GDP-Mannose 3',5'-Epimerase Forms GDP-L-gulose, a Putative Intermediate for the de Novo Biosynthesis of Vitamin C in Plants*

Beata A. Wolucka‡§§ and Marc Van Montagu§

From the ‡Department of Molecular Microbiology, Flanders Interuniversity Institute for Biotechnology (VIB), Kasteelpark Arenberg 31, B-3001 Leuven-Heverlee, Belgium and §Department of Plant Systems Biology, VIB, Ghent University, K. L. Ledeganckstraat 35, B-9000, Belgium

Despite its importance for agriculture, bioindustry, and nutrition, the fundamental process of L-ascorbic acid (vitamin C) biosynthesis in plants is not completely elucidated, and little is known about its regulation. The recently identified GDP-Man 3',5'-epimerase catalyzes a reversible epimerization of GDP-D-mannose that precedes the committed step in the biosynthesis of vitamin C, resulting in the hydrolysis of the highly energetic glycosyl-pyrophosphoryl linkage. Here, we characterize the native and recombinant GDP-Man 3',5'-epimerase of Arabidopsis thaliana. GDP and GDP-D-glucose are potent competitive inhibitors of the enzyme, whereas GDP-L-fucose gives a complex type of inhibition. The epimerase contains a modified version of the NAD binding motif and is inhibited by NAD(P)H and stimulated by NAD(P)*. A feedback inhibition of vitamin C biosynthesis is observed apparently at the level of GDP-Man 3',5'-epimerase. The epimerase catalyzes at least two distinct epimerization reactions and releases, besides the well known GDP-L-galactose, a novel intermediate: GDP-L-gulose. The yield of the epimerization varies and seems to depend on the molecular form of the enzyme. Both recombinant and native enzymes co-purified with a Hsp70 heat-shock protein (Escherichia coli DnaK and A. thaliana Hsc70.3, respectively). We speculate, therefore, that the Hsp70 molecular chaperones might be involved in folding and/or regulation of the epimerase. In summary, the plant epimerase undergoes a complex regulation and could control the carbon flux into the vitamin C pathway in response to the redox state of the cell, stress conditions, and GDP-sugar demand for the cell wall/glycoprotein biosynthesis. Exogenous L-gulose and L-gulono-1,4-lactone serve as direct precursors of L-ascorbic acid in plant cells. We propose an L-gulose pathway for the de novo biosynthesis of vitamin C in plants.

Received for publication, August 18, 2003, and in revised form, September 3, 2003
Published, JBC Papers in Press, September 3, 2003, DOI 10.1074/jbc.M309135200

Vitamin C (L-ascorbic acid (L-AA)†) acts as an enzyme cofactor and an antioxidant. In plants it may represent one of the major soluble carbohydrates and is involved in crucial physiological processes such as biosynthesis of the cell wall, phytohormones, and secondary metabolites, cell division and growth, and stress resistance and photoprotection (for review, see Ref. 1). Large variations in vitamin C content (from 0.003 to 1% of fresh weight; w/w), reported for different plant species, organs, and tissues (2), are intimately linked to the vitamin biosynthesis, stability, and function. Plants, algae, and the majority of animals are able to synthesize vitamin C. Humans, however, lack L-gulono-1,4-lactone oxidase, the last enzyme of the vitamin C pathway in animals, and require L-AA as an essential micronutrient. L-AA biosynthetic genes can be engineered to increase vitamin C content of plants (3–4), in view of improving the nutritional value and stress resistance of crops, but also potentially exploited for the industrial production of vitamin C (5).

The biosynthesis of vitamin C in plants is not completely elucidated, and its regulation is largely unknown. Two distinct pathways for vitamin C biosynthesis in plants were proposed (6–7). The salvage pathway involves pectin-derived D-galacturonic acid (6) that is reduced at C1 to L-galactonic acid by the recently identified D-galacturonic acid reductase (4), and the resulting L-galactono-1,4-lactone is oxidized to L-AA by the mitochondrial L-galactono-1,4-lactone dehydrogenase (8–9). Conversion of D-galacturonic acid into L-galactonic acid results in the inversion of carbon numbering. However, labeling studies of Loewus and Kelly (10) indicate that a non-inversion pathway, in which a hexose is converted into L-AA without reversion of the carbon chain, predominates in plants. The second pathway (7) is a non-inversion, energy-dependent biosynthesis that involves the conversion of GDP-D-mannose to GDP-L-galactose catalyzed by a GDP-D-Man 3',5'-epimerase (11). L-Galactose, released from the nucleotide through some poorly understood steps, is then oxidized at C1 to L-galactono-1,4-lactone by an L-galactose dehydrogenase (12); the latter compound is converted to vitamin C by the L-galactono-1,4-lactone dehydrogenase.

Recently, we obtained a highly purified GDP-Man 3',5'-epimerase preparation from Arabidopsis thaliana cell suspensions and identified the corresponding gene (11). Only one copy of the epimerase gene is present in the Arabidopsis genome, and the gene is highly conserved among plant species (>88% identity at the protein level and >78% identity at the DNA level). A database search revealed the presence of the epimerase sequence in cDNA libraries obtained from mature tomato fruits and potato tubers but also from salt- and pathogen-stressed ice plant and potato leaves, respectively.

The unique double epimerization reaction of the activated form of D-Man catalyzed by GDP-Man 3',5'-epimerase precedes the committed step in the biosynthesis of L-AA and glycoconjugates, which results in the irreversible hydrolysis of the highly energetic glycosyl-pyrophosphoryl linkage. Therefore, the epimerization step must be tightly controlled. Here, we
show that the GDP-Man 3′,5′-epimerase of A. thaliana undergoes a complex regulation that is linked to the cell wall biosynthesis and the redox state of the cell. In particular, we demonstrate the formation of a novel intermediate, GDP-L-gulose, and propose that this compound could be dedicated for the de novo biosynthesis of vitamin C in plants.

EXPERIMENTAL PROCEDURES

Reagents—d-[U-14C]Mannose (specific activity 286 mCi/mmol) and guanosine diphospho-d-[U-14C]mannose were purchased from Amer sham Biosciences. Nickel nitrotriacetic acid Superflow resin was obtained from Qiangen (Hilden, Germany). Glutathione S-transferase (GST) affinity resin was from Stratagene (Madison, WI). All reagents were of analytical grade. Guanosine diphospho-l-fucose, guanosine diphospho-l-glucose, adenosine diphospho-l-glucose, l-gulose, and l-gulono-1,4-lactone were purchased from Sigma-Aldrich.

Plasmids—The GATEWAYTM (Invitrogen) plasmids containing the GDP-Man 3′-epimerase gene of A. thaliana, pDEST15_Epim and pDEST17_Epim, were prepared as described (11) for the bacterial expression of GST- and His-tagged epimerase (N-terminal fusions), respectively.

Plant Material—A. thaliana (L.) Heynh ecotype Columbia cell suspensions were grown as described (13). White potato (Solanum tuberosum L. cv. Irish Cobbler) tubers were stored at 13 °C until use.

Heterologous Bacterial Expression of the Recombinant Epimerase—The GDP-Man 3′,5′-epimerase gene and l-AAA were measured by the HPLC method as described (13), with the exception that the concentration of methanol in solvent A was 0.5%, and the flow rate was 0.8 ml/min.

In Vivo Labeling of A. thaliana Cell Suspensions with d-[U-14C]-Man—In vivo labeling of A. thaliana cells was performed as described (13). Cell suspensions were pre-adapted to labeling conditions for 20 h in the presence or absence of exogenous l-AAA or its precursors (2.5 mM) followed by a 2-h labeling with 1 μCi of d-[U-14C]-Man. l-AAA was extracted with 5% metaphosphoric acid containing 2 mM dithiothreitol and 1 mM EDTA.

Characterization of the GDP-Man 3′,5′-Epimerase—Heterologous expression of the recombinant epimerase in Escherichia coli was performed to a “reversed” heat shock (a shift from 37 to 26 °C just before the induction) was performed as described (11). Cells were re-suspended in 3 volumes of 50 mM Tris-HCl buffer, pH 7.7, containing 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 20% glycerol in buffer A. Crude extracts and 90% ammonium sulfate precipitates were prepared as described (11).

Nickel Nitrotriacetic Acid Metal Affinity Chromatography of the Recombinant GDP-Man 3′,5′-Epimerase—A crude extract containing the His-tagged epimerase protein was loaded on a 2-mL nickel nitrotriacetic acid Superflow column equilibrated with 5 mM imidazole in 25 mM Tris-HCl buffer, pH 7.7 containing 1 mM phenylmethylsulfonyl fluoride (buffer B). The column was washed with 10 volumes of the equilibration buffer followed by 5 volumes of 20 mM imidazole in buffer B. The elution was carried out with 3 volumes of 300 mM imidazole in buffer B.

GST Affinity Chromatography of the Recombinant GDP-Man 3′,5′-Epimerase—A crude extract containing the GST-tagged epimerase was applied to a 2-mL GST-affinity column equilibrated with buffer A. The column was washed with 15 volumes of buffer A, and the recombinant epimerase was eluted with 3 volumes of 10 mM glutathione (reduced form) in buffer A.

Extraction and Assay of l-Gulono-1,4-lactone Dehydrogenase Activity—The l-gulono-1,4-lactone dehydrogenase activity was extracted from white potato tubers essentially as described (14), except that gel filtration was performed on NAP-25 columns (Amersham Biosciences) and the obtained high molecular weight fraction was separated by centrifugation (20,000 × g for 20 min) into the cytosolic (supernatant) and the mitochondrial (pellet) fractions. The dehydrogenase activity was measured spectrophotometrically by following the l-gulono-1,4-lactone dependent reduction of cytochrome c (14).

PAGE—Proteins were separated by SDS/PAGE using 12.5% mini-gels and the buffer system described by Laemmli (15). Gels were stained with Coomassie Brilliant Blue R-250.

Peptide Sequencing and Protein Identification—Tryptic peptides prepared in-gel digested protein bands were analyzed by nanoelectrospray tandem mass spectrometry, and the obtained sequence information was submitted to database searching as described (11).

Protein Determination—Protein concentration was determined by the method of Bradford (16) using bovine serum albumin as standard.

Sugar Analysis—GDP-13C-labeled hexoses of the epimerase reaction mixtures were hydrolyzed in 50 mM HCl at 100 °C for 20 min. For HPLC analysis the acid-released 13C-labeled hexoses together with cold sugar standards were converted to the corresponding 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatives (17). The HPLC system with on-line UV and radioactivity detection (13) was used. The solvent was 18% acetonitrile in 0.1% phosphate buffer, pH 7.0 or pH 5.0 (for separation of PMP-derivatives of Man and Gal) at a flow rate of 0.8 ml/min. To identify allose, free sugars were separated by TLC on silica gel 60 aluminum sheets (pre-impregnated with 0.3 mM NaH2PO4) in acetone/n-butanol/water (8:1:1, v/v/v) and detected as described (13).

RESULTS

Characterization of the GDP-Man 3′,5′-Epimerase—To gain an insight into the regulation of the de novo biosynthesis of vitamin C, we have characterized the native and recombinant epimerase of A. thaliana. The epimerase belongs to the short chain dehydrogenase/reductase family (18). The native enzyme is a homodimer of 43 kDa subunits (11) and possesses two potential NAD binding sites and two potential substrate binding sites per dimer (19). The epimerase has a low Km for the GDP-Man substrate (4.4 μM) (Fig. 1A) compared to the Chlorella epimerase (96 μM) (20) and to the related bacterial enzymes, GDP-Man 4′,6′-dehydratase (19) and GDP-L-Fuc synthetase (21–22) (280 and 38.6 μM, respectively). In contrast to the GDP-Man 4′,6′-dehydratase (23) and GDP-L-Fuc synthetase (24) of E. coli, the epimerase is strongly inhibited by GDP and GDP-Glc in a competitive manner, with respective Ki values of 0.7 μM (Fig. 1C) and 5 μM (data not shown). The enzyme recognizes the purine moiety of GDP-derivatives since an adenine derivative, ADP-L-fucose, had no effect on the enzyme activity. Surprisingly, only a partial inhibition was observed with GDP-L-Fuc (K0.5 = 70 μM; Fig. 1D), even at 1 mM concentration (Table I). The sigmoidal-shaped affinity curve with l-AAA (Fig. 1D) is reminiscent of a feedback regulation observed in the biosynthesis of NDP-6-deoxyhexoses in bacteria (25–27). Like GDP-Glc 4′,6′-dehydratase of Yersinia pseudotuberculosis (28), the purified epimerase was stimulated by exogenously added oxidized forms of nicotinamide-adenine dinucleotides, NAD+ and NADP+ (145 and 110% of control, respectively), and inhibited by their reduced forms, NADH and NADPH (78 and 88% of control, respectively) (Table I). Physiological concentrations (1 mM) of l-AAA, a reducing agent and the end product of the pathway, inhibited the epimerase activity by 15% (Table I). In agreement with that, a feedback inhibition of the l-AAA biosynthesis was clearly observed in vivo, since feeding A. thaliana cells with exogenous l-AAA resulted in an increased level of the intracellular l-AAA and a decreased incorporation of the [14C]Man label into l-AAA (Table II). The partial inhibition of the epimerase by l-AAA might be explained in terms of “reductive inhibition” (29), i.e. as an l-AAA-dependent reduction of the enzyme-NAD+ complex in the absence of the nucleotide sugar. However, the same degree of inhibition (14%) was observed in the presence of 1 mM l-galactono-1,4-lactone (Table I), whereas D-isoascorbic acid and l-galactose had no effect. These facts suggest the existence of a stereospecific mechanism of the enzyme inhibition by sugar lactones.

We cloned the epimerase gene of A. thaliana (11) and affinity-purified the recombinant His- and GST-tagged proteins from E. coli host submitted to a “reversed” heat-shock. Of the recombinant epimerase protein (3 mg/liter of culture), 90% were found in the soluble fraction, but only 30% of the total activity were retained on the affinity columns probably because of interactions with other proteins. Analysis of the affinity-purified enzyme by SDS-PAGE (Fig. 1B) followed by mass spectrometry identification of the protein bands revealed the presence of the major GDP-Man 3′,5′-epimerase band and of a weak 70-kDa band corresponding to DnaK, a Hsp70 chaperone
of *E. coli* (the tryptic peptides identified are TTPSIIAYTQDG-ETLVGQPAK, IINEPTAAALAYGLDK, and SLGQFNLDGIN-PAPR). Thus, the recombinant epimerase could interact with a Hsp70 molecular chaperone. During purification of the native GDP-Man 3',5'-epimerase from *A. thaliana* cell suspensions (11), a 70-kDa chaperone (DnaK ortholog) co-purified with the epimerase throughout the whole procedure and was identified in the NaCl eluate from Blue-Sepharose as a cytosolic Hsc70.3 heat-shock cognate protein of *Arabidopsis* (At3g-09440; the identified tryptic peptides are NQVAMNPINTVFD-AK, NAVVTVPAYFNDSQR, DAGVIAGLNVMR, VQQLLVDF-FNGK, and FELSGIPPAKR). The majority of the Hsc70 protein was separated from the epimerase by gel filtration.

![FIG. 1.](image)

**A** Steady-state parameters of GDP-Man 3',5'-epimerase of *A. thaliana*

| Enzyme form                       | \(K_m\) for GDP-Man (\(\mu\)M) | \(V_{max}\) (\(\mu\)mol h\(^{-1}\cdot\)mg\(^{-1}\)) | \(k_{cat}\) (s\(^{-1}\)) | \(k_{cat}/K_m\) (s\(^{-1}\).mM\(^{-1}\)) |
|-----------------------------------|-------------------------------|---------------------------------|----------------|------------------|
| Native (purified from *A. thaliana*\(^a\)) | 4.5                           | 1.76                            | 0.041          | 9.1              |
| Recombinant (N-terminal GST-tag)\(^b\) | 31                            | 0.43                            | 0.010          | 0.3              |
| Recombinant (N-terminal His-tag)\(^b\) | 18                            | 0.31                            | 0.007          | 0.4              |

**B** SDS-PAGE of the affinity-purified recombinant His-tagged epimerase. Proteins were identified by nanoelectrospray ionization-tandem mass spectroscopy of in-gel tryptic digests. *Left*, molecular mass standards. *Right*, 0.3 M imidazole-eluted fraction from nickel nitrilotriacetic acid-Sepharose. Proteins were identified by Coomassie Blue staining. A, steady-state parameters of the native (purified from *A. thaliana* cell suspensions) and recombinant enzyme. \(^a\), the hydroxyapatite fraction of native epimerase (11) was used. \(^b\), the affinity-purified recombinant enzymes were used. *Left*, SDS-PAGE of the affinity-purified recombinant His-tagged epimerase. Proteins were visualized by Coomassie Blue staining. Left, molecular mass standards. Right, 0.3 M imidazole-eluted fraction from nickel nitrilotriacetic acid-Sepharose. Proteins were identified by nanoelectrospray ionization-tandem mass spectroscopy of in-gel tryptic digests. C, competitive inhibition of the native epimerase by GDP. Double reciprocal plots were shown, and each line represents a fixed GDP concentration: circles, 0 \(\mu\)M; triangles, 2 \(\mu\)M; squares, 4 \(\mu\)M. \(V_o\) is pmol of GDP-L-Gal-L-Gul produced/min. GDP-Man concentrations were from 1.1 to 5.5 \(\mu\)M. Inset, secondary plot of slopes versus GDP concentration was used to determine the \(K_i\) value for GDP. D, partial inhibition of the native epimerase by GDP-L-Fuc. Incubations contained GDP-[\(^{14}\)C]Man (3.4 \(\mu\)M), increasing amounts of GDP-L-Fuc, and an aliquot of the hydroxyapatite fraction of epimerase.

**TABLE I** GDP-Man 3',5'-epimerase activity in the presence of effectors

| Compound                  | Epimerase activity (\% of control) |
|---------------------------|-----------------------------------|
| No addition               | 100                               |
| GDP                       | 0                                 |
| GDP-\(\alpha\)-glucose    | 0                                 |
| GDP-L-fucose              | 37                                |
| NADH                      | 78                                |
| NADPH                     | 88                                |
| NADP                      | 110                               |
| NAD                       | 145                               |
| L-ascorbic acid           | 85                                |
| L-galactono-1,4-lactone   | 86                                |

**TABLE II** Effect of exogenous L-ascorbic acid L-AA and precursors on its de novo biosynthesis and cellular content in *A. thaliana* cell suspensions

4-day-old *A. thaliana* cell suspensions were preincubated for 20 h with 2.5 \(\mu\)M L-AA or its precursors and then labeled for 2 h with \[^{14}\text{C}]\text{GDP-Man}. \[^{14}\text{C}]-labeled and cold total L-AA in acid extracts was measured by HPLC. fw, fresh weight.
This step resulted in a 10-fold decrease of the epimerase activity, possibly because of not only the partial loss of the NAD cofactor (11) but also the disruption of interactions with the Hsc70 chaperone. The steady-state parameters of the recombinant GST- and His-tagged epimerase were comparable with those of the native enzyme, although the recombinant enzymes were catalytically less efficient (Fig. 1A). The $V_{\text{max}}$ of the native enzyme was low, suggesting a rate-limiting enzyme. The overall catalytic efficiency of GDP-Man 3',5'-epimerase, expressed as $k_{\text{cat}}/K_m$ (9.1 s$^{-1}$ mM$^{-1}$), was fairly good and comparable with...
that reported for the GDP-L-Fuc synthetase of E. coli (21).

Formation of GDP-L-Gulose by the GDP-Man 3',5'-Epimerase of A. thaliana—The most intriguing observation was the variation of the apparent equilibrium constant $K_{eq}$ (from 0.1 to 1.5) measured for different preparations of the recombinant epimerase. Similar variations of $K_{eq}$ but in a narrower range (from 0.1 to 0.4) were observed with preparations of the native epimerase from A. thaliana. Also, an unexplained anomaly with the measured $K_{eq}$ values for the reverse reaction was reported for the epimerase of Chlorella sp. (20). Fig. 2A, panel a, shows the HPLC profile of the reaction products at the equilibrium obtained with the affinity-purified epimerase. The measured ratio ($K_{eq}$) of the epimerization product(s) to the GDP-Man substrate was 0.6. If GDP-L-Gal were the only epimerization product, then a similar ratio (0.6) should be obtained for the mild acid-released 14C-labeled L-Gal versus D-Man. The measured L-Gal to D-Man ratio was only 0.35 (Fig. 2A, panel b), fact that indicated that the Man peak contained an unknown component. This component was separated from D-Man and co-migrated with l-gulose standard (Fig. 2A, panel c). Therefore, we conclude that the epimerase reaction mixture contained at equilibrium GDP-D-Man, GDP-L-Gal, and GDP-L-Gul in a ratio of 1:0.4:0.2. A similar analysis of the epimerization products obtained with an epimerase-containing 55–70% ammonium sulfate fraction from A. thaliana cell suspensions revealed the presence of GDP-D-Man, GDP-L-Gal, and GDP-L-Gul in a ratio of 1:0.18:0.09 (results not shown).

Fig. 2B shows the HPLC profiles of the epimerase reaction products obtained with a crude recombinant enzyme (90% ammonium sulfate fraction) and the relative ratios of the GDP-hexoses formed. In this case the reaction was shifted toward the GDP-L-Gul formation and the relative ratios of GDP-D-Man, GDP-L-Gal, and GDP-L-Gul at the equilibrium were 1:0.4:1.1 (Fig. 2B).

Our results demonstrate that the GDP-Man 3',5'-epimerase reaction can be dissected into at least two distinct epimerization reactions leading to the formation of two discrete products: GDP-L-Gal and GDP-L-Gul (Fig. 3). The fate of the epimeriza-

![Scheme 1](image1)

**Scheme 1**

5\(^{-}\)-epimerization

GDP-D-mannose $\rightleftharpoons$ GDP-L-gulose $\rightleftharpoons$ GDP-L-galactose

3\(^{-}\)-epimerization

![Scheme 2](image2)

**Scheme 2**

5\(^{-}\)-epimerization

GDP-L-gulose $\rightleftharpoons$ GDP-D-altrose

3\(^{-}\)-epimerization

GDP-D-mannose $\rightleftharpoons$ GDP-L-galactose

**Fig. 3. Dissection of the GDP-Man 3',5'-epimerase reaction; possible paths for reversible interconversions between GDP-D-Man, GDP-L-Gal, and GDP-L-Gul.** In scheme 1, the first step is a 5'-epimerization of GDP-D-Man, and thus, the resulting GDP-L-Gul is an obligate intermediate in the formation of GDP-L-Gal from GDP-D-Man. In scheme 2, GDP-L-Gul and GDP-L-Gal are formed independently. The 5'-epimerization of GDP-D-Man leads to the formation of GDP-L-Gul. The 3'-epimerization of GDP-D-Man would lead to GDP-D-altrose (which was undetectable and, thus, is shown in brackets) followed by the 5'-epimerization of the latter and formation of GDP-L-Gal. A possible interconversion between GDP-L-Gul and GDP-L-Gal through a 3'-epimerization reaction is shown by dotted arrows.

GDP-L-gulose in Vitamin C Biosynthesis
tion seems to depend on the molecular form of the enzyme, probably as a result of its interactions with other proteins.

Synthesis of l-AA from L-Gulose and L-Gulono-1,4-lactone by Plant Cells—In contrast to l-Gal, which is a minor constituent of plants, L-Gul, as far as we know, has never been reported in plants. Therefore, GDP-L-Gul might be dedicated to the biosynthesis of l-AA. To test this hypothesis, we supplied A. thaliana cells with cold L-Gul or l-gulono-1,4-lactone and measured the level of cold l-AA in the cells. As reported earlier for cress seedlings, preincubation with l-gulono-1,4-lactone (30) but also with l-Gul resulted in an increased level of vitamin C, and l-gulono-1,4-lactone was as efficient a precursor of l-AA as was l-galactono-1,4-lactone (Table II). Moreover, in the presence of cold precursors, a decreased incorporation of the [14C]Man label into vitamin C was observed (Table II), as expected for activity (0.66 milliunit/g of tissue) in the mitochondrial fraction. In contrast to L-Gal, which is a minor constituent of plants. In the presence of high concentrations of GDP-L-Fuc, the cellular level of the GDP-l-Gal precursor will be low, thus resulting in a lesser incorporation of l-Gal into glycoconjugates. Indeed, in the l-Fuc-deficient mur1 mutant of Arabidopsis, which lacks the GDP-Man 4’6’-dehydratase activity (32) catalyzing the first step in the GDP-l-Fuc pathway (Fig. 4), L-Gal replaces l-Fuc in xyloglucans (33) and Φ-glycans (34).

We observed that the epimerase is stimulated by the oxidized forms of nicotinamide adenine dinucleotides (NAD+/NADP+) but inhibited by their reduced forms (NADH/NADPH) and by L-AA (Table I). Because the NAD binding motif (GAGGFI) present within the epimerase sequence is a modified version of the common Rossmann consensus (GXGXG) found in other members of the short chain dehydrogenase/reductase family (35), this feature could be responsible for a lower affinity of the enzyme for the dinucleotides and the corresponding stimulation/inhibition effects. Moreover, our in vivo experiments demonstrated a feedback inhibition in the vitamin C pathway (Table II). A feedback inhibition was suggested by others (36), but no potential target for it was indicated. The late steps of the

**TABLE III**

| Substrate          | l-gulono-1,4-lactone dehydrogenase activity (milliunits/g of tissue) |
|--------------------|---------------------------------------------------------------------|
| l-gulono-1,4-lactone | 4.51                                                                 |
| l-galactono-1,4-lactone | 4.06                                                                 |

**Fig. 4. Proposed l-gulose pathway in de novo biosynthesis of vitamin C in plants.** The l-galactose pathway proposed by Wheeler et al. (7) and the de novo biosynthesis of GDP-l-Fuc are also shown. The relative fluxes into the pathways are not known. The scheme shows a dual role of GDP-Man 3’,5’-epimerase, which converts GDP-u-Man into GDP-l-Gal and GDP-l-Gul. We speculate that interactions of the epimerase with a molecular chaperone(s) might increase the enzyme activity and/or favor the formation of GDP-l-Gul, thus linking the vitamin biosynthesis to stress response. GDP-l-Gul is then channeled exclusively to the vitamin C path; after release from the nucleotide, l-Gul is oxidized to l-AA with l-gulono-1,4-lactone as an intermediate.

**DISCUSSION**

The biochemical characterization of the GDP-Man 3’,5’-epimerase of A. thaliana has brought new insights into the de novo biosynthesis of l-AA and its regulation. The unexpected partial inhibition of the epimerase by GDP-l-Fuc (Fig. 1D) could be of paramount importance because, even at high concentration of GDP-l-Fuc in the cell, the epimerase would still supply GDP-l-Gal/GDP-l-Gul substrates necessary for the de novo synthesis of l-AA. The complex type of inhibition by GDP-l-Fuc could also play a role in the regulation of the cell wall biosynthesis in plants. In the presence of high concentrations of GDP-l-Fuc, the cellular level of the GDP-l-Gal precursor will be low, thus resulting in a lesser incorporation of l-Gal into glycoconjugates. Indeed, in the l-Fuc-deficient mur1 mutant of Arabidopsis, which lacks the GDP-Man 4’6’-dehydratase activity (32) catalyzing the first step in the GDP-l-Fuc pathway (Fig. 4), L-Gal replaces l-Fuc in xyloglucans (33) and Φ-glycans (34).

We observed that the epimerase is stimulated by the oxidized forms of nicotinamide adenine dinucleotides (NAD+/NADP+) but inhibited by their reduced forms (NADH/NADPH) and by l-AA (Table I). Because the NAD binding motif (GAGGFI) present within the epimerase sequence is a modified version of the common Rossmann consensus (GXGXG) found in other members of the short chain dehydrogenase/reductase family (35), this feature could be responsible for a lower affinity of the enzyme for the dinucleotides and the corresponding stimulation/inhibition effects. Moreover, our in vivo experiments demonstrated a feedback inhibition in the vitamin C pathway (Table II). A feedback inhibition was suggested by others (36), but no potential target for it was indicated. The late steps of the
vitamin C biosynthesis are not inhibited by the end product because exogenous L-galactose/L-gulose and L-galactono-1,4-lactone are efficiently converted into L-AA (Table II). Therefore, the observed feedback inhibition must take place earlier in the pathway. GDP-Man 3′,5′-epimerase is partially inhibited by both L-CAA and L-galactono-1,4-lactone in vitro (Table I). Inhibition of the reversible epimerase reaction by the end product could be of great importance because it would save the energy of the glycosyl-pyrophosphoryl linkage of the sugar nucleotide, otherwise irreversibly lost during the subsequent hydrolytic steps. We propose, therefore, that the GDP-Man 3′,5′-epimerase catalyzing the first specific step in the biosynthesis of vitamin C could undergo a feedback inhibition, sense the redox state of the cell, and play an important role in the regulation of vitamin C and cell wall/glycoproteins biosynthesis.

We have shown that GDP-Man 3′,5′-epimerase produces a novel intermediate, GDP-L-Gul, in addition to the well known GDP-L-Gal (Fig. 2). Thus, the unique epimerization catalyzed by the epimerase is a complex reaction involving at least two distinct epimerization steps (Fig. 3). To our knowledge, this is the first report of a sugar epimerase releasing two different epimerization products (37).

Both recombinant and native GDP-Man 3′,5′-epimerase of A. thaliana co-purified with Hsp70 heat-shock proteins (E. coli DnaK and A. thaliana Hsc70.3, respectively). The Hsc70 heat-shock protein of Arabidopsis is constitutively expressed and stress-inducible (38). The highly conserved, ubiquitously Hsp70 chaperones play a key role in protection and adaptation to stress by participating in folding and unfolding of misfolded and native-state proteins (39), disassembly of regulatory complexes (40), and regulation of protein/enzyme activity (41–44). Significantly, increased vitamin C levels of Arabidopsis leaves in response to heat shock were reported (45), and an overexpression of the bacterial DnaK chaperone improved the salt and heat tolerance of transgenic tobacco (46–47). On the basis of its chromatographic behavior and enzymatic properties (Km, Vmax), we could detect different molecular forms of the epimerase, although the nature of these forms is not understood. Given the known functions of molecular chaperones and the fact that epimerase could interact with Hsp70 heat-shock proteins, we speculate that the Hsc70.3 protein of A. thaliana might be implicated in folding and/or regulation of the epimerase.

1-Gulose is an extremely rare sugar. It is present in two bacterial products, a glycolipid of Thermoplasma acidophilum (archaeobacteria) (48) and in the bleomycin of Streptomyces verticillus (Actinomycetales) (49), the synthesis of which is inhibited by both L-CAA and L-galactono-1,4-lactone (archaeobacteria) (48) and in the bleomycin of Thermoplasma acidophilum (50). Consequently, some of these proteins could be responsible for the observed conversion of L-gulono-1,4-lactone to L-CAA.

In summary, the first step of the de novo pathway for vitamin C in plants, catalyzed by GDP-Man 3′,5′-epimerase, undergoes a complex control and supplies two distinct products: GDP-L-Gal and GDP-L-Gul. We propose that, in contrast to GDP-L-Gal, which is used for the biosynthesis of glycoconjugates, GDP-L-Gul would be channeled directly into the vitamin C pathway (Fig. 4). After release, L-Gul could be oxidized to L-gulono-1,4-lactone by the L-galactose dehydrogenase (12) or a similar enzyme. The last step of the proposed L-Gul branch, oxidation of L-gulono-1,4-lactone by an organelle-specific dehydrogenase, may take place at different cellular locations and, thus, produce L-CAA in situ with no need for its intracellular transport. Further studies will be necessary to unravel the nature and specificity of the hydrolytic step(s) responsible for L-Gal-L-Gul release and to determine whether both branches, involving either L-Gal or L-Gul, function in the de novo biosynthesis of vitamin C in plants.

Acknowledgment—We thank Rebecca Verbanck for graphics.

REFERENCES
1. Smirnoff, N., and Wheeler, G. L. (2000) Crit. Rev. Plant Sci. 19, 267–290
2. Davies, M. B., Austin, J., and Partridge, D. A. (1991) Vitamin C Its Chemistry and Biochemistry, p. 81, Royal Society of Chemistry, Cambridge, UK
3. Jönsson, A., and Nessler, C. G. (2000) Mol. Breeding 6, 73–78
4. Agius, F., González-Lamothe, R., Caballero, J. L., Muñoz-Blanco, J., Botella, M. A., and Valpuesta, V. (2003) Nat. Biotechnol. 21, 177–181
5. Hancek, R. D., and Viola, R. (2002) Trends Biotechnol. 20, 299–305
6. Isherwood, F. A., Chen, Y. T., and Mapson, L. W. (1953) Nature 171, 348–349
7. Wheeler, G. L., Jones, M. A., and Smirnoff, N. (1998) Nature 393, 365–369
8. Östergaard, J., Persson, G., Davey, M. W., Bause, G., and Van Montagu, M. (1997) J. Biol. Chem. 272, 30099–30106
9. Imai, T., Kariita, S., Shiraito, G.-i., Hattori, M., Nunome, T., Oba, K., and Hirai, M. (1998) Plant Cell Physiol. 39, 1550–1558
10. Loomis, F. A., and Kelly, S. L. (1961) Nature 189, 1059–1061
11. Wolucka, B. A., Persson, G., Van Doorselaere, J., Davey, M. W., Demol, H., Vandekerckhove, J., Van Montagu, M., Zabeau, M., and Boerjan, W. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 14843–14848
12. Gatzek, S., Wheeler, G. L., and Smirnoff, N. (2002) Plant J. 30, 541–553
13. Wolucka, B. A., Davey, M., and Boerjan, W. (2001) Anal. Biochem. 294, 161–168
14. Oba, K., Fukui, M., Imai, I., Iriyama, S., and Nogami, K. (1994) Plant Cell Physiol. 35, 473–478
15. Laemmli, U. K. (1970) Nature 227, 685–688
16. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
17. Honda, S., Akao, E., Suzuki, S., Okuda, M., Kakehi, K., and Nakamura, J. (1989) Anal. Biochem. 180, 351–357
18. Jornwall, H., Persson, B., Krook, M., Atrian, S., González-Duarte, R., Jeffery, J., and Ghosh, D. (1995) Biochemistry 34, 6003–6013
19. Somozza, R. J., Menon, S., Schneider, H., Joseph-McCarthy, D., Dessen, A., and Ghosh, D. (1995) J. Mol. Biol. 19, 6003–6013
20. Menon, S., Stahl, M., Kusman, R., Xu, G.-Y., and Sullivan, F. (1999) J. Biol. Chem. 274, 26743–26750
21. Melo, A., and Glaser, L. (1965) J. Biol. Chem. 240, 398–405
22. Kornfeld, R. H., and Ginsburg, V. (1966) Biochem. Biophys. Acta 117, 79–87
23. Benford, W., Asuncion, M., Lam, J. S., and Naismith, J. H. (2000) EMBO J. 19, 6652–6663
24. He, X., Thorson, J. S., and Liu, H.-W. (1996) Biochemistry 35, 4721–4731
25. Mehta, O. (1978) Trends Biochem. Sci. 3, 193–195
26. Isherwood, F. A., Chen, Y. T., and Mapson, L. W. (1954) Biochem. J. 56, 1–15
27. Shigeoka, S., Nakano, Y., and Kitaoaka, S. (1979) Agric. Biochem. 43, 2187–2188
28. Bonin, C. P., Potter, I., Vanzan, G. F., and Reiter, W.-D. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 2085–2090
29. Zabackis, R., York, W. S., Pauly, M., Hantus, S., Reiter, W.-D., Chappell,
GDP-1-gulose in Vitamin C Biosynthesis

C. C. S., Albersheim, P., and Darvill, A. (1996) Science 272, 1808–1810
34. Rayon, C., Cabanes-Macheteau, M., Loutelier-Bourhis, C., Salliot-Maire, I., Lemoine, J., Reiter, W.-D., Lerouge, P., and Faye, L. (1999) Plant Physiol. 119, 725–733
35. Scrutton, N. S., Berry, A., and Perham, R. N. (1990) Nature 343, 38–43
36. Pallanca, J. R., and Smirnoff, N. (2000) J. Exp. Bot. 51, 669–674
37. Allard, S. T. M., Giraud, M.-F., and Naismith, J. H. (2001) Cell. Mol. Life Sci. 58, 1650–1665
38. Wu, S.-H., Wang, C., Chen, J., and Lin, B.-L. (1994) Plant Mol. Biol. 25, 577–583
39. Hartl, F. U., and Hayer-Hartl, M. (2002) Science 295, 1852–1858
40. Freeman, B. C., and Yamamoto, K. R. (2002) Science 296, 2232–2235
41. Morishima, Y., Murphy, P. J. M., Li, D.-P., Sanchez, E. R., and Pratt, W. B. (2000) J. Biol. Chem. 275, 18054–18060
42. Haus, U., Trommler, P., Fisher, P. R., Hartmann, H., Lottspeich, F., Noegel, A. A., and Schleicher, M. (1993) EMBO J. 12, 3763–3771
43. Lutz, W., Kohno, K., and Kumar, R. (2001) Biochem. Biophys. Res. Commun. 282, 1211–1219
44. Yan, W., Frank, C. L., Korth, M. J., Sopher, B. L., Novoa, I., Ren, D., and Katze, M. G. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 15920–15925
45. Panchuk, I. I., Volkov, R. A., and Schoff, F. (2002) Plant Physiol. 128, 838–853
46. Sugino, M., Hihino, T., Tanaka, Y., Niu, N., Takabe, T., Takabe, T. (1999) Plant Sci. 137, 81–88
47. Ono, K., Hihino, T., Kohinata, T., Suzuki, S., Tanaka, Y., Nakamura, T., Takabe, T., Takabe, T. (2001) Plant Sci. 160, 455–461
48. Swain, M., Brusen, J.-A., Sprout, G. D., Cooper, F. P., Patel, G. B. (1997) Biochim. Biophys. Acts 1345, 56–64
49. Da, L., Sánchez, C., Chen, M., Edwards, D. J., and Shen, B. (2003) Chem. Biol. 10, 623–642
50. Mengelle, R., and Sumper, M. (1992) FEBS Lett. 298, 14–16
51. Burkhard, B., Melkonian, M., and Kemelring, J. P. (1998) J. Phycol. 34, 779–787
52. Wagner, C., Sefkow, M., and Kopka, J. (2003) Phytochemistry 62, 887–900