Supply and consumption of glucose 6-phosphate in the chloroplast stroma

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Running Title: **Supply and consumption of plastidic glucose 6-phosphate**

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Highlight

Glucose 6-phosphate stimulates glucose-6-phosphate dehydrogenase. This enzyme is less active

during the day but retains significant activity that is very sensitive to the concentration of glucose

6-phopshate.

Abstract

Fructose 6-phosphate is an intermediate in the Calvin-Benson cycle and can be acted on by

phosphoglucoisomerase to make glucose 6-phosphate (G6P) for starch synthesis. A high

concentration of G6P is favorable for starch synthesis but can also stimulate G6P dehydrogenase

initiating the glucose-6-phosphate shunt an alternative pathway around the Calvin-Benson cycle.

A low concentration of glucose 6-phosphate will limit this futile cycle. In order to understand the

biochemical regulation of plastidic glucose 6-phosphate supply and consumption, we

characterized biochemical parameters of two key enzymes, phosphoglucoisomerase (PGI) and

G6P dehydrogenase (G6PDH). We have found that the plastidic PGI in has a higher K_m for G6P

compared to that for fructose 6-phosphate. The K_m of G6PDH isoform 1 is increased under

reducing conditions. The other two isoforms exhibit less redox regulation; isoform 2 is the most

inhibited by NADPH. Our results support the conclusion that PGI restricts stromal G6P synthesis

limiting futile cycling via G6PDH. It also acts like a one-way valve, allowing carbon to leave the

Calvin-Benson cycle but not reenter. We found flexible redox regulation of G6PDH that could

regulate the glucose-6-phosphate shunt.

Keywords: Calvin-Benson cycle, glucose-6-phosphate dehydrogenase, glucose 6-phosphate,

glucose 6-phosphate shunt, hydrogen peroxide, phosphoglucoisomerase, redox regulation

Abbreviations:

6PG   6-phosphogluconic acid

At   *Arabidopsis thaliana*

DHAP   dihydroxacetone phosphate

E4P   erythrose 4-phosphate

F6P   fructose 6-phosphate

FBP   fructose 1,6-bisphosphate

G6P   glucose 6-phosphate

G6PDH   glucose-6-phosphate dehydrogenase

PGA   3-phosphoglyceric acid

PGI   phosphoglucoisomerase

PGM   phosphoglucomutase

So   *Spinacia oleracea*

Xu5P   xylulose 5-phosphate

Introduction

Glucose 6-phosphate (G6P) is the first product out of the Calvin-Benson cycle in the starch synthesis pathway. However, it can also enter the oxidative pentose phosphate pathway creating a G6P shunt that bypasses the nonoxidative branch of the pentose phosphate pathway reactions that make up a significant part of the Calvin-Benson cycle. This pathway is generally considered to occur only in the dark (Anderson *et al.*, 1974; Buchanan, 1980; Buchanan *et al.*, 2015; Heldt and Piechulla, 2005; Scheibe *et al.*, 1989). However, a high G6P concentration, favorable for starch synthesis, could cause the shunt to occur in the light. Generally, the G6P concentration in the plastid is low, much lower than its concentration in the cytosol (Gerhardt *et al.*, 1987; Sharkey and Vassey, 1989; Szecowka *et al.*, 2013) but under some conditions the plastid G6P concentration might increase depending on the production and consumption of plastid G6P.

Four different enzymes in the plastid can produce or consume G6P (Fig. 1). First, G6P can be produced by phosphoglucoisomerase (PGI). This enzyme reversibly isomerizes fructose 6-phosphate (F6P) and G6P. Analysis of mutant lines of *Clarkia xantiana* indicated that PGI is not in great excess (Kruckeberg *et al.*, 1989). There are two isoforms of PGI in Arabidopsis, one targeted to the plastid and the other found in the cytosol. The plastid PGI in particular is likely limiting given that G6P/F6P ratios in the plastid are significantly displaced from equilibrium and much lower than in the cytosol (Backhausen *et al.*, 1997; Gerhardt *et al.*, 1987; Schnarrenberger and Oeser, 1974; Sharkey and Vassey, 1989; Szecowka *et al.*, 2013). Plants with loss-of-function mutants in the plastidic enzyme have 98.5% less starch in leaves (Yu *et al.*, 2000). Loss-of-function mutants in the cytosolic enzyme results in increased starch and decreased sucrose (Kunz *et al.*, 2014).

The second enzyme affecting G6P in the chloroplast is phosphoglucomutase. This enzyme converts G6P to glucose 1-phosphate. This reaction is an important step in starch synthesis. Third, G6P can be transported across the chloroplast membrane by GPT2, a glucose-6-phosphate/phosphate antiporter in the chloroplast membrane. GPT2 is not normally present green tissue (Kammerer *et al.*, 1998; Kunz *et al.*, 2010) and this is corroborated by the large concentration gradient in G6P between the chloroplast and cytosol (Gerhardt *et al.*, 1987; Sharkey and Vassey, 1989; Szecowka *et al.*, 2013). However, GPT2 is important in acclimation to light (Dyson *et al.*, 2015) and is expressed in plants grown in high CO_2 (Leakey *et al.*, 2009)

and is increased when starch synthesis is repressed by knocking out starch synthesis genes (Kunz

et al. 2010). When GPT2 is present, the gradient of G6P would result in G6P import into the

plastid (Gerhardt *et al.*, 1987; Sharkey and Vassey, 1989; Szecowka *et al.*, 2013). Finally,

glucose-6-phosphate dehydrogenase (G6PDH) can oxidize G6P to 6-phosphoglucanolactone, the

first step in the oxidative branch of the pentose phosphate pathway. There are six isoforms of

G6PDH in Arabidopsis. Four of these are predicted to be targeted to the chloroplast where three

are functional (Meyer *et al.*, 2011; Wakao and Benning, 2005). It has been hypothesized that

during the day, G6PDH initiates a G6P shunt around the Calvin-Benson cycle (Sharkey and

Weise, 2016). The G6P shunt oxidizes and decarboxylates G6P to synthesize xylulose 5-

phosphate (Xu5P). While the G6P shunt is a futile cycle, it has been proposed to play an

important role in stabilization of photosynthesis.

Our goal is to understand the kinetic regulation of the stromal G6P pool, specifically its

production by PGI and its consumption by G6PDH. We will not further investigate the roles of

PGM and GPT2 since PGM has been characterized because of its key role in starch synthesis

(Hattenbach and Heineke, 1999; Najjar, 1948; Ray and Roscelli, 1964), and GPT2 is usually not

present in green photosynthetic tissue (Kammerer *et al.*, 1998; Kunz *et al.*, 2010). We repeated

critical measurements of PGI kinetics and found that while the isomerization of F6P and G6P is

reversible, PGI has a greater affinity for F6P than G6P. Novel findings describing the regulation

of G6PDH indicate that G6PDH can remain fairly active during the day. We conclude that a G6P

shunt is allowed and even likely in light of the kinetic parameters of G6PDH and that its activity

could be modulated during the day to regulate flux through the G6P shunt.

Materials and Methods

Overexpression and purification of recombinant enzymes

His-tagged (N-terminal) *Arabidopsis thaliana* plastidic and cytosolic PGI, and C-terminal *Strep*-

tagged (Wakao and Benning, 2005; Wendt *et al.*, 2000) plastidic G6PDH1, 2, and 3 genes were

commercially synthesized by GenScript (https://www.genscript.com). All of the plasmid

constructs were overexpressed in *E. coli* strain BL21. Cells were grown at 37ºC to an OD_{600} of

0.6 to1 and induced with 0.5 mM isopropyl β-D-1 thiogalactopyranoside at room temperature.

Cells were centrifuged and resuspended in lysis buffer (5 ml lysis buffer/g of pellet; 50 mM

sodium phosphate, pH 8.0, 300 mM NaCl) containing 1 mg ml^{-1} lysozyme, 1 µg ml^{-1} of DNAseI,

and 1x protease inhibitor cocktail (Sigma, www.sigmaaldrich.com). Cells were then lysed by

sonication (Branson Sonifier 250, us.vwr.com). The sonicator was set at 50% duty cycle and an

output level of 1. The cells were sonicated using five steps where each step consisted of 15 s

pulses and 15 s on ice. The lysate was centrifuged and supernatant collected. For plastidic and

cytosolic PGI, Ni-NTA resin (Qiagen, https://www.qiagen.com) was added to the crude lysate

with gentle stirring for 1 hr. The mixture was loaded onto a column and washed with wash buffer

(50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole) until the OD_{280} of the

effluent was less than 0.05. Protein was eluted with elution buffer (50 mM sodium phosphate pH

8.0, 300 mM NaCl, 250 mM imidazole) containing 1x protease inhibitor cocktail. The Ni-NTA

column purification was performed in a cold room at 4°C. For G6PDH1, 2, and 3, harvested

proteins were resuspended in cold Buffer W (IBA, www.iba-lifesciences.com) with protease

inhibitor cocktail, 1 mg ml^{-1} lysozyme, and 2.73 kU DNAseI and lysed as described above.

Protein was purified on a *Strep*-Tactin column (IBA) following the manufacturer's instructions.

For all purified proteins, SDS-PAGE was carried out and fractions containing >95% of total

protein of interest were combined and concentrated using Amicon Ultra 0.5 ml centrifugal filters

(molecular weight cut off 3 kDa). Glycerol was added to the concentrated protein to obtain a

final protein solution with 15% glycerol. The glycerol stock of the proteins was aliquoted into

small volumes, frozen in liquid nitrogen, and stored at -80°C. The concentration of the proteins

was determined using Pierce 660 nm protein assay reagent kit (ThermoFisher Scientific,

www.thermofisher.com) using a bovine serum albumin standard. Final preparations of purified

protein were run on an SDS-polyacrylamide gel and stained with Coomassie Blue to check the

purity of the enzymes. Molecular weights were estimated from the protein construct using Vector

NTI (ThermoFisher Scientific, www.thermofisher.com).

Coupled spectrophotometric assay for PGI (F6P to G6P reaction) and G6PDH

The activity of the purified plastidic and cytosolic PGI and G6PDH1, 2, and 3 was studied using

coupled spectrophotometric assays. Concentrations of G6P and F6P were determined using

NADPH-linked assays measured spectrophotometrically. All assays were validated by

demonstrating linear product formation, proportional to the time of the assay and amount of

enzyme added. All coupling enzymes were added in excess so that no change in product

formation was seen when varying the coupling enzyme. PGI assays were done in 50 mM bicine

buffer pH 7.8, containing 4.8 mM DTT, 0.6 mM NADP$^+$, 2 U G6PDH (from *Leuconostoc*

mesenteroides), varying concentrations of F6P, and 1.31 ng plastidic or cytosolic PGI. The

concentrations used to study the K_m of G6PDH for F6P were 0-4.8 mM. For G6PDH assays, the

assay was done in 150 mM Hepes buffer pH 7.2 containing varying concentrations of $NADP^+$,

varying concentrations of G6P, and G6PDH. 8.3 ng of G6PDH1, 20 ng of G6PDH2, and 44 ng

of G6PDH3 were used. The concentrations used to study the K_m of G6PDH for G6P were 0 –

44.2 mM, and the concentrations used to study K_m for $NADP^+$ were 0 – 11 µM. When G6PDH

was assayed varying G6P, 0.6 mM $NADP^+$ was added. When G6PDH was assayed varying

$NADP^+$, 7.6 mM G6P was added for G6PDH1 and 3 and 15.4 mM for G6PDH2. Under these

conditions, less than 5% of the non-limiting substrate was consumed over the duration of the

assay. The assay mixtures were prepared by adding all the components except the enzyme.

Activity was recorded with a dual wavelength filter photometer (Sigma ZFP2) as the change in

absorbance at 334 – 405 nm caused by $NADP^+$ reduction to NADPH using an extinction

coefficient of 6190 M^{-1} cm^{-1}. These wavelengths were used because they correspond to mercury

lamp emission wavelengths of the lamp used in the filter photometer. When assaying redox

sensitivity, G6PDH was incubated with 10 mM DTT or hydrogen peroxide at room temperature

before addition to the assay. The G6P concentration was 0.3 mM. For G6P protection assays,

G6PDH1 was assayed at 5 mM G6P. After getting a stable baseline with G6PDH and $NADP^+$,

the reaction was initiated by addition of G6P and incubated at room temperature. Activity was

measured 30 or 60 minutes later to allow time for DTT deactivation. Less than 5% of added G6P

and $NADP^+$ (non-limiting substrate) was consumed within 60 minutes.

Mass spectrometry assay for PGI (G6P to F6P reaction)

The activity of the purified plastidic and cytosolic PGI in the G6P to F6P direction was studied

using a coupled mass spectrometer assay. The assay mixture contained 50 mM Tris pH 7.8, 2.5

mM $MgCl_2$, 1 mM ATP, 5 mM DTT, 0.15 U phosphofructokinase (from *Bacillus*

stearothermophilus), varying concentrations of G6P, and 1.6 ng of plastidic or cytosolic PGI.

The assay mixtures were prepared by adding all the components except the enzyme. The reaction

was initiated with the enzyme. After five min, the reaction was quenched with four volumes of

100% ice-cold methanol. Production of FBP was shown to be linear for up to ten min. Five nmol

of D-[UL-$^{13}C_6$] fructose 1,6-bisphosphate was added as an internal standard for quantification,

and the sample was heated for 5 minutes at 95°C. Six volumes of 10 mM tributylamine, pH 5.0,

was added and the sample was filtered through a Mini-UniPrep 0.2 µm Syringeless Filter Device

(GE Healthcare Life Sciences, Whatman). LC/MS-MS was carried out on a Waters Quattro

Premier system and was operated in electrospray negative ion mode with both multiple and

selected reaction monitoring. The capillary voltage was 2.75 kV; the cone voltage, 50 V; the

extractor voltage, 5 V. The source temperature was 120°C and the desolvation temperature was

350°C. Gas flow for the desolvation and cone was set to 800 and 50 $l\,hr^{-1}$, respectively. The

syringe pump flow was 10 µl min^{-1}. MassLynx software and the Acquity UPLC Console were

used to control the instrument. Samples were passed through an Aquity UPLC BEH Column

(Waters) with a multi-step gradient with eluent A (10 mM tributylamine, adjusted to pH 6 with

500 mM acetic acid) and eluent B (methanol): 0-1 min, 95-85% A; 1-3 min, 85%-65% A; 3-3.5

min, 65-40% A; 3.5-4 min, 40-0% A; 4-8.50 min, 0% A; 8.5-10 min, 100% A. The flow rate was

0.3 ml min^{-1}. FBP peaks were integrated using MassLynx software and the concentration of the

metabolites was quantified by comparing the peak response to a calibration curve.

Kinetic characterization

Enzymes were assayed at varying concentrations of substrate while keeping the concentration of

other substrates (if applicable) constant as described above. The K_m values for plastidic and

cytosolic PGI were determined by fitting the data with non-linear regression using the Hill

function in OriginPro 8.0 (OriginLab Corporation). All G6PDH isoforms showed substrate

inhibition, therefore we estimated regression lines and kinetic constants by finding the minimum

of the sum of the squared residuals from the following equation using Solver in Excel, where v is

the specific activity of the enzyme in µmol mg^{-1} min^{-1} (Gray *et al.*, 2011):

200
$$v = \frac{V_{max} + V_i \left(S^x / K_{is}^x \right)}{1 + \frac{K_m^H}{S^H} + \frac{S^x}{K_{is}^x}} \quad \text{Eq. 2}$$

Inhibition studies

Different metabolites of the Calvin-Benson cycle were tested for their effect on both PGI and

G6PDH activity. All the metabolites were purchased from Sigma Aldrich. In metabolite

screening assays, metabolites were assayed at a 1:1 ratio with the substrate. To determine the K_i

of G6PDH or PGI for different metabolites, the assay was carried out in presence of various

concentrations of F6P or G6P and other metabolites. Assay mixtures were prepared as described

above with different concentrations of substrate. For PGI studies, 0-0.98 mM F6P or 0-1.5 mM

G6P was used and 0-3.8 mM G6P was used for G6PDH studies. The concentration of NADP$^+$ in

G6PDH assays was held constant at 600 µM. The concentration range used to study the K_i of

PGI for E4P were 0-0.05 mM and that for 6PG were 0-1.5 mM. For G6PDH1 and 3 assays 0-0.3

mM NADPH was used and 0-14.5 µM NADPH was used for the G6PDH2 assays. The

mechanism of inhibition was determined from Hanes-Woolf plots. The K_i was determined from

the non-linear least squares fitting of the activity vs. F6P concentration plot using Solver in Excel

using the standard equation for competitive inhibition as described below.

$$v = \frac{V_{max}*S}{K_m\left(1+\frac{I}{K_i}\right)+S} \qquad \text{Eq. 3}$$

where V_{max} is the maximum velocity, S is the F6P concentration, K_m is the Michaelis constant,

and K_i is the inhibition constant. For non-competitive inhibition, the below equation was used.

$$v = \frac{V_{max}*S/\left(1+\frac{I}{K_i}\right)}{\left(K_m\left(1+\frac{I}{K_i}\right)/\left(1+\frac{I}{K_i}\right)+S\right)} \qquad \text{Eq. 4}$$

Midpoint potential of G6PDH1

A series of oxidation-reduction titrations was done with purified G6PDH1. Fully reduced DTT

was prepared daily by combining 100 mM DTT with 200 mM sodium borohydride. The mixture

was incubated on ice for 20 minutes and then neutralized by adding concentrated HCl to a final

concentration of 0.2 M. The mixture was brought to a pH of 8 and diluted to a final concentration

of 50 mM DTT. Oxidized DTT and buffers used in the assay were also pH 8. We used mixtures

of oxidized and reduced DTT at different redox potentials, ranging from -420 to -124 mV. The

total concentration of DTT was 1-8.5 mM. 4.1 ng of G6PDH1 was incubated in the DTT mixture

and 1 mg/ml BSA, pH 8 for 1 hr at 25°C in an anaerobic environment. Activity of G6PDH was

measured as described in 'Chloroplast isolation' with 0.3 mM G6P. The data were fit to the

Nernst equation for a two-electron process. We used the E_m of DTT as determined by Hutchison

and Ort (1995), -391 mV at pH 8. Oxidized and reduced DTT was quantified using modified

protocols from Cho *et al.* (2005) and Charrier and Anastasio (2013) to calculate the potential.

$$E_h = E_m + 2.303\left(RT/nF\right) * log_{10}(DTT_{ox}/DTT_{red}) \qquad \text{Eq. 5}$$

Leaf extract assays

Wild type *Arabidopsis* was grown in a 12 hr photoperiod at 120 µmol m^{-2} s^{-1} of light. Day

temperature was 23˚C and night temperature was 21˚C. Approximately 300 mg of leaf samples

were collected in a 2 ml microfuge tube and immediately frozen by plunging in liquid nitrogen.

Samples were ground in a Retsch mill with 4 mm silicone carbide particles (BioSpec Products,

www.biospec.com). One ml of extraction buffer (45 mM Hepes, pH 7.2, 30 mM NaCl, 10 mM

mannitol, 2 mM EDTA, 0.5% Triton-X-100, 1% polyvinylpolypyrrolidone, 0.5% casein, 1%

protease inhibitor cocktail) was added to the sample and vortexed for 30 s. The sample was

centrifuged for 30 s at maximum speed and immediately placed on ice. G6PDH activity was

assayed as described in "Coupled spectrophotometric assay for phosphoglucoisomerase (F6P to

G6P reaction) and G6PDH". Assays that used leaf extracts were normalized by mg of

chlorophyll added to the assay mixture.

Chloroplast isolation

Fresh spinach was purchased at a local market for use that day. Spinach was either dark or light

treated for 1.5 hours before beginning isolation and petioles were kept in water to prevent

wilting. Arabidopsis Col-0 was grown on soil in a growth chamber at a 12 h light at 120 µmol m^{-2} s^{-1},

23˚C and 12 h dark at 21˚C. Plants were harvested either midday for light samples or

midnight for dark samples.

Chloroplasts were isolated using a Percoll gradient (Weise *et al.*, 2004). Leaves were placed in a

chilled blender with grinding buffer (330 mM mannitol, 50 mM Hepes, pH 7.6, 5 mM MgCl$_2$, 1

mM MnCl$_2$, 1 mM EDTA, 5 mM ascorbic acid, 0.25% BSA), blended, and then filtered through

four layers of cheese cloth. Filtered liquid was centrifuged and the pellet was resuspended in

resuspension buffer (330 mM mannitol, 50 mM Hepes, pH 7.6, 5 mM MgCl$_2$, 1mM MnCl$_2$,

1mM EDTA, 0.25% BSA). The resuspended pellet was layered on top of a 20-80% Percoll

gradient which was centrifuged at 1200 g for 7 min. The bottom band in the gradient containing

the intact chloroplasts was collected. One volume of resuspension buffer was added to collected

chloroplasts and centrifuged at 1200 g for 2 min. The pellet was resuspended in 50 µl of water

and vortexed to lyse the chloroplasts. One volume of 2x buffer (100 mM Hepes, pH 7.6, 10 mM

MgCl$_2$, 2 mM MnCl$_2$, 2 mM EDTA, 2 mM EGTA, 60% glycerol, 0.2% Triton X-100, 0.2%

PVPP) was added. Samples were stored at -80˚C until used for further analysis. Chlorophyll was

quantified by lysing 50 µl of purified chloroplasts by sonication and adding supernatant to 1 ml

of 95% ethanol. OD_{654} was used to calculate the chlorophyll concentration (Wintermans and

DeMots, 1965):

$$\text{mg Chl} = OD * 0.0398 * 0.050 \; \mu l \quad \text{Eq. 1}$$

When chloroplast isolations were used to assess activity of fully oxidized and reduced plastidic

G6PDH, oxidized or reduced DTT was added at a concentration of 10 mM to each solution used

in the isolation. Assays that used isolated chloroplasts were normalized by mg of chlorophyll

added to the assay mixture.

Results

Purification of recombinant PGI and G6PDH

Final concentration of purified plastidic AtPGI was 15.3 mg/ml and that of cytosolic AtPGI was

13.8 mg/ml (Supplemental Fig. S1). The molecular weight of His-tagged recombinant plastidic

and cytosolic AtPGI were ~62.9 kDa and ~62.5 kDa respectively. The specific activity was 787

μmol mg^{-1} protein min^{-1} for plastidic AtPGI and 1522 µmol mg^{-1} protein min^{-1} for cytosolic

AtPGI. The final concentration of AtG6PDH1 was 1.66 mg/ml, AtG6PDH2 was 1.90 mg/ml, and

AtG6PDH3 was 0.177 mg/ml (Supplemental Fig. S1). The molecular weight of Strep-tagged

recombinant AtG6PDH1 was ~65.2 kDa, AtG6PDH2 was ~70.2 kDa, and AtG6PDH3 was 70.5

kDa. The maximum specific activity was 28.0 µmol mg^{-1} protein min^{-1} for AtG6PDH1, 18.7

µmol mg^{-1} protein min^{-1} for AtG6PDH2, and 7.1 µmol mg^{-1} protein min^{-1} for AtG6PDH3.

Kinetic characterization of plastidic and cytosolic PGI

Table 1 shows the K_m (for both F6P and G6P) of plastidic and cytosolic AtPGI (Supplemental

Fig. S2). For plastidic AtPGI, the K_m value for G6P was ~2.9-fold higher than that for F6P. The

K_m's for F6P and G6P of the cytosolic enzyme were the same. DTT did not significantly

influence the specific activity of plastidic or cytosolic PGI (Supplemental Fig. S3).

E4P and 6PG inhibition of PGI

We tested different metabolites for their effect on PGI activity. Inhibition with either limiting

F6P or G6P was similar for both plastidic and cytosolic AtPGI. Erythrose 4-phosphate (E4P), 3-

phosphoglyceric acid (PGA), dihydroxacetone phosphate (DHAP), and 6-phosphogluconic acid

(6PG) were screened (Fig. 2). Only E4P and 6PG showed significant inhibition. Inhibitory

effects were not different between plastidic and cytosolic AtPGI. Fig. 3 shows the activity of

plastidic AtPGI over a range of F6P and E4P concentrations. Activity of cytosolic AtPGI was

analyzed in a similar manner as shown for plastidic AtPGI (Supplemental Fig. S4). The

calculated K_i values of E4P and 6PG are shown in Table 1. The K_i values for 6PG were between

31-203 µM, depending on the isoform and substrate. E4P was shown to be more inhibitory with

K_i's between 1.5- 6 µM. Based on the Hanes-Woolf plots (Supplemental Fig. S5), E4P was

shown to be competitive, except above 0.04 mM, with G6P. 6PG was identified as competitive

with F6P, except above 1.0 mM, and non-competitive with G6P.

Regulation of PGI in isolated chloroplasts

Plastidic SoPGI activity from chloroplasts from dark-treated spinach leaves had a higher K_m for

G6P compared to light-treated chloroplasts (Fig. 4). The K_m of SoPGI for F6P did not change in

the light or dark.

Kinetic characterization of G6PDH

All three AtG6PDH isoforms showed substrate inhibition (Fig. 5a). The AtG6PDH K_m for G6P

for isoforms 1 and 3 was 0.3 mM, while the K_m for isoform 2 was approximately 34-fold higher

(10.3 mM). Table 2 shows the K_m (for both G6P and $NADP^+$), k_{cat}, and G6P K_i of all three

AtG6PDH isoforms. The catalytic efficiency of AtG6PDH1 for G6P was 190 mM^{-1} s^{-1}, of

AtG6PDH2 was 3.8 mM^{-1} s^{-1}, and of AtG6PDH3 was 48.7 mM^{-1} s^{-1}. For $NADP^+$, the catalytic

efficiency of AtG6PDH1 was 81.4 mM^{-1} s^{-1}, of AtG6PDH2 was 30.3 mM^{-1} s^{-1}, and of

AtG6PDH3 was 29.2 mM^{-1} s^{-1}.

Identification and characterization of inhibitors

We tested ribulose 1,5-bisphosphate (RuBP), ribulose 5-phosphate (Ru5P), F6P, PGA, DHAP,

E4P, NADPH, and 6PG for their effect on G6PDH activity. Only NADPH showed inhibition.

While NADPH inhibited all three isoforms, AtG6PDH2 was the most inhibited. The calculated

K_i values of NADPH are shown in Table 2. NADPH was found to be competitive for all

isoforms based on the Hanes-Woolf plots, except above 14.5 µM for G6PDH2 and above 0.15

mM for G6PDH3 (Supplemental Fig. S5).

Redox regulation

All isoforms of AtG6PDH were susceptible to deactivation by DTT, but AtG6PDH1 was the

most deactivated after two hours, losing approximately 90% of its activity (Fig. 6a). Kinetic

characterization of AtG6PDH1 incubated with 10 mM DTT showed that decreased activity in

AtG6PDH1 was due to both a decrease in k_{cat} and an increase in K_m and occurred over

approximately 45 minutes (Fig. 6b). However, the k_{cat} was less affected than the K_m (Table 3).

Comparison of our results to those of Née *et al.* (2009), who used thioredoxins to deactivate

AtG6PDH1, show that DTT is an acceptable mimic of thioredoxins to deactivate AtG6PDH1.

Both results show that AtG6PDH1 will lose ~90% of activity when fully reduced. AtG6PDH2

and 3 showed a decrease in k_{cat}, but not an increase in K_m. AtG6PDH2 retained ~60% of activity

and AtG6PDH3 retained ~80% of activity. Redox deactivation of AtG6PDH can be rescued by

addition of hydrogen peroxide equimolar to DTT *in vitro* (Fig. 7a). AtG6PDH1 activity reached

approximately 64% activity while 79% of the DTT was still reduced (Fig. 7b). The calculated E_m

at this time point was -407 mV. Based on our determined midpoint potential of AtG6PDH1 (see

Midpoint potential of G6PDH1), we predict AtG6PDH1 would have < 5% activity at the redox

potential of the DTT. Therefore, we conclude the addition of hydrogen peroxide did not result in

the re-activation of G6PDH1 by oxidizing DTT but that hydrogen peroxide was directly

activating G6PDH1. Redox deactivation of G6PDH1 was decreased when G6P was present.

When G6P was present at K_m concentration during incubation with DTT, the activity of reduced

AtG6PDH was higher than when G6P was not present (Fig. 8).

Midpoint potential of G6PDH1

We determined the activity of AtG6PDH1 in a series of oxidation-reduction potentials (Fig. 9).

The data was fit with the Nernst equation for a two-electron process. Incubation of AtG6PDH1 at

higher redox potentials (-300 to -140 mV) did not increase activity any further. The midpoint

potential of G6PDH1 at pH 8 was -378 mV. This corresponds to a midpoint potential of -318 mV

at pH 7.

Activity of G6PDH in isolated chloroplasts and leaf extracts

We used rapid leaf extract assays and chloroplast isolations to determine the activity of redox-

regulated SoG6PDH and AtG6PDH compared to total activity in the plastid and the whole leaf.

After illumination at 500 µmol m^{-2} s^{-1} for one hr, G6PDH activity spinach leaf extracts decreased

by about 35% (Fig. 10a). We also isolated chloroplasts in fully oxidizing or fully reducing

conditions. Fully reduced chloroplast activity was about 50% of fully oxidized chloroplast

activity in both spinach and *Arabidopsis* (Fig. 10b).

Discussion

Regulation of production of G6P

High concentration of G6P in the chloroplast has been proposed to cause a G6P shunt (Fig. 12).

The results of this study of key enzymes regulating the stromal G6P concentration support the

hypothesis of the G6P shunt.

PGI

We propose that PGI is a key regulatory point in carbon export from the Calvin-Benson cycle.

PGI acts as a one-way valve, going from F6P to G6P. The G6P/F6P ratio at equilibrium has been

reported to vary from 3.70 at 10°C to 2.82 at 40°C (Dyson and Noltmann, 1968). However, *in*

vivo measurements show the ratio of G6P/F6P in the stroma to be close to 1 (Backhausen *et al.*,

1997; Gerhardt *et al.*, 1987; Schnarrenberger and Oeser, 1974; Sharkey and Vassey, 1989;

Szecowka *et al.*, 2013). This disequilibrium is not seen for the cytosolic PGI where G6P/F6P

ratios are 2.4-4.7 (Gerhardt *et al.*, 1987; Sharkey and Vassey, 1989; Szecowka *et al.*, 2013).

Kinetic hydrogen isotope effects in starch, but not sucrose, also support the conclusion that

plastidic PGI, but not cytosolic PGI, has insufficient activity to maintain equilibrium (Schleucher

et al., 1999).The high K_m for G6P for the plastidic enzyme makes this reaction functionally

irreversible, helping to explain the kinetic isotope effects only seen in starch. The difference in

K_m is seen in both recombinant plastidic Arabidopsis PGI and in isolated plastidic spinach PGI.

We previously assumed that PGA is a strong inhibitor of PGI (eg Sharkey and Weise 2016)

based on the report by Dietz (1985). Surprisingly, we did not observe this to be the case.

Examination of data from Dietz (1985) shows that during PGA inhibition assays, 6PG was also

present in the reaction mixture at 50 µM. The G6P/F6P disequilibrium in chloroplasts was

proportional to PGA (Dietz, 1985)(see his Table I) but PGA was not tested alone for its effect on

PGI. We found that the K_i of plastidic PGI for 6PG with limiting F6P was 31 µM or with limiting

G6P was 203 µM. Based on our findings, we propose that PGI is not inhibited by PGA, and the

previously seen inhibition can be explained by presence of 6PG or E4P. *In vivo* plastidic

concentrations of 6PG are not known, therefore, extent of inhibition of PGI *in vivo* by 6PG

cannot be currently determined.

PGI is inhibited by µM concentrations of E4P (Backhausen *et al.*, 1997; Grazi *et al.*, 1960; Salas

et al., 1964). E4P may be inhibitory to both isoforms of PGI because it is a competitive inhibitor

and the active sites of both isoforms may be similar (Backhausen *et al.*, 1997). Presumably there

is no E4P in the cytosol since it lacks crucial enzymes in the non-oxidative branch of the pentose

phosphate pathway (Schnarrenberger *et al.*, 1995). Measurements and estimations of plastidic

E4P concentrations *in vivo* show E4P to be ~17-20 µM (Backhausen *et al.*, 1997; Bassham and

Krause, 1969; Heldt *et al.*, 1977). This is well above the K_i of E4P for plastidic PGI. Backhausen

et al. (1997) propose that this regulation is necessary in order to keep photosynthetic pool sizes

stable during changes in light intensity.

In addition to stabilizing the Calvin-Benson cycle, we propose that inhibition of PGI by E4P can

provide insight into the phenomenon of reverse sensitivity to CO_2 and O_2 of photosynthetic CO_2

assimilation rate observed by Sharkey and Vassey (1989). They found that when potato leaves

were switched to decreased partial pressure of oxygen, rates of photosynthetic CO_2 assimilation

decreased as a result of decreased starch synthesis. Sharkey and Vassey (1989) proposed this was

an effect of PGA inhibition of PGI, but because we did not find PGA to be inhibitory, we now

suggest that the decrease in starch synthesis is due to an increase in E4P concentrations (or

possibly 6PG).

We conclude that PGI is an important regulatory enzyme in central carbon metabolism, keeping

G6P concentration lower than would be present at equilibrium thereby regulating the rate of the

G6P shunt but also starch synthesis. Overexpression of phosphoglucomutase significantly

increased starch synthesis confirming that starch synthesis is regulated at PGI in addition to the

well-known regulation at ADPglucose pyrophosphorylase (Uematsu *et al.*, 2012).

GPT2

If GPT2 is present it can import G6P from the cytosol to the chloroplast. Niewiadomski *et al.*

(2005) showed that GPT2 could restore starch accumulation to plants lacking PGI. Expression of

GPT2 is present in leaves when starch synthesis is blocked by loss of starch synthesis enzymes,

when plants are grown in high CO_2, or exposed to an increase in light intensity (Dyson *et al.*,

2015; Kunz *et al.*, 2010; Leakey *et al.*, 2009). It also is expressed in CAM plants, which require

high rates of starch synthesis (Cushman *et al.*, 2008; Neuhaus and Schulte, 1996). Thus, we

believe that much of the time plants rely on PGI alone to supply G6P for starch synthesis and

regulate PGI to regulate the supply of G6P to control the rate of the shunt. When higher rates of

starch synthesis are needed GPT2 is expressed, increasing the supply of G6P (Fig. 11) but

making the plant vulnerable to high rates of the G6P shunt.

Regulation of consumption of G6P by G6PDH

Stromal G6P is primarily thought of as an intermediate in starch synthesis. It is converted by

phosphoglucomutase to glucose 1-phosphate. However, there are additional reactions involving

stromal G6P can participate in in the plastid. Here, we investigated consumption of G6P by

G6PDH. G6PDH is competitively inhibited by its product NADPH and redox regulation that

results mostly in an increase in K_m, which reduces futile cycling in leaves in the light (Scheibe *et*

al., 1989; Wakao and Benning, 2005). However, while the enzyme is less active in the light

(Anderson *et al.*, 1974; Buchanan, 1980; Buchanan *et al.*, 2015; Heldt and Piechulla, 2005;

Scheibe *et al.*, 1989) our results show that it retains significant activity. Two factors that will

modulate G6PDH activity in the light are the sensitivity of G6PDH to G6P and the redox

regulation of G6P.

Sensitivity to G6P

The activity of G6PDH (and thus the G6P shunt) is sensitive to stromal G6P concentration by

three mechanisms.

- Deactivation of G6PDH in reducing conditions is primarily due to an increase in K_m
(Scheibe *et al.*, 1989, Fig 6).
- G6P protects G6PDH from deactivation in reducing conditions (Fig 8).
- G6P has been shown to relieve the inhibition of G6PDH by NADPH, as well as decrease
the K_m and increases the k_{cat} of G6PDH in assays where NADP is varied (Olavarría *et*
al., 2012; Shreve and Levy, 1980).

In conditions where G6P concentrations in the stroma may increase, such as those discussed

above in "Production of G6P", flux through G6PDH (and the G6P shunt) would also increase.

Redox Regulation

We have determined the midpoint potential of G6PDH to be -378 mV at pH 8. This agrees with

results from Née *et al.* (2009) and is close to the midpoint potential of other redox regulated

enzymes in the Calvin-Benson cycle and electron transport (Cammack *et al.*, 1977; Hirasawa *et*

al., 1998; Hirasawa *et al.*, 2000; Hirasawa *et al.*, 1999; Knaff, 2000; Née *et al.*, 2009; Strand *et*

al., 2016). Assuming equilibrium and the midpoint potential of G6PDH1 at pH 8 as a reference,

using the Nernst equation, we calculate that all Calvin-Benson enzymes and electron transport

proteins are almost fully reduced and thus active while G6PDH maintains 50% of its activity

(Table 4). Exceptions are ferredoxin and malate dehydrogenase (MDH), which are predicted to

be oxidized at -378 mV. Although there may be deviations from redox equilibrium within the

stroma, from these approximations we conclude that the midpoint potential of AtG6PDH1 is in a

range to allow dynamic regulation of G6PDH and that it is theoretically possible to have flux

through the Calvin-Benson cycle and the G6P shunt at the same time.

We have also shown that G6PDH can be activated upon addition of hydrogen peroxide. Brennan

and Anderson (1980) and Née *et al.* (2009) previously demonstrated a role for hydrogen

peroxide regulation of G6PDH both *in vivo* and *in vitro* in the presence of thioredoxin. In

conditions where hydrogen peroxide can accumulate, such as high light, G6PDH deactivation

could be reversed to modulate the consumption of G6P by the G6P shunt. The activity of

G6PDH can be modulated by redox status of the plastid, G6P concentration, and hydrogen

peroxide.

Redox regulation of dominant isoforms of G6PDH is found in many species, including

Arabidopsis, pea, potato, spinach, and barley (Scheibe *et al.*, 1989; Schnarrenberger *et al.*, 1973;

Semenikhina *et al.*, 1999; Wenderoth *et al.*, 1997; Wendt *et al.*, 2000; Wright *et al.*, 1997). We

have shown that plastidic G6PDH from isolated Arabidopsis chloroplasts retains approximately

50% of its total activity, even in high light conditions. Additionally, cytosolic G6PDH is not

redox regulated and makes up 33% of whole leaf G6PDH activity. G6P in the cytosol could be

converted to pentose phosphate and be imported into the plastid by the XPT transporter (Eicks *et*

al., 2002).

Oxidative stress might also stimulate the G6P shunt. Drought or high light can result in an

accumulation of hydrogen peroxide and other ROS products (see Suzuki *et al.* (2011) for a

review). Based on current findings, we propose that, with accumulation of hydrogen peroxide,

the K_m of G6PDH1 can decrease, increasing the flux through the G6P shunt. Sharkey & Weise

(2016), proposed that the shunt can induce cyclic electron flow, which may help protect PSI.

Photoprotective mechanisms of PSII, for example state transitions of the antenna complex or

energy dependent quenching, are usually sufficient to safely dissipate excess excitation energy at

PSII (Derks *et al.*, 2015). However, with high light, in fluctuating light (Allahverdiyeva *et al.*,

2014), and at low temperature (Sonoike, 2011), excess energy or electrons could still be passed

on to PSI and result in PSI photoinhibition. Unlike PSII, the proteins of PSI have a low turnover

rate and damage to PSI is considered more severe (Scheller and Haldrup, 2005; Sonoike, 2011).

Coupling ATP consumption in the G6P shunt with cyclic electron flow would dissipate light

energy at PSI (Miyake *et al.*, 2004; Munekage *et al.*, 2004; Strand and Kramer, 2014).

Conclusion

Our data supports the conclusion that production and consumption of plastidic G6P is carefully

regulated. Plastidic PGI activity is not adequate to bring F6P and G6P to equilibrium, preventing

an accumulation of G6P, and G6PDH is partially deactivated reducing loss of carbon while still

maintaining regulatory flexibility to increase and decrease the G6P shunt (Fig. 12) as needed.

Supplementary data

Supplemental Fig. S1- SDS page of the purified G6PDH and PGI proteins, stained with

Coomassie blue.

Supplemental Fig. S2- Kinetics of plastidic and cytosolic AtPGI at different F6P and G6P

concentrations.

Supplemental Fig. S3- Effect of 10 mM DTT on plastidic and cytosolic AtPGI

Supplemental Fig. S4- Hanes-Woolf plots of E4P and 6PG inhibition of plastidic AtPGI

Supplemental Fig. S5- Hanes-Woolf plots of NADPH effect on G6PDH1

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Author Contributions

A.L.P. designed and carried out the experiments and analyzed the data. A.B. designed and carried out the PGI experiments. N.F. did the calculations for the midpoint potential experiments. A.L.P. wrote the manuscript. T.D.S. supervised the project and edited the manuscript. All authors discussed the results and provided critical feedback.

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	F6P $\rightarrow$ G6P		G6P $\rightarrow$ F6P	
	Plastidic PGI	Cytosolic PGI	Plastidic PGI	Cytosolic PGI
K_m (µM)	73 ± 46	203 ± 7	164 ± 25	158 ± 49
E4P K_i (µM)	2.3	1.5	6.0	3.7
6PG K_i (µM)	31	106	245	44

Table 1. Kinetic constants and inhibition constants for plastidic and cytosolic AtPGI as determined by NADPH-linked spectrophotometric assays and LC-MS/MS assays. Each number was determined from the fitted curve as described in the methods.

	G6PDH1	G6PDH2	G6PDH3
G6P K_m (mM)	0.3	10.3	0.3
G6P K_i (mM)	18.9	30.0	37.0
NADP K_m (µM)	0.7	1.3	0.6
k_{cat} (s^{-1})	57.0	39.4	14.6
Catalytic Efficiency G6P (mM^{-1} s^{-1})	190.0	3.8	48.7
Catalytic Efficiency NADP (µM^{-1} s^{-1})	81.4	30.3	29.2
NADPH K_i (µM)	59	0.9	112

Table 2. Kinetic constants and inhibition constants of AtG6PDH1, 2, and 3 as determined by NADPH-linked spectrophotometric assays. Each number was determined from by a modified Michalis-Menten equation which includes substrate inhibition. Data points used in model fitting were n=3 different preparations. For inhibition constants, each number was determined from the fitted curves as described in the methods.

		G6PDH1	G6PDH2	G6PDH3
Oxidized	G6P K_m (mM)	0.3	10.3	0.3
	k_{cat} (s^{-1})	57.0	39.4	14.6
Reduced	G6P K_m (mM)	3.4	8.6	0.6
	k_{cat} (s^{-1})	52.2	20.4	11.0

Table 3. Kinetic constants of oxidized and reduced AtG6PDH1, 2, and 3 determined by NADPH-linked spectrophotometric assays. Each number was determined from by a modified Michalis-Menten equation which includes substrate inhibition. Data points used in model fitting were n=3 different preparations.

Enzyme or metabolite	Midpoint potential, E_m (mv) at pH 8	% reduced at -378 mV
G6PDH	-378	50.0
Ferredoxin	-410	7.0
NADPH	-380	46.0
Thioredoxin f	-350	90.6
Thioredoxin m	-360	81.1
NADP-MDH	-390	27.5
FBPase	-375	56.0
PRK	-355	86.5
Cyclic electron flow	-330	98.0

Table 4. Midpoint potentials and percent reduction of key Calvin-Benson cycle enzymes and electron transport proteins at -378 mV at pH 8, assuming equilibrium. Calvin-Benson cycle enzymes are mostly active at the midpoint potential of G6PDH. One electron chemistry is assumed for ferredoxin and two electron chemistry for all others.

Fig 1. Production and consumption of plastidic G6P. Plastidic G6P can be produced by PGI isomerization of F6P, transported across the plastidic membrane by GPT2, consumed by PGM for starch synthesis, or consumed by G6PDH to enter the G6P shunt.

Fig 2. Comparison of specific activity of plastidic (a and b) and cytosolic (c and d) AtPGI with various metabolites Each bar represents mean and error bars represent S.E. (n=3). All metabolites were screened at 1:1 F6P substrate to metabolite. Data with an asterisk (*) are significantly different from the control as determined by Student's t-test ($P < 0.05$).

Fig 3. Effect of E4P (a, b) and 6PG (c, d) on plastidic AtPGI. Different symbols represent different concentrations of inhibitor. PGI was more inhibited by E4P than by 6PG. Lines represent data fit to Eq. 3.

Fig 4. Comparison of G6P and F6P K_m in plastidic SoPGI in dark and light-treated isolated spinach chloroplasts. Each bar represents mean and error bars represent S.E. (n=3). The K_m for G6P increased in dark treated compared to light treated isolated chloroplasts. Bars with a cross (+) are significantly different from corresponding light treated samples as determined by Student's t-test (P < 0.1).

Fig 5. Kinetics of AtG6PDH1, 2, and 3 at different G6P (a) and NADP$^+$ (b) concentrations. Each data point represents mean and error bars represent S.E. (n=3). All three isoforms of oxidized G6PDH showed substrate inhibition for G6P. G6PDH1 and 3 showed the greatest affinity for G6P and G6PDH3 had the greatest affinity for NADP$^+$. During the G6P experiments NADP$^+$ was 0.6 mM and during the NADP$^+$ experiment G6P concentration was 7 mM for G6PDH1 and 3 and 15 mM for G6PDH2. In (a) lines represent data fit to Eq. 2 and in (b) lines represent data fit to the Michaelis-Menten equation.

Fig 6. Activity of AtG6PDH 1, 2, and 3 with and without DTT treatment (a) and K_m shift with DTT in G6PDH 1 (b). Each bar or data point represents mean and error bars represent S.E. (n=3). G6PDH1 was the most affected by DTT treatment. White bars indicate controls incubated without DTT for 30 minutes and shaded bars represent incubation with 10 mM DTT for 30 minutes. Assays were done with 5 mM G6P for G6PDH1 and G6PDH3, and 15 mM for G6PDH2. The lines represent data fit to Eq. 2. Bars with an asterisk (*) are significantly different from corresponding controls as determined by Student's t-test (P < 0.05).

Fig 7. Activation of DTT-deactivated AtG6PDH1 with hydrogen peroxide treatment.
G6PDH1 deactivation by DTT could be recovered by addition of equimolar hydrogen peroxide.
(a). Reactivation is not through DTT oxidation, but rather hydrogen peroxide directly effects
G6PDH1 (b). Assays were done with 5 mM G6P. Each bar or data point represents mean and
error bars represent S.E. (n=3). Bars with an asterisk (*) are significantly different as determined
by two tailed Student's t-test ($P < 0.05$).

Fig 8. AtG6PDH1 protection from deactivation by G6P. G6PDH1 is less deactivated by DTT after 30 and 60 min when G6P is present at 5 mM. Each bar represents the mean and error bars represent S.E. (n=3). Bars with asterisk (*) are significantly different as determined by two tailed Student's t-test (P < 0.05).

Fig 9. AtG6PDH1 midpoint potential. The midpoint potential of G6PDH1 was determined to be -378 mV at pH 8. Assays were done at the K_m concentration of G6P for oxidized G6PDH1, 0.3 mM. Each data point represents the mean and error bars represent S.E. (n=3). The dashed line represents the Nernst equation for one electron. The solid line represents the Nernst equation for two electrons.

Fig 10. Whole leaf and chloroplast activity of G6PDH in Arabidopsis, and spinach. Whole leaf activity of AtG6PDH decreased 35% after illumination (a). This represents the total redox sensitive G6PDH fraction in Arabidopsis leaves. Chloroplast G6PDH activity decreased 50% after reduction by DTT (b). Samples were normalized by µmol min^{-1} of activity per mg of chlorophyll added to the assay mixture. Assays were done with 5 mM G6P. Each bar represents the mean and error bars represent S.E. (n=3). Bars with a plus sign (+) are significantly different as determined by two tailed Student's t-test (P < 0.1). One asterisk (*) signifies statistical difference as determined by two tailed Student's t-test (P < 0.05)

Fig 11. Model of production and consumption of glucose 6-phosphate in the chloroplast stroma through the G6P shunt. Under normal conditions, there may be flux through the G6P shunt as well as flux to starch synthesis (a). Flux through the G6P shunt can by modulated either by an increase in G6P substrate (b) or an increase in G6PDH activity (c). Arrows represent activity of enzymes and changes in thickness represent relative changes in flux. Red represents changes in the steady-state conditions.

Fig 12. The glucose 6-phosphate shunt. G6PDH is consumed by G6PDH to enter the G6PDH shunt. G6P re-enters the Calvin-Benson cycle as Ru5P. Overall, the shunt consumes three ATP and two NADP$^+$ and produces two NAPDH. One CO_2 molecule is lost for every G6P that enters the shunt.