hydroxyl group at C2 is in the “L” form as in L-GulL and L-GalL (Ashwell et al., 1961; Figure 1). D-mannono-1,4-lactone has a similar chemical configuration as L-GulL except that the hydroxyl group at C5 is opposite to that of L-GulL. The same is the relationship between D-ALtrono-1,4-lactone and L-GalL (Figure 1). D-mannono-1,4-lactone and D-ALtrono-1,4-lactone were used at low efficiency by some isoforms (Table 2). However, the enantiomer, L-mannono-1,4-lactone was not oxidized by cauliflower and *Arabidopsis* GLDHs (Østergaard et al., 1997; Leferink et al., 2008b).

D-glucono-1,4 and D-glucono-1,5-lactones, the substrates for *P. griseoroseum* and *P. notatum*, were not substrates for any other aldonolactone oxidoreductases tested. Conversely, L-GulL, L-GalL or D-AL, the animal, plant or yeast substrates, were not utilized by GUO of *P. notatum* and *P. griseoroseum* (Table 2).

L-fucono (6-deoxy-GalL) and D-threono-1,4-lactones served as substrates for *S. cerevisiae* ALO (Bleeg and Christensen, 1982). L-Idono, L-talono, and D-ALlono-1,4-lactones are some of the stereoisomers tested and they were not substrates for rat and chicken GulLOs (Kiuchi et al., 1982), *S. cerevisiae* ALO (Nishikimi et al., 1978), *G. frondosa*, and *L. edodes* GulLOs (Okamura, 2001). D-Glucurono-1,4-lactone was used with low efficiency by GulLDH of *G. oxydans* (Sugisawa et al., 1995) and cauliflower GLDH (Mapson and Breslow, 1958). It was not a substrate for rat GulLO (Ashwell et al., 1961; Nakagawa and Asano, 1970), ALOs (Nishikimi et al., 1978; Huh et al., 1994) or *M. tuberculosis* GulLDH (Wolucka and Communi, 2006).

Isomers among pentono lactones, D-xylono and D-lyxono-1,4-lactones were not substrates for *G. frondosa*, *L. edodes* GulLOs (Okamura, 2001) and *S. cerevisiae* ALO (Bleeg and Christensen, 1982). D-xylono-1,4-lactone was not oxidized by cauliflower GLDH as well (Østergaard et al., 1997). However, D-lyxono and L-xylono-1,4-lactones were substrates for

rat GulLO at 59% and 38% of L-GuLL , respectively (Ashwell et al., 1961). *C. albicans* ALO oxidized $\text{L-xylono-1,4-lactone}$ at a rate equal to L-GalL (Huh et al., 1994).

4.3 Sugar acids and sugars

The lactone ring was essential for aldonolactone oxidoreductases as no reaction was observed with precursor acids that are in the AsA biosynthetic pathway of animals and plants. D-Glucuronic acid was found not to be a substrate for rat GulLO (Nakagawa and Asano, 1970) and bacterial GulLDHs (Sugisawa et al., 1995; Wolucka and Communi, 2006). Neither D-Glucuronic acid nor $\text{D-galacturonic acid}$ were a substrate for plant GLDHs from spinach (Mutsuda et al., 1995), tobacco (Yabuta et al., 2000) and *Arabidopsis* (Leferink et al., 2008b). Other precursors such as L-Galactonic acid and L-gulonic acid were also not substrates for rat GulLO (Nakagawa and Asano, 1970), *S. cerevisiae* ALO (Nishikimi et al., 1978) and cauliflower GLDH (Mapson and Breslow, 1958).

GulLDH of *G. oxydans*, however, can use D-xyllose in addition to L-GuLL at high efficiency. D-glucose can also be used at about 25% efficiency. D-Mannose was used less efficiently but D-galactose and L-gulose were not substrates for this enzyme (Sugisawa et al., 1995). D-Arabinose and D-xyllose did not serve as substrates for *M. tuberculosis* GulLDH (Wolucka and Communi, 2006).

4.4 Enantiomers

The enantiomers of preferred substrates for animals (L-GuLL), plants, protozoa (L-GalL) and yeast (D-AL) enzymes are D-GuLL , D-GalL and L-AL , respectively (Figure 1). The enantiomers were never a substrate for any of the aldonolactone oxidoreductases tested.

The enantiomers, D-GalL and D-GuLL were not substrates to rat GulLO (Ashwell et al., 1961), ALOs of *C. albicans* (Huh et al., 1994) and *S. cerevisiae* (Nishikimi et al., 1978), *G. frondosa*, *L. edodes* GulLOs (Okamura, 2001), GLDHs of cauliflower (Østergaard et al., 1997; Mapson and Breslow, 1958), sweet potato (Ôba et al., 1995), spinach (Mutsuda et al., 1995), tobacco (Yabuta et al., 2000), strawberry (Nascimento et al., 2005) or *Arabidopsis* (Leferink et al., 2008b). *P. griseoroseum* GUO (Takahashi et al., 1976) was not tested with D-GuLL but it cannot oxidize D-GalL . Isoforms of GulLDHs from bacteria and *E. gracilis* were not tested for D-GalL and D-GuLL . L-AL was not a substrate for *C. albicans* (Huh et al., 1994).

4.5 Configurational specificity

Animal GulLOs have a configurational specificity for the hydroxyl group at C2 and C4 in the substrates and the C5 hydroxyl group being in the “*b*” arrangement makes it more efficient (Figure 1). Ascomycete enzymes have a configurational specificity for the hydroxyl group at C2, C3, and C4, (Figure 1). Protozoan enzymes are similar to ascomycete enzymes with a substrate specificity for C2 and C3 hydroxyl group (Figure 1). In plants, the specificity is for the hydroxyl group at C2, C3, C4 and C5. Basidiomycete enzymes from *G. frondosa* and *L. edodes* and bacterial enzymes are similar to plant enzymes but the difference is the hydroxyl group at C2 is opposite to that of L-GalL .

S. cerevisiae and *C. albicans* ALOs (Nishikimi et al., 1978; Huh et al., 1994) use substrates as five or six carbon sugar acid lactones with hydroxyl groups in “D” form at C2 and C3. Molecules with hydroxyl groups on the opposite side in C3 to the above can also be substrates. However, GulLO of *G. frondosa* and *L. edodes* are specific for both the hydroxyl groups.

4.6 Product formed

GulLOs of animals such as the ones from rat, goat (Nishikimi et al., 1976), chicken (Kiuchi et al., 1982), mouse (Ha et al., 2004) and pig (Hasan et al., 2004) and a basidiomycetes fungus, *G. frondosa* (Okamura, 2001) produce AsA when L-GulL or L-GalL are used as a substrate (Kiuchi et al., 1982; Okamura, 2001; Figure 1). Similarly, GulLDHs from bacteria (Sugisawa et al., 1995; Wolucka and Communi, 2006) produce AsA as well, when L-GulL is the substrate. The enzyme products of *G. oxydans* were D-xylosone and D-glucosone when D-xylose and D-glucose were used as substrates, respectively (Sugisawa et al., 1995). Plant GLDHs also produced AsA as product with L-GalL as the starting reactant (Mapson and Breslow, 1958; Leferink et al., 2008b; Figure 1).

The reaction product of ascomycete fungal ALOs from *S. cerevisiae* and *C. albicans* was D-erythroascorbic acid when D-AL was used as the substrate (Bleeg and Christensen, 1982; Huh et al., 1994; Figure 1). D-erythroascorbic acid was found to be the naturally occurring AsA analogue in *C. albicans* (Huh et al., 1994). These enzymes can also make AsA when L-GalL was used as the substrate (Nishikimi et al., 1978; Huh et al., 1994). Interestingly, this property was exploited to make a yeast strain to produce AsA (Hancock et al., 2000). GUO enzymes from *P. notatum* and *P. griseoroseum* yield D-araboascorbic acid (D-erythorbic acid) as their enzyme reaction products (Takahashi, 1969; Takahashi et al., 1976; Figure 1).

5. Electron acceptor of aldonolactone oxidoreductases

Aldonolactone oxidoreductases are called “oxidases” when the oxidation of an aldonolactone uses molecular oxygen as electron acceptor. Hydrogen peroxide is the byproduct of this reaction. These enzymes are called “dehydrogenases”, if the reaction involves alternative electron acceptors such as cytochrome C, phenazine methosulfate, 2,6-dichloroindophenol or benzoquinone.

Almost all characterized aldonolactone oxidoreductases reported as oxidases have also dehydrogenase activity. Various enzyme assays exist to determine oxidase activity. The activity staining technique in native gel electrophoresis is based on the dehydrogenase property of the enzyme using phenazine methosulfate (Nishikimi et al., 1976). Animal GulLOs, fungal ALOs and GUO have both oxidase and dehydrogenase activities. Animal enzymes from rat, goat (Nishikimi et al., 1976), chicken (Kiuchi et al., 1982) and fungal enzymes: *C. albicans* (Huh et al., 1994), *S. cerevisiae* (Nishikimi et al., 1978), *P. griseoroseum* (Takahashi et al., 1976; Salusjärvi et al., 2004) and *G. frondosa* (Okamura, 2001) were all reported to use oxygen and alternative electron acceptors, i.e. to have both oxidase and dehydrogenase activities.

Plant GLDHs have been reported to be exclusive dehydrogenases in cauliflower (Mapson and Breslow, 1958; Østergaard et al., 1997), spinach (Mutsuda et al., 1995), tobacco (Yabuta et al., 2000) and *Arabidopsis* (Leferink et al., 2008b). Recently, an alanine residue was found in AtGLDH to function as a gatekeeper in not allowing oxygen to reach the reduced flavin (Leferink et al., 2009b; Figure 2). A glycine or proline is present in nearly all oxidases of the VAO family near the C4a locus of the isoalloxazine ring of flavin, whereas other residues are present in dehydrogenases (Leferink et al., 2009b). Exceptions to this rule are also found in the analysis of different VAO family members. For example, alditol oxidase from *Streptomyces coelicolor* has an alanine and it functions as an oxidase while eugenol hydroxylase from *Pseudomonas* sp. strain HR199 functions as a dehydrogenase although it has a proline residue (Leferink et al., 2009b). Among aldonolactone oxidoreductases, all members obey this rule except *M. tuberculosis* GulLDH (Wolucka and Communi, 2006) and AtGulLO5 (At2g46740; Aboobucker et al., submitted; Figure 2). Nevertheless, changing the Ala-113 to Gly in AtGLDH gained oxidase activity without any loss in the dehydrogenase activity (Leferink et al., 2009b; amino acid numbered based on mature peptide as in Leferink et al., 2008b). AtGLDH modified to use oxygen is the first enzyme to use both oxygen and cytochrome C as electron acceptors among the plant GLDHs.

Enzymes that function exclusively as dehydrogenases have also been reported in bacteria such as *G. oxydans* (Sugisawa et al., 1995) and *M. tuberculosis* (Wolucka and Communi, 2006). The amino acid sequence for *M. tuberculosis* GulLDH also has a glycine at this position (Leferink et al., 2009b), but it was reported not to use molecular oxygen (Wolucka and Communi, 2006). It is worth noting that the recombinant *M. tuberculosis* GulLDH did not have a flavin and this could be the reason for its non-reactivity to molecular oxygen. *G. oxydans* GulLDH is an interesting protein as it has a covalently bound FAD and functions exclusively as a dehydrogenase (Sugisawa et al., 1995). However, its amino acid sequence is unknown to date.

Protozoan enzymes from *T. brucei*, *T. cruzi* and *L. donovani* were reported to have dehydrogenase activity but their oxidase activity was not tested, even though they were named “oxidases” (Biyani and Madhubala, 2011; Logan et al., 2007; Wilkinson et al., 2005). Recently, a GalLO from *T. cruzi* was tested for its oxidase activity and found to operate as an oxidase as well (Kudryashova et al., 2011). The authors reported the absence of an alanine residue in *T. cruzi*, similar to AtGLDH (Figure 2; Kurdyashova et al., 2011). Similarly, this alanine residue is absent in *T. brucei* GalLO and *L. donovani* ALO and they both may function as oxidases as well (Figure 2).

A single protein component is responsible for both the activities in all cases examined. The presence of two components for oxidase and dehydrogenase activity in rat GulLO and their separation was reported (Isherwood et al., 1960; Bublitz, 1961). Later studies of the enzyme found the presence of both the activities, but were unable to separate them (Eliceiri et al., 1969; Nakagawa and Asano, 1970). However, the presence of two active sites in one component for both oxidase and dehydrogenase activities was postulated (Nakagawa and Asano, 1970). Nevertheless, the above studies were based on partial purification of the enzyme. It was later confirmed in a homogeneous preparation that only one component was

responsible for both oxidase and dehydrogenase activities in rat GulLO (Nishikimi et al., 1976).

One interesting observation is that rat GulLO (Eliceiri et al., 1969), GUO from *P. cyaneofulvum* (Takahashi et al., 1976) and GulLO from *G. frondosa* (Okamura, 2001) cannot take cytochrome C but use molecular oxygen and phenazine methosulfate as electron acceptors. The use of cytochrome C as an electron acceptor was not tested in other animal or yeast enzymes. GLDH from plants, on the other hand, can use cytochrome C and phenazine methosulfate but not molecular oxygen as acceptors. Enzyme catalysis occurs via flavin when cytochrome and oxygen are acceptors as demonstrated by inhibitor studies, but the inhibitors do not interfere in the reaction via phenazine (Mapson and Breslow, 1958). Cytochrome C was used as an electron acceptor by plant enzymes but not animal isoforms. The residue in charge of allowing the molecular oxygen to enter the active site is known (Leferink et al., 2009b). However, the interaction between cytochrome C and AtGLDH is dynamic, involving low affinity and electrostatic protein-protein interaction rather than a single residue (Hervás et al., 2013).

The GulLDH from *E. gracilis* uses only phenazine methosulfate among other alternative electron acceptors tested including cytochrome C (Shigeoka et al., 1979) differing from animal, yeast and plant isoenzymes. The GulLDH did not have oxidase activity either, similar to plants and bacterial enzymes.

6. Active site, inhibitors, and functional residues of aldonolactone oxidoreductases

A flavin group or cofactor is found to be involved in the enzyme catalysis of many characterized aldonolactone oxidoreductases. Its involvement is observed by the disappearance of 450 nm peak when the substrate is added to the enzyme solution such as in GulLOs of rat (Nakagawa et al., 1975), chicken (Kiuchi et al., 1982) and *G. frondosa* (Okamura, 2001) as well as in the GUO of *P. griseoroseum* (Takahashi et al., 1976), *S. cerevisiae* ALO (Nishikimi et al., 1980; Bleeg and Christensen, 1982), *T. brucei* GalLO (Wilkinson et al., 2005) and in the GLDHs of cauliflower (Mapson and Breslow, 1958) and *Arabidopsis* (Leferink et al., 2008b). Flavin involved reactions proceed as two half reactions. In the first reductive half, flavin is reduced by the substrate and the latter is oxidized. The flavin is reoxidized, in the second half, by electron acceptors such as oxygen (oxidase) or alternative electron acceptors (dehydrogenases). Hydrogen peroxide is produced as a result of the transfer of electrons from flavin to oxygen. Both half reactions can be studied separately due to characteristic spectral properties exhibited during oxidation and reduction cycles of the flavin group (Leferink, 2009a). Flavin involvement in the catalysis of L-GalL by AtGLDH was studied in detail. During the first reductive half, the flavin was reduced by L-GalL to a two electron reduced state. The second oxidative half proceeds in two steps as cytochrome C is a one-electron acceptor. The appearance of a semiquinone radical intermediate was monitored during the oxidative half (Leferink et al., 2008b).

Inhibitor studies have been conducted on many aldonolactone oxidoreductases to determine the functional groups and they are summarized in Table 3. The involvement of flavin through cytochrome C had also been determined using flavoprotein inhibitors such as atebtrin, riboflavin, and acriflavine in cauliflower, sweet potato, and tobacco GLDHs, *E. gracilis* GulLDH, and *G. frondosa* GulLO (Table 3). In another observation, riboflavin did not inhibit any activity of GLDH in cauliflower (Østergaard et al., 1997). Interestingly, the reaction proceeding through phenazine methosulfate of cauliflower GLDH was not inhibited by flavoprotein inhibitors (Mapson and Breslow, 1958). Rat GulLO was not inhibited by flavoprotein inhibitors and notably the authors were unable to find flavin as a prosthetic group in this particular preparation (Nakagawa and Asano, 1970). GulLDH of *G. oxydans* (Sugisawa et al., 1995) and cauliflower GLDH (Mapson and Breslow, 1958) were reported to have a heme component in addition to flavin.

Another important feature of aldonolactone oxidoreductases is the participation of a thiol group. Enzyme activity of all members of this family was inhibited by sulfhydryl group modifiers with two exceptions (Table 3). *P. griseoroseum* GUO is one of the exceptions whose sequence has been deduced (Salusjärvi et al., 2004). Interestingly, a Cys residue, conserved among other isoenzymes, is not present in *P. griseoroseum* GUO, which was the lead to identify the functional Cys residue in AtGLDH (Figure 2; Leferink et al., 2009d) as will be discussed later. *p*-Chloromercuribenzoate (PCMB) or *p*-chloromercurisulfate (PCMS) inhibition was reversible with glutathione, 2-mercaptoethanol or potassium cyanide. Reversal of enzyme activity with glutathione was observed in rat GulLO (Nakagawa and Asano, 1970), *P. notatum* GUO (Takahashi, 1969) and cauliflower GLDH (Mapson and Breslow, 1958). 2-Mercaptoethanol reversal was observed in ALOs of *S. cerevisiae* (Nishikimi et al., 1978), *C. albicans* (Huh et al., 1994) and sweet potato GLDH (Ôba et al., 1995). Potassium cyanide reversed inhibition was seen in *E. gracilis* GulLDH (Shigeoka et al., 1979) and ALOs of *C. albicans* (Huh et al., 1994) and *S. cerevisiae* (Bleeg and Christensen, 1982). *N*-Ethylmaleimide (NEM) inhibition, on the other hand, was reversible with glutathione in sweet potato GLDH (Imai et al., 1998) but irreversible in *C. albicans* ALO (Huh et al., 1994) and tobacco GLDH (Yabuta et al., 2000). Prior incubation with glutathione decreased inhibition by Hg²⁺ or Ag²⁺ in *P. griseoroseum* GUO (Takahashi et al., 1976).

The inhibition of rat GulLO activity by PCMS and Hg²⁺ was through inhibition of flavin reduction as observed by the disappearance of the peak at 450 nm corresponding to flavin. Sulfite, however, did not inhibit flavin reduction although it inhibited the oxidase activity. Therefore, the authors postulated the presence of two active sites for oxidase and dehydrogenase activities (Nakagawa and Asano, 1970). This idea may be correct as indeed, an alanine residue (Ala-113) was found to prevent oxygen from entering the active site and thereby lacking oxidase activity in AtGLDH (Leferink et al., 2009b). When replaced with glycine, AtGLDH was able to utilize oxygen without any reduction in the dehydrogenase activity (Leferink et al., 2009b) supporting the previous finding. The same was observed in GalLO of *T. cruzi*, which had both oxidase and dehydrogenase activities and a gatekeeper alanine residue is absent (Kudryashova et al., 2011).

Sulfhydryl group inhibition was studied in detail in *S. cerevisiae* ALO (Noguchi et al., 1981) and in AtGLDH (Leferink et al., 2009d). Both the studies found that the sulfhydryl group is not playing a role in the catalysis itself but it was involved in substrate binding. The same was observed in cauliflower GLDH (Mapson and Breslow, 1958). Also, only one sulfhydryl group was involved in this function in both the studies and it was identified as Cys-340 in AtGLDH (Leferink et al., 2009d). This residue is conserved in all aldonolactone oxidoreductases except *P. griseoroseum* GUO and *M. tuberculosis* GuILDH (Figure 2). In AtGLDH, a Cys-340 was found to be buried away from the flavin and not directly involved in the catalysis as the flavin spectrum remain unchanged (Leferink et al., 2009d). Noguchi et al. (1981) suggested the sulfhydryl group may be situated near flavin and that the sulfhydryl group is provoked during catalytic process to be more reactive with the sulfhydryl modifying reagents. Replacing Cys-340 with Ser or Ala strongly weakened substrate binding in AtGLDH, while the catalytic activity was retained. In support of this, pre-incubation with the substrate slowed down inhibition by thiol modifying agents, as was observed in AtGLDH (Leferink et al., 2009d), *S. cerevisiae* ALO (Noguchi et al., 1981) and also in cauliflower GLDH (Mapson and Breslow, 1958). The sulfhydryl group is required for phenazine activity in addition to substrate binding in cauliflower GLDH (Mapson and Breslow, 1958).

In addition to the two active sites for oxidase and dehydrogenase activities, reactions proceeding with phenazine methosulfate may have a different active site and flavin is probably not involved. In cauliflower GLDH, cytochrome C activity was inhibited with flavoprotein inhibitors such as atebirin or riboflavin while phenazine activity was not inhibited (Mapson and Breslow, 1958). Similarly, in *M. tuberculosis*, GuILDH activity was higher with phenazine methosulfate than with cytochrome C and it did not have a flavin prosthetic group (Wolucka and Communi, 2006). A contrasting observation, in GuILDH of *E. gracilis*, is that only phenazine was used as an electron acceptor but not cytochrome C and flavin participation was also indicated by riboflavin inhibition (Shigeoka et al., 1979).

Metal ions inhibited most of the characterized aldonolactone oxidoreductases (Table 3). Hg^{2+} , Zn^{2+} and Cu^{2+} were found as common inhibitors and they were implicated to inhibit through sulfhydryl groups (Mapson and Breslow, 1958; Biyani and Madhubala, 2011). None of these enzymes are considered metalloproteins as chelating agents had no effect on their activity except *M. tuberculosis* GuILDH and cauliflower GLDH (Table 3). KCN inhibition suggests the presence of iron-like groups in the GuILDH enzyme of *M. tuberculosis* (Wolucka and Communi, 2006). The involvement of an iron-sulfur cluster in catalysis was also suggested for *S. cerevisiae* ALO (Bleeg and Christensen, 1982).

Substrate analogs were also inhibitory in some cases (Table 3) and they were mainly the “b” forms of the aldonolactone substrates. Preferred substrates were also found to be inhibitory for *T. brucei* GalLO and many plant GLDHs at above certain concentrations (Table 3). Quinoprotein inhibitors were tested on GuILDH of *G. oxydans* and found not to be inhibitory (Sugisawa et al., 1995). Anions were inhibitory to cytochrome activity of cauliflower GLDH but not to phenazine activity (Mapson and Breslow, 1958). Lycorine is a specific inhibitor for GLDH (Imai et al., 1998), but no inhibition was found with AtGLDH (Leferink et al., 2008b) or cauliflower GLDH (Østergaard et al., 1997).

Amino acid residues involved in flavin binding have also been identified. A two-domain folding topology is common in VAO family members and they are FAD-binding domain (PFAM Id: 01565) and a catalytic domain (PFAM Id: 04030) found at the interface of the two domains is the active site. The catalytic domain in the C terminus is where the substrate binds (Fraaije et al., 1998; Leferink et al., 2008a). Although the sequence similarity among aldonolactone oxidoreductases from different species is low, the presence of these two characteristic features is shared. His-61 of VAO was shown to promote covalent flavin binding as no covalent FAD was found in His-61Thr mutant (Fraaije et al., 2000). The flavin is covalently linked to His-104 in the N-terminus in berberine bridge enzyme (Kutchan and Dittrich, 1995) and 6-hydroxy-*n*-nicotine oxidase (Brandsch et al., 1987), two members of VAO family. The N terminus histidine of FAD-binding domain is found in a stretch of VGSGHS sequences in aldonolactone oxidoreductases (Imai et al., 1998; Wolucka and Communi, 2006). All covalently bound FAD containing isoenzymes have a histidine, whereas a leucine or lysine is present in enzymes with non-covalent FAD (Figure 2). An exception to this is GULLDH of *M. tuberculosis* that has a histidine residue but it was found to be without flavin (Table 1; Wolucka and Communi, 2006). It was thought that FAD is covalently linked to this histidine residue in VAO (Fraaije et al., 1998). However, it is not known if the flavin is linked to the histidine in N or C termini in aldonolactone oxidoreductases.

Attempts were made to generate enzymes with covalently bound FAD by replacing leucine with histidine in AtGLDH (Leferink et al., 2008) and lysine with histidine in *T. cruzi* GalLO (Logan et al., 2007). No covalent linkage was observed, rather FAD was loosely bound to AtGLDH (Leferink et al., 2008b) and flavin binding was dramatically reduced in *T. cruzi* GalLO (Logan et al., 2007). Leferink et al. (2008b) explained that the His-422 is activated by His-61 in VAO for covalent flavinylation. The presence of similar His-61 is not known in AtGLDH due to the lack of structural information for aldonolactone oxidoreductases. However, it is not known if flavin binding in these enzymes is in the C terminus, similar to VAO. Attempts to solve the crystal structure of AtGLDH failed as all crystals for AtGLDH, including variants, diffracted to 3.5-4 Å and it was not enough to solve the structure (Leferink, 2009a).

The His-422 residue in the C terminus of VAOs is where the FAD is covalently bound (Fraaije et al., 1999). This histidine is conserved among all aldonolactone oxidoreductases in a HWXK motif present in the catalytic domain (Figure 2; Logan et al., 2007). The functional role of the HWXK motif was studied in *T. cruzi* GalLO by site-directed mutagenesis (Logan et al., 2007). His-447 and Trp-448 were individually mutated to glycine and absence of flavin binding and enzyme activity were observed. When, Lys-450 was mutated to Gly, the enzyme retained half of the flavin but not the activity (Logan et al., 2007). The authors proposed that His-447 and Trp-448 are involved directly or indirectly in interactions with FMN while Lys-450 has a role in activity independent of flavin binding. Kudryashova et al. (2011) reported after examining *T. cruzi* GalLO with alditol oxidase (AldO) that His-447 and Lys-450 are located close enough to the flavin for direct interaction. They also proposed that the flavin moiety could be binding to His-447. Also, the lysine residue is part of the active site of AldO and Lys-450 in *T. cruzi* may have a similarly important role.

Kudryashova et al. (2011) suggested that loss of flavin binding and activity in the Trp448Gly mutant could be due to incorrect protein folding. The X in this motif is an alanine in all of the enzymes except AtGulLO5, *M. tuberculosis* GullLDH and *P. griseoroseum* GUO (Figure 2). It is interesting to note that L-GalL is not a substrate for AtGulLO5, *M. tuberculosis* GullLDH, and the resting cells of *P. notatum* (Aboobucker et al., submitted; Takahashi et al., 1976; Wolucka and Communi, 2006). The substrate, L-GalL was not tested with *P. griseoroseum* GUO.

Comparison of the structure of VAO with several of its family members including rat GulLO, *S. cerevisiae* ALO and cauliflower GLDH enabled the identification of residues interacting with parts of the FAD molecule (Fraaije et al., 1998). These residues are conserved in all aldonolactone oxidoreductases whose sequences are known (Figure 2). AtGulLO5 may not have the complete stretch of sequences that interact with pyrophosphate but part of it is still present (Figure 2). A similar analysis was done for the sequence of *T. cruzi* and *T. brucei* GalLOs and FAD was predicted to be the cofactor in *T. cruzi* (Kudryashova et al., 2011). Experimental evidence also proved that the cofactor was FAD (Kudryashova et al., 2011), which was previously reported as FMN (Logan et al., 2007).

The functional role of the Glu-Arg pair in the catalytic domain, conserved among aldonolactone oxidoreductases, was studied in AtGLDH by site-directed mutagenesis (Leferink et al., 2009c). It was demonstrated that this pair is essential for optimal catalysis. Glu-386 is involved in substrate binding and determining substrate specificity. Glu386Asp variant lost its specificity for L-GalL but used L-GulL with more efficiency. The Arg-388 residue is essential for the stabilization of negative charge resulting from flavin reduction. An Arg388Lys variant showed some activity while the Arg388Ala variant was essentially inactive (Leferink et al., 2009c). This pair is also widely conserved among all aldonolactone oxidoreductases with the exception of AtGulLO5 and *P. griseoroseum* GUO (Figure 2).

7. Flavín binding in recombinantly expressed aldonolactone oxidoreductases

There is a discrepancy about covalent flavin attachment in recombinantly produced GulLO isoenzymes in *E. coli*. Three enzymes were expressed in *E. coli*, viz. ALO from *S. cerevisiae* (Lee et al., 1999), GUO from *P. griseoroseum* (Salusjärvi, 2006) and GullLDH from *M. tuberculosis* (Wolucka and Communi, 2006). Native ALO (Kenney et al., 1979) and GUO (Harada et al., 1979) were experimentally found to have a covalently bound FAD as well as a histidine residue in the FAD-binding domain in N terminus. GullLDH from *M. tuberculosis* was predicted to have a covalently bound FAD, as a histidine is found in the N terminal FAD-binding domain. The recombinant enzymes from *S. cerevisiae* (ALO) and *M. tuberculosis* (GullLDH) were active but did not have a flavin prosthetic group. The GUO from *P. griseoroseum* was not active and Salusjärvi (2006) suggested it could be due to the absence of covalent flavin attachment. However, GUO was found to have covalently linked FAD when expressed in *Pichia pastoris* (Salusjärvi et al., 2004). Rat GulLO and AtGulLO2, 3, 4 and 5 were not able to be successfully expressed in *E. coli* (Maruta et al., 2010).

The rat GulLO has been expressed in monkey cells (Yagi et al., 1991) and BmN4 silkworm cells (Nishikimi et al., 1994) and found to be covalently linked to FAD. A similar observation was made with mouse GulLO expressed in guinea pig cells (Ha et al., 2004). Rat GulLO was expressed as both holo and apo forms in a riboflavin rich and free media, respectively. The apo form only made non-covalent interaction when FAD was added to it (Nishikimi et al., 1994), but the activity was as in the holo form with covalent FAD. This finding is in contrast to 6-hydroxy-D-nicotine-oxidase (Brandsch and Bichler, 1991), a VAO family member, wherein the apo form made covalent FAD, when exogenous FAD was added.

On the other hand, protozoan GulLO isoenzymes from *T. brucei* (Wilkinson et al., 2005) and *T. cruzi* (Logan et al., 2007; Kudryashova et al., 2011) as well as AtGLDH from *Arabidopsis* (Leferink et al., 2008b) and GLDH from tobacco (Yabuta et al., 2000) were expressed in *E. coli* and found to have a non-covalent flavin as predicted from their amino acid sequences. It is interesting to note that *T. cruzi* GalLO was expressed in the inclusion bodies of *E. coli* and successfully refolded in a reverse micellar system to a native-like structure in the presence of FAD but not with FMN (Kudryashova et al., 2011). *L. donovani* GalLO (Biyani and Madhubala, 2011), predicted to have non-covalently bound FAD, was found to be in the apo form when expressed in *E. coli* as no activity was detected without FAD addition.

Expression of aldonolactone oxidoreductases in *E. coli* did not produce an enzyme with covalently linked FAD. This could be because the recombinant proteins may not have correctly folded, since several VAO members were successfully expressed as holo proteins in *E. coli*, including VAO (Benen et al., 1998), such as eugenol oxidase (Jin et al., 2007), alditol oxidase (Heuts et al., 2007a), and chitoooligosaccharide oxidase (Heuts et al., 2007b). Incorrect folding of recombinant *M. tuberculosis* GulLDH, expressed in *E. coli* may have been the reason for producing a non flavoprotein.

8. Subcellular localization of aldonolactone oxidoreductases

Aldonolactone oxidoreductases from animals such as rat (Eliceiri et al., 1969), goat (Nishikimi et al., 1976), mouse (Braun et al., 1999), chicken (Kiuchi et al., 1982), and pig (Hasan et al., 2004) were localized to microsomes and they all are integral membrane proteins. Several possible membrane associating regions were identified in rat GulLO amino acid sequence (Koshizaka et al., 1988). The active site of rat GulLO was found to be intraluminal and the enzyme releases its products towards the lumen of endoplasmic reticulum (Puskás et al., 1998).

Plant GLDHs are localized in the mitochondria in cauliflower (Mapson and Breslow, 1958; Østergaard et al., 1997), spinach leaves (Mutsuda et al., 1995), tobacco (Yabuta et al., 2000), white potato tubers and leaves (Ôba et al., 1994; Bartoli et al., 2000). About one-fifth of GLDH activity was also observed in microsomes of white potato tubers (Ôba et al., 1994) and leaves (Bartoli et al., 2000). A mitochondrial transit peptide is found in all plant GLDHs whose cDNA sequences have been identified in cauliflower (Østergaard et al., 1997), tobacco (Yabuta et al., 2000), *Arabidopsis* (Tamaoki et al., 2003), sweet potato (Imai et al.,

1998), strawberry (Nascimento et al., 2005), muskmelon (Pateraki et al., 2004), wheat (Bartoli et al., 2005), *Rosa roxburghii* (An et al., 2007), tomato (Alhag Dow et al., 2007) and rice (Yu et al., 2010).

Detailed localization studies of GLDH in white potato (Bartoli et al., 2000) and kidney beans (Siendones et al., 1999) found that GLDH is located at the inner mitochondrial membrane. Bartoli et al. (2000) proposed that the catalytic site is on the outside of the inner mitochondrial membrane by predicting the location of the FAD-binding site, which was thought to be Leu-137. It is worth noting that the Leu-137 was only thought to be a putative FAD binding site (Imai et al., 1998). Recent identification of the HWXK motif towards the C terminus of *T. cruzi* GalLO suggests that the FAD binding site could be the histidine in this motif (Logan et al., 2007; Kudryashova et al., 2011). Nevertheless, the prediction by Bartoli et al. (2000) could still be correct as the HWXK motif is also present on the outside of the inner mitochondrial membrane in the GLDH from white potato leaves.

S. cerevisiae (Nishikimi et al., 1978; Blegg and Christensen, 1982) and *C. albicans* (Huh et al., 1994) ALOs are localized in the mitochondria, where they are predicted to be integral membrane proteins (Huh et al., 1998; Huh et al., 2001). The subcellular localization of *G. frondosa* GulLO was not determined but it may be a membrane protein (Okamura, 2001). Interestingly, *P. griseoroseum* GUO was isolated as a soluble enzyme (Takahashi et al., 1976) and the native signal peptide was sufficient to be secreted when expressed in *P. pastoris* (Salusjärvi et al., 2004). The *G. oxydans* GulLDH was also isolated from the soluble fraction (Sugisawa et al., 1995) and that of *E. gracilis* was localized to the cytosol by sucrose density gradient centrifugation, although about 12% of the activity was also found in the mitochondria (Shigeoka et al., 1979). GulLDH of *M. tuberculosis* (Mawuenyega et al., 2005) and *Arabidopsis* AtGulLO5 (Aboobucker et al., submitted), however, are localized to the cell wall.

Trypanosomal enzymes are localized in glycosome and they have a characteristic SHL motif at the C terminus of their amino acid sequence which may be targeted to the glycosomes (Wilkinson et al., 2005; Logan et al., 2007). *L. donovani* ALO is also localized to the glycosomes as well as to the cytosol. The amino acid sequence lacks the SHL motif (Biyani and Madhubala, 2011).

9. Plants have GulLOs, in addition to GLDHs

It is absolutely clear from the characterization of GLDHs of many plant species that they are highly specific to L-GalL and cannot use L-GulL as a substrate, except at a very low efficiency in some cases (Table 2). In a recent article, Wheeler et al. (2015) conclude that plants have been replaced with the GLDH enzyme instead of GulLO. However, the following evidences demonstrate the existence of a GulLO enzyme in plants in addition to GLDH.

First, multiple precursor feeding studies have reported the use of L-GulL as a substrate since 1954 (Isherwood et al., 1954) including our observation in the detached leaves of *Nicotiana benthamiana* (wood tobacco; Lisko et al., 2014). The increase in AsA when fed with L-GulL is different than with L-GalL or L-Gal feeding. The substrates L-GalL or L-Gal are readily

converted to AsA while L-GuLL is converted less efficiently. This activity of converting L-GuLL to AsA can be attributed to the existence of a GuLLO enzyme since GLDH is strictly specific to L-GalL (Table 2). The possibilities of differential substrate uptake (Baig et al., 1970; Davey et al., 1999) and the stimulation of AsA synthesis by a general increase in carbohydrate pool (Loewus, 1963; Baig et al., 1970) have been ruled out. Interestingly, when Wheeler et al. (2015) fed L-Gal or L-GuLL to the rhodophyte algae *Galdieria sulphuraria*, the phenomenon of AsA increase observed was just as it has been observed in many plant species as mentioned above (Isherwood et al., 1954; Lisko et al., 2014). Rightfully so, Wheeler et al. (2015) concluded that *G. sulphuraria* has a GuLLO enzyme instead of a GLDH. For this same reason, we argue the idea of a GuLLO enzyme in plants in addition to GLDH based on the precursor feeding experiments with L-GuLL. Second, the enzyme activity responsible for this catalysis has been observed in hypocotyl homogenates of kidney beans (Siendones et al., 1999), cytosolic and mitochondrial fractions of *Arabidopsis* cell culture (Davey et al., 1999) and potato tubers (Ôba et al., 1994; Wolucka and van Montagu, 2003). Third, rat GuLLO, the enzyme known to use both L-GalL and L-GuLL, has been successfully expressed in multiple plant species including lettuce, tobacco (Jain and Nessler, 2000), potato (Hemavathi et al., 2010), *Arabidopsis* (Radzio et al., 2003), and tomato (Lim et al., 2012) resulting in elevated AsA levels. It may be possible that these observed increases were due to rat GuLLO using L-GalL, as the enzyme can also use this as a substrate (Table 2). However, it might not have been the case as the level of L-GalL is reduced in the vitamin C deficient (*vtc*) mutants and expression of rat GuLLO rescued AsA level in these mutants (Radzio et al., 2003; Gallie, 2014). Overexpression of GLDH has failed to increase AsA in tobacco plants (Imai et al., 2009). Bartoli et al. (2005) did not find an increase in AsA level with increase in GLDH activity in wild type wheat. This further strengthens the idea for the requirement of an enzyme to use L-GuLL as a substrate. Multiple authors (Davey et al., 1999; Wolucka and van Montagu, 2003; Leferink et al., 2008b) have insisted on the existence of a GuLLO isoenzyme in plants. Baig et al. (1970) went on to predict that this isoenzyme will be specific to L-GuLL. Seven putative GuLLOs were predicted in *Arabidopsis* based on the homology to rat GuLLO (Wolucka and van Montagu, 2003) and two ESTs were identified for GuLLOs in kiwi (Crowhurst et al., 2008). Therefore, we and others strongly support the idea of a GuLLO enzyme using L-GuLL as a substrate in plants, distinct from GLDH.

Recently, Maruta et al. (2010) expressed four of the seven putative GuLLOs from *Arabidopsis* in BY2 cell suspension cultures. They found that transgenic cell cultures expressing three of the GuLLOs had increased AsA content when L-GuLL was fed for 48 h. This marks the first observation of plant genes encoding proteins with GuLLO activity. While enzyme activity was present, no detailed characterization was reported. Recently, we characterized, for the first time, a recombinant AtGuLLO5 (TAIR: At2g46740) expressed in a *Nicotiana benthamiana* based transient expression system (Aboobucker et al. submitted). AtGuLLO5 has an absolute specificity for L-GuLL and it cannot use L-GalL as predicted by Baig et al. (1970). The enzyme did not have oxidase activity and it behaved more like bacterial GuLDHs in terms of its substrate specificity and the use of electron acceptor. AtGuLLO5 is localized in the cell wall (Boudart et al., 2005; Aboobucker and Lorence, unpublished observation). This provides further evidence for the existence of a GuLLO in

addition to GLDH in plants thereby to the existence of alternate routes for AsA biosynthesis in plants.

10. Conclusions and perspectives

Animal and fungal GulLO and ALO have covalently bound FAD while plants and protozoans have non-covalently bound FAD. There is a strong correlation between the presence of histidine and lysine/leucine in amino acid sequences in the N terminus of FAD-binding domain and covalent and non-covalent FAD interactions, respectively. A second histidine residue, involved in FAD binding in VAO, is also found in a widely conserved HWXK motif among all aldonolactone oxidoreductases. Flavin is assumed to be bound to aldonolactone oxidoreductases in the N terminus as in two of VAO family members, berberine bridge enzyme and 6-hydroxy-D-nicotine oxidase. Rather, the flavin group may be binding to the HWXK motif in the C terminus as in VAO. Resolving the crystal structure of aldonolactone oxidoreductases will provide a clear answer.

All animal and yeast aldonolactone oxidoreductases are oxidases, but in addition they also possess dehydrogenase-like activity utilizing phenazine methosulfate but not cytochrome C as electron acceptor. Protozoan enzymes are both oxidases and dehydrogenases as well and they use both cytochrome C and oxygen. Plant GLDHs are exclusive dehydrogenases using cytochrome C and phenazine methosulfate. An amino acid responsible for oxygen reactivity is found in AtGLDH which is present in all plant GLDHs. Bacterial enzymes are exclusive dehydrogenases as well. An isoenzyme from algae is unique to use only phenazine methosulfate and neither cytochrome C nor oxygen as an electron acceptor *in vitro*.

The flavin moiety is involved in enzyme catalysis in many aldonolactone oxidoreductases. The thiol group is essential for these enzymes as all isoenzymes except GUO of *P. griseoroseum* were inhibited by sulfhydryl groups. The thiol group participates in substrate binding but not in the catalysis itself. Only one sulfhydryl group is involved in this function and it was identified as Cys-340 in AtGLDH. *M. tuberculosis* GulLDH was the only enzyme identified as a metalloenzyme among all. Site-directed mutagenesis studies assisted to understand the roles of some amino acid residues in these enzymes. Although, multiple studies have been done to understand the roles of residues involved in enzyme function in these families, molecular determinants for substrate specificity are yet to be found.

Animal GulLOs and an algal GulLDH use L-GulL as well as L-GalL as substrates. Plant GLDHs are highly specific to L-GalL and cannot use L-GulL. Similarly, protozoan enzymes can use L-GalL and/or D-AL but not L-GulL. Ascomycete fungal ALOs prefer D-AL, in addition they catalyze L-GalL at a significant rate and L-GulL is also a substrate although at a low rate. An exception to this is GUO of *Penicillia* that is specific to D-gluconolactone and doesn't oxidize L-GalL, L-GulL or D-AL. Conversely, animal, yeast, plant and bacterial enzymes don't catalyze D-gluconolactone. A basidiomycete GulLO isoenzyme and bacterial GulLDHs are very specific to L-GulL and L-GalL is not a substrate for them. So, three kinds of enzymes exist, one can use only L-GulL, the second can use both L-GulL and L-GalL, and the third can use only L-GalL.

Animal GulLOs are localized to microsomes. Plant GLDHs and ascomycete fungal ALOs are localized to the mitochondria except GUO of *P. griseoroseum* that was isolated from the soluble fraction. Bacterial GulLDHs were found to be localized in the soluble fraction in *G. oxydans* and in cell wall in *M. tuberculosis* while the algal GulLDH was localized in both cytosol and mitochondria.

Plants, in addition to GLDH, have GulLO with an absolute specificity for L-GulL, similar to bacterial enzymes. It is not known why plants have both GLDH and GulLO. The differential expression of these genes spatially and temporally may be involved in regulating the synthesis of AsA, which is yet to be studied. Also, GLDH is a single copy gene in many species whereas GulLO exists as a gene family at least in *Arabidopsis* and the reason for this is unclear.

In the past, enzymes from the AsA biosynthetic and recycling pathways have been overexpressed to increase AsA in plants (reviewed in Lisko et al., 2014). Ascorbate biosynthetic pathways in plants converge to either L-GulL or L-GalL as the immediate precursor. Substrate availability is the limiting factor for AsA synthesis through L-GalL in plants (Imai et al., 2009). The L-GulL pool may be exploited to increase AsA in the plants. Understanding the regulation of GulLO enzyme(s) in plants may provide opportunities to increase AsA through L-GulL.

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Abbreviations

AtGLDH	<i>Arabidopsis thaliana</i> L-galactono-1,4-lactone dehydrogenase
AtGulLO	<i>A. thaliana</i> L-gulono-1,4-lactone oxidase
D-AL	D-Arabinono-1,4-lactone
L-AL	L-Arabinono-1,4-lactone
ALO	D-Arabinono-1,4-lactone oxidase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GalLO	L-galactono-1,4-lactone oxidase
L-GalL	L-Galactono-1,4-lactone
D-GalL	D-Galactono-1,4-lactone
GLDH	L-Galactono-1,4-lactone dehydrogenase
D-GulL	D-Gulono-1,4-lactone

l-GulL	l-Gulono-1,4-lactone
GulLDH	l-Gulono-1,4-lactone dehydrogenase
GulLO	l-Gulono-1,4-lactone oxidase
GUO	D-Gluconolactone oxidase
Man/Gal	D-Mannose/l-Galactose
VAO	Vanillyl alcohol oxidase

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Highlights

- A flavin cofactor is a characteristic feature present in almost all aldonolactone oxidoreductases.
- An HWXK motif is present in all aldonolactone oxidoreductases for which amino acid sequences are known.
- All aldonolactone oxidoreductases that are oxidases also have dehydrogenase activity however, exclusive dehydrogenases do exist.
- Aldonolactone oxidoreductases with absolute specificity to *L*-galactono lactone or *L*-gulono lactone and promiscuous enzymes are found.
- Plants have both *L*-gulono-1,4-lactone oxidases (GulLOs) and *L*-galactono-1,4-lactone dehydrogenases (GLDHs) involved in ascorbate biosynthesis

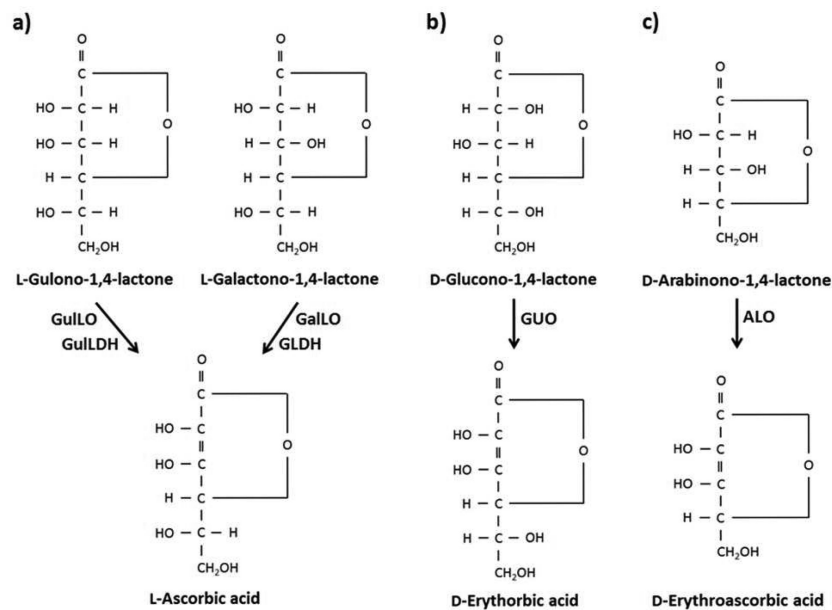


Figure 1.

Aldonolactone oxidoreductases catalyzing the terminal step in the biosynthesis of ascorbate or its analogues in (a) plants, animals, protozoa and bacteria, (b) *Penicillia* and (c) yeast from their preferred substrates. ALO, *D*-arabinono-1,4-lactone oxidase; GalLO, *L*-galactono-1,4-lactone oxidase; GLDH, *L*-galactono-1,4-lactone dehydrogenase; GulLDH, *L*-gulono-1,4-lactone dehydrogenase; GulLO, *L*-gulono-1,4-lactone oxidase; GUO, *D*-gluconolactone oxidase.

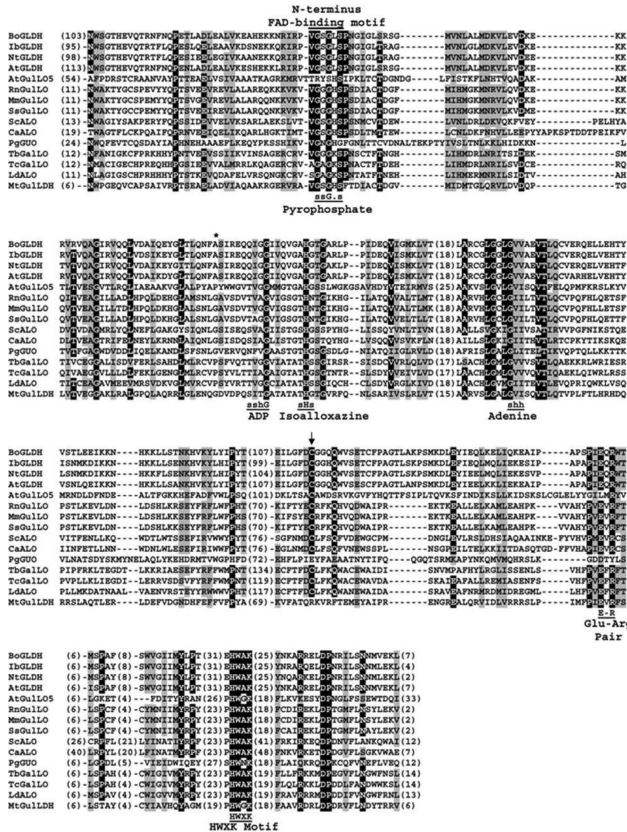


Figure 2. Multiple sequence alignment of all characterized aldolactone oxidoreductases including Arabidopsis GulLO (AtGulLO5 or At2g46740)

Conserved residues interacting with the parts of FAD in the alignment are shown as in Fraaije et al (1998). (G, glycine; H, histidine; s, small: G, A, S, T; h, hydrophobic: I, L, V; -, any residue). Numbers in parenthesis indicate the residues present in gaps and termini. The residue indicating oxygen reactivity is marked by asterisk (*); Leferink et al. 2009b). Arrow indicates the cysteine residue known to be involved with thiol modifying agents in AtGLDH (Leferink et al. 2009d). The Glu-Arg pair identified in the catalytic site of AtGLDH (Leferink et al. 2009c) is also highlighted. The sequences used are: BoGLDH (cauliflower; Swiss-Prot: O47881), IbGLDH (sweet potato; GenBank: BAA34995), NtGLDH (tobacco; GenBank: BAB13368), AtGLDH (Arabidopsis; Swiss-Prot: Q9SU56), AtGulLO5 (Arabidopsis; GenBank: AEC10747), RnGulLO (rat; Swiss-Prot: P10867), MmGulLO (mouse; GenBank: AAR15891), SsGulLO (pig; GenBank: AAN63634), ScALO (S. cerevisiae; Swiss-Prot: P54783), CaALO (C. albicans; GenBank: AAC98913), PgGUO (P. griseoroseum; Swiss-Prot: Q671X8), TbGalLO (T. brucei; Swiss-Prot: Q57ZU1), TcGalLO (T. cruzi; Swiss-Prot: Q4DPZ5), LdALO (L. donovani; GenBank: ACV04074), MtGulLDH (M. tuberculosis; Swiss-Prot: O06804).

Table 1

Properties of flavin characterization in aldono-lactone oxidoreductases

Source	Spectral peaks (nm)	Mode of flavin attachment	Covalent flavin linkage	Residue in N terminus FAD domain	Presence of HWXX motif	FAD or FMN	Fluorescence in SDS-PAGE	References
Animals	Rat	Covalent	8 α -N(1)-histidyl	His	Yes	FAD	Yes	Nakagawa et al., 1975 Kenney et al., 1976
	Chicken	Covalent	8 α -N(1)-histidyl	ND	ND	FAD	Yes	Nishikimi et al., 1977
	Goat	Covalent	ND	ND	ND	ND	ND	Kiuchi et al., 1982
	Mouse	Covalent	ND	His	Yes	ND	ND	Nishikimi et al., 1976
	Pig	ND	Covalent	ND	His	Yes	ND	Ha et al., 2004 Hasan et al., 2004
Fungi	<i>S. cerevisiae</i>	Covalent	8 α -N(1)-histidyl	His	Yes	FAD	Yes	Nishikimi et al., 1978 Kenney et al., 1979 Huh et al., 1998 Huh et al., 1994
	<i>C. albicans</i>	Covalent	8 α -N(3)-histidyl	His	Yes	FAD	Yes	Huh et al., 2001 Takahashi et al., 1976 Shimizu et al., 1977
	<i>P. griseoroseum</i>	Covalent	8 α -N(3)-histidyl	His	Yes	FAD	Yes	Harada et al., 1979
	<i>G. frondosa</i>	Covalent	ND	ND	ND	ND	Yes	Salusjärvi et al., 2004 Okamura, 2001

Source	Spectral peaks (nm)	Mode of flavin attachment	Covalent flavin linkage	Residue in N terminus FAD domain	Presence of HWXK motif	FAD or FMN	Fluorescence in SDS-PAGE	References
Bacteria								
<i>M. tuberculosis</i>	Nil	Nil	ND	His	Yes	Nil	No	Wolucka and Communi, 2006
<i>G. oxydans</i>	ND	Covalent	ND	ND	ND	ND	Yes	Sugisawa et al., 1995
<i>T. brucei</i>	ND	Non-covalent	ND	Lys	Yes	FAD (predicted)	ND	Wilkinson et al., 2005 Logan et al., 2007
<i>T. cruzi</i>	ND	Non-covalent	ND	Lys	Yes	FAD	ND	Logan et al., 2007 Kudryashova et al., 2011
<i>L. donovani</i>	ND	Non-covalent (predicted)	ND	Lys	Yes	ND	ND	Biyani and Madhubala, 2011
Protozoa								
Cauliflower	375, 455	Non-covalent	ND	Leu	Yes	FMN	ND	Mapson and Breslow, 1958 Østergaard et al., 1997
Sweet potato	373, 448	Non-covalent	ND	Leu	Yes	ND	ND	Óba et al., 1995 Imai et al., 1998
Tobacco	ND	Non-covalent	ND	Leu	Yes	ND	ND	Yabuta et al., 2000
<i>Arabidopsis</i>	375, 450	Non-covalent	ND	Leu	Yes	FAD	ND	Leferink et al., 2008b

ND: not determined

Table 2

Substrate specificity of aldonolactone oxidoreductases towards each other's preferred substrates

Source	L-Gulono-1,4-lactone	D-Mannono-1,4-lactone	L-Galactono-1,4-lactone	D-Altrono-1,4-lactone	D-Arabinono-1,4-lactone	D-Glucono-1,4-lactone	References
Rat	100	68	87	47	22	ND ^d	Ashwell et al., 1961
Rat	100 (0.066 mM)	61	70	15	ND	<3	Kiuchi et al., 1982
Chicken	100 (0.007 mM)	64	90	16	ND	<3	Nishikimi et al., 1976
Goat	100 (0.15 mM)	ND	ND	ND	ND	ND	Kiuchi et al., 1982
							Nishikimi et al., 1976
Fungi							
<i>S. cerevisiae</i>	32	<3	100	68	ND	<3	Nishikimi et al., 1978
<i>S. cerevisiae</i>	0	ND	100 (0.30 mM)	65 (2.0 mM)	97 (0.16 mM)	ND	Bleeg and Christensen, 1982
<i>C. albicans</i>	24.7	ND	87.6 (53.7 mM)	ND	100 (44.1 mM)	ND ^d	Huh et al., 1994
<i>P. griseoroseum</i>	0	0	ND	ND	ND	100 ^e (1.7 mM)	Takahashi et al., 1976
<i>P. notatum</i>	ND	ND	0	0	ND	100	Takahashi, 1969
<i>G. frondosa</i>	100 (24 mM)	25	2	0	0	ND ^d	Okamura, 2001
<i>L. edodes</i>	100	24	0	0	0	ND ^d	Okamura, 2001
Bacteria							
<i>M. tuberculosis</i>	100 (5.5 mM)	ND	0	ND	ND	ND	Wolucka and Communi, 2006
<i>G. oxydans</i>	100 (34.8 mM)	ND	0	ND	ND	ND ^d	Sugisawa et al., 1995
Protozoa							
<i>T. brucei</i>	3	ND	77 (0.154 mM)	ND	100 (0.055 mM)	ND	Wilkinson et al., 2005
<i>T. cruzi</i>	0	ND	100 (0.161 mM)	ND	96 (0.285 mM)	ND	Logan et al., 2005
<i>L. donovani</i>	0	ND	ND	ND	100 (0.039 mM)	ND	Biyani and Madhubala, 2011

Source	L-Gulono-1,4-lactone	D-Mannono-1,4-lactone	L-Galactono-1,4-lactone	D-Altrono-1,4-lactone	D-Arabinono-1,4-lactone	D-Glucono-1,4-lactone	References
Algae							
<i>E. gracilis</i>	71	ND	100	ND	ND	ND	Shigeoka et al., 1979
Cauliflower	0	0	100 (2 and 4 mM) ^d	10	ND	0	Mapson and Breslow, 1958
Cauliflower	0	ND	100 (3.3 mM)	ND	ND	ND	Østergaard et al., 1977
White potato	19.5	ND	100 (0.08 and 0.23 mM) ^b	ND	ND	ND	Óba et al., 1994
Sweet potato	1	ND	100 (0.12 mM)	ND	ND	ND	Óba et al., 1995
Spinach	0	ND	100 (0.192 mM)	ND	ND	ND	Mutsuda et al., 1995
Tobacco	7	ND	100 (0.06 and 0.08 mM) ^c	ND	ND	ND	Yabuta et al., 2000
Strawberry	0	ND	100	ND	ND	ND	Nascimento et al., 2005
<i>Arabidopsis</i>	(13.1 mM)	ND	100 (0.17 mM)	ND	10.2 mM	ND	Leferink et al., 2008b Leferink et al., 2009c

^a with different electron acceptors

^b with different methods

^c with native and recombinant enzymes

^d No or very less activity with Dglucono-1,5-lactone

^e similar result with D-glucono-1,5-lactone; ND, not determined. Numbers in the parantheses indicate the substrate affinity values (K_m).

Table 3
A comparison of the effects of different classes of inhibitors on aldonolactone oxidoreductases

Source	Sulfhydryl group inhibitors	Metal ions	Metal Chelating agents	Substrate analogs	Flavin inhibitors	Reference
Animals						
Rat	Sulfide, sulfite, PCMS	1-Hg ²⁺ 2-Cd ²⁺	Nil	ND	Nil	Nakagawa and Asano, 1970
Fungi						
<i>S. cerevisiae</i>	PCMS, iodoacetamide, NEM, sulfite, sulfide	1-Hg ²⁺ , Cd ²⁺	Nil	L-Gull, D-Gall	ND	Bleeg and Christensen, 1982
<i>S. cerevisiae</i>	PCMB	ND	Nil	Nil	ND	Nishikimi et al., 1978
<i>C. albicans</i>	PCMB, NEM, iodoacetic acid, iodoacetamide	1-Cd ²⁺ , Hg ²⁺ , Mn ²⁺ , Zn ²⁺	Nil	D-Glucono-1,5-lactone, L-AL, D-Gall, D-Gull	ND	Huh et al., 1994
<i>P. griseoroseum</i>	Nil	2-Cu ²⁺ , Mg ²⁺ 1-Hg ²⁺ , Ag ²⁺ , Fe ³⁺ , Fe ²⁺ , Cu ²⁺ 2-Co ²⁺ , Mn ²⁺ , Zn ²⁺ , Cd ²⁺ , Mg ²⁺ , Ca ²⁺ , As ²⁺	ND	ND	ND	Takahashi et al., 1976
<i>P. notatum</i>	PCMB	ND	Nil	ND	ND	Takahashi, 1969
<i>G. frondosa</i>	Nil	ND	ND	ND	Acriflavine	Okamura, 2001
Bacteria						
<i>M. tuberculosis</i>	NEM	1-Cu ²⁺ , Zn ²⁺ 2-Mg ²⁺ , Ca ²⁺ 3-Mn ²⁺	EDTA, KCN	ND	Nil	Wolucka and Communi, 2006
<i>G. oxydans</i>	Nil	1-Cu ²⁺ , Mn ²⁺ 3-Fe ⁶⁺ , Mo ⁶⁺ , Ti ⁺ , Zn ²⁺ , Ni ²⁺	Nil	ND	ND	Sugisawa et al., 1995
Protozoa						
<i>T. brucei</i>	ND	3-Cu ²⁺	ND	Substrate >4mM	ND	Wilkinson et al., 2005
<i>T. cruzi</i>	PCMB, NEM	1-Hg ²⁺ , Zn ²⁺	ND	ND	ND	Logan et al., 2007
<i>L. donovani</i>	NEM	1-Zn ²⁺ , Hg ²⁺	ND	ND	ND	Biyani and Madhubala, 2011
Algae						
<i>E. gracilis</i>	PCMB, NEM	1-Cu ²⁺ , Zn ²⁺ , Fe ²⁺	Nil	ND	Riboflavin	Shigeoka et al., 1979
Plants						
Cauliflower	PCMB, <i>o</i> -Iodosobenzoate	1-Cu ²⁺ 2-Mn ²⁺ , Zn ²⁺ , Fe ³⁺ , MoO ₄ ²⁻	<i>o</i> -phenanthroline	D-Gull, D-Gall	Atebrin, Riboflavin	Mapson and Breslow, 1958
Cauliflower	PHMB, NEM, monoiodoacetate	Nil	Nil	L-Gall - 32.6 mM	Nil	Østergaard et al., 1997

Source	Sulfhydryl group inhibitors	Metal ions	Metal Chelating agents	Substrate analogs	Flavin inhibitors	Reference
Sweet potato	PCMB, NEM*	1- Cu ²⁺ , Zn ²⁺ *	ND	l-GalL 4 mM	Acridflavine	Óba et al., 1995; * Imai et al., 1998
Spinach	PCMB, NEM	ND	ND	l-GalL > 1 mM	ND	Mutsuda et al., 1995
Tobacco	PCMB, NEM	Cu ²⁺ , Zn ²⁺	ND	ND	Acridflavine	Yabuta et al., 2000
Strawberry	ND	ND	ND	l-GalL > 10 mM	ND	Nascimento et al., 2005
<i>Arabidopsis</i>	DTNB, NEM	ND	ND	l-GalL > 2 mM*	ND	* Leferink et al., 2008b; Leferink, 2009a

1: inhibitory, **2:** not inhibitory, **3:** slightly inhibitory, Nil: no inhibition found, DTNB: 5,5'-dithiobis(2-nitrobenzoic acid), NEM: *N*-ethylmaleimide, PCMB: *p*-chloromercuribenzoate, PCMS: *p*-chloromercurisulfate, PHMB: *p*-hydroxymercuribenzoate. ND: not determined