Photosynthesis: Dark Reactions

Dieter Heineke, *Albrecht-von-Haller-Institut für Pflanzenwissenschaften, Universität Göttingen, Germany*

Introductory article

Article Contents

- Introduction
- Fixation of CO_2 is Catalysed by the Ribulose-bisphosphate Carboxylase
- Reduction of 3-Phosphoglycerate
- Regeneration of Ribulose Bisphosphate
- Light-dependent Regulation of the Calvin Cycle

Light reactions occur in the thylakoid membrane of chloroplasts that provide reducing power in the form of NADPH and energy as ATP. These products are consumed in the so-called 'dark reactions' to synthesize carbohydrates from carbon dioxide. The reactions are organized in a cyclic metabolic pathway called the Calvin cycle.

Introduction

In the thylakoid membrane of chloroplasts there occur light reactions that provide reducing power in the form of NADPH and energy as ATP. These products are consumed in the so-called 'dark reactions' to synthesize carbohydrates from CO_2. These reactions are organized in a cyclic metabolic pathway called the 'reductive pentose phosphate pathway' or the 'Calvin cycle'. The products of these chloroplast reactions are triose phosphates, which are exported into the cytosol by specific transport to be converted to sucrose. Surplus carbon is transiently stored in the chloroplast stroma as starch.

The Calvin cycle can be subdivided into three sections: (i) the carboxylation of ribulose 1,5-bisphosphate (RuBP), leading to the formation of two molecules of 3-phosphoglycerate (3PGA); (ii) the reduction of 3PGA; and (iii) the regeneration of the primary CO_2 acceptor RuBP (**Figure 1**). Regulation of Calvin cycle enzymes by light-dependent activation processes ensures that, despite its name, the dark reactions do not proceed during darkness. The Calvin cycle is only active when the required NADPH and ATP are supplied by the light reactions.

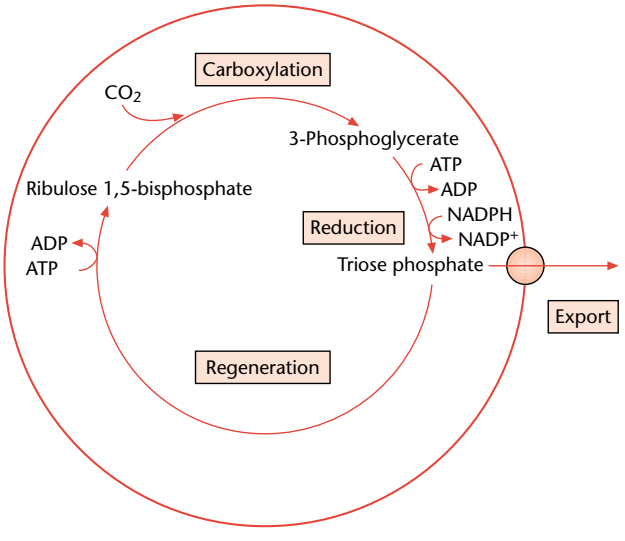

Figure 1 Overview of the basic reactions of the Calvin cycle.

Fixation of CO_2 is Catalysed by the Ribulose-bisphosphate Carboxylase

The fixation of atmospheric CO_2 is the key reaction of photosynthetic CO_2 assimilation. One molecule of ribulose 1,5-bisphosphate and one of CO_2 are converted to two molecules of 3-phosphoglycerate. This reaction is catalysed by the enzyme ribulose-bisphosphate carboxylase/oxygenase (Rubisco E.C.4.1.1.39). Beside the carboxylation reaction, this enzyme reacts with oxygen to form one molecule of 2-phosphoglycolate and one of 3-phosphoglycerate (**Figure 2**). The affinity of Rubisco differs greatly to the two substrates (K_m value for CO_2 is 9 μmol L^{-1}; for O_2 it is 535 μmol L^{-1}), but as the atmospheric concentration of O_2 is 21% and that of CO_2 is 0.0316%, during photosynthesis in a leaf at 25°C the ratio of oxygenation to carboxylation is in the range of 1:4 to 1:2. The recycling of the byproduct 2-phosphoglycolate in the photorespiratory cycle requires 10 enzymatic steps in three different subcellular compartments (chloroplasts, peroxisomes and mitochondria) and consumes a considerable amount of photosynthetic energy in form of ATP and NADPH. Whereas the fixation of 1 mol CO_2 expends 3 mol ATP and 2 mol NADPH, the oxygenation reaction costs 5 mol ATP

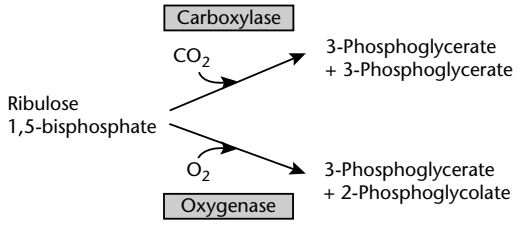

Figure 2 Carboxylase and oxygenase reaction of Rubisco.

and 3 mol NADPH per mol O_2. These data illustrate that the consumption of ATP and NADPH, required to compensate for the consequences of oxygenation, is much higher than the ATP and NADPH expenditure for carboxylation. If the ratio of oxygenation to carboxylation amounts to 1:2, 83% more ATP and 75% more NADPH are consumed than in CO_2 fixation without oxygenation.

In plants and cyanobacteria, Rubisco consists of eight identical large subunits with molecular mass 51–58 kDa and eight identical small subunits of 12–18 kDa. The large subunit is encoded in the plastid genome and the small subunit in the nucleus. The large subunit carries the catalytic centre. In purple bacteria, Rubisco consists of a dimer two large subunits only. Evidently, the function of the small subunit is not essential for catalysis. It has been proposed that the eight small subunits stabilize the complex of the eight large subunits.

The catalysis of the carboxylation of RuBP is very slow. The turnover number for each subunit amounts to 3.3 s^{-1}, which is 10^3 to 10^5 times lower than for other enzymes such as dehydrogenases or carbonic anhydrase, respectively. This unfavourable kinetic behaviour requires large amounts of enzyme to catalyse the fluxes necessary to allow rapid photosynthesis. In leaves, Rubisco can amount to 50% of the total soluble protein and the concentration of catalytic centres is as high as about 4 mmol L^{-1}. The concentration of the substrate CO_2 is about 10 μmol L^{-1}, leading to the abnormal situation that the concentration of catalytic centres is orders of magnitude above the concentration of its substrate.

Activation of ribulose-bisphosphate carboxylase

Rubisco is only active when the ε-amino group of lysine 201 reacts with CO_2 to form a carbamate to which an Mg^{2+} ion is bound. Activation by carbamoylation leads to a change in the conformation of the large subunit. The active conformation is stabilized by the complex formation with Mg^{2+}. This carbamoylation is prerequisite for the activity of all known Rubisco proteins. It should be noted that the CO_2 bound as carbamate is different from the CO_2 that is a substrate of the carboxylation reaction of Rubisco.

When carbamoylation occurs in a nonenzymatic process, it is very slow and requires an unphysiologically high CO_2 concentration. In chloroplasts, carbamoylation is accelerated by the enzyme Rubisco activase upon consumption of ATP. The mechanism of the action of Rubisco activase is not fully understood. The noncarbamoylated Rubisco binds RuBP very tightly and this binding prevents carbamoylation. The activase probably promotes activation of Rubisco by facilitating the release of the tightly bound RuBP.

2-Carboxyarabinitol 1-phosphate (CA1P), a strong inhibitor of Rubisco, was found in a number of plant species. Its affinity to the RuBP binding site is thousandfold higher than that of RuBP. CA1P accumulates in leaves during the night and inactivates the enzyme by blocking the binding sides. Release of CA1P during the day is facilitated by the Rubisco activase. This mechanism explains the inactivation of Rubisco during the night, but since CA1P is not formed in all plant species, its physiological role is still a matter of debate.

Reduction of 3-Phosphoglycerate

The products of the light reactions, NADPH and ATP, are required for the reduction of 3-phosphoglycerate (3PGA) to form D-glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, collectively termed triose phosphates. Three enzymes are involved in this reaction sequence (**Figure 3**). First, 3PGA is phosphorylated by the enzyme phosphoglycerate kinase to form glycerate 1,3-bisphosphate. This reaction requires one ATP per molecule of 3-PGA. The phosphate group is bound to the carboxyl group and forms a mixed anhydride. As the free energy of this anhydride is high and similar to that of the phosphate anhydride in ATP, the phosphoglycerate kinase reaction is reversible.

The product glycerate 1,3-bisphosphate is reduced by the NADP-dependent glyceraldehyde phosphate dehydrogenase. When the carboxyl group is reduced to an aldehyde group by the consumption of NADPH, the bound phosphate residue is released. The product glyceraldehyde 3-phosphate is in equilibrium with dihydroxyacetone phosphate catalysed by the triose-phosphate isomerase. Owing to the equilibrium constant of this reaction, about

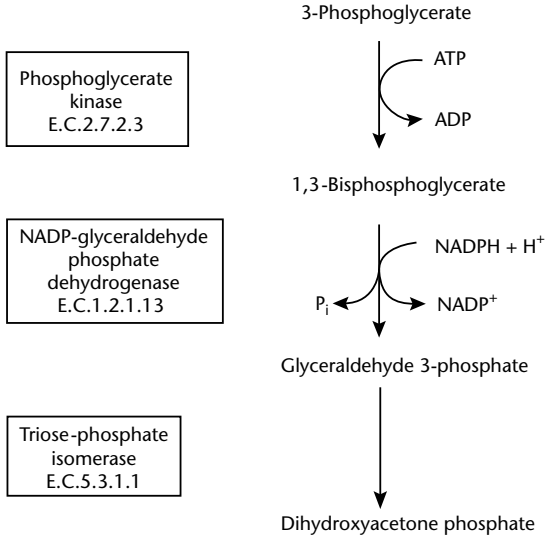

Figure 3 Conversion of 3-phosphoglycerate to triose phosphate.

96% of the triose phosphates are in the form of dihydroxyacetone phosphate.

Isoenzymes of these three enzymes occur in the glycolytic pathway. The main difference is that in the glycolytic pathway the cofactor of the glyceraldehyde-phosphate dehydrogenase is NAD, and in the chloroplast Calvin cycle it is NADP. In the cytosol, the direction of this reaction sequence is mainly towards oxidation of glyceraldehyde 3-phosphate, whereas in the chloroplast glycerate 1,3-bisphosphate is reduced. This is an example of the different roles of NAD and NADP. Usually the ratio of NADPH to NADP is about a hundred times higher than that of NADH to NAD. NADPH donates reducing equivalents for synthesis processes, as NAD collects reducing equivalents from substrate oxidation.

Regeneration of Ribulose Bisphosphate

The metabolism of triose phosphates is a branching point of the Calvin cycle. Only 1/6 of the triose phosphates are actual gain and can be provided to the cell for various biosynthetic processes. This portion of the triose phosphates is exported from the chloroplast via the triose phosphate translocator, the main protein of the inner chloroplast envelope membrane (**Figure 1**), and is further metabolized in the cytosol, e.g. to sucrose to be exported from the mesophyll cells by phloem transport. The other 5/6 is needed to regenerate the CO_2 acceptor RuBP. Three types of enzymes are involved to convert five molecules of triose phosphates into three pentose phosphates: aldolase, bisphosphatases and transketolase (**Figure 4**). The reactions of aldolase and transketolase are reversible, those of the bisphosphatases irreversible. Aldolase catalyses the condensation of an aldose phosphate and a ketose phosphate, forming a ketose bisphosphate. The phosphoryl group of the C1 position is cleaved by a bisphosphatase. Transketolase transfers a C2 unit from a ketose phosphate to an aldose phosphate, releasing an aldose phosphate with two missing carbons and a ketose phosphate with two additional carbon atoms.

The first step is the condensation of dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (Gald3) by aldolase (E.C.4.1.2.13) resulting in the formation of fructose 1,6-bisphosphate. As an intermediate of this reaction, a protonated Schiff base is formed between a lysine residue in the active centre of the enzyme and the keto group of the dihydroxyacetone phosphate. The enzyme fructose bisphosphatase (E.C.3.1.3.11) catalyses the release of the P of the C1 position forming fructose 6-phosphate. Transketolase (E.C.2.2.11) transfers a C2 unit to a second Gald3, releasing the aldose phosphate erythrose 4-phosphate and the first pentose phosphate, xylulose 5-phosphate. In this reaction thiamin pyrophosphate is involved as a prosthetic group that binds the C2 group and transfers it to the aldose phosphate.

One of the products of the transketolase reaction, erythrose 4-phosphate, is substrate for the second aldolase reaction. It condenses with DHAP, which leads to sedoheptulose 1,7-bisphosphate. This is dephosphorylated by the action of sedoheptulose bisphosphatase (E.C.3.1.3.37) and subsequently a C2 group of the product sedoheptulose 7-phosphate is transferred to Gald3 by transketolase. This leads to the formation of the two pentose phosphates, xylulose 5-phosphate and ribose 5-phosphate.

The three pentose phosphates formed are then converted to ribulose 5-phosphate. The OH-group of the C3 position of xylulose 5-phosphate is epimerized by ribulose-5-phosphate epimerase (E.C.5.1.31) and the aldose ribose 5-phosphate is converted to the ketose ribulose 5-phosphate by ribose-phosphate isomerase (E.C.5.3.1.6). Finally an ATP-dependent phosphorylation is catalysed

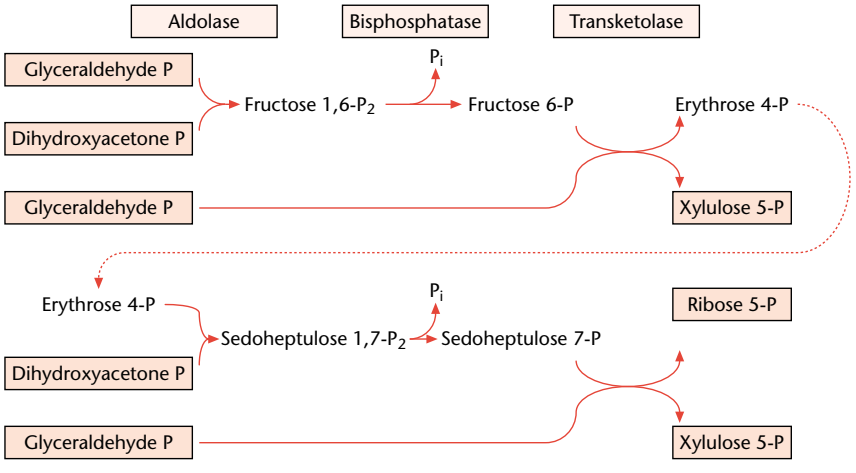

Figure 4 Regeneration of pentose phosphates from triose phosphates.

by ribulose-phosphate kinase (E.C.2.7.1.19). This reaction is irreversible as the energy-rich anhydride in the ATP is converted to a phosphate ester in ribulose 1,5-bisphosphate.

Light-dependent Regulation of the Calvin Cycle

In leaf cells NADPH and ATP can be generated by the light-dependent electron transport or by the oxidation of carbohydrates. When CO_2 assimilation in the Calvin cycle would proceed at the expense of carbohydrate oxidation, a futile cycle would occur, wasting energy without net CO_2 uptake. This is prevented by metabolic regulation, which ensures that key enzymes of the Calvin cycle are active only during illumination, when light-dependent NADPH and ATP production proceeds. Main targets of this light modulation are those enzymes that are catalysing irreversible reactions: Rubisco, fructose bisphosphatase, sedoheptulose bisphosphatase and ribulose-5-phosphate kinase. Possible mechanisms for the light-dependent activation of Rubisco are discussed in a previous section of this article and will be excluded in the following.

Redox modulation of enzymes by thioredoxin

An important signal for illumination is the thioredoxin-dependent modulation of Calvin cycle enzymes. Thioredoxins form a family of small soluble proteins of about 100 amino acids. Common to this family is an amino acid sequence of cysteine-glycine-proline-cysteine in their catalytic centre. The cysteine groups can be present in two different redox states: the reduced thioredoxin with two SH groups and the oxidized form in which the two cysteines form a disulfide (S–S) bridge. Electrons for the reduction of thioredoxins origin from the electron transport chain and are transferred from ferredoxin by the ferredoxin–thioredoxin reductase. Reduced thioredoxins function as protein disulfide oxidoreductases (**Figure 5**).

Four enzymes of the Calvin cycle are activated by light-dependent reduction with thioredoxin: three enzymes that catalyse irreversible reactions, namely fructose bisphosphatase, sedoheptulose bisphosphatase and ribulose-phosphate kinase, and additionally NADP-glyceraldehyde phosphate dehydrogenase. Two other chloroplast enzymes, F-ATP synthase (E.C.3.6.1.34) and NADP–malate dehydrogenase (E.C.1.1.1.82) are also activated by reduced thioredoxin. The F-ATP synthase uses the proton gradient of the thylakoid membrane to generate ATP. The light regulation prevents ATP being hydrolysed by reversible reaction of F-ATP synthase during darkness, when the proton gradient is diminished. The NADP-malate dehydrogenase is involved in the export of surplus reducing equivalents out of the chloroplast. When the ATP

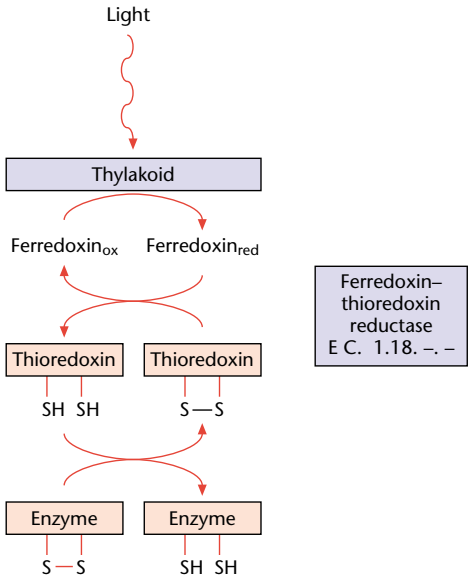

Figure 5 The thioredoxin-mediated light modulation of chloroplast enzymes.

demand of the chloroplast exceeds its production by the electron transport, NADPH accumulates. Under this condition NADPH is reoxidized by the NADP–malate dehydrogenase and the reducing equivalents are exported into the cytosol.

Reduction of enzymes by thioredoxin can also lead to inactivation. Glucose-6-phosphate dehydrogenase, the key enzyme of the oxidative pentose phosphate pathway providing NADPH by oxidation of glucose phosphates, is inactivated in this way. The thioredoxin-dependent inactivation of glucose-6-phosphate dehydrogenase ensures that the oxidative pentose phosphate pathway does not operate simultaneously with the reductive pentose phosphate pathway, thus avoiding a futile cycle.

Mechanism of thioredoxin activation

For an understanding of the mechanism of the thioredoxin-mediated activation, a comparison has been made of the amino acid sequences between the chloroplast and cytosolic isoenzymes of fructose bisphosphatase and malate dedydrogenase and of the mitochondrial F-ATPase. These studies show that most of the amino acid sequences of the corresponding enzymes are homologous, but the chloroplast isoenzymes possess additional sections at the end or in the inner region of their sequence containing two cysteine residues. The SH groups of these cysteine residues can be converted by oxidation to a disulfide and represent the targets for the thioredoxin-dependent reduction. The extrachloroplastic isoenzymes, which lack these cysteine groups, are often more active. If

one of these regulatory cysteine residues of the chloroplast form is modified by genetic engineering, the resulting enzyme is as active as the extrachloroplastic form and the activity is independent on the presence of thioredoxin. From these mutation studies it can be concluded that oxidation of the corresponding enzymes forces them into a conformation in which the catalytic centre is inactivated. Reduction of the disulfide bridge by thioredoxin releases this blockage and the enzymes become active.

This light-activation mechanism is not only an on/off switch for enzymes. When an enzyme is reduced by thioredoxin, it is simultaneously reoxidized by oxygen in a nonenzymatic reaction. That portion of the enzyme that is in the reduced state varies with changing redox state of thioredoxin. A continuous reduction and reoxidation of the enzyme allows coordination between the supply of redox equivalents by the electron transport chain and the activation state of the target enzymes in the Calvin cycle.

Effect of magnesium and stromal pH on light activation

Redox activation is an efficient but not the sole way to transfer the light signal to target enzymes of the Calvin cycle. Other light-dependent signals generated by the electron transport chain in the thylakoid membrane are decrease of the proton concentration and increase in Mg^{2+} concentration in the stroma. These changes influence the activation state of some key enzymes in the Calvin cycle. When isolated chloroplasts are illuminated, acidification of the thylakoid space is accompanied by an alkalization of the stroma from pH 7.2 in the dark to pH 8.0 in the light. Additionally, the concentration of Mg^{2+} in the stroma increases from 3 mmol L^{-1} to 7 mmol L^{-1}. Fructose and sedoheptulose bisphosphatases, which are regulated by thioredoxin, respond to changing pH and Mg^{2+} concentrations. Both are nearly inactive at pH 7.2 and low Mg^{2+} and their activity increase with rising pH and Mg^{2+} concentration. Together with the thioredoxin system, these mechanisms of light activation are very efficient for switching Calvin cycle enzymes on and off according to demand.

Flux control of activated enzymes is achieved by metabolites

When the Calvin cycle is active as the result of the light-dependent activation of the key enzymes, the phosphorylated intermediates of the cycle accumulate in the chloroplast and the content of inorganic phosphate (P_i) decreases. On the other hand, P_i is required as the substrate for the F-ATP synthase. To avoid inhibition of photophosphorylation by substrate limitation, a certain level of P_i has to be maintained in the stroma. This requires a coordination of activities of the key enzymes, which is achieved by regulatory effects of intermediates.

The stromal enzymes fructose and sedoheptulose bisphosphatase are activated by their substrates and allosterically inhibited by their products. High levels of fructose 1,6-bisphosphate or sedoheptulose 1,7-bisphosphate indicate that the activation states of the enzymes are too low to hydrolyse their substrates and lead to higher activities. Fructose 6-phosphate or sedoheptulose 7-phosphate allosterically inhibit the activity of the corresponding enzyme to avoid excessive product accumulation and sequestration of phosphate.

Ribulose phosphate kinase is inhibited by its two metabolites 3PGA and ADP. In the Calvin cycle two reactions compete for ATP: ribulose-phosphate kinase, which catalyses an irreversible reaction, and the reversible reaction catalysed by phosphoglycerate kinase. When ATP supply is limited, the irreversible phosphorylation of ribulose 5-phosphate at the expense of the 3PGA phosphorylation would be an advantage. This would result in an accumulation of 3PGA and ADP. As both metabolites inhibit ribulose-phosphate kinase, phosphorylation of phosphoglycerate kinase can proceed even under limiting ATP supply and accumulation of 3PGA is prevented.

The regulation of Rubisco is not well understood. The degree of activation characterized by the degree of carbamoylation is changing with changes in light intensity. This is probably achieved by the action of Rubisco activase, which is dependent on the ATP to ADP ratio (see above). But other observations suggest that this cannot be the only mechanism involved. Rubisco activity increases with higher pH and Mg^{2+} concentration and is inhibited by its product, 3PGA. These effects indicate that Rubisco activity is modulated by similar signals to those for the other regulated enzymes, but a conclusive mechanism for light activation and fine regulation is still missing.

Further Reading

Heldt HW (1997) *Plant Biochemistry and Molecular Biology*. Oxford: Oxford University Press.

Portis AR (1992) Regulation of ribulose 1,5-bisphosphate carboxylase/oxygenase activity. *Annual Review of Plant Physiology and Plant Molecular Biology* **43**: 415–437.

Quick WP and Neuhaus E (1997) The regulation and control of photosynthetic carbon assimilation. In: Foyer CH and Quick WP (eds) *A Molecular Approach to Primary Metabolism in Higher Plants*, pp. 41–62. London: Taylor and Francis.

Scheibe R (1992) Light/dark modulation: regulation of chloroplast metabolism in a new light. *Botanica Acta* **103**: 327–334.

Sharkey TD (1998) Photosynthetic carbon reduction. In: Raghavendra AS (ed.) *Photosynthesis*, pp. 111–122. Cambridge: Cambridge University Press.