Functional assignment of Glu386 and Arg388 in the active site of L-galactono-γ-lactone dehydrogenase

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The flavoenzyme L-galactono-γ-lactone dehydrogenase (GALDH) catalyzes the terminal step of vitamin C biosynthesis in plants. Little is known about the catalytic mechanism of GALDH and related aldolactone oxidoreductases. Here we identified an essential Glu–Arg pair in the active site of GALDH from Arabidopsis thaliana. Glu386 and Arg388 variants show high Km values for L-galactono-1,4-lactone and low turnover rates. Arg388 is crucial for the stabilization of the anionic form of the reduced FAD cofactor. Glu386 is involved in productive substrate binding. The E386D variant has lost its specificity for L-galactono-1,4-lactone and shows the highest catalytic efficiency with L-gulono-1,4-lactone.

1. Introduction

L-Ascorbate (vitamin C) is an important antioxidant, redox buffer and enzyme cofactor for many organisms. Plants and most animals can synthesize ascorbate to meet their daily requirements, while humans and other primates have lost this ability during evolution. Ascorbate is particularly abundant in plants, the main dietary source of vitamin C for humans. The terminal step of ascorbate biosynthesis in plants is catalyzed by the flavoenzyme L-galactono-γ-lactone dehydrogenase (GALDH; EC 1.3.2.3). GALDH is localized in the mitochondrial intermembrane space where it catalyzes the oxidation of L-galactono-1,4-lactone into L-ascorbate with the concomitant reduction of cytochrome c [1,2]. GALDH is also required for the proper functioning of plant mitochondria and for the correct assembly of plant respiratory complex I [3,4].

GALDH and related aldolactone oxidoreductases involved in vitamin C biosynthesis belong to the vanillyl-alcohol oxidase (VAO) flavoprotein family. Members of this family share a two-domain folding topology, comprising a conserved N-terminal FAD-binding domain and a C-terminal cap domain that determines the substrate specificity. The active site is located at the interface of the domains [5,6].

Aldolactone oxidoreductases have been isolated from various sources, including several plants, animals and fungi, but are relatively poorly characterized. No three-dimensional structure is known for any aldolactone oxidoreductase, hence little is known about the nature of the active site and the reaction mechanism. GALDH shows respectively 25% and 20% sequence identity with alditol oxidase (AldO) [7] and cholesterol oxidase (CO) [8]. These VAO-family members with known three-dimensional structure contain a Glu–Arg pair in the cap domain that is conserved among aldolactone oxidoreductases (Fig. 1). In AldO, Glu320 is involved in extensive hydrogen bonding with the polyol substrate [7]. Removal of the positive charge of Arg477 in CO resulted in a dramatic loss of activity [9]. Furthermore, the Glu–Arg pair of CO is located at the bottom of a tunnel that extends from the surface to the active site, and is implicated to function as a gate to control oxygen access [8,9].

Here we addressed the role of the Glu–Arg pair in GALDH from Arabidopsis thaliana. Biochemical analysis of Glu386 and Arg388 variants revealed that both residues are essential for catalysis.
Removal of the positive charge of Arg388 results in a nearly inactive GALDH variant that is unable to stabilize negative charges in the active site. Mutation of Glu386 into Asp creates a GALDH variant with a preference for L-gulono-1,4-lactone.

2. Materials and methods

2.1. Materials

L-Galactono-1,4-lactone was synthesized from D-galacturonic acid by borohydride reduction according to the method described by Mapson and Breslow [10]. The crude product was purified on a silica column (particle size 40–63 μm) running in ethyl acetate/methanol (6:4). The purified product was re-crystallized from ethanol. All other chemicals were from commercial sources and of the purest grade available.

2.2. Site-directed mutagenesis

GALDH Glu386 and Arg388 variants were constructed using pET-GALDH-His6 [11] as template using the QuikChange II protocol (Stratagene, USA). Oligonucleotides used were (only sense primers are shown, changed codons are underlined): 5’-GCACCTGCTCCAATAGCGCAGGCAATGACACGC-3’ (E386A); 5’-GCACCTGCTCCAATAGCGCAGGCAATGACACGC-3’ (E386D); 5’-GCACCTGCTCCAATAGCGCAGGCAATGACACGC-3’ (E386D); 5’-GCTCCAATAGCGCAGGCAATGACACGC-3’ (R388A) and 5’-GCTCCAATAGCGCAGGCAATGACACGC-3’ (R388K). Successful mutagenesis was confirmed by automated sequencing.

2.3. Enzyme production and purification

Escherichia coli BL21(DE3) cells, harboring a pET-GALDH plasmid, were grown in Luria–Bertani medium supplemented with 100 μg/ml ampicillin until an OD₆₀₀ of 0.7 was reached. GALDH expression was induced by addition of 0.4 mM isopropyl-thio–D-galactopyranoside and the incubation was continued for 16 h at 37 °C. The His₆-tagged GALDH variants were purified as described before [11].

2.4. Spectral analysis

Absorption spectra were recorded at 25 °C on a Hewlett-Packard 8453 diode array spectrophotometer in 50 mM sodium phosphate, pH 7.4. The molar absorption coefficients of protein-bound flavin for the mutant proteins were determined by recording absorption spectra in the absence and presence of 0.1% (w/v) SDS, assuming a molar absorption coefficient for free FAD of 11.3 mM⁻¹ cm⁻¹ at 450 nm.

Photoreduction of GALDH variants (10 M) in the presence of EDTA and 5-deazaflavin was performed in 50 mM sodium phosphate, pH 7.4 as described [12]. Catalytic amounts of glucose oxidase and 10 mM α-D-glucose were added to scavenge final traces of oxygen, and catalase was added to remove hydrogen peroxide formed during the reaction. Solutions were made anaerobic by alternate evacuation and flushing with oxygen-free argon. Absorption spectra were recorded at regular intervals during illumination until complete reduction was achieved.

Titration of GALDH variants (10 μM) with sodium sulfite was carried out in 50 mM sodium phosphate, pH 7.4. A 1 M sodium sulfite stock solution in 50 mM sodium phosphate, pH 7.4 was freshly prepared before use; suitable dilutions were made in the same buffer before addition to the enzyme solution. Absorption spectra were recorded until no further change was observed before the next addition was done. The dissociation constant (K₅₆) for the enzyme-sulfite complex was calculated from the change in absorbance at 450 nm [11].
2.5. Activity measurements

GALDH activity was routinely assayed by following the reduction of bovine heart cytochrome c at 550 nm [11]. Initial velocities were calculated using a molar difference absorption coefficient \((550)\) of 21 mM\(^{-1}\) cm\(^{-1}\) for reduced minus oxidized cytochrome c. One unit of enzyme activity is defined as the amount of enzyme that oxidizes 1 mol of L-galactono-1,4-lactone per min, which is equivalent to the reduction of 2 mol of cytochrome c [13].
3. Results and discussion

3.1. Spectral properties of GALDH variants

The GALDH Glu386 and Arg388 variants were expressed as soluble holoproteins in *E. coli*, and purified in similar yields as wild-type GALDH [11]. The specific activities of the purified mutant proteins were all less than 1% of the wild-type enzyme, which has a specific activity of 76 U mg⁻¹ [11].

The UV/VIS absorption spectral properties of the Glu386 variants were very similar to that of wild-type GALDH (Fig. 2A). The molar absorption coefficients of protein-bound flavin were determined to be 13.6 ± 0.1 mM⁻¹ cm⁻¹ at 451 nm for E386D and 13.1 ± 0.1 mM⁻¹ cm⁻¹ at 452 nm for E386A, compared to 12.9 ± 0.1 mM⁻¹ cm⁻¹ at 450 nm for wild-type GALDH [11]. Small perturbations with respect to wild-type GALDH were observed in the absorption spectra of the Arg388 variants (Fig. 2B). The absorption maximum near 375 nm is about 7 nm blue-shifted in both absorption spectra of the Arg388 variants (Fig. 2B). The absorption maximum near 375 nm is about 7 nm blue-shifted in both mutants, and the spectrum of R388A shows a more pronounced shoulder at 466 nm, similar to the CO variant R477A [9]. The molar absorption coefficients were determined to be 13.6 ± 0.1 mM⁻¹ cm⁻¹ at 450 nm for R388K and 13.5 ± 0.1 mM⁻¹ cm⁻¹ at 450 nm for R388A.

The ability to form a flavin N5-sulfite adduct is characteristic for many flavoprotein oxidases, and correlates with the ability of the protein to stabilize the red anionic semiquinone. Although wild-type GALDH reacts poorly with molecular oxygen [14,15], it readily forms a covalent flavin N5-sulfite adduct with a *Kₐ* of 18 μM [11]. Mutation of Glu386 did not have a significant effect on the formation of the sulfite adduct. Both E386D (*Kₐ* = 14 μM, Fig. 3A) and E386A (*Kₐ* = 5 μM) stabilize the sulfite adduct in a similar way as wild-type GALDH. Mutation of Arg388 strongly influenced the formation of the sulfite adduct. Replacing Arg388 by Lys gave a *Kₐ* of 0.44 mM for the enzyme-sulfite complex (Fig. 3B) whereas removal of the positive charge (R388A) resulted in a *Kₐ* of >20 mM, a more than 1000-fold increase compared to wild-type enzyme. Thus, the dissociation constants for the GALDH-sulfite adducts are directly related to the presence of a positive charge in close proximity of the flavin. This indicates that the ability of a flavoprotein to form a flavin-sulfite adduct is not related to its oxidase activity, as previously proposed [16], but to the nature of the active site. Flavocytochrome *b₂* also forms the flavin-sulfite adduct, and is poorly active with oxygen [17]. The crystal structure of AldO in complex with sodium sulfite shows that the sulfite adduct is stabilized by Arg322 and Lys375 [7], which correspond to Arg388 and Lys455 in GALDH. The adduct is formed on the *si*-face of the flavin, the same side as the substrate binding pocket, while the reaction of VAO-family members with molecular oxygen presumably takes place on the *re*-face of the flavin [15]. In flavocytochrome *b₂* also an Arg residue is involved in both catalysis and stabilization of the N5-sulfite adduct [18].

During anaerobic photoreduction wild-type GALDH stabilizes the red anionic flavin semiquinone [11]. Both Glu386 variants fully retained this property (Fig. 4A). In contrast, R388K only partially stabilizes the red semiquinone (Fig. 4B), while R388A undergoes reduction to the flavin hydroquinone without any semiquinone stabilization (not shown).

The above results show that replacement of Arg388 by Ala results in a variant that is unable to stabilize negative charges in the active site. The fact that the side chain of Arg388 is essential for the stabilization of the anionic flavosemiquinone suggests that it is located near the N1–C2 locus of the isoalloxazine ring of the flavin. A positive charge in this region will stabilize the anionic flavosemiquinone (Fig. 4B). The above results show that replacement of Arg388 by Ala results in a variant that is unable to stabilize negative charges in the active site. The fact that the side chain of Arg388 is essential for the stabilization of the anionic flavosemiquinone suggests that it is located near the N1–C2 locus of the isoalloxazine ring of the flavin. A positive charge in this region will stabilize the anionic flavosemiquinone (Fig. 4B).
ingly, the non-covalently bound FAD of GALDH has a relatively high redox potential (−44 mV) [15].

3.2. Catalytic properties of GALDH variants

The Glu386 variants are catalytically far less efficient than wild-type GALDH (Table 1). The Glu386 variants show about a 100-fold increase in the Michaelis–constant for the γ-galactono-1,4-lactone substrate and a significant reduction in turnover rate. Interestingly, replacing Glu386 with Asp in GALDH results in a variant that is more active with γ-gulono-1,4-lactone than the wild-type enzyme (Table 1). Presumably, the shorter side chain of residue 386 provides a better geometry for the interaction with γ-gulono-1,4-lactone, which has a different stereochemical configuration of the hydroxyl substituent at C3 than γ-galactono-1,4-lactone. Wild-type GALDH shows considerable activity with ω-arabinono-1,4-lactone (kcat = 51 s−1), which has the same stereochemical configuration at C2 and C3 as γ-galactono-1,4-lactone but a shorter side chain. The rather low affinity of GALDH for this substrate (Km = 10.2 mM), indicates that the length and stereochemistry of the side chain is important for proper binding and aligning of the substrate lactone. This suggests that all hydroxyl groups are involved in substrate binding, participating in a well defined hydrogen-bonding network as was proposed for AldD [7]. For the Glu386 variants activity with ω-arabinono-1,4-lactone could only be detected with E386D, but again the mutant protein is far less efficient than the wild-type enzyme.

Replacement of Arg388 has a dramatic effect on GALDH catalysis. Whereas R388K shows significant activity, removal of the charge in R388A results in a protein that is essentially inactive. Oxidation of γ-gulono-1,4-lactone and ω-arabinono-1,4-lactone by the Arg388 variants could not be detected, even at substrate concentrations up to 100 mM. Arg388A critically interacts with the substrate, replacement by either Lys or Ala gives a 1000-fold increase in the Michaelis–constant for γ-galactono-1,4-lactone. In AldD, the corresponding Arg322 is involved in hydrogen bonding with substrate C1, the site of oxidation, and flavin C4=O, suggesting a similar critical role [7].

In conclusion, we have shown that the conserved Glu–Arg pair in the active site of GALDH is required for optimal catalysis. GALDH is involved in substrate binding and proper catalytic alignment and Arg388 seems crucial for the stabilization of negative charge that is generated as a result of flavin reduction. Glu386 is also involved in determining the substrate specificity, replacement by Asp causes loss of specificity for γ-galactono-1,4-lactone, but increases the catalytic efficiency with γ-gulono-1,4-lactone. Previously we identified a Cys residue (Cys340) that seems also involved in substrate binding of GALDH. This Cys is strictly conserved in aldonoalactone oxidoreductases but not in other VAO-members [21]. Thus, aldonoalactone reductases share certain catalytic properties with other VAO-family members but evolved as a separate clade to discriminate between different aldonoalactones.

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