



FOCUS PAPER

Redox regulation of carbon storage and partitioning in response to light and sugars

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Abstract

Redox signals generated by the photosynthetic electron transport chain are known to be involved in regulating the Calvin cycle, ATP synthesis, and NADPH export from chloroplasts in response to light. The signal cascade involves transfer of electrons from photosystem I via the ferredoxin–thioredoxin system to target enzymes that are activated by reduction of regulatory disulphide bonds. The purpose of this review is to discuss recent findings showing that this concept can be extended to the regulation of carbon storage and partitioning in plants. Starch is the major carbon store in plants, and ADP-glucose pyrophosphorylase (AGPase) is the key regulatory enzyme of starch synthesis in the plastid. It has been shown that AGPase from potato tubers is subject to post-translational redox modification, and here experimental data will be provided showing that the isozyme from pea leaf chloroplasts is activated by reduced thioredoxin *f* or *m* in a similar way. Recent reports will be summarized providing *in planta* evidence that this mechanism regulates storage starch synthesis in response to light and sugars. Post-translational redox activation of AGPase in response to sugars is part of a signalling mechanism linking the rate of starch synthesis to the availability of carbon in diverse plant tissues. Some of the components of the signalling pathway reporting changes in the cytosolic sugar status to the plastid have been postulated, but detailed work is in progress to confirm the exact mode of action. Recent evidence will be discussed showing that key enzymes of *de novo* fatty acid synthesis (acetyl-CoA carboxylase) and ammonium assimilation (glutamine synthetase and glutamine:oxoglutarate amino transferase) are regulated by reversible disulphide-bond formation

similar to AGPase. Redox regulation is proposed to be the preferred strategy of plastidial enzymes to regulate various metabolic processes such as carbon fixation, starch metabolism, lipid synthesis, and amino acid synthesis in response to physiological and environmental inputs.

Key words: Acetyl-CoA carboxylase, ADP-glucose pyrophosphorylase, amino acids, *Arabidopsis*, lipids, potato (tuber), redox (regulation), signalling (SNF1), starch, sucrose, thioredoxin.

Introduction

Redox reactions are central to biological energy conversion and critical for the redox state of a cell, which determines and regulates many cellular functions. Redox regulation as a concept linking CO₂ assimilation and related photosynthetic processes to light was established more than two decades ago and since then has been the subject of many reviews (Buchanan *et al.*, 1979, 2002; Buchanan, 1980; Scheibe, 1991; Schürmann and Jacquot, 2000). Light-dependent photosynthetic electron transport leads to reduction of ferredoxin, and reducing groups are then transferred by ferredoxin:thioredoxin reductase to thioredoxins *f* and *m*, which react with their targets. Enzymes of the Calvin cycle, ATP synthesis and NADPH export from chloroplasts are activated by reduction of cysteine residues, while chloroplast glucose-6-P dehydrogenase becomes inactivated. In the following review, recent findings showing the general relevance of reversible disulphide-bridge formation for the regulation of plastidial carbon metabolism will be discussed. By contrast to many cytosolic enzymes that are regulated by the protein-kinase/protein-phosphatase system, an increasing number of plastidial enzymes are

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regulated by redox modulation not only in green tissues, but also in heterotrophic organs such as tubers and seeds.

ADP-glucose pyrophosphorylase is activated by thioredoxin *f* and *m* via reduction of a regulatory disulphide bond

During photosynthesis, inorganic CO₂ is converted to triose-phosphates, which are exported to the cytosol where they are used to synthesize sucrose. Some of the reduced carbon is retained in the chloroplast to synthesize starch as a transient carbon store, which is remobilized during the night to support non-photosynthetic leaf metabolism and sucrose export (Fig. 1). ADP-glucose pyrophosphorylase (AGPase) catalyses the conversion of glucose-1-phosphate and ATP to ADPGlc and PPi, which is the first committed step in the pathway of starch synthesis (Preiss, 1988; Martin and Smith, 1995). Studies with *Arabidopsis* mutants have demonstrated that this enzyme represents a key site for the control of starch synthesis in leaves (Neuhaus and Stitt, 1990). The enzyme is a heterotetramer that contains two large subunits (AGPS, 51 kDa) and two slightly smaller subunits (AGPB, 50 kDa) (Morell *et al.*, 1987; Okita *et al.*, 1990) and is exquisitely sensitive to allosteric regulation, with 3-phosphoglycerate (3-PGA) acting as an activator and Pi as an inhibitor (Sowokinos, 1981; Sowokinos and Preiss, 1982; Preiss, 1988).

Studies with isolated chloroplasts led to the conclusion that the allosteric properties of AGPase are important to regulate starch synthesis in response to a transient imbalance between photosynthesis and triose-phosphate export, which goes along with changes in the 3PGA to Pi ratio

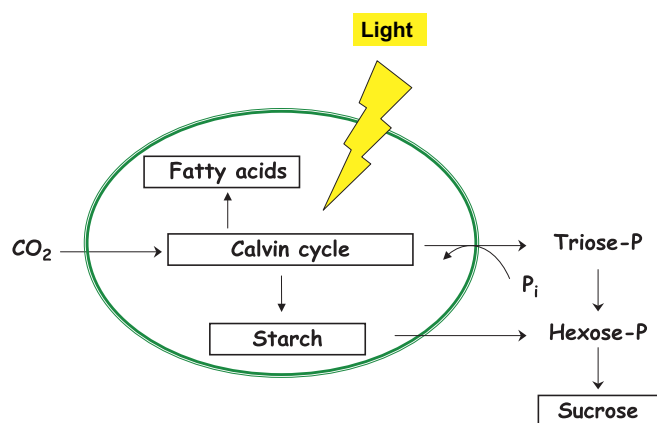


Fig. 1. Schematic representation of photosynthetic CO₂ assimilation and carbon partitioning in leaves. In the light, operation of the Calvin cycle converts inorganic CO₂ to phosphorylated intermediates which are used for starch and fatty acid synthesis in the plastid or sucrose synthesis in the cytosol. The transitory starch that accumulates during the day is degraded in the following night to maltose and glucose, which are exported to support cytosolic metabolism (Zeeman *et al.*, 2004). Fatty acids are used to synthesize lipids via different routes that are located inside or outside the plastid (Ohlrogge and Browes, 1995).

(Heldt *et al.*, 1977). However, the significance of changes in metabolite levels for the regulation of starch synthesis in response to light–dark transitions has not yet been clearly demonstrated. Early studies by Kaiser and Bassham (1979a) showed that the kinetics of changes in 3PGA and Pi levels when compared with the kinetics of starch formation were too slow to account for the fast light–dark regulation of starch formation observed in experiments with isolated chloroplasts. The authors' results with intact *Arabidopsis* leaves showed that the short-term decrease in the ADPGlc level upon light–dark transition was not reflected by a decrease in 3PGA (P Geigenberger and Y Gibon, unpublished results). Gerhardt *et al.* (1987) used a non-aqueous fractionation technique to measure subcellular metabolite levels in spinach leaves during the diurnal cycle. Interestingly, stromal 3PGA was present in the dark in considerable amounts and rose only slightly after illumination. Crucially, no increase in stromal 3PGA was observed under conditions where sucrose synthesis was inhibited and starch was accumulating. More recent studies showed that there is a strong stimulation of starch synthesis in the absence of any increase in 3PGA when leaf sugar levels are increased by interruption of phloem transport (Krapp and Stitt, 1995; Geigenberger *et al.*, 1996) or by external sugar supply (Krapp *et al.*, 1991). Furthermore, transgenic plants with increased 3PGA levels, due to antisense inhibition of cytosolic phosphoglycerate mutase, did not show any increase in starch in their leaves (Westram *et al.*, 2002). Overall, these studies show that additional factors must be involved in the light–dark control of starch formation, besides changes in metabolite levels.

Already in 1979, Kaiser and Bassham (1979b) proposed that light-mediated changes in sulphhydryl groups might be involved in the regulation of AGPase, as was already described for Calvin cycle enzymes at that time. They showed that incubation of extracts from lysed chloroplasts with the sulphhydryl-reductant dithiothreitol (DTT) leads to a strong increase in the rate of ADPGlc formation. The significance of this finding was, however, unclear since quite high concentrations of DTT (10–50 mM) were used and regulatory cysteines were not investigated. Almost 20 years later, Fu *et al.* (1998) found that an intermolecular bridge forms between the Cys-82 residues of the two AGPB subunits of the potato AGPase, when heterologously over-expressed in *Escherichia coli*. To obtain active enzyme it was necessary to incubate the complex with 1–5 mM DTT to break this link. The change in redox state could be detected from a modification of the electrophoretic mobility of the small subunit in non-reducing SDS gels, with AGPB running as a dimer in the oxidized form and as a monomer in the reduced form. Reduction of the intermolecular disulphide bond led to a dramatic increase in activity due to a decrease in the *K_m* (ATP) and increased sensitivity to activation by 3PGA (Fig. 2). Further studies showed that reduced forms of both thioredoxins *f* and *m* from spinach

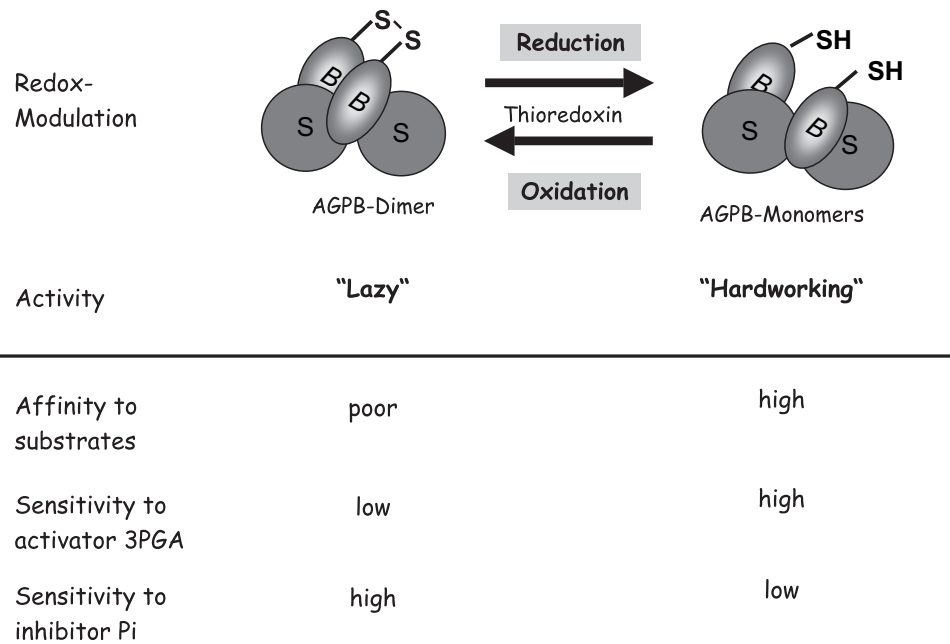


Fig. 2. ADP-glucose pyrophosphorylase (AGPase), catalysing the first committed step of starch synthesis in the plastid, is subject to post-translational redox modification. The redox regulation of potato tuber AGPase by thioredoxin *f* or *m* involves reduction of a disulphide bond between the Cys82 of the two AGPB subunits of the tetrameric protein, which leads to a change in the kinetic properties of the enzyme (Fu *et al.*, 1998; Ballicora *et al.*, 2000; Tiessen *et al.*, 2002).

leaves were able to activate the recombinant AGPase from potato tubers by up to 4-fold (Ballicora *et al.*, 2000). Fifty per cent activation was obtained at 4.5 and 8.7 μM for reduced thioredoxins *f* and *m*, respectively, being two orders of magnitude lower than for DTT. Oxidized thioredoxins reversed the activation. DTT led to monomerization of AGPB and an increase of AGPase activity also in potato tuber extracts (Tiessen *et al.*, 2002).

Post-translational redox modification of AGPase regulates starch synthesis in leaves in response to a light-dependent signal

The cysteine shown by Fu *et al.* (1998) to be involved in the formation of the regulatory disulphide bridge in the recombinant potato tuber enzyme corresponds to Cys82 of the full-length gene (Genbank X61186). The QTCLDP motif is conserved in the N termini of almost all dicot plant AGPB isoforms, including those from leaves. First attempts to investigate whether AGPase from leaves can be redox-regulated in a similar way to the potato tuber enzyme were hampered, since addition of DTT alone or together with thioredoxins to whole leaf extracts seriously interfered with the AGPase assay, leading to a loss of AGPase activity (A Tiessen, JHM Hendriks, A Kolbe, and P Geigenberger, unpublished results). A desalted extract of stromal proteins prepared from chloroplasts of dark-adapted pea leaves was therefore used for incubation experiments with reduced

thioredoxin *f* or *m*, which had been purified from spinach leaves. Stromal proteins were incubated with different concentrations of reduced thioredoxins for 15 min before samples were rapidly quenched in non-reducing sampling buffer to analyse AGPB dimerization using non-reducing SDS gels. Figure 3A and B shows that AGPB was completely dimerized in control incubations, while incubation with 20 μM of reduced thioredoxin *f* or *m* led to the appearance of AGPB monomers, indicating that the enzyme had been partly converted into its activated reduced form. To investigate whether changes in monomerization were causally related to changes in activity, the stromal protein fraction was incubated with 20 μM of thioredoxin *f* and AGPase activity measured after 15 min in the ADPGlc synthesis direction using an enzymatic test (Fig. 3C). Thioredoxin *f* led to an increase of up to 3-fold in AGPase activity, due to a marked increase in the sensitivity of the enzyme to activation by 3PGA.

Results shown in Fig. 3 indicate that pea-leaf AGPase is regulated in a similar way to that of the potato tuber enzyme, although the AGPB dimer was not completely converted to monomers, even when higher concentrations of thioredoxin *f* and *m* up to 100 μM were supplied (Fig. 3). One explanation for this is that other thioredoxin isoforms might be more effective to activate AGPase than thioredoxin *f* and *m*. The degree of monomerization that was obtained after incubation with thioredoxin *f* and *m* is, however, similar to that usually observed in illuminated leaves (see below). A second explanation is that interaction of thioredoxins with

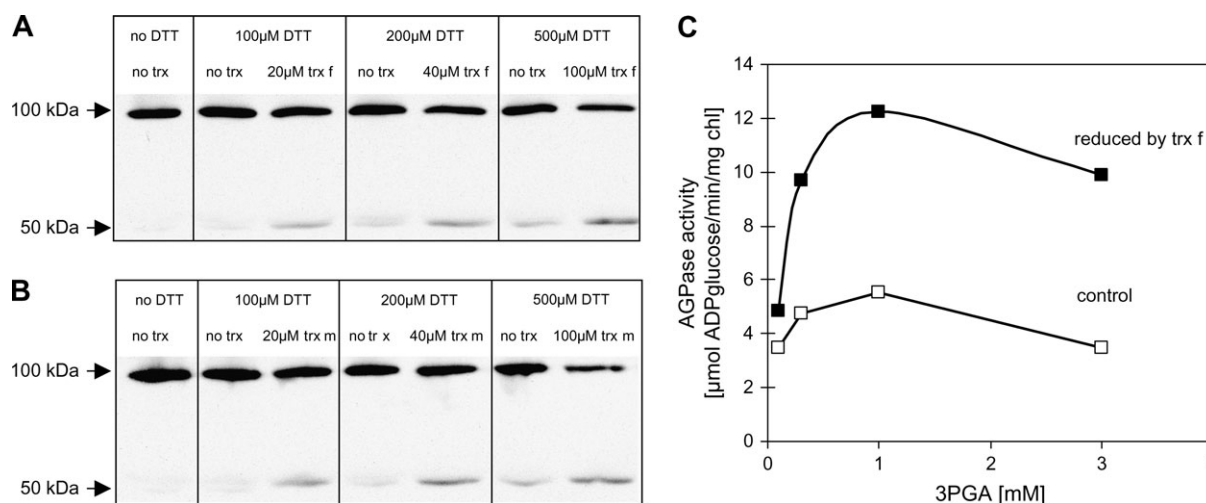


Fig. 3. ADP-glucose pyrophosphorylase (AGPase) from pea-leaf chloroplasts is redox-activated by thioredoxins. Chloroplasts were prepared from pea leaves as in Hendriks *et al.* (2003), lysed by osmotic shock and subsequently desalted on Sepharose-50 columns, before incubation with reduced thioredoxins for 15 min. (A, B) Stromal proteins were subsequently extracted in non-reducing sample buffer, separated using non-reducing SDS gels and AGPB detected by immunoblotting according to Hendriks *et al.* (2003). Thioredoxin *f* (A) and *m* (B) led to a partial conversion of AGPB from dimer (100 kDa) to monomer (50 kDa). (C) To investigate whether changes in monomerization were causally related to changes in activity, the stromal protein fraction incubated with 20 μM of thioredoxin *f* was used to measure AGPase activity in the ADPGlc synthesis direction using an enzymatic test according to Hendriks *et al.* (2003). Thioredoxin *f* led to an increase of up to 3-fold in AGPase activity, due to a marked increase in the sensitivity of the enzyme to activation by 3PGA. Thioredoxin *f* and *m* purified from spinach leaves were kindly provided by Professor Renate Scheibe (Osnabrück). They were reduced by incubation with DTT in concentrations that were 5-fold higher than the thioredoxin concentration according to Sasaki *et al.* (1997). Control incubations containing DTT but no thioredoxin are shown for comparison.

AGPase is modulated by additional factors allowing a further degree of fine control. It has been found for photosynthetic enzymes that activation by thioredoxin is modified by pH, Mg^{2+} , and the levels of substrates and products, all of which show marked changes upon light–dark transitions (Scheibe, 1991). Interestingly, *in vitro* activation of potato AGPase by DTT requires the presence of substrates, which are needed to attain the active conformation after reduction (Fu *et al.*, 1998). More detailed studies are necessary using purified enzyme to investigate the affinities of leaf AGPase for different thioredoxin isoforms, and to investigate whether interaction of AGPase with thioredoxins is modified by low molecular weight factors.

While the results presented above show that AGPase from chloroplast preparations is redox-regulated by thioredoxins, recent reports provide *in vivo* evidence that this type of regulation is relevant for starch synthesis in intact leaves (Hendriks *et al.*, 2003). When pea leaves were extracted under either dark or light conditions, using trichloroacetic acid to quickly denature AGPase before separation on non-reducing SDS gels, AGPB was found to be completely dimerized in the dark and partly converted to monomer in the light. Similar results were found for potato and *Arabidopsis* leaves. Appearance of the monomer was accompanied by an increase in AGPase activity due to changes in the kinetic properties of the enzyme. Darkening of leaves rapidly reversed the light-dependent monomerization of AGPB within minutes, while sugar levels

remained unaltered. AGPB occurred almost exclusively as a dimer when intact isolated chloroplasts were incubated in the dark and was converted to monomer within 6 min of illumination, paralleling an increase in plastidial fructose-1,6-bisphosphatase activity. Light-dependent monomerization of AGPB was promoted when chloroplasts were externally supplied with 3PGA to refill Calvin cycle pools. This is consistent with previous reports showing that substrate levels modulate the activation of thioredoxin-regulated enzymes (Scheibe, 1991) and could provide a mechanism to prevent a depletion of phosphorylated intermediates due to excessive activation of AGPase.

Taken together, these studies show that AGPase is redox-regulated by a light-dependent signal in leaves, analogous to the way in which enzymes of the Calvin cycle and related photosynthetic processes are regulated (Fig. 4). This allows assimilation and storage of carbon to be regulated in a co-ordinated way without the 3PGA to Pi ratio as a necessary intervening step. While *in vitro* experiments show that AGPase is activated by thioredoxin *f* and *m* (see above), the thioredoxin isoforms that are responsible for the *in vivo* regulation of AGPase are presently not known. Plants contain a large multigene family of thioredoxins, of which several are targeted to the plastid (Schürmann and Jacquot, 2000). Further studies will be necessary to assess their *in vivo* importance in the regulation of AGPase and carbon partitioning in leaves. This may involve thioredoxin knock-out mutants or transgenic approaches.

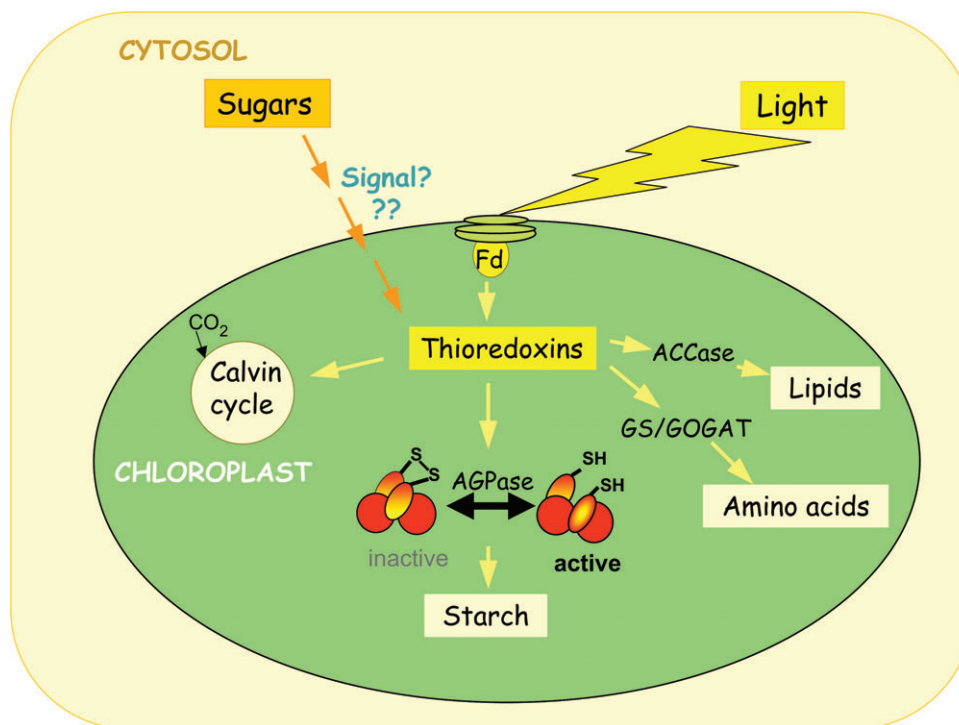


Fig. 4. Carbon metabolism in leaf chloroplasts is regulated by post-translational redox regulation in response to light and sugars. Several key enzymes of the Calvin cycle are known to be regulated by redox modification via the ferredoxin/thioredoxin system in response to light (Buchanan, 1980). In addition to photosynthetic carbon fixation, key enzymes of starch, lipid, and amino acid synthesis have been shown to be regulated in a similar way (see text for further details and references). It has been proposed that this concept not only applies to light–dark regulation, but that it also allows the plastid to respond to cytosolic sugar levels. The focus of further research will be to identify the components that are mediating this response. Fd=ferredoxin; AGPase=ADPglucose pyrophosphorylase, ACCase=acetyl-CoA carboxylase, GS=glutamine synthetase, GOGAT=glutamine:oxoglutarate aminotransferase.

Redox-regulation of AGPase regulates starch synthesis in response to the carbon status

In addition to light, sugars provide a second input leading to redox activation of AGPase in leaves (Fig. 4). Studies by Hendriks *et al.* (2003) showed that monomerization of AGPB is increased by supplying exogenous sucrose to wild-type leaf material in the dark, and is also increased in a phosphoglucosyltransferase-deficient *Arabidopsis* mutant in the light and at the beginning of the night when this mutant contains higher sugar levels than the wild-type. Despite the higher AGPase activation, the leaves of this mutant do not accumulate starch because it cannot supply the glucose-1-phosphate substrate for the enzyme. Comparison of the relationships between light, internal sugar levels, and AGPase monomerization showed that light and sugars act in an additive manner to increase AGPase activation in leaves, leading to an almost complete monomerization of AGPB in the mutant at the end of the day. In addition to direct light-activation of starch synthesis, post-translational redox regulation provides a mechanism that allows starch synthesis to be increased when sucrose accumulates in the leaf due to a decreased export to the growing sinks. This might provide a way to link changes in the carbon status of the sinks to storage processes in the leaf (Gibson *et al.*, 2004).

Studies with potato tubers show that redox activation of AGPase is part of a general mechanism for regulation of starch synthesis in response to the carbon status (Tiessen *et al.*, 2002, 2003). By contrast to leaves, potato tubers represent a non-photosynthetic plant tissue that uses a large amount of imported sucrose to synthesize starch as the major carbon store (Mares and Marschner, 1980). Interruption of sucrose supply leads to a short-term inhibition of the rate of starch synthesis that cannot be explained by changes in metabolite levels or in the expression of AGPase (Geigenberger *et al.*, 1994; Geiger *et al.*, 1998). Tiessen *et al.* (2002) showed that inhibition of starch synthesis in response to decreased sucrose import is due to redox modification of AGPase, leading to a complete dimerization of AGPB and a change in the kinetic properties of the enzyme, resulting in a decreased affinity for its substrates and decreased sensitivity to activation by 3PGA. Moreover, redox activation of AGPase *in planta* was correlated closely with the tuber sucrose content across a range of physiological and genetic manipulations, indicating that redox-modulation of AGPase is responsible for channelling incoming sucrose towards synthesis of storage starch. Crucially, this mechanism allows starch synthesis to be stimulated by a sucrose-related signal independently of any

increase in glycolytic intermediates, specifically hexose-phosphates or 3PGA. The effect of sugars on AGPase redox activation could be mimicked by feeding low concentrations of the reductant DTT to tissue slices from potato tubers (Tiessen *et al.*, 2002) or darkened *Arabidopsis* leaves (A Kolbe and P Geigenberger, unpublished results) to increase the redox state of the tissue. Feeding DTT for 1 h led to redox activation of AGPase in a concentration-dependent manner, which was accompanied by a corresponding stimulation in the rate of starch synthesis and a decrease in glycolytic metabolite levels.

The importance of redox regulation of AGPase for the synthesis of starch in other heterotrophic plant tissues still has to be investigated. Starch is the major carbon store in cereal seeds such as maize, rice, and wheat, which are also of great economical importance. The pathway of starch synthesis in the cereal endosperm is different from other plant species and tissues such as tubers and leaves, in that it involves a cytosolic AGPase in addition to the plastidic isoform (Tetlow *et al.*, 2004). The regulatory cysteine identified by Fu *et al.* (1998) is missing in these cytosolic AGPB isoforms, indicating that the cytosolic AGPase of cereal endosperm is redox-insensitive. However, more studies are needed to confirm this interpretation.

What is the link between sugar and redox signals?

The studies summarized above show a very good correlation between sucrose levels, AGPase redox state and starch synthesis in such diverse tissues as photosynthesizing leaves and heterotrophic tubers, providing evidence for a general link between sugars and redox activation of AGPase in the plastid. Studies in potato tubers show that sucrose and glucose lead to redox activation of AGPase via two different signalling pathways involving a SNF1-like protein kinase and an endogenous hexokinase, respectively (Tiessen *et al.*, 2003). Antisense inhibition of a SNF1-kinase homologue in potato tubers strongly attenuated the reductive activation of AGPase after supplying sucrose, but not after supplying glucose. Glucose-dependent redox activation of AGPase required phosphorylation of hexoses by an endogenous hexokinase, while sucrose dependent activation could be mimicked by non-metabolizable sucrose analogues, showing that it is not necessarily linked to sucrose metabolism or cleavage into glucose and fructose (Fig. 5). This is in line with previous studies in tubers (Geiger *et al.*, 1998; Fernie *et al.*, 2001) and seeds (Borisjuk *et al.*, 1998), showing that sucrose provides a signal that leads to a direct stimulation of storage starch synthesis without requiring an intervening increase in metabolite levels.

Both hexokinase and SNF1-kinase are part of a regulatory network that controls the expression and phosphory-

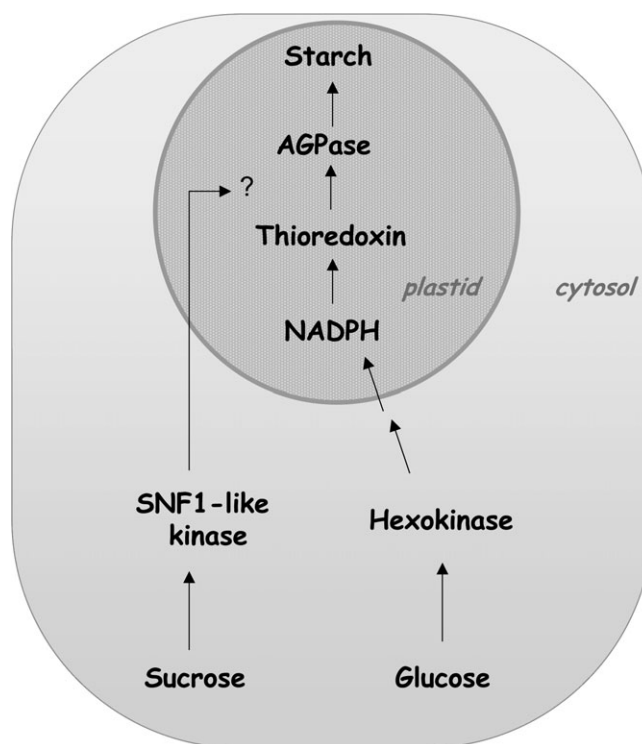


Fig. 5. Redox regulation of ADPGlc pyrophosphorylase (AGPase) is part of a novel mechanism that links storage starch synthesis to the supply of sugars. In heterotrophic potato tubers two separate sugar signalling pathways have been identified that lead to post-translational redox regulation of AGPase. A glucose-dependent pathway requires hexose-phosphorylation by an endogenous hexokinase, whereas a sucrose-dependent pathway proceeds via SNF1-related protein kinase (Tiessen *et al.*, 2003). Glucose feeding leads to an increase in the overall NADPH:NADP⁺ ratio of the cell, which in turn will affect plastidial redox regulation. It is not known how the signal is transferred between the cytosol and the plastid, but results imply the existence of additional factors influencing the redox transfer between thioredoxins and their specific targets.

lation of cytosolic enzymes in response to sugars (Smeekens, 2000). It will be interesting to determine by what kind of mechanisms they are linked to the reductive activation of AGPase in the plastid. Measurements of NADPH:NADP⁺ ratios after supplying either glucose or sucrose to potato tuber tissue showed that increased redox activation of AGPase involved a 2-fold increase in the overall redox state of the tissue in response to glucose, but not to sucrose (A Tiessen and P Geigenberger, unpublished results). This is consistent with results showing glucose feeding to increase and sucrose to decrease the flux of carbon into respiratory pathways (Geiger *et al.*, 1998).

The increased redox state in response to glucose may be indicative of a stimulation of the oxidative pentose phosphate cycle or other processes leading to increased reduction of NADP⁺ to NADPH. This probably involves a plastidic isoform of glucose-6-P dehydrogenase (P2-G6PDH), identified recently in potato, which is much less susceptible to inactivation by thioredoxin and to

end-product inhibition by NADPH (Wendt *et al.*, 2000). It has been proposed that P2-G6PDH could play a role in the provision of reductant in heterotrophic plastids, where photochemical processes are absent, or contribute to the capacity of chloroplasts to generate NADPH (Debnam *et al.*, 2004). An elevated NADPH:NADP⁺ ratio will lead to an increase in the reduction state of thioredoxins via the NADP/thioredoxin reductase or the ferredoxin/NADP reductase systems, and hence activate AGPase. Interestingly, overexpression of P2-G6PDH in transgenic tobacco plants led to an increase in the starch to soluble sugar ratio in leaves (Debnam *et al.*, 2004). More studies are needed to identify the thioredoxin isoforms in heterotrophic plastids and the way they are reduced by the activity of the oxidative pentose phosphate pathway.

By contrast to glucose, sucrose did not lead to changes in the NADPH:NADP⁺ ratio in tubers (see above). This indicates that sucrose leads to an increase in reductive activation of AGPase even though the plastidial redox-state does not increase. The underlying mechanism still has to be resolved, but may involve modulation of redox-transfer from NADPH to AGPase by metabolites. Recent studies with transgenic *Arabidopsis* plants over-expressing trehalose-6-P metabolizing enzymes provided genetic and biochemical evidence for a role of trehalose-6-P in this response (A Kolbe, A Tiessen, H Schlueppmann, M Paul, S Ulrich, and P Geigenberger, unpublished results). Trehalose-6-P is an intermediate of trehalose synthesis and has been shown to be an indispensable regulator of sugar utilization in plants (Schlueppmann *et al.*, 2003).

A similar regulatory network might also be relevant to regulate carbon utilization in other organisms. Interestingly, insulin signalling in mammals involves changes in cellular redox-potential that may be linked to thioredoxins (Mahadev *et al.*, 2004). Moreover, a thioredoxin-interacting protein has been identified in mice that inhibits thioredoxin-mediated redox transfer from NADPH to sulphhydryl groups and affects the metabolic response to nutritional signals (Hui *et al.*, 2004; Shet *et al.*, 2005).

Is there a role of redox-signals to co-ordinate the antagonistic processes of starch synthesis and degradation?

Whilst redox-related signals are crucial for the regulation of starch synthesis, they may also be operating to regulate the antagonistic process of starch degradation (Fig. 1). In leaves, starch mobilization is inhibited during the day and stimulated during the night (Zeeman *et al.*, 2002). However, the proteins involved in this process such as α -amylase, β -amylase, isoamylase, D-enzyme and other glucan-degrading enzymes reveal hardly any regulatory properties and their extractable activities do not change substantially during the day–night cycle (Zeeman *et al.*,

2004). Recently, an enzyme that catalyses the phosphorylation of starch-granules (glucan water dikinase; GWD) was found to be essential for the initiation of starch degradation in leaves and tubers (Lorberth *et al.*, 1998; Yu *et al.*, 2001; Ritte *et al.*, 2002, 2004). It was shown that a major fraction of GWD is attached to the surface of the starch granule when isolated from dark-adapted plants, whereas the protein is predominantly found in a soluble form upon isolation from illuminated plants (Ritte *et al.*, 2000). More recent studies of Mikkelsen *et al.* (2005) showed that GWD is subject to redox activation mediated by thioredoxin *f* and *m*, which is accompanied by the reversible reduction of a specific intramolecular disulphide bond, as determined by disulphide-linked peptide mapping and confirmed by site-directed mutagenesis. Evidence is provided that the redox state of GWD affects binding of the enzyme to starch granules in a selective and reversible manner which would promote granule association of GWD in the dark (Mikkelsen *et al.*, 2005). More studies are needed to clarify the *in vivo* relevance of this mechanism to initiate granule degradation at the onset of darkness. Similar to GWD, other enzymes involved in starch degradation such as β -amylase or pullulanase have been found to be activated upon reduction (Spradlin and Thoma, 1970; Dauville *et al.*, 2001; Schindler *et al.*, 2001; Wu *et al.*, 2002) or to interact with thioredoxins (Balmer *et al.*, 2003), but whether this affects granule binding or multi-protein complex formation was not investigated in these earlier studies. Degradation of the crystalline starch granule may involve a co-ordinated association of several enzymes to the surface of the granule that is triggered by a decrease in the reduction state of thioredoxins. This may link starch degradation to light and the carbon status of the leaf.

Redox-regulation of lipid synthesis

Similar to the metabolism of starch, the reactions of *de novo* fatty acid synthesis are also localized in the plastid (Ohlrogge and Browse, 1995). Fatty acids are essential components of the cellular membranes of all plant cells and serve as substrates for storage lipid synthesis in seeds. In the chloroplasts of leaves, the carbon used for fatty acid synthesis mainly derives from CO₂ fixation, using a pathway that converts 3PGA to acetyl-CoA, but also free acetate deriving from hydrolysis of mitochondrial acetyl-CoA can serve as the carbon source (Bao *et al.*, 2000). Acetyl-CoA carboxylase (ACCase) catalyses the first committed step of *de novo* fatty acid synthesis in the plastid, converting acetyl-CoA and CO₂ to malonyl-CoA, and has been proposed to play a major role in the regulation of this pathway (Hunter and Ohlrogge, 1998). Reducing agents led to an increase in ACCase activity in spinach chloroplasts (Sauer and Heise, 1984) and preparations of partially purified pea ACCase (Sasaki *et al.*, 1997). Reduced thioredoxin activated the

enzyme more efficiently than DTT alone, and thioredoxin *f* was more effective than thioredoxin *m* and *E. coli* thioredoxin (Sasaki *et al.*, 1997).

Chloroplast ACCase from most species is a heteromer composed of a dissociable complex of biotin carboxylase, catalysing the carboxylation of biotin on the biotin carboxyl carrier protein subunit and the two carboxyltransferase subunits, which catalyse the transfer of CO₂ from carboxybiotin to acetyl-CoA. Interestingly, the activity of carboxyltransferase was influenced by DTT, whereas that of biotin carboxylase was not (Kozaki and Sasaki, 1999). *In vitro* studies, using site-directed mutagenesis of recombinant carboxyltransferase, indicated that two cysteines, which form an intermolecular disulphide bridge between the two carboxyltransferase subunits, are involved in redox regulation (Kozaki *et al.*, 2001). Immunoblots from chloroplasts isolated from dark- or light-adapted plants showed that such disulphide–dithiol exchange could be relevant during light–dark changes *in vivo*, similar to the regulation of AGPase.

More studies are needed to clarify the physiological importance of ACCase redox regulation *in planta*. It has been shown in leaves that fatty acid synthesis is stimulated in response to light (Browse *et al.*, 1981), but whether this is mainly due to light-induced changes in metabolite concentrations or redox activation of ACCase is a matter of debate (Hunter and Ohlrogge, 1998). Furthermore, fatty acid synthesis is also required in the absence of light, suggesting that additional factors linked to the metabolism of sugars will be involved in regulating ACCase. It has been shown that ACCase is redox-activated in the dark when *Arabidopsis* leaves are externally supplied with DTT, and a similar response was observed after supplying sucrose (A Kolbe, P Waldeck, H Vigeolas, and P Geigenberger, unpublished results). Crucially, under both conditions, increased redox activation of ACCase correlated with a decrease in the level of its substrate acetyl-CoA and an increase in the rate of lipid synthesis, providing *in vivo* evidence that reductive activation of ACCase is regulating lipid synthesis in response to a sugar-related signal in a similar way to the regulation of AGPase and starch synthesis (see above).

Although these studies document the importance of redox regulation of ACCase for the regulation of lipid synthesis in leaves, less is known concerning its role in lipid-storing seeds. In developing seeds of oil-seed rape or *Arabidopsis*, sucrose is imported from the phloem and converted to fatty acids, which are subsequently used for the synthesis of triacylglycerol as the major storage compound (Ohlrogge and Browse, 1995). These seeds are green and photosynthetically active, and lipid synthesis has been shown to be stimulated in response to light in developing rape seeds (Ruuska *et al.*, 2004). The major factors that are involved in this regulatory process still have to be resolved in seeds, but it may involve redox activation

of ACCase. Recent studies show that both redox activation of ACCase and flux to storage lipids were increased in a similar manner when rape seeds were subjected to light or to external feeding of DTT or sucrose *in planta* (A Kolbe, P Waldeck, H Vigeolas, and P Geigenberger, unpublished results). Recently, a new γ -subtype of plastidial thioredoxins (AtTrx γ 2) was identified in *Arabidopsis* that is mainly expressed in seeds at the stage of major accumulation of storage lipids, which suggests that this isoform may be involved in the regulation of lipid synthesis (Collin *et al.*, 2004). Interestingly, AtTrx *f*1 and AtTrx *f*2 mRNA levels showed very similar patterns to that of AtTrx γ 2.

In summary, these studies show that the two major processes of carbon storage, starch and lipid synthesis, are both subject to redox regulation, providing a general mechanism for allowing carbon storage to be regulated in response to light and sugars without the requirement of large changes in metabolite levels.

Redox-regulation of plastidial nitrogen metabolism

While the ferredoxin/thioredoxin system has been shown to be involved in the regulation of CO₂ fixation and storage of reduced carbon in plants, its role in the assimilation of inorganic nitrate to organic amino acids in leaves is discussed controversially. The initial step of nitrate assimilation, the conversion of nitrate to nitrite, is catalysed by nitrate reductase in the cytosol. Nitrate reductase is known to be regulated by reversible protein phosphorylation involving calcium-dependent and SNF1-related kinases, which leads to activation of the enzyme in response to elevated sugar and hexose-phosphate levels during photosynthesis (Sugden *et al.*, 1999; Kaiser and Huber, 2001). Moreover, regulation of nitrate reductase in response to light–dark transitions may also be attributable to changes in NADH levels (Kaiser and Huber, 2001).

The subsequent reactions of nitrite reductase, glutamine synthetase (GS), and ferredoxin-dependent glutamine: oxoglutarate amino transferase (Fd:GOGAT) are located in the chloroplast where they are involved in the reduction of nitrite to ammonium, the ATP-dependent conversion of ammonium and glutamate to glutamine, and the conversion of glutamine and 2-oxoglutarate to glutamate, respectively. Fd:GOGAT was found to be significantly stimulated by DTT and thioredoxins when isolated from spinach chloroplasts, with thioredoxin *m* being more efficient than thioredoxin *f* in the activation of the enzyme, leading to an increase in the affinity to 2-oxoglutarate (Lichter and Häberlein, 1998). In the same study, nitrite reductase and GS from spinach revealed no thioredoxin-dependent activation. This is in contrast to the study of Choi *et al.* (1999) which shows that the chloroplast isoform of glutamine synthetase (GS2) from *Canavalia lineata* is activated by

DTT. In this latter study it was shown by site-directed mutagenesis that the redox-sensitivity of GS2 is due to two conserved cysteine residues, which are not present in the redox-insensitive cytosolic isoform GS1. Moreover, glutamine synthetase was among the enzymes isolated in proteomic approaches using resin-bound mutant thioredoxins to capture target proteins (Motohashi *et al.*, 2001; Balmer *et al.*, 2003).

While these studies provide evidence that the two enzymes responsible for the assimilation of ammonium in the chloroplast, Fd-GOGAT and GS2, are subject to thioredoxin-dependent redox regulation, the *in vivo* relevance of these findings still has to be determined. The flux of ammonium through the photorespiratory nitrogen cycle has been estimated to be 10 times higher than that resulting from primary assimilation (Lam *et al.*, 1996). Therefore, a link between the GS/GOGAT cycle and photosynthetic light reactions will be required for a co-operated control during illumination. When DTT was fed to *Arabidopsis* leaves to increase the redox state of the tissue in the dark, the level of 2-oxoglutaric acid decreased while the levels of most of the amino acids increased, which is indicative of reductive activation of the GS/GOGAT cycle *in situ* (A Kolbe, AR Fernie, JHM Hendriks, and P Geigenberger, unpublished results). Further studies will be needed to pinpoint the redox-regulated steps of this pathway and the underlying signal mechanisms *in planta*. The findings that low sugar levels lead to an inhibition of ammonium assimilation at GOGAT and a general inhibition of amino acid synthesis (Stitt *et al.*, 2002) may indicate that redox regulation of GOGAT is also important to co-ordinate nitrogen metabolism with the availability of sugars.

Conclusions and perspectives

More than 20 years ago, the ferredoxin–thioredoxin system was discovered to link the activity of enzymes of the Calvin cycle and of other photosynthetic processes to light. In this review recent findings showing that this concept can be extended to the regulation of key enzymes of carbon storage and nitrogen metabolism in plants are discussed. In addition to light, sugars provide a second input that modulates reductive activation of plastidial enzymes in such diverse tissues as photosynthesizing leaves and heterotrophic tubers. The components of the signalling pathway linking cytosolic and plastidial metabolism have to be identified and their exact roles confirmed. The redox modification of AGPase opens a new way to explore sugar-signalling pathways, since we are looking for signal transduction components that lead to defined processes at the end of the signalling chain. Redox-regulation of plastidial metabolism may emerge as a general concept to regulate processes such as starch, lipid, and amino acid synthesis by sugars. This may only be the tip of the iceberg,

since recent proteomic studies identified a large number of new thioredoxin targets that are involved in a variety of metabolic processes inside and outside the plastid (Motohashi *et al.*, 2001; Balmer *et al.*, 2003, 2004; Lemaire *et al.*, 2004; Marchand *et al.*, 2004; Wong *et al.*, 2004), although it remains to be established whether these new thioredoxin targets identified *in vitro* are also subject to redox regulation *in vivo*. In *E. coli* (Leichert and Jakob, 2004) and *Arabidopsis* (Lee *et al.*, 2004), proteomic techniques were used to profile for proteins with disulphide bonds, providing a more general and global overview of thiol-modified proteins *in vivo*. The recent development of metabolic profiling and DNA-micro-array techniques will allow a comprehensive systemic strategy to identify new targets of redox regulation *in vivo*.

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