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RESEARCH PAPER

The pathway of L-ascorbic acid biosynthesis in the colourless microalga *Prototheca moriformis*

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Abstract

When mutant strain UV77–247 of *Prototheca moriformis* Kruger was fed D-[1-^{13}C]Glc, it synthesized L-ascorbic acid (AA) with approximately three-quarters of the label at the C-1 position and the remaining label at the C-6 position, showing that AA is made by a non-inversion (retention) pathway, i.e. C-1 of Glc becomes C-1 of AA. The label present at C-6 is consistent with the glycolytic conversion of Glc to 3-carbon intermediates and subsequent gluconeogenesis. Compounds suggested as intermediates in inversion-type pathways were not converted to AA. Most strains converted Man to AA at a rate greater than they did Glc. Enzyme activities leading from Fru-6-P to the formation of GDP-Man were identified in all strains, but none of these activities correlated with the mutants' abilities to accumulate AA. However, there was a strong correlation between GDP-Man-3,5-epimerase activity and AA accumulation. Wild-type *P. moriformis* ATCC 75669 and mutant strains of varying AA-synthesizing abilities rapidly converted L-Gal or L-galactono-1,4-lactone to AA. Based on this data, a biosynthetic pathway from Glc to AA is proposed in which the epimerase is the rate-limiting activity in AA synthesis.

Key words: GDP-Mannose-3,5-epimerase, L-ascorbic acid, microalga, *Prototheca*.

Introduction

L-ascorbic acid (AA; vitamin C) is essential for the growth and nutrition of humans and other animals. Currently, AA is produced commercially by either the Reichstein process, which entails one bacterial fermentation and several chemical synthesis steps (Jaffe, 1984; Reichstein and Grussner, 1934) or by fermentation to produce 2-keto-L-gulonic acid, followed by a chemical conversion to AA (Hancock and Viola, 2001). A one-step fermentation process has been described for the production of AA using the heterotrophic green microalga *Chlorella pyrenoidosa* (Running *et al*., 1994). That work resulted in increases in intracellular AA content of more than 70-fold, and in cells containing more than 2% of their dry weight as AA (Doncheck *et al*., 1996). Virtually none of the AA produced was extracellular. It has also been shown that *C. protothecoides* and members of all *Prototheca* species synthesize AA from Glc (Running *et al*., 2002).

The genus *Prototheca* is a member of the microalgal Class Chlorophyceae and is related to the genus *Chlorella* (Huss and Sogin, 1990; Kessler, 1982; Pore, 1985), especially *C. protothecoides* (Huss *et al*., 1999). *Prototheca* has been called a 'colourless *Chlorella*' (Casselton and Stacey, 1969; Walker *et al*., 1975), but its members have characteristics which distinguish them from members of most *Chlorella* species: (1) they do not have photosynthetic pigments (Pore, 1993); (2) they cannot use nitrate as a sole nitrogen source (Pore, 1985, 1993); (3) they are all thiamine auxotrophs (Pore, 1993); (4) they are acidophilic (Huss *et al*., 1988); and (5) they can use a variety of hydrocarbons as sole carbon sources (Koenig and Ward, 1983; Walker *et al*., 1975; Walker and Pore, 1978).

Strain-improvement efforts using *Prototheca* generated many mutant strains with increased abilities to accumulate AA (Running *et al*., 2002). A number of mutants accumulated less AA than the wild-type (wt) strain; AA

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Abbreviations: AA, L-ascorbic acid; GMP, GDP-Mannose pyrophosphorylase; L-GalL, L-galactono-1,4-lactone; L-GalDH, L-galactose dehydrogenase; PMI, phosphomannose isomerase; PMM, phosphomannomutase.

was undetectable in some. These mutants were used to aid in identifying a pathway for AA biosynthesis that involves Man-containing intermediates. This pathway, identified in *P. moriformis* earlier (Berry *et al*., 1999), is similar to that recently described in higher plants (Wheeler *et al*., 1998). Both proposals incorporate the key observation that the carbon chain of metabolized Glc is not inverted in the synthesized AA (see Results and discussion), unlike the pathways described for animals and some algae (Grün and Loewus, 1984; Helsper *et al*., 1982; Isherwood *et al*., 1954*b*; Shigeoka *et al*., 1979; see Results and discussion). Two recent reviews provide detailed descriptions of the AA synthesis pathways in various organisms (Loewus, 1999; Smirnoff *et al*., 2001).

Materials and methods

Strains, growth media and culture conditions

Wild-type strains were obtained from the American Type Culture Collection, Manassas, VA (ATCC), the Culture Collection of Algae and Protozoa at the University of Texas at Austin (UTEX) or from Professor R Scott Pore (West Virginia University Medical School, Charleston, WV). Strains were grown in a Glc minimal medium containing (l^{-1}): 1.3 g KH_2PO_4, 3.8 g K_2HPO_4, 7.7 g Na_3-citrate, 3.7 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4.7H_2O$, 1.5 mg $FeSO_4.7H_2O$, 3–6 mg thiamine HCl, 30–60 g Glc, and 2 ml of trace metals solution. The trace metal solution contained (l^{-1}): 20 ml HCl, 156 mg $CoCl_2.6H_2O$, 914 mg H_3BO_3, 1.76 g $ZnSO_4.7H_2O$, 1.23 g $MnSO_4.H_2O$, 48 mg $Na_2MoO_4.2H_2O$, 11.4 g $CaCl_2.2H_2O$, 80 mg $VOSO_4.2H_2O$; 40 mg $Ni(NO_3)_2.6H_2O$, and 40 mg Na_2SeO_3. For Mg-limiting growth, the $MgSO_4.7H_2O$ was reduced to 20 mg l^{-1}. The standard growth temperature was 35 °C.

AA analysis

For colorimetric analysis of AA, a modification of the dipyridyl method (Omaye *et al*., 1979) was used. HPLC analysis was based on that of Grün and Loewus (1983). Supernates were chromatographed on a Bio-Rad HPX-87H organic acid column (Bio-Rad Laboratories, Richmond, CA) with 13 mM nitric acid as solvent, at a flow rate of 0.7 ml min^{-1} at room temperature. Detection was at 254 nm. This system can distinguish between AA and D-erythorbic acid, the 5-epimer.

[^{13}C]labelling

Shake flask cultures were grown to moderate cell density under Mg-limiting conditions, with D-[1-^{13}C]labelled Glc (Cambridge Isotope Laboratories, Andover, MA) as 10% of the total Glc (40 g l^{-1}). The combined culture supernates (90 ml) were clarified by the addition of four parts ethanol and overnight storage at –20 °C, with precipitated solids removed by centrifugation. The supernate was evaporated to approximately 350 ml, 500 ml of water was then added, and the resulting solution was again evaporated to 350 ml. The pH of 80 ml aliquots was brought to 2.0 with HCl, 5 g of AG501-X8 mixed bed resin (Bio-Rad Laboratories, Richmond, CA) was added, and the pH of the mixture was kept between 2.0 and 3.0 by periodic additions of HCl until the pH, AA content (as measured with test strips; EM Science, Gibbstown, NJ), and conductivity had stabilized. The resin was removed by filtration and the samples were lyophilized. Samples were subjected to [^{13}C]nuclear magnetic resonance analysis using a Varian XL-400 spectrometer. Isotopic enhancements were determined by integrating relevant peaks, subtracting the contribution from natural abundance ^{13}C and dividing by the latter.

Whole cell conversions of potential AA precursors

Shake flask-grown cells were washed and resuspended in buffer (5 mM citrate/20 mM phosphate, pH 4.5) or fresh growth medium. Cell suspensions were supplemented with carbon sources, as indicated in the results. Supplemented cell suspensions were incubated for 24–30 h at 35 °C with shaking. The entire suspension was extracted with 5% trichloroacetic acid and assayed for AA.

Phosphomannose isomerase (PMI) and phosphomannomutase (PMM) assays

Magnesium-limited cells, washed and resuspended in 50 mM TRIS/10 mM $MgCl_2$, pH 7.5, were broken in a French press and centrifuged at 30 000 *g* for 30 min. The resulting supernates were desalted using Pharmacia PD-10 columns (Pharmacia Biotech, Uppsala, Sweden). Activities of PMI and PMM were determined according to the methods of Sa-Correia *et al*. (1987). Protein in the original extracts was determined by the method of Bradford (1976). All biochemicals were purchased from Sigma Chemical Co. (St Louis, MO).

GDP-Man pyrophosphorylase (GMP) assay

Cells in 50 mM phosphate buffer, pH 7.0, containing 20% (v:v) glycerol and 0.1 M NaCl (3 ml buffer g^{-1} wet cells) were broken in a French press. The crude extracts were centrifuged at 15 000 *g* for 15 min. The reactions were carried out in the forward direction by adding various volumes of extracts to solutions of 50 mM phosphate/4 mM $MgCl_2$ buffer, pH 7.0, containing 1 mM GTP. The reactions were initiated by addition of 1 mM (final) Man-1-P. The final reaction volume was 0.1 ml. The reaction mixtures were incubated at 30 °C for 10 min, filtered through 0.45 µm PVDF syringe filters and analysed for GDP-Man by HPLC. A Supelcosil SAX1 column (4.6×250 mm; Supelco, Bellefonte, PA) was used with solvent mixtures (flow rate 1 ml min^{-1}) of (A) 6 mM potassium phosphate, pH 3.6 and (B) 500 mM potassium phosphate, pH 4.5. The step gradient was: 0–3 min, 100% (A); 3–10 min, 79% (A); 10–15 min, 29% (A). Column temperature was 30 °C.

GDP-Man-3,5-epimerase assay

Cells were harvested after 43–48 h growth under Mg-limiting conditions, washed and resuspended in 50 mM MOPS/5 mM EDTA, pH 7.2. Cells were disrupted in a French press and centrifuged at 20 000 *g* for 20 min. Fifty µl aliquots of dilutions of the resulting extracts were added to 10 µl of 6.3 mM Na-[U-^{14}C]GDP-Man (NEN Life Science Products, Boston, MA). This substrate had an activity of 16 µCi ml^{-1} before dilution into the reaction mixture. The reactions were stopped after 10 min by transferring 20 µl of each mixture into a microfuge tube containing 20 µl of 250 mM trifluoroacetic acid (TFA) and 1.0 g l^{-1} each Man and Gal. These tubes were sealed and boiled for 10 min, cooled, spun at 10 000 *g* for 60 s, and 5 µl of each hydrolysate was spotted on plastic-backed EM Science (Gibbstown, NJ) silica gel 60 thin-layer chromatography plates, with 1 cm lanes created by scoring with a blunt stylus. After drying, plates were twice chromatographed for 2.5 h in ethyl acetate:isopropanol:water, 65:22.3:12.7 (plates were dried between runs). Spots of free sugars were visualized with a *p*-anisaldehyde spray (Krebs *et al*., 1969), followed by heating at 105 °C for about 15 min. Spots corresponding to Gal and Man were cut from the plates and counted in a scintillation counter (Model LS2800, Beckman Instruments, Inc, Irvine, CA). For time-zero control counts, 16.7 µl of each extract dilution was added to 23.3 µl of the labelled substrate above, which had been diluted 1 to 7 with the TFA/Man/Gal solution.

Biochemicals

D-Glucosone was prepared from Glc using pyranose-2-oxidase prepared from *Polyporus obtusus* by the method of Koths and Halenbeck (1986). L-sorbosone and L-idonic acid were gifts from Frank Loewus, Washington State University, Pullman, WA. All other chemicals were of the highest grade commercially available.

Results and discussion

^{13}C-NMR

When cultures of strain UV77-247 were grown on D-[1-^{13}C]Glc and the AA isolated from those cultures was analysed by quantitative [^{13}C]NMR, about three-quarters of the label was found at the C-1 position, and the remaining label was found at C-6 (Table 1). Further analyses suggested that the label at C-6 was due to catabolism of the Glc to 3-carbon intermediates and subsequent gluconeogenesis, similar to that found in carrots by Krook *et al*. (1998, 2000). Isotopic enhancements in the 'mirror image' positions were seen in AA recovered from cultures grown with D-[2-^{13}C]Glc or D-[3-^{13}C]Glc; about one-fifth of the labels were found at C-5 and C-4, respectively.

The small, but significant, amount of enhancement observed in other, 'non-mirror image', positions is consistent with flux through the pentose phosphate pathway, the overall balanced equation for which is:

$$3\ \text{Glc-6-P} \rightarrow 2\ \text{Fru-6-P} + \text{Glyceraldehyde-3-P} + 3\ CO_2$$

Based on the known biochemistry, it would be expected that carbon flux through this pathway would result in AA with isotopic enhancement at positions (1) and (3) when cells were grown on D-[2-^{13}C]Glc and enhancement at position (2) when cells were grown on D-[3-^{13}C]Glc. This was indeed observed. That there is twice as much enhancement at C-1 as there is at C-3 after growth on D-[2-^{13}C]Glc is also predicted.

Conversion of putative pathway intermediates by strain UV77-247

When resting cells of strain UV77-247 were incubated with various compounds, some of which have been proposed as intermediates in the AA biosynthetic pathways in animals, plants and other algae, AA accumulated to various extents, depending on the substrate (Table 2). L-Gal and L-galactono-1,4-lactone (L-GalL) were converted to AA more rapidly than was Glc, suggesting that these compounds are intermediates in the biosynthetic pathway from Glc, with the rate-limiting steps occurring upstream of those compounds.

Saito *et al*. (1990) proposed a pathway in bean and spinach in which Glc is converted to AA through D-glucosone and L-sorbosone. Strain UV77-247 converted D-glucosone to AA better than it did L-sorbosone. In the absence of transport considerations, this is the opposite of what one might expect if the D-glucosone pathway is correct, since L-sorbosone is downstream from D-glucosone in that pathway. The importance of this pathway has also been questioned by Pallanca and Smirnoff (1999).

GlcUA and D-glucurono-6,3-lactone, intermediates in the biosynthetic pathway in rats (Isherwood *et al*., 1954*a*, *b*) were not converted to AA. Neither was GalUA, proposed as an intermediate in early descriptions of the AA biosynthetic pathway for plants (Isherwood *et al*., 1954*a*) and algae (Shigeoka *et al*., 1979). Other conceivable precursors not converted to significant levels of AA included D-gluconolactone, D-gulono-1,4-lactone,

Table 1. *Labelling pattern in AA after cells were fed D-[1-^{13}C], [2-^{13}C] or [3-^{13}C]Glc*

Cells of strain U77–247 were grown to moderate density in Mg-limiting medium with D-[1-^{13}C], [2-^{13}C] or [3-^{13}C]Glc as 10% of the total Glc (40 g l^{-1}). The clarified, deionized culture broth was analysed by nmr to determine the labelling pattern in synthesized AA.

Carbon position in AA	% of label in AA carbons when cells were fed Glc labelled at:		
	C-1	C-2	C-3
1	73.4	7.3	2.8
2	0	73.0	6.3
3	0	3.6	69.7
4	0	0	19.7
5	0	16.1	1.4
6	26.6	0	0

Table 2. *AA concentrations in resting-cell suspensions of* P. moriformis *strain UV77–247 incubated with potential AA precursors*

Cells from Glc-depleted flask cultures were washed, resuspended in complete medium with Glc replaced by one of the listed compounds (50 mM), incubated for 24–30 h with shaking at 35 °C and whole broths were assayed for AA.

Substrate	AA (mg l^{-1}), less no-substrate control	
	Trial 1	Trial 2
L-Gal	623	430
L-GalL	476	393
Man		174
Fru	248	120
D-glucosone	247	174
Glc	242	116
Gal	200	73
D-glucono-1,5-lactone	40	
D-gulono-1,4-lactone	24	
GlcUA	22	
L-sorbosone	0	
2-keto-D-gluconic acid	–1	
D-glucurono-6,3-lactone	–13	
D-gluconic acid	–33	
D-GalUA	–45	
L-idonate	–46	

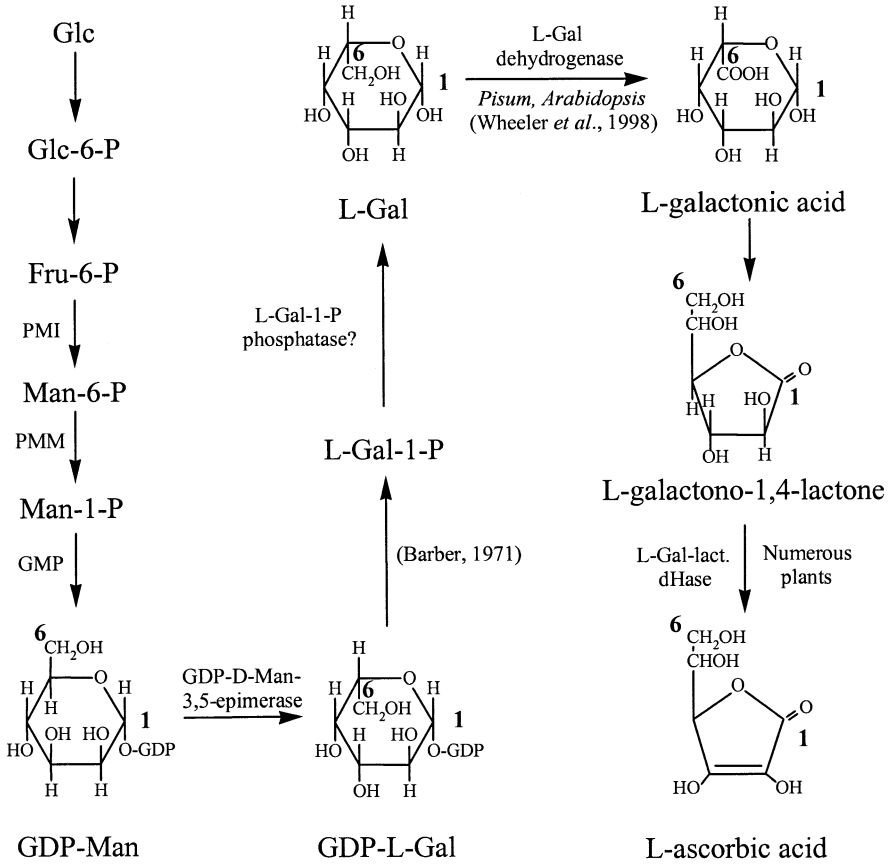

Fig. 1. Proposed pathway from Glc to AA in *P. moriformis*. The activities of PMI, PMM, GMP and GDP-Man-3,5-epimerase were assayed in mutant strains. PMI, phosphomannose isomerase; PMM, phosphomannomutase; GMP, GDP-Man pyrophosphorylase.

2-keto-D-gluconic acid, D-gluconic acid and L-idonic acid. The lack of conversion of the last two is consistent with the results of ^{14}C labelling studies in beans by Saito and Loewus (1989). In a second trial, UV77-247 converted Man and D-glucosone to AA at greater rates than it did Glc. Again, L-Gal and L-GalL were rapidly converted. These data are consistent with the pathway shown in Fig. 1.

Conversion of potential AA biosynthesis intermediates by mutant P. moriformis *strains*

Mutants with varying abilities to accumulate AA were tested for their abilities to convert Glc, Man, L-Gal and L-GalL into AA (Table 3). Three strains (designated type I) that overproduce AA in Glc-fed culture and are typified by ATCC PTA-111 converted Man to AA as well or better than they did Glc, and converted L-Gal and L-GalL to AA at higher rates than they converted either D-sugar. Five other strains (type II), exemplified by the immediate descendant of ATCC PTA-111, UV244–1, did not convert either Glc or Man to AA, yet readily converted L-Gal and L-GalL, strongly suggesting blocks in the pathway upstream from L-Gal. Strain UV244–15 (type III) converted Glc and Man to AA, but did not convert either L-Gal or L-GalL. This strain is a moderately good producer of AA, and this result suggests an inability to transport these two exogenously supplied compounds into the cell. Strain UV244–16 (type IV) converted Glc to AA and converted the other three compounds less well. This strain may well have a lesion that reduces the efficiency of the last enzymatic step in the pathway and this would explain its overall weak AA productivity from Glc.

Enzyme activities in mutants

Magnesium-limited cells of the wt and 14 mutant isolates ultimately derived from it were assayed for four key enzyme activities shown in Fig. 1. Table 4 summarizes the results of these assays. All of the strains that produced measurable amounts of AA had measurable epimerase activity. The converse was not true. Four of the strains that synthesized trace amounts or no AA had significant epimerase activities. Since these strains can synthesize AA from L-Gal and L-GalL, their genetic lesions are likely to lie in genes that affect the conversion of GDP-L-Gal to L-Gal. As shown in Fig. 2, there is a strong correlation between AA accumulation and GDP-Man-3,5-epimerase activity. This argues strongly for GDP-Man-3,5-epimerase

Table 3. *AA concentrations in resting cell suspensions of strain ATCC PTA-111 and three immediate descendants of various AA-synthesizing abilities after incubation with potential AA precursors*

Strain	Specific AA formation (mg l^{-1} culture A_{620}^{-1})	Substrate			
		Glc	Man	L-Gal	L-GalL
		AA, mg l^{-1}, less no-substrate control			
Type I					
ATCC PTA-111	73.7	44	80	138	120
Type II					
UV244-1	0.0	0	0	148	146
Type III					
UV244-15	24.5	51	68	4	0
Type IV					
UV244-16	5.0	46	25	25	25

Table 4. *Specific enzyme activities (mU)[a] of selected mutant* Prototheca *strains*

Strain	Specific AA formation (mg l^{-1} culture A_{620}^{-1})	PMI[b]	PMM[b]	GMP[b]	Epimerase[b]
UV164-6	78.4				0.79
ATCC PTA-111	73.7	10.8	69.6	2.6	0.78
UV140-1	69.9				0.78
ATCC 209681	61.4				0.58
UV77-247	44.4				0.52
ATCC PTA-112	40.1	11.1	45.8	4.3	0.39
UV244-15	24.5	14.3	41.5	3.1	0.42
NA21-14	23.6	12.1	60.3	2.4	0.27
ATCC 75669[c]	21.9				0.28
UV244-16	5.0	16.5	85.6	4.3	
ATCC PTA-113	2.0	17.7	47.0	2.0	0.03
UV218-1	0.4	15.9	72.1	2.7	0.83
UV213-1	0.1	19.7	47.7	3.2	0.60
UV82-21	0.0	14.6	70.6	4.1	0.15
UV244-1	0.0	18.2	51.1	5.5	0.15

[a] Units: PMI and PMM, nmoles NADP reduced min^{-1} mg^{-1} protein; GMP, nmoles GDP-Man formed min^{-1} mg^{-1} protein; epimerase, nmoles GDP-L-Gal formed min^{-1} mg^{-1} protein.
[b] PMI: phosphomannose isomerase; PMM: phosphomannomutase; GMP: GDP-Man pyrophosphorylase; Epimerase: GDP-Man-3,5-epimerase.
[c] Wild-type strain.

activity being the rate-limiting step in the biosynthesis of AA in these cells and supports the AA synthesis pathway depicted in Fig. 1.

Non-inversion nature of AA pathway in Prototheca

Relying on labelling studies by Horowitz and colleagues (Horowitz *et al*., 1952; Horowitz and King, 1953), Isherwood *et al*. (1954*b*) proposed an AA biosynthetic pathway in animals in which the carbon chain of Glc is inverted (C-1 of Glc becomes C-6 of AA; Fig. 3). They found that cress seedlings can synthesize small amounts of AA from D-GalUA methyl ester (Mapson and Isherwood, 1956), but the poor conversion argued against this compound as a natural substrate. Recent results (Davey *et al*., 1999) have revived interest in this route as an alternative AA biosynthetic pathway in plants. Loewus' group has shown by tracer studies that cress seedlings and parsley (Loewus, 1963), strawberries (Loewus, 1963; Loewus *et al*., 1956, 1958; Loewus and Kelly, 1961), and the green alga *C. pyrenoidosa* (Renstrøm *et al*., 1982/1983) synthesize AA from Glc without inverting the carbon chain configuration (C-1 of Glc becomes C-1 of AA).

Studies of AA biosynthesis in other algae have indicated an inversion pathway (Fig. 3) that involves GalUA (Grün and Loewus, 1984; Helsper *et al*., 1982; Shigeoka *et al*., 1979). One of these, *Euglena gracilis*, is a flagellated, photosynthetic protist. The other two are heterokont algae (Daugbjerg and Anderson, 1997), the flagellated chrysophyte *Ochromonas danica*, and the diatom, *Cyclotella cryptica* (Bacillariophyta). Their possession of the inversion pathway has phylogenetic implications, allying them biochemically with animals and separating them from organisms that use a non-inversion pathway, such as higher plants (Wheeler *et al*., 1998) and green algae (Renstrøm *et al*., 1982/1983). Indeed, many diatom genes more

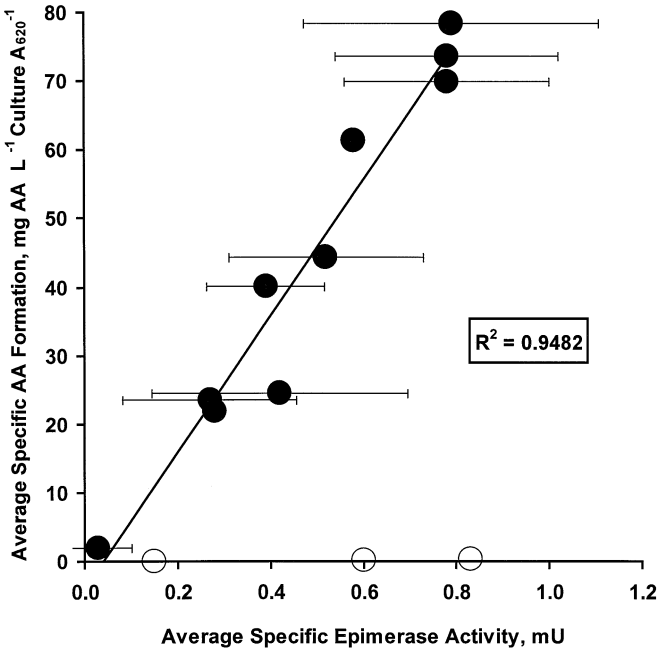

Fig. 2. Correlation of average specific epimerase activities with average whole broth AA specific formations of *P. moriformis* ATCC 75669 and fourteen mutants ultimately derived from it. The AA non-producing strains represented by open symbols probably are affected in steps between GDP-L-Gal and L-Gal.

closely resemble animal genes than those of higher plants (Qiu and Palmer, 1999). Since the colourless genus *Prototheca* fits into the clade encompassing higher plants and chlorophytes (Chesnick *et al*., 1996), and chlorophytes are the algae most closely related to higher plants (Hicks *et al*., 2001), it is not surprising that the AA biosynthesis pathway in *Prototheca* (Berry *et al*., 1999) is similar to the pathway in higher plants (Wheeler *et al*., 1998).

The [^{13}C]labelling data in support of the non-inversion nature of AA synthesis in *Prototheca* are consistent with the inability of this organism to convert into AA compounds which are associated with an inversion pathway (Table 2). This study's [^{13}C]labelling results agree with the [^{14}C]labelling results reported by Loewus *et al.* for higher plants (Loewus, 1963; Loewus *et al*., 1956, 1958; Loewus and Kelly, 1961) and *Chlorella* (Renstrøm *et al*., 1982/1983). About three-quarters of the label originating in C-1 of Glc was found in C-1 of AA made by *P. moriformis*. In line with the similar results of Krook *et al*. (1998, 2000), it is believed that the remaining label found at C-6 of AA is due to metabolism of Glc through triose-phosphate intermediates. After the formation of glyceraldehyde-3-P (Gly-3-P) and dihydroxyacetone-P (DHAP), and the conversion of the DHAP into a second molecule of Gly-3-P, the C-1 and C-6 carbons of Glc both become C-3 of Gly-3-P. When C-1-labelled Glc is metabolized to triose phosphates in glycolysis and then re-formed during subsequent gluconeogenesis, the newly formed Glc would be labelled at either or both C-1 and C-6. If this re-formed Glc were then used to make AA, one would expect the AA to be similarly labelled. Consistent with this type of 'isotopic mixing' is the observation that Suc obtained from D-[1-^{13}C]labelled Glc was labelled at positions 1, 6 and 1′, and presumably 6′ (data not shown), with the C-1 to C-6 ratio consistent with that observed for AA. Similar isotopic enrichment of Suc was also seen by Krook *et al*. (2000).

The non-inversion nature of the pathway is also supported by the C-2 and C-3 labelling data (Table 2). In both cases, about three quarters of the label in Glc remained at that same carbon in AA, and about one-fifth of the label was detected in the 'mirror-image' positions in AA, consistent with glycolytic isotopic mixing. Small amounts of label were also detected in 'non-mirror image' AA carbons, consistent with a small amount of the labelled Glc passing through the pentose phosphate pathway before gluconeogenesis.

Reactions in proposed pathway

Roberts (1971) fed labelled Man to corn root tips and found the label in polysaccharide, specifically in the residues of Man, L-Gal and Fuc. No label was detected in Glc, Gal, Ara, or Xyl. Man is converted to AA by *Prototheca* cells as well or better than Glc. This argues for Man being downstream from Glc in the pathway. The enzymes involved in the conversion of Fru-6-P to GDP-Man (PMI, PMM, GMP) have been well characterized in plants (Feingold and Avigad, 1980; Oesterhelt *et al*., 1997), yeast (Boles *et al*., 1994; Hashimoto *et al*., 1997), filamentous fungi (Smith and Payton, 1994), red algae (Oesterhelt *et al*., 1996), and bacteria (Koplin *et al*., 1992; Sa-Correia *et al*., 1987). These enzyme activities were identified in all mutants of *P. moriformis*, but there was no correlation between any of these activities and their abilities to accumulate AA. Barber (1971, 1975) identified in *C. pyrenoidosa* enzyme activities for the conversion of GDP-Man to GDP-L-Gal (GDP-Man-3,5-epimerase) and conversion of GDP-L-Gal to L-Gal-1-P. The present study found a strong correlation between mutants' GDP-Man-3,5-epimerase activities and AA synthesizing abilities (Fig. 2), implicating this enzyme activity as the rate-limiting step in AA biosynthesis. These reactions logically link Man to the compounds which are rapidly converted to AA in *Prototheca*, namely L-Gal and L-GalL. It is highly probable that the four strains that showed little or no AA accumulation, but measurable epimerase activity (Fig. 2; Table 4), have genetic lesions affecting activities between GDP-L-Gal and L-Gal, since their abilities to convert L-Gal to AA are unimpaired (Table 3, 'Type II').

Based on their discovery of an L-Gal dehydrogenase (L-GalDH), Wheeler *et al*. (1998) have recently proposed a similar pathway in higher plants. The same authors (Gatzek *et al*., 2002) saw no increase in AA content

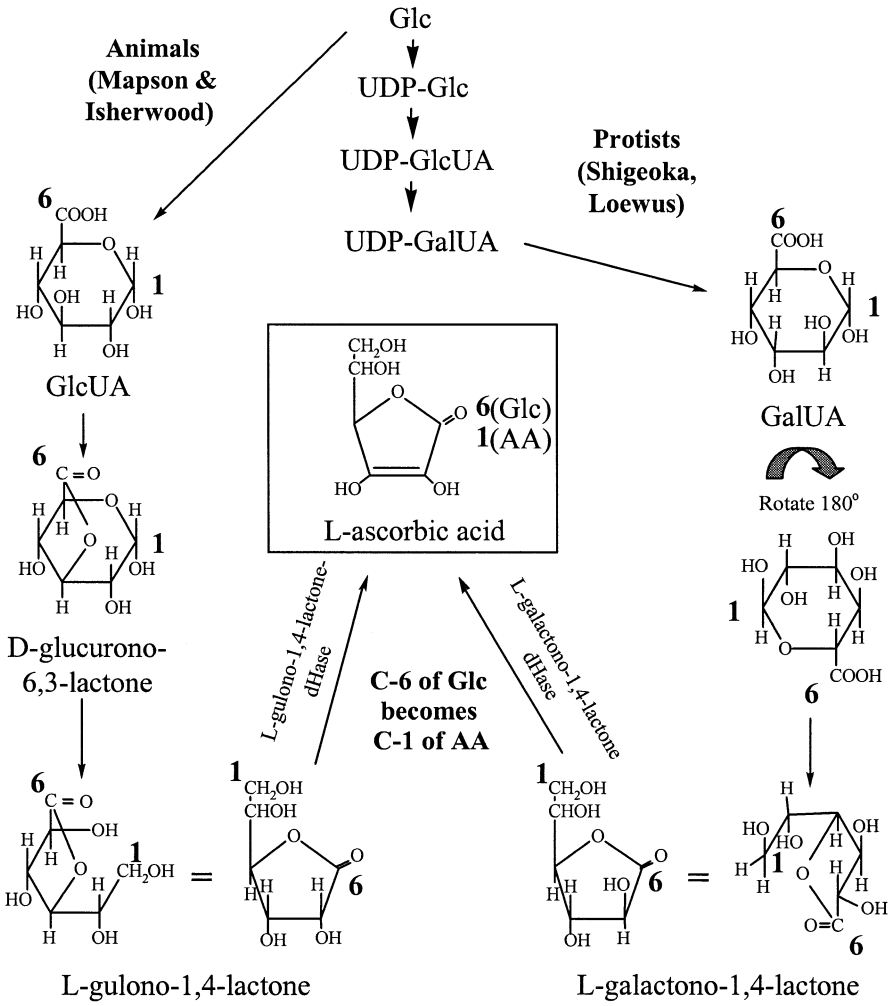

Fig. 3. Proposed biosynthetic pathways from Glc to AA in non-chlorophyte algae and animals. Inversion of the carbon chain was shown in the algae *Cyclotella* (Grün and Loewus, 1984), and *Poteriochromonas* (Helsper *et al.*, 1982). The latter saw AA enhancement with free GalUA, as did Shigeoka *et al*. in *Euglena* (Shigeoka *et al.*, 1979), but it is thought that UDP intermediates are the natural precursors. The inversion route in animals is based on results in rats (Isherwood *et al.*, 1954*b*).

when that *Arabidopsis* enzyme was overexpressed in tobacco. Although AA pool size increased under high illumination, L-GalDH activity did not. Peltzer *et al.* (1999) saw differences in the total AA and reduced/oxidized AA ratios in illuminated/darkened leaf parts of *Coleus*, and concluded that the L-GalDH may be light-modulated. They saw no difference in L-GalDH activities of light- and dark-harvested leaves, at physiological pHs. If the reaction rates in the *Coleus* AA pathway are similar to those in *Prototheca*, it is difficult to see how L-GalDH is responsible for the 30–50% increase in total AA levels seen under illumination. It is plausible that the difference in AA levels reflect an increase in the activity of another pathway enzyme under illumination. Although one must be wary of translating *in vitro* enzyme reaction rates to *in vivo* conditions, Table 4 shows epimerase rates that are more than an order of magnitude lower than those measured for PMM and PMI, and three or more times lower than the GMP rates. Figure 2 clearly shows that the increase in productivity in the *Prototheca* mutants was due to increases in epimerase activity, implicating this enzyme as the rate-limiting step. Wolucka *et al.* (2001) have also demonstrated a strong correlation between epimerase activity and AA content of *Arabidopsis* cells. The proposed pathway, first described by Berry *et al.* (1999) and Wheeler *et al.* (1998), clears up decades of speculation as to the main route of AA synthesis in plants. These discoveries bring closer the possibilities of genetically engineering plants for increased AA content, and industrial microbes for the fermentative production of AA at large scale.

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