

The Pathway via D-Galacturonate/L-Galactonate Is Significant for Ascorbate Biosynthesis in *Euglena gracilis*

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF ALDONOLACTONASE^{*[5]}

Received for publication, May 22, 2008, and in revised form, August 5, 2008. Published, JBC Papers in Press, September 9, 2008, DOI 10.1074/jbc.M803930200

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We have previously proposed that *Euglena gracilis* possesses a pathway for the production of ascorbate (AsA) through D-galacturonate/L-galactonate as representative intermediates (Shigeoka, S., Nakano, Y., and Kitaoka, S. (1979) *J. Nutr. Sci. Vitaminol.* 25, 299–307). However, genetic evidence proving that the pathway exists has not been obtained yet. We report here the identification of a gene encoding aldonolactonase, which catalyzes a penultimate step of the biosynthesis of AsA in *Euglena*. By a BLAST search, we identified one candidate for the enzyme having significant sequence identity with rat gluconolactonase, a key enzyme for the production of AsA via D-glucuronate in animals. The purified recombinant aldonolactonase expressed in *Escherichia coli* catalyzed the reversible reaction of L-galactonate and L-galactono-1,4-lactone with zinc ion as a cofactor. The apparent K_m values for L-galactonate and L-galactono-1,4-lactone were 1.55 ± 0.3 and 1.67 ± 0.39 mM, respectively. The cell growth of *Euglena* was arrested by silencing the expression of aldonolactonase through RNA interference and then restored to the normal state by supplementation with L-galactono-1,4-lactone. *Euglena* cells accumulated more AsA on supplementation with D-galacturonate than D-glucuronate. The present results indicate that aldonolactonase is significant for the biosynthesis of AsA in *Euglena* cells, which predominantly utilize the pathway via D-galacturonate/L-galactonate. The identification of aldonolactonase provides the first insight into the biosynthesis of AsA via uronic acids as the intermediate in photosynthetic algae including *Euglena*.

Vitamin C (L-ascorbic acid (AsA)²) represents a group of essential nutrients for humans and is a hydrophilic antioxidant

* This work was supported in part by The Sumitomo Foundation (to T. I.) and Grant-in-aid for Scientific Research 19208031 from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to S. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBankTM/EBI Data Bank with accession number(s) AB306917.

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² The abbreviations used are: AsA, L-ascorbic acid; ALase, aldonolactonase; L-GalA, L-galactonate; D-GalUA, L-galacturonate; L-Gall, L-galactono-1,4-

synthesized by all animals except primates and photosynthetic organisms, including plants and algae (2). AsA has a pivotal role both as an antioxidant and as an enzyme cofactor in animals. In plants, AsA has been documented to play multiple roles in the control of photosynthesis, cell expansion and growth, and transmembrane electron transport in addition to its well characterized role as an antioxidant (3, 4).

With regard to the biosynthesis of AsA in living organisms, the major pathways differ between animals and plants. In animals, the committed step in the biosynthesis utilizes D-glucuronate (D-GlcUA), L-gulonate (L-GulA), and L-gulono-1,4-lactone (L-GulL) as a direct precursor of AsA (5). This pathway is a branch of the D-GlcUA pathway via the pentose phosphate pathway. A microsome-localized enzyme, L-GulL oxidase, catalyzes the terminal step to produce AsA. Genetic evidence that L-GulL is synthesized directly from L-GulA, not mediated by D-glucuronolactone, has been provided by the identification of SMP30 in rats as gluconolactonase (6).

In contrast to animals, plants utilize L-galactono-1,4-lactone (L-GalL) as the terminal precursor for the biosynthesis of AsA, which is produced from L-galactose via GDP-D-mannose and GDP-L-galactose as the intermediates (Man/Gal pathway) (4, 7, 8). The L-GalL is finally oxidized by the action of L-GalL dehydrogenase located in the mitochondrial inner membrane, resulting in the production of AsA (9, 10). Recently, an overall picture of the pathway was completed with the identification and analysis of a causal gene in low-AsA *vtc* mutants in *Arabidopsis thaliana* (11, 12). Genetic evidence indicates that the Man/Gal pathway is of critical importance. A null *Arabidopsis* mutant of the *CYT1* gene, which encodes the same protein as *VTC1* (GDP-Man pyrophosphorylase, an enzyme required for production of GDP-Man from Man 1-P), showed lethality in seedlings as well as caused reduced AsA levels (13). Dowdle *et al.* (14) also reported that *VTC2* encodes a GDP-L-Gal phosphorylase enzyme that breaks down GDP-L-Gal phosphorolytically, producing L-Gal 1-P, and the knock-out mutant of two *VTC2* genes shows growth arrest immediately upon germination and the cotyledons subsequently bleach. These findings

lactone; L-GlcUA, L-glucuronate; L-GulA, L-gulonate; L-GulL, L-gulono-1,4-lactone; PIPES, 1,4-piperazinediethanesulfonic acid; RNAi, RNA interference; dsRNA, double-stranded RNA.

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indicated that the Man/Gal pathway is the only physiologically significant source of AsA in *Arabidopsis* plants.

Although the Man/Gal pathway appears to be the predominant pathway, several other possible biosynthetic pathways have been proposed via uronic acid intermediates (15, 16). The isolation of an aldo-keto reductase specific for D-galacturonate (D-GalUA) from ripening strawberry fruits and the generation of transgenic plants overexpressing its gene in *Arabidopsis* indicated the contribution of the alternative pathway via D-GalUA to the production of AsA (15). In the pathway, although the conversion of L-galactonate (L-GalA), the product derived from the reduction of D-GalUA, to L-GalL is indispensable, aldono-lactonase (ALase), a key catalyzing enzyme, is still missing. In addition to the pathway via D-GalUA, other possible pathways via D-GlcUA or L-gulose are also proposed, although most of the enzymes related to these pathways have not been identified yet. Therefore, it is still unclear how these pathways contribute to the biosynthesis of AsA *in planta*.

In contrast to animals and plants, although some eukaryotic microorganisms also contain significant concentrations of cellular AsA, only scarce data are available on the way of biosynthesis. *Chlorella* and *Prototheca* contained appreciable amounts of AsA, and adding Man, L-GalA and L-GalL to the *Prototheca* medium resulted in significantly increased levels of AsA, suggesting the presence of a Man/Gal pathway similar to that in plants (17, 18). It was recently identified that a protozoan parasite, *Trypanosoma brucei*, possesses a functional gene encoding D-arabinono-1,4-lactone oxidase that can also oxidize L-GalL, although the exact pathway arriving at the final precursor has not been clarified yet (19). In *Euglena gracilis* containing high concentrations of AsA, the biosynthetic pathway was first proposed by Shigeoka *et al.* (1). Radioisotope tracer studies revealed that the biosynthesis of AsA in *Euglena* proceeded via the conversion of UDP-D-GlcUA to UDP-D-GalUA, with production of D-GalUA and reduction to L-GalA (Fig. 1). L-GalL was the final precursor in the production of AsA. These findings indicate that *Euglena* has developed a pathway analogous to the alternative pathway of AsA biosynthesis via D-GalUA, which is unclear in plants. Detection of L-GalL dehydrogenase activity in the *Euglena* cells (20) and characterization of purified D-GalUA reductase (21) strongly supported the contribution of the pathway via D-GalUA/L-GalA in promoting AsA biosynthesis in this alga.

In this study, to understand the biosynthesis of AsA in *Euglena* in detail, we have identified and characterized ALase, catalyzing the penultimate step from L-GalA to L-GalL. We report that *Euglena* cells silenced of the expression of ALase by RNAi are unable to grow unless supplemented with L-GalL. Based on the present findings, we discuss the significance of AsA biosynthesis via D-GalUA/L-GalA and the physiological role of AsA in this organism.

EXPERIMENTAL PROCEDURES

Materials—L-GalL, L-GulL, and D-GulL were purchased from Sigma-Aldrich. D-GalL and D-glucono- δ -lactone were purchased from Wako Pure Chemical (Osaka, Japan). L-GalA and L-GulA were prepared by the hydrolysis of L-GalL and L-GulL, respectively, under basic conditions (21). Twenty mil-

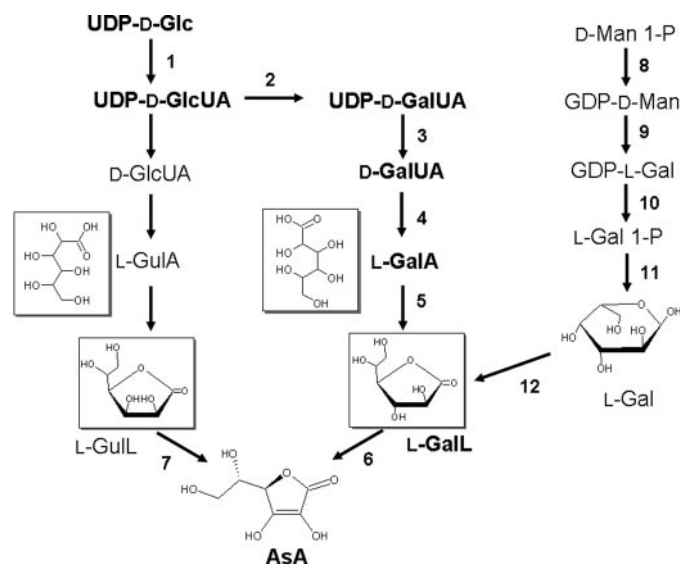


FIGURE 1. The pathway of AsA biosynthesis in *Euglena* and plants. A radio-tracer experiment provides evidence for the biosynthesis of AsA via uronic acids in *Euglena*. The unnumbered reactions are uncertain; however, enzyme 4 catalyzes the conversion of D-GlcUA to L-GulA (21). The pathway from D-Man 1-P to L-GalL (Man/Gal pathway, reactions 8–12) mainly operates in higher plants. Enzymes catalyzing the numbered reactions are: 1, UDP-Glc dehydrogenase; 2, UDP-Glc-4-epimerase; 3, UDP-D-GalUA pyrophosphatase/phosphorylase; 4, D-GalUA reductase; 5, ALase; 6, L-GalL dehydrogenase; 7, L-GulL oxidase/dehydrogenase; 8, GDP-D-Man pyrophosphorylase (VTC1); 9, GDP-Man-3',5'-epimerase; 10, GDP-L-Gal phosphorylase (VTC2/VTC5); 11, L-Gal 1-P phosphatase (VTC4); 12, L-Gal dehydrogenase.

liliters of 0.3 M NaOH was added to 100 μ l of 10 mM L-GalL or L-GulL, the mixture was vigorously agitated by vortexing for 20 s, and 20 μ l of 0.3 M hydrochloric acid was added to neutralize the solution.

Strain and Culture—*E. gracilis* strain Z was grown in Koren-Hutner medium under continuous light at a photosynthetic photon flux density of 24 μ mol $m^{-2} s^{-1}$ at 26 $^{\circ}C$ for 6 days, by which time the stationary phase was reached (22).

Enzyme Assay—The activity of lactonase was assayed based on the change in absorbance of a *p*-nitrophenol pH indicator using a modified version of a previously described method (23). The reaction mixture contained 10 mM PIPES buffer, pH 6.5, 250 μ M *p*-nitrophenol, 5 mM D-glucono- δ -lactone, 75 μ M ZnCl₂, and an enzyme in a total volume of 1 ml. Decolorization of *p*-nitrophenol by acidification was monitored at 405 nm and quantified by titration with known amounts of HCl.

Cloning of ALase cDNA and Expression of Recombinant Protein in *Escherichia coli*—Based on the *Euglena* EST data base from the Proteome EST Program, a gene-specific primer was designed to amplify the missing 3' end of the ALase EST (ELL00003818). The primer was 5'-TGACTGCCAGTCTGGGGACC-3' and was used sequentially for 3'-rapid amplification of cDNA ends (3'-RACE) with a 5'/3'-RACE kit (Roche Applied Science). The complete sequence has been deposited with the DDBJ data bank under accession number AB306917. Then the full-length coding sequence of *Euglena* ALase was amplified by PCR with rALase-F (5'-CTCGAGATGCGAACGCTGGCCACCGTC-3') and rALase-R (5'-AAGCTTACACGAGTTCAGCACAGCC-3'). The upstream and downstream primers contained XhoI and HindIII restriction sites, respectively. PCR amplification was carried out using

Blend Taq (Toyobo, Osaka, Japan). The template was from a single-strand cDNA pool that was made from 6-day-old *Euglena* cells. The amplified product was cloned in the vector pGEM-T (Promega, Madison, MI), and the sequence was verified by DNA sequencing. The XhoI/HindIII fragment was extracted and subcloned into the corresponding restriction site of the expression vector pCold TF (Takara, Shiga, Japan). *E. coli* cells (BL21-CodonPlus (DE3)-RIL, Stratagene) were transformed with the resulting constructs. The cells were grown in LB medium supplemented with 34 $\mu\text{g ml}^{-1}$ chloramphenicol and 50 $\mu\text{g ml}^{-1}$ ampicillin at 37 °C. When the culture reached an absorbance at 0.4–0.5 at 600 nm, 1 mM isopropyl β -D-thiogalactopyranoside was added, and the cells were grown further at 15 °C for 20 h.

Site-directed Mutagenesis—The *Euglena* ALase mutants, N116A, N161A, D219A, and S262A, were constructed using ALase cDNA as a template with a two-step PCR by KOD-Plus polymerase (Toyobo). The oligonucleotides used were: N116A-F 5'-GAAAGGTTTCATACCGGAGCGCAGATGGAAAATGTG-3', N116A-R 5'-CACATTTTCCATCTGCGCTCCGGTATGAACCTTTC-3', N161A-F 5'-GTGTCACCATCGGAGCCGGTCTTGCCTGGAG-3', N161A-R 5'-CTCCAGGCAAGACCGGCTCCGATGGTGACAC-3', D219A-F 5'-GAGGCGGCCCGCTGGGATGACCATCGAC-3', D219A-R 5'-GTTCGATGGTTCATCCAGCGGGGGCCGCTC-3', S262A-F 5'-GCCAAGTACACGACCGCCGTGGCGCTCGGG-3', and S262A-R 5'-CCCGAGCGCCACGGCGGTCGTGACTTCGC-3'. Changed nucleotides are underlined. Successful mutagenesis was confirmed by DNA sequencing. The resultant mutants were subcloned into the pCold TF expression vector (Takara) and then introduced into *E. coli* cells as described above.

SDS-PAGE—SDS-PAGE was performed in a 12.5% polyacrylamide slab gel using a Tris/Gly buffer system. The gels were stained with Coomassie Blue G-250.

Purification of Recombinant *Euglena* ALase—All the procedures for the recombinant proteins were performed at 4 °C. *E. coli* cells were resuspended in 50 mM potassium phosphate buffer, pH 7.0, containing 0.3 M NaCl and 10 mM imidazole and lysed by sonication. The His-tagged recombinant ALase was purified on a column packed with TALON Metal Affinity resin (Clontech). The trigger factor with a His tag was removed from recombinant ALase by a 20-h digestion with HRV 3C protease (Takara) at 4 °C. To obtain the truncated recombinant ALase, the reaction mixture was applied to the TALON Metal Affinity resin column, and the flow-through protein was collected. The active fraction was stored at –20 °C after the addition of 20% (w/v) glycerol until used.

RNAi Experiments—Silencing of ALase by RNAi was performed as described by Iseki *et al.* (24). An ~450-bp length of partial ALase cDNA was PCR-amplified with the addition of the T7 RNA polymerase promoter sequence at one end. The primer sequences were: EgALase RNAi-F 5'-TAATACGACTCACTATAGGGGAGCTGCAGCCAGAGCTTCCGAAAGG-3' and EgALase RNAi-R 5'-GTTTCCTTCTCCACCACACGGGGATATCACTCAGCATAAT-3' (T7 RNA polymerase promoter sites are underlined). Then the sense and antisense RNAs were synthesized using the PCR products as templates

(MEGAscript RNAi kit, Ambion). After purification of transcribed RNA with DNase I digestion followed by phenol/chloroform extraction and ethanol precipitation, double-stranded RNA (dsRNA) was made by annealing equimolar amounts of the sense and antisense RNAs. *Euglena* cells of 2-day-old cultures were collected and resuspended in culture medium containing 4.2 mM $\text{Ca}(\text{NO}_3)_2$, 3.7 mM KH_2PO_4 , and 2.1 mM MgSO_4 . Forty-five microliters of the cell suspension ($\sim 1 \times 10^6$ cells) was transferred to a 0.4-cm-gap cuvette and electroporated with 5 μl of RNA solution (25 μg of ALase-dsRNA in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA) using Gene Pulser II (Bio-Rad) at 1.2 kV and 25 microfarads. After a 30-min incubation at room temperature, the cell suspension was diluted with fresh Koren-Hutner medium (22) or the medium supplemented with precursors related to AsA biosynthesis and cultured at 27 °C for restoration.

Northern Analysis—Total RNA was extracted from the cells using RNAiso (Takara). The total RNA (10 $\mu\text{g/lane}$) was separated by electrophoresis through a 1.2% agarose/formaldehyde gel, blotted onto a nylon membrane (Hybond-N⁺, Amersham Biosciences), and hybridized with a ³²P-labeled ALase cDNA prepared by random priming.

Measurement of AsA Concentration—*Euglena* cells were homogenized in 0.1 M HCl and 1 mM EDTA (~ 0.1 g flesh weight ml^{-1}) and centrifuged at 12,000 $\times g$ for 5 min. Total AsA content was measured by iron(III) reduction (25).

Data Analysis—The significance of differences between data sets was evaluated by *t* test. Calculations were carried out with Microsoft Excel software.

RESULTS

The Presence of a Homologue of the Rat Gluconolactonase Gene in *Euglena* Cells—Our previous identification and characterization of D-GalUA reductase from *Euglena* indicated that the occurrence of ALase is essential for the conversion of L-GalA, the resultant product of D-GalUA reduction, to L-Gall in this organism (21). Therefore, a BLASTp search was performed against the *Euglena* EST data base of the Protest EST Program using the amino acid sequence of the rat gluconolactonase involved in the biosynthesis of AsA in mammals (6) as a query. An EST clone encoding the putative *Euglena* ALase had the cluster ID ELL00003818. The corresponding full-length cDNA was cloned by the rapid amplification of cDNA ends method and submitted to the DDBJ/GenBank/EMBL data base (accession number AB306917). The predicted protein product, designated EgALase, displayed significant sequence identity (30.5%) and similarity (70.2%) with the rat gluconolactonase (Fig. 2).

The EgALase Protein Has Lactonase Activity—To clarify the function of the EgALase isolated from the single-strand cDNA pool in 6-day-old *Euglena* cells, the recombinant EgALase was expressed in *E. coli* with the addition of N-terminal His tags followed by the trigger factor. The recombinant protein was purified by nickel-affinity chromatography and treated with HRV 3C protease to remove the additional tag proteins. The final product migrated in the SDS-polyacrylamide gel as a single band with an apparent molecular mass of ~ 34 kDa (Fig. 3). The recombinant enzyme non-stereospecifically hydrolyzed aldo-

TABLE 2
Kinetic parameters of purified recombinant ALase

 The values are the mean \pm S.E. for three independent experiments.

ALase/substrate	K_m mM	V_{max} $\mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$	k_{cat} s^{-1}	k_{cat}/K_m $s^{-1} \text{mM}^{-1}$
rEgALase				
L-Galatono- γ -lactone	1.67 \pm 0.39	333 \pm 23.3	450 \pm 32	270
L-Galactonic acid	1.55 \pm 0.30	455 \pm 212	615 \pm 287	397
L-Gulono- γ -lactone	3.05 \pm 0.75	769 \pm 140	1040 \pm 189	341
L-Gulonic acid	4.55 \pm 0.23	1111 \pm 226	1503 \pm 306	330
D-Glucono- σ -lactone	2.08 \pm 0.45	120 \pm 22.7	160 \pm 31	77
SMP30 ^a				
D-Glucono- σ -lactone	9.40	345	192	20

^a Kondo *et al.* (6).

TABLE 3
Effect of various compounds on purified recombinant ALase from *Euglena*

 The values are the mean \pm S.E. for three independent experiments.

Compound	Concentration mM	Relative activity	
		L-GalA	L-GalL
None		100	100
EDTA	1	1.2	0.0
AsA	1	94.7	98.8
Dithiothreitol	1	97.3	105
H ₂ O ₂	0.1	123	118
	0.5	132	127
N-Ethylmaleimide	0.4	52.5	0.0
	4.0	33.3	0.0

δ -lactone were 2.08 \pm 0.45 mM and 120 \pm 22.7 $\mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$, respectively, and the optimum pH was 6.5 (data not shown). The K_m values for L-GalL and L-GulL were 1.67 \pm 0.39 and 3.05 \pm 0.75 mM, respectively, whereas the K_m values for L-GalA and L-GulA were 1.55 \pm 0.3 and 4.55 \pm 0.23 mM, respectively. Although the K_m values for L-GulL and L-GulA were two to three times higher than those for L-GalL and L-GalA, the V_{max} values for them were approximately two times higher than those for L-GalL and L-GalA. These findings indicated the catalytic efficiency (K_{cat}/K_m) of *Euglena* ALase for these substrates to be almost equal and that the reaction between uronic acid and lactone is reversible.

Various compounds listed in Table 3 were examined as to their effects on the ALase activity, with L-GalL and L-GalA as substrates. EDTA, a chelating reagent, at 1 mM strongly inhibited the activity in both directions. Reducing reagents, such as AsA and dithiothreitol, had no effect on the enzyme. On the other hand, the activity was inhibited by treatment with *N*-ethylmaleimide, a sulfhydryl reagent. Interestingly, the activity toward L-GalL as a substrate was completely inhibited by 0.4 mM *N*-ethylmaleimide, whereas that toward L-GalA was only inhibited 47.5% at the same concentration compared with activity levels without *N*-ethylmaleimide. These results suggest that a Cys residue is involved in the recognition of substrates by the *Euglena* ALase. In contrast to the results for *N*-ethylmaleimide, 0.5 mM H₂O₂ caused a 27 and 32% activation with L-GalL and L-GalA as the substrate, respectively.

Identification of Zn²⁺ Binding Residues in *Euglena* ALase—A homology model of the *Euglena* lactonase was generated using the molecular graphics package Molecular Operating Environment (Chemical Computing Group Inc., Montreal, Canada) on the basis of the crystal structure of Drp35, a Ca²⁺-dependent

lactonase hydrolyzing aromatic lactones from *Staphylococcus aureus* (PDB codes 2DG1) as a template (26). Drp35 has a six-bladed β -propeller structure with two calcium ions. One calcium ion binds at the center of the β -propeller structure and is essential for the lactonase activity (26). Interestingly, although the primary structure of *Euglena* ALase had comparatively little homology (11% identity) with that of *Staphylococcus* Drp35 (Fig. 2), the calculated topology of *Euglena* ALase was quite similar to that of Drp35 (Fig. 5A). Comparison of the center of the β -propeller structure of *Euglena* ALase with that of *Staphylococcus* Drp35 suggests the presence of four residues coordinating with the zinc ion (Fig. 5B). Among them, Asn-116, Asn-161, and Asp-219 in *Euglena* ALase corresponded to Asp-138, Asn-185, and Asp-236, respectively, in *Staphylococcus* Drp35. Additionally, Ser-262 of *Euglena* ALase was estimated to be involved in coordinating with the zinc ion. To test the significance of the corresponding residues in *Euglena* ALase, these four residues were individually substituted with Ala, then the activity of each purified recombinant mutant was determined. As a result, none of mutants showed activity even at up to 150 μM concentrations of zinc ion (Fig. 6), indicating that these four residues, including the novel Ser-262, are essential for the ALase activity and participate in the binding of zinc ion.

Silencing of ALase Expression Results in Growth Defects—To examine the physiological significance of the ALase to the biosynthesis of AsA in *Euglena*, we transiently silenced the expression of ALase by RNA-mediated interference. A dsRNA synthesized from part of the *Euglena* ALase sequence was introduced into *Euglena* cells by electroporation. Interestingly, the cells grew in medium supplemented with L-GalL (an intermediate that is downstream of ALase) 1 week after the introduction of the dsRNA (Fig. 7A). The AsA level in the dsRNA-containing cells after supplementation with L-GalL was 25 μmol of 10⁻⁹ cells. The cells did not grow in KH medium without any supplements at least within the study period. Northern analysis and an assay of ALase activity of the dsRNA-containing cells showed no significant detectable signal and activity of ALase, respectively, confirming the complete suppression of endogenous ALase by the introduction of dsRNA (Fig. 7B). These results clearly indicate that the expression of ALase is essential for the biosynthesis of AsA in *Euglena*, and AsA plays a pivotal role in cell growth.

Effect of Treatment with Precursors on the Total AsA Level in *Euglena* Cells—To evaluate the effect of intermediates involved in the two possible pathways via D-GalUA/L-GalA or D-GlcUA/L-GulA on cellular AsA accumulation, *Euglena* cells were

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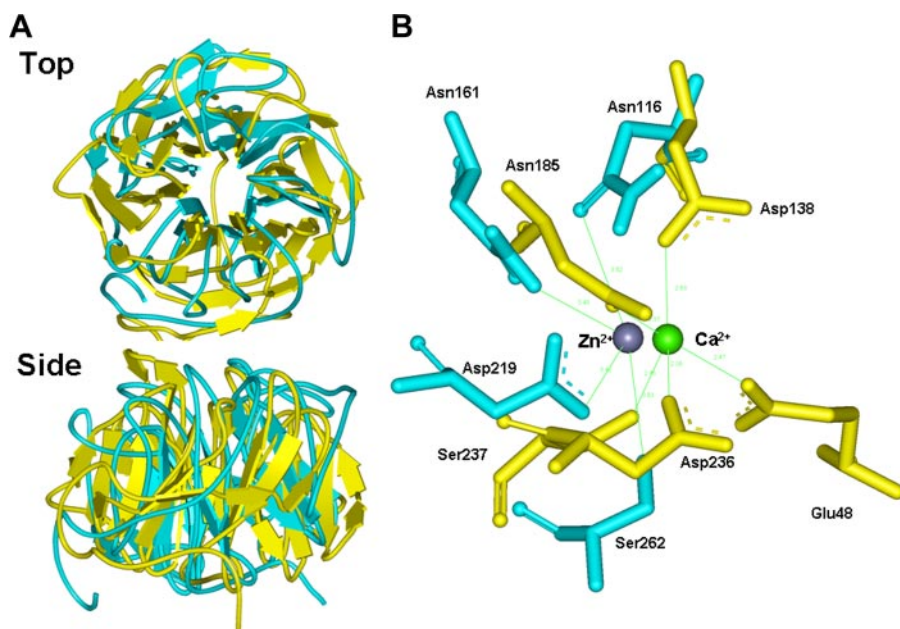


FIGURE 5. **Structural comparison of *Staphylococcus* Drp35 and predicted model of *Euglena* ALase.** *A*, superimposition of *Staphylococcus* Drp35 and *Euglena* ALase. The protein structure of Drp35 and ALase is shown in yellow-green and pale blue, respectively. *B*, Ca^{2+} binding center in Drp35 and prediction of Zn^{2+} -binding residues in *Euglena* ALase. The residues in Drp35 and ALase are shown in yellow-green and pale blue, respectively.

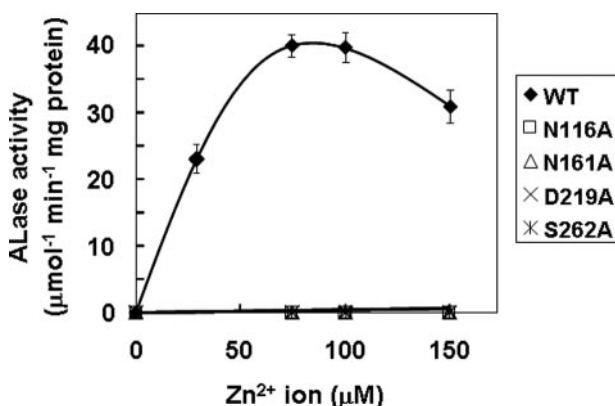


FIGURE 6. **Effect of amino acid substitution on *Euglena* ALase activity.** ALase activities of affinity-purified mutant enzymes were determined at the indicated concentrations of ZnCl_2 . Values are expressed as the mean \pm S.E. for three independent experiments. WT, wild type.

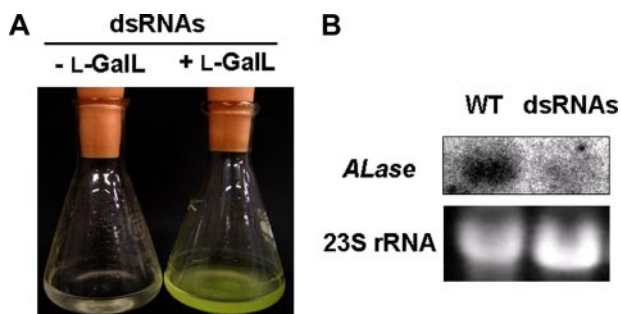


FIGURE 7. **Phenotypic characterization of ALase-silenced *Euglena* cells.** *A*, growth phenotype of dsRNA-introduced cells in a medium supplemented with L-GalL. Cells were restored and grown for 1 week in the presence of 1 mM L-GalL after the introduction of dsRNAs for ALase. Cultures of representative cells grown in a medium supplemented with 1 mM L-GalL were photographed 1 week after the dsRNA was introduced. *B*, Northern blot analysis. Each lane was loaded with 10 μg of total RNA from 1-week-old wild type (WT) cells and dsRNA-introduced cells. The RNA blot was hybridized with a ^{32}P -labeled ALase cDNA probe. The bottom shows ethidium bromide-stained rRNA as a RNA-loading control.

treated with 5 mM D-GalUA, L-GalL, D-GlcUA, or L-GulL under light at $55 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 6 h. As shown in Fig. 8, D-GalUA and L-GalL, the precursors for the pathway via D-GalUA/L-GalA, were converted to AsA even more effectively than D-GlcUA and L-GulL for the pathway via D-GlcUA/L-GulA. The result strongly confirms the possibility that the pathway via D-GalUA/L-GalA is an effective route to highly maintain the cellular total AsA level in *Euglena* cells.

Effect of Illumination on ALase Expression in *Euglena*—It has been reported that the cellular levels of total AsA in *Euglena* cells markedly depended on light (1, 27), and in particular, the activity of L-GalL dehydrogenase in dark-grown *Euglena* was augmented by illumination (20). Therefore, we investigated the relationship between

ALase expression and AsA accumulation under light. When *Euglena* cells grown in the dark were moved into light, cellular AsA levels increased steadily for 24 h, reaching a maximum of $11 \mu\text{mol} \cdot 10^{-9}$ cells, which was comparable with the level attained by green cells illuminated continuously (Fig. 9). The ALase activity eventually increased ~ 3 -fold with illumination for 24 h. In contrast to the ALase activity, the transcript levels remained constant, suggesting that ALase is post-transcriptionally regulated in response to light, reflecting the cellular level of AsA in *Euglena* cells.

DISCUSSION

Our previous analysis of intermediates for AsA biosynthesis with radio-tracer experiments in *Euglena* has provided evidence that this organism utilizes a pathway via D-GalUA/L-GalA for the biosynthesis (1). In the present study we have successfully identified ALase and demonstrated that it is the *bona fide* enzyme in the biosynthesis of AsA in *Euglena*.

Characterization of *Euglena* ALase—The recombinant *Euglena* ALase recognized a broad range of substrates including various types of aldonate lactone, with a requirement for zinc ion as a cofactor. Such properties are well consistent with the rat enzyme (6). Zinc ion was the most effective divalent cation and played a critical role in the catalysis (Fig. 4). This was confirmed by the finding that EDTA inhibits the activity (Table 3). The requirement of divalent cation is common to most lactone hydrolases (28). Recent progress in the structural analysis of various lactonases has identified the residues coordinating with the divalent cation (26, 29). For example, *N*-acyl-L-homoserine lactonase from *Erwinia* requires zinc ions for its activity, and a structural analysis revealed that it contains two Zn^{2+} molecules coordinating partly with a conserved Zn^{2+} binding motif, HXHDXH_nH, at the center of the catalytic site (29). The *Euglena* ALase had low identity (7.7%) and similarity (43%) with

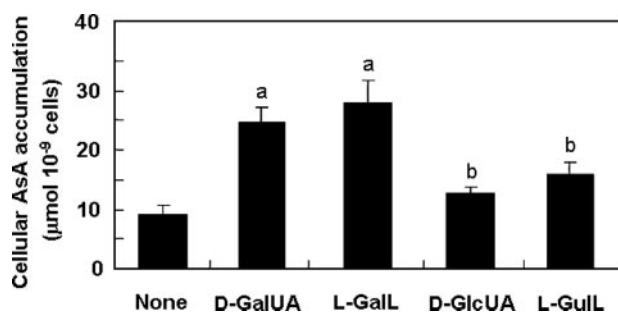


FIGURE 8. Effect of precursors on AsA formation in *Euglena*. *Euglena* cells were incubated with 5 mM concentrations of each compound under light ($55 \mu\text{mol m}^{-2} \text{s}^{-1}$) at 25 °C for 6 h. Values are expressed as the mean \pm S.E. for three independent experiments. Values that were significantly different between control and treated cells are indicated; ^a, $p < 0.05$; ^b, $p < 0.01$.

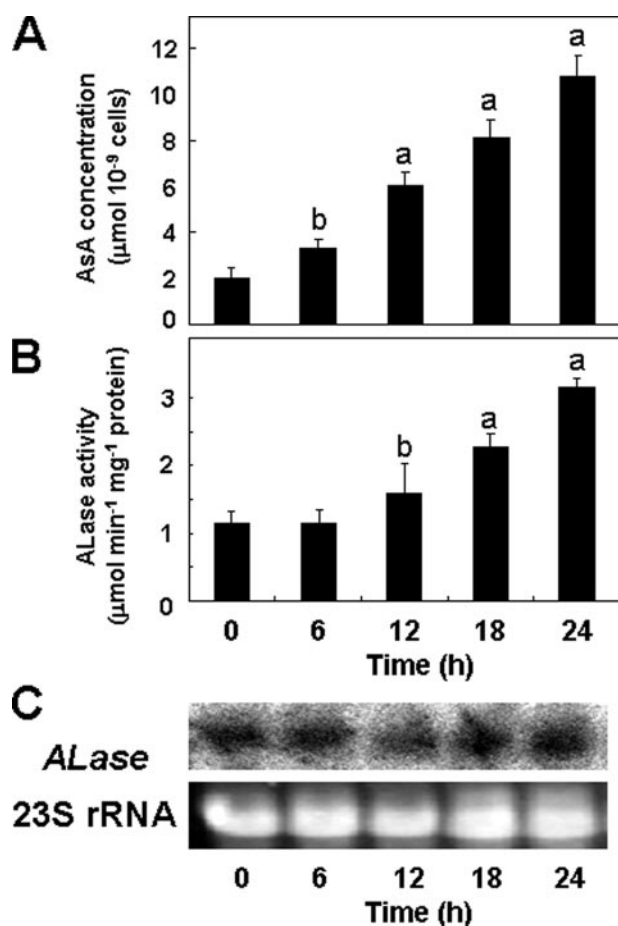


FIGURE 9. AsA level and expression of ALase under light in dark-grown *Euglena*. The cells were illuminated at an intensity of $55 \mu\text{mol m}^{-2} \text{s}^{-1}$. Samples were taken out at the indicated times and used for the determination of AsA, the assay of ALase, and Northern hybridization. Values are expressed as the mean \pm S.E. for three experiments. A, AsA level. B, ALase activity. C, Northern blot analysis. Ten micrograms of total RNA extracted from each sample was electrophoresed through a formaldehyde-containing agarose gel, capillary blotted onto a nylon membrane, and hybridized with ³²P-labeled cDNA of *Euglena* ALase. Ethidium bromide staining of the rRNA is shown for the equality of loading. Values that were significantly different between control and illuminated cells are indicated. ^a, $p < 0.05$; ^b, $p < 0.01$.

the *Erwinia* enzyme and lacked a Zn²⁺ binding motif conserved within the primary sequence. Although the *Erwinia* enzyme was not suitable as a template for structural modeling of *Euglena* ALase, the *Staphylococcus* Drp35, a drug-responsive protein with Ca²⁺-dependent aromatic lactone hydrolyzing

activity (26), provided useful information about the residues coordinating with the zinc ion of the *Euglena* enzyme. Based on a structural comparison with Drp35 and a mutational analysis, four residues (Asn-116, Asn-161, Asp-219, and Ser-262) in *Euglena* ALase were identified as significant for the activity (Figs. 5 and 6). These four residues were completely conserved in the rat gluconolactonase (Fig. 2). Three of them (Asn-116, Asn-161, and Asp-219) coordinate with Ca²⁺ in Drp35 (26). More importantly, the residue corresponding to Glu-48 in Drp35 was not found in *Euglena* ALase, whereas Ser-262 in *Euglena* ALase was not conserved in Drp35, assuming that this difference accounts for the coordination with zinc ion, but not calcium ion, in this type of lactonase.

In higher plants, feedback regulation of L-Gal dehydrogenase and GDP-D-Man-3,5-epimerase has been reported to be one of the regulatory mechanisms in AsA biosynthesis (30, 31). Treatment with AsA had no effect on the ALase activity (Table 3), indicating a lack of feedback regulation of the activity in the biosynthesis of AsA in *Euglena*. On the other hand, *Euglena* ALase showed redox sensitivity caused by H₂O₂ treatment (Table 3). Activation of D-GalUA reductase by H₂O₂ has also been observed in our previous study (21). Because AsA levels in *Euglena* cells increased under oxidative stress (20, 27) and light (Fig. 8), the activation of the enzymes in response to H₂O₂ levels seems to be a key regulatory mechanism promoting AsA biosynthesis in *Euglena* cells under stress.

The Pathway via D-GalUA/L-GalA Is the Significant Route for AsA Biosynthesis in Euglena—Silencing of *Euglena* ALase resulted in an arrest of cell growth in the normal KH medium, but the cells grew in medium supplemented with L-GalL (Fig. 7). This result provides compelling evidence that ALase is essential for AsA biosynthesis, and the pathway via uronic acid is the major source of AsA in *Euglena* cells. We previously reported that a D-GalUA reductase purified from *Euglena* had almost the same catalytic efficiency with two uronic acids, D-GalUA and D-GlcUA (21). Judging from the enzymology parameters of both D-GalUA reductase and ALase, it is difficult to understand which is the more effective route to promote the level of AsA in *Euglena* cells. However, it is clear from Fig. 8 that L-GalL is the most effective precursor of AsA. L-GulL and D-GlcUA are ~41 and 48% less effective as precursors in the biosynthesis of AsA than L-GalL and D-GalUA, respectively. These findings are well consistent with the results of the tracer experiment reported by Shigeoka *et al.* (1). Additionally, crude extracts prepared from *Euglena* cells had significant dehydrogenase activity with L-GalL, but not L-GulL, as a substrate, whereas they had no oxidase activity with L-GulL or L-GalL.³ Consequently, we conclude that the pathway via D-GalUA/L-GalA is the major route for the biosynthesis of AsA in this organism. It is worth noting that, from a direct comparative tracer experiment, the mean ratio of the conversion of D-[U-¹⁴C]glucose to D-GalUA was ~2-fold higher than that to D-GlcUA (1), suggesting the key point controlling the flux of the pathway is the epimerization of UDP-D-GlcUA to UDP-D-GalUA.

³ T. Ishikawa, H. Nishikawa, and S. Shigeoka, unpublished results.

Identification of Ascorbate Biosynthesis Pathway in *Euglena*

Physiological Role of AsA in *Euglena* Cells—Little is known about the physiological significance of AsA in photosynthetic algae including *Euglena*, except that it is an important antioxidant and essential donor for reactions of AsA peroxidase (EC 1.11.1.1.) (8, 32, 33). In addition to confirming the pathway via D-GalUA/L-GalA as the significant route of AsA biosynthesis, our results showed the importance of AsA in *Euglena* cells. A delay of cell growth has also demonstrated by an analysis of *Trypanosoma* mutants with disrupted orthologs encoding plant L-GalL dehydrogenase (19). SMP30, gluconolactonase, knock-out mice showed typical symptom of vitamin C deficiency including scurvy and did not grow well (6). This was due to a lack of collagen biosynthesis caused by a defective activity of AsA-dependent 2-oxoglutarate-dependent dioxygenase that is required for hydroxyproline synthesis. In *Arabidopsis* plants, Dowdle *et al.* (14) have reported that the simultaneous disruption of two genes encoding GDP-L-Gal phosphorylase (*vtc2/vtc5*), a key enzyme of the Man/Gal pathway, resulted in growth arrest upon germination, and the mutants resumed their growth on supplementation with L-Gal or AsA, indicating the function of AsA to be essential for plant growth. They also assumed that the growth arrest of the mutants reflects the role of AsA in growth, possibly due to the decrease in synthesis of extracellular matrix hydroxyproline-rich glycoproteins and/or plant hormones such as ethylene, gibberellic acid, and abscisic acid by suppression of AsA-dependent peptidyl-prolyl hydroxylase and dioxygenase activities.

In the case of *Euglena*, the reason for the growth arrest caused by the suppression of AsA biosynthesis would be different from that observed in *Arabidopsis* plants. It is possible that the AsA deficiency affects the regulation of the cell cycle in *Euglena*. Periodic variations in cAMP levels play a major signaling role in the progression of the *Euglena* cell cycle and the cAMP and enzymes adenylate cyclase and phosphodiesterase which control the level constitute a relay of the endogenous circadian clock (34). It is also suggested that cGMP serves as an upstream effector that mediates the cAMP oscillation by regulation of the metabolic enzymes (35). It is worth mentioning that AsA augmented cGMP levels in human lymphocytes (36), although the phenomenon is yet to be confirmed in *Euglena*. It has been reported that the level of AsA in *Euglena* is well synchronized with circadian rhythms (20, 27), also supporting a positive relationship between AsA biosynthesis and the regulation of the cell cycle. Although further investigations needed to conclude the effect of AsA levels on the control of the cell cycle, the silencing of ALase provides a good model with which to analyze the physiological role of AsA in *Euglena* cells.

The Possibility That the Pathway via D-GalUA/L-GalA Is Present in Other Photosynthesizing Organisms—The ALase plays a central role in the biosynthesis of AsA in *Euglena*. This article describes the first example to our knowledge of the biosynthesis of AsA via uronic acid in photosynthetic algae. To detect an ALase in other algae and plants, we performed a BLASTp search using both the *Euglena* and rat enzymes as a query sequence. The search found no orthologs in the genomes of higher plants, at least in *Arabidopsis*, rice, and *Populus*, whereas additional putative orthologs of ALase, which display significant sequence identity (~30%) with the *Euglena* and rat

ALase proteins, were found in a limited number of eukaryotic algae, including *Ostreococcus lucimarinus*, *Thalassiosira pseudonana*, and *Phaeodactylum tricoratum* (supplemental Fig. S1). A sequence alignment among these homologous proteins showed that four amino acid residues coordinating with the zinc ion in *Euglena* ALase (Asn-116, Asn-161, Asp-219, and Ser-262) are completely conserved at the appropriate sequence position in some species (*O. lucimarinus* and *T. pseudonana*), indicating the possibility that these algae contain an active ALase with properties similar to those of *Euglena* and the rat. In contrast, no significant orthologs were found in *Cyanidioschyzon merolae*, *Chlamydomonas reinhardtii*, *Volvox carterii*, and *Ostreococcus tauri*. It is worth noting that all the algae listed above possess predicted orthologs encoding L-GalL dehydrogenase. It is, therefore, assumed that algae have developed diverse pathways for the biosynthesis of AsA, including the pathway via D-GalUA/L-GalA in *Euglena* and the Man/Gal pathway in higher plants. Because the information available on the biosynthesis of AsA in algae is quite limited at the present moment, further investigation is needed to understand the distribution of these pathways among photosynthesizing algae.

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