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

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF ALDONOLACTONASE

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 Km 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-galactonate 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)) represents a group of essential nutrients for humans and is a hydrophilic antioxidant 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-GluUA 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 glucuronolactonase (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 CYTI 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...
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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 milliliters of 0.3 M NaOH was added to 100 μl of 10 mM L-Gall or L-Gull, the mixture was vigorously agitated by vortexing for 20 s, and 20 μl 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 μmol m⁻² s⁻¹ at 26 °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 μM p-nitrophenol, 5 mMD-glucuronic acid, 75 μM 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 Protest EST Program, a gene-specific primer was designed to amplify the missing 3' end of the ALase EST (ELL00003818). The primer was 5'-TGACTGCAGTCTGGGACC-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 database under accession number AB306917. Then the full-length coding sequence of Euglena ALase was amplified by PCR with rALase-F (5'-CTCGAGATGC-  GACGCTGGCCACCCTGTC-3') and rALase-R (5'-AAGCTTACAGAGGTCGACAGGAC-3'). The upstream and downstream primers contained Xhol and HindIII restriction sites, respectively. PCR amplification was carried out using...
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Blond 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, WI), 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 mg ml⁻¹ chloramphenicol and 50 mg ml⁻¹ ampicillin at 37 °C. When the culture reached an absorbance of 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'-GAAAGGTTCATACGCGGAGCGCATG-3', N116A-R 5’-CACATTTCCATCTGCCGGA-3', N161A-F 5’-GTCGATGGTCATCCCAGCGGGGGCCGCCTC-3', N161A-R 5’-GCAAGACCGGCTCCGATGGTGACAC-3', D219A-F 5’-GAGGGCCGCCCGCTGGGATGACCTCGAC-3', D219A-R 5’-GTCCATTGCTATCCCAGCGGCGCGCTCTC-3', S262A-F 5’-GGAAATACAGCCGCCGGCGCTCGGG-3', and S262A-R 5’-CCCGAGGGCCACGCCGCGTCGTAATTCTTCG-3'. Changed nucleotides are underlined. Successful mutagenesis was confirmed by DNA sequencing. The XhoI/HindIII fragment was purified by nickel-affinity chromatography and treated 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 μg/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 probe 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 x 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 database of the Protost 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 EELL0000381. The corresponding full-length cDNA was cloned by the rapid amplification of cDNA ends method and submitted to the DDBJ/GenBank/EMBL database (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-
nate lactones, such as D-glucono-δ-lactone, D-/L-GalL, and D-/L-GulL, indicating that the isolated cDNA encodes an aldonolactonase (Table 1). The enzyme required a divalent metal ion for maximum activity which was obtained by the addition of Zn^{2+} at a concentration of 75 μM (Fig. 4). Other divalent ions, Mn^{2+}, Co^{2+}, Mg^{2+}, and Ca^{2+}, had a significant low effect on the activity to 19, 9, 8, and 7%, respectively, of the enzyme activity given by the case of Zn^{2+}. Therefore, Zn^{2+} was used at 75 μM for the subsequent experiments to study the catalytic properties of the Euglena lactonase.

**Catalytic and Other Properties of the Recombinant Euglena ALase**—Normal hyperbolic kinetics were obtained for all substrates listed in Table 2. The Km and Vmax values for D-glucono-δ-lactone were determined in the presence of 75 μM concentrations of various divalent metal ions. B, ALase activity was determined at the indicated concentrations of ZnCl\(_2\). Values are expressed as the mean ± S.E. of three independent experiments.

**TABLE 1**

| Substrate Specificity | rEgALase | SMP30 | rEgALase | SMP30 |
|-----------------------|----------|-------|----------|-------|
|                       | μmol min\(^{-1}\) mg\(^{-1}\) of protein | | μmol min\(^{-1}\) mg\(^{-1}\) of protein | |
| D-Glucono-δ-lactone   | 39.0 ± 0.8 (100%) | 217 ± 3.6 (100%) | 217 ± 3.6 (100%) | 217 ± 3.6 (100%) |
| L-Glucono-δ-lactone   | 5.4 ± 2.2 (2.49%) | 5.4 ± 2.2 (2.49%) | 5.4 ± 2.2 (2.49%) | 5.4 ± 2.2 (2.49%) |
| L-Galactono-γ-lactone | 24.5 ± 3.3 (62.8%) | 4.0 ± 0.0 (0.37%) | 24.5 ± 3.3 (62.8%) | 4.0 ± 0.0 (0.37%) |
| D-Galactono-γ-lactone | 27.3 ± 2.3 (70.0%) | 15.4 ± 0.9 (6.93%) | 27.3 ± 2.3 (70.0%) | 15.4 ± 0.9 (6.93%) |
| L-Gulono-γ-lactone    | 40.9 ± 0.1 (105%) | 2.7 ± 0.1 (1.24%) | 40.9 ± 0.1 (105%) | 2.7 ± 0.1 (1.24%) |
| D-Gulono-γ-lactone    | 20.1 ± 0.5 (51.5%) | 12.0 ± 0.9 (5.53%) | 20.1 ± 0.5 (51.5%) | 12.0 ± 0.9 (5.53%) |

a Kondo et al. (6).
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The values are the mean ± S.E. for three independent experiments.

| Compound         | Concentration | Relative activity | r-GalA | r-Gall |
|------------------|---------------|-------------------|--------|--------|
| None             | 100           | 100               | 100    | 100    |
| EDTA             | 1             | 1.2               | 0.0    |        |
| AsA              | 1             | 94.7              | 98.8   |        |
| Dithiothreitol   | 1             | 97.3              | 105    |        |
| H2O2             | 0.1           | 123               | 118    |        |
| N-Ethylmaleimide | 0.05          | 0.5               | 127    |        |
|                 | 0.4           | 52.5              | 0.0    |        |
|                 | 4.0           | 33.3              | 0.0    |        |

TABLE 2
Kinetic parameters of purified recombinant ALase

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

| ALase/substrate          | Km [mM] | Vmax [μmol min⁻¹ mg⁻¹ protein] | kcat [s⁻¹] | kcat/Km [s⁻¹ mM⁻¹] |
|--------------------------|---------|-------------------------------|------------|---------------------|
| rEgALase                 |         |                               |            |                     |
| L-GalA                   | 1.67 ± 0.39 | 333 ± 23.3                | 450 ± 32   | 270                 |
| L-Galactonic acid        | 1.55 ± 0.30 | 455 ± 212                | 615 ± 287  | 397                 |
| L-Gulono-γ-lactone       | 3.05 ± 0.75 | 769 ± 140               | 1040 ± 189 | 341                 |
| L-Gulonic acid           | 4.55 ± 0.23 | 1111 ± 226              | 1503 ± 306 | 330                 |
| D-Glucono-α-lactone      | 2.08 ± 0.45 | 120 ± 22.7               | 160 ± 31   | 77                  |
| SMP30α                   | 9.40     | 345                          | 192       | 20                  |

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

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

δ-lactone were 2.08 ± 0.45 mM and 120 ± 22.7 μmol min⁻¹ mg⁻¹ protein, respectively, and the optimum pH was 6.5 (data not shown). The Km values for L-GalL and L-GulL were 1.67 ± 0.39 and 3.05 ± 0.75 mM, respectively, whereas the Km values for L-GaLA and L-GuLA were 1.55 ± 0.3 and 4.55 ± 0.23 mM, respectively. Although the Km values for L-GuL and L-GuLA were two to three times higher than those for L-GaL and L-GaLA, the Vmax values for them were approximately two times higher than those for L-GaL and L-GaLA. These findings indicated the catalytic efficiency (kcat/Km) 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-GaL 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, 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, confirming the complete suppression of endogenous ALase by the introduction of dsRNA (Fig. 7). 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. AsA plays a pivotal role in cell growth.

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-GaL (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-GaL 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-GluUA/L-GaLA or D-GlcUA/L-GuL on cellular AsA accumulation, Euglena cells were
treated with 5 mM D-GalUA, L-GalL, D-GlCUA, or L-GulL under light at 55 μmol m⁻² 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-Gul 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 mol 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-acetyl-L-homoserine lactonase from Erwinia requires zinc ions for its activity, and a structural analysis revealed that it contains two Zn²⁺ molecules coordinating partly with a conserved Zn²⁺ binding motif, HXHDXHXX, at the center of the catalytic site (29). The Euglena ALase had low identity (7.7%) and similarity (43%) with...
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The cells were incubated with 5 mM concentrations of each compound under light (55 μmol m⁻² s⁻¹) at 25 °C for 6 h. Values are expressed as the mean ± S.E. for three independent experiments. Values that were significantly different between control and treated cells are indicated: *, p < 0.05; **, p < 0.01.

**FIGURE 8. Effect of precursors on AsA formation in Euglena.** Euglena cells were incubated with 5 mM concentrations of each compound under light (55 μmol m⁻² s⁻¹) at 25 °C for 6 h. Values are expressed as the mean ± S.E. for three independent experiments. Values that were significantly different between control and treated cells are indicated: *, p < 0.05; **, 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 μmol m⁻² 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 ± S.E. for three experiments. Values that were significantly different between control and treated cells are indicated: *, p < 0.05; **, p < 0.01.

The cells were illuminated at an intensity of 55 μmol m⁻² s⁻¹.

**FIGURE 10. ALase activity with L-GalL and D-GalUA.** The cells were illuminated at an intensity of 55 μmol m⁻² s⁻¹.

**FIGURE 11.** The Pathway via d-GalUA/L-GalL 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. Consequently, we conclude that the pathway via d-GalUA/L-GalL 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.

**FIGURE 12.** The Pathway via d-GalUA/L-GalL 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. Consequently, we conclude that the pathway via d-GalUA/L-GalL 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.
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 (vct2/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 CAM 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 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.

Identification of Ascorbate Biosynthesis Pathway in Euglena

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