6

The Calvin cycle catalyzes photosynthetic CO_2 assimilation

Chapters 3 and 4 showed how the electron transport chain and the ATP synthase of the thylakoid membrane use the energy from light to provide reducing equivalents in the form of NADPH, and chemical energy in the form of ATP. This chapter will describe how NADPH and ATP are used for CO_2 assimilation.

6.1 CO_2 assimilation proceeds via the dark reaction of photosynthesis

It is relatively simple to isolate chloroplasts with intact envelope from leaves (see section 1.7). Upon transfer of these chloroplasts to an isotonic medium containing an osmoticum, a buffer, bicarbonate, and inorganic phosphate, and the light is switched on, the generation of oxygen can be observed. By the action of light water is split and oxygen evolved, and the resulting reducing equivalents are used for CO_2 assimilation (Fig. 6.1, see also Chapter 3). There is no oxygen evolution with intact chloroplasts in the absence of CO_2 or phosphate, demonstrating that the light reaction in the intact chloroplasts is (a) coupled to CO_2 assimilation and (b) the product of this assimilation contains phosphate. The main assimilation product of the chloroplasts is **dihydroxyacetone phosphate**, a **triose phosphate**. Figure 6.2 shows that the synthesis of triose phosphate from CO_2 requires energy as ATP and reducing equivalents as NADPH, which have been provided by the **light reaction of photosynthesis**. The reaction chain for the formation of triose phosphate from CO_2, ATP, and NADPH was formerly called

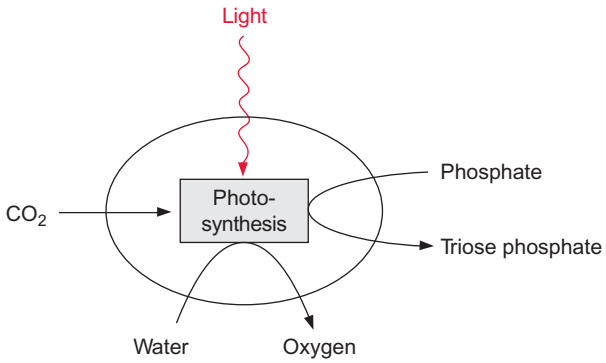


Figure 6.1 Schematic presentation of photosynthesis in a chloroplast.

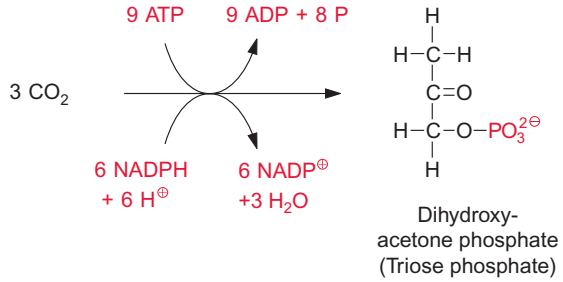


Figure 6.2 Overall reaction of photosynthetic CO_2 fixation.

the **dark reaction of photosynthesis**, as it requires no light *per se* and theoretically it should also be able to proceed in the dark. The fact is, however, that in leaves this reaction does not proceed during darkness, since some of the enzymes of the reaction chain, due to regulatory processes, are active only during illumination (section 6.6).

Between 1946 and 1953 Melvin Calvin and his collaborators Andrew Benson and James Bassham, in Berkeley, California, resolved the mechanism of photosynthetic CO_2 assimilation. In 1961 Calvin was awarded the Nobel Prize in Chemistry for this fundamental discovery. A prerequisite for the elucidation of the CO_2 fixation pathway was the discovery of the radioactive carbon isotope ^{14}C in 1940, which, as a by-product of nuclear reactors, was available in larger amounts in the United States after 1945. Calvin chose the green alga *Chlorella* for his investigations. He added radioactively labeled CO_2 to illuminated algal suspensions, killed the algae after a short incubation period by adding hot ethanol, and used paper chromatography to analyze the radioactively labeled products of the CO_2 fixation. By successively shortening the incubation time, he was able to show that 3-phosphoglycerate was synthesized as the first stable product of CO_2 fixation. More detailed studies revealed that CO_2 fixation proceeds by a cyclic process,

which has been named the **Calvin cycle** after its discoverer. **Reductive pentose phosphate pathway** is another term that will be used in some sections of this book. This name derives from the fact that a reduction occurs and pentoses are formed in the cycle.

The Calvin cycle can be subdivided into three sections:

1. The **carboxylation** of the C_5 sugar ribulose 1,5-bisphosphate leading to the formation of two molecules 3-phosphoglycerate;
2. The **reduction** of the 3-phosphoglycerate to triose phosphate; and
3. The **regeneration** of the CO_2 acceptor ribulose 1,5-bisphosphate from triose phosphate (Fig. 6.3).

As a product of photosynthesis, triose phosphate is exported from the chloroplasts into the cytosol by specific transport. However, most of the triose phosphate remains in the chloroplasts to regenerate ribulose 1,5-bisphosphate. These reactions will be discussed in detail in the following sections.

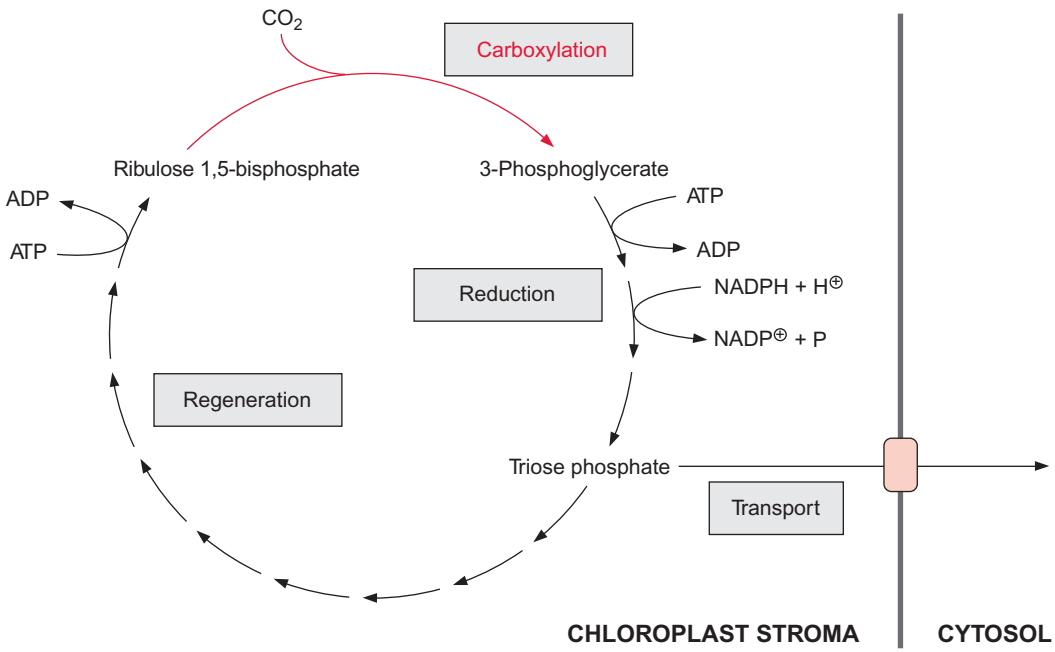


Figure 6.3 Simplified overview of the reactions of the Calvin cycle (without stoichiometries).

6.2 Ribulose bisphosphate carboxylase catalyzes the fixation of CO_2

The key reaction for photosynthetic CO_2 assimilation is the binding of atmospheric CO_2 to the acceptor ribulose 1,5-bisphosphate (RuBP) to synthesize two molecules of 3-phosphoglycerate. The reaction is very exergonic (ΔG^o − 35 kJ/mol) and therefore virtually irreversible. It is catalyzed by the enzyme ribulose bisphosphate carboxylase/oxygenase (abbreviated **RubisCO**). It is also called oxygenase because the same enzyme also catalyzes a side-reaction in which the ribulose bisphosphate reacts with O_2 (Fig. 6.4).

Figure 6.5 shows the reaction sequence of the **carboxylase reaction**. Keto-enol isomerization of RuBP yields an enediol, which reacts with CO_2 to form the intermediate 2-carboxy 3-ketoarabinitol 1,5-bisphosphate, which is cleaved to two molecules of 3-phosphoglycerate. In the **oxygenase reaction**, an unavoidable by-reaction, probably O_2, reacts in a similar way as CO_2 with the enediol to form a peroxide as an intermediate. In a subsequent cleavage of the O_2 adduct, one atom of the O_2 molecule is released in the form of water and the other is incorporated into the carbonyl group of 2-phosphoglycolate (Fig. 6.6). The final products of the oxygenase reaction are 2-phosphoglycolate and 3-phosphoglycerate.

Ribulose bisphosphate-carboxylase/oxygenase **is the only enzyme that enables the fixation of atmospheric CO_2 for the formation of biomass**. This

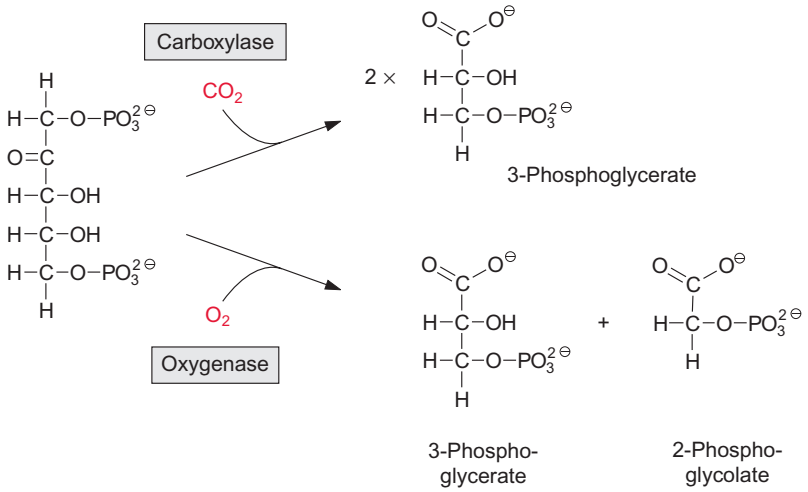


Figure 6.4 Ribulose bisphosphate carboxylase catalyzes two reactions with the substrate RuBP: the carboxylation, which is the actual CO_2 fixation reaction; and the oxygenation, an unavoidable side-reaction.

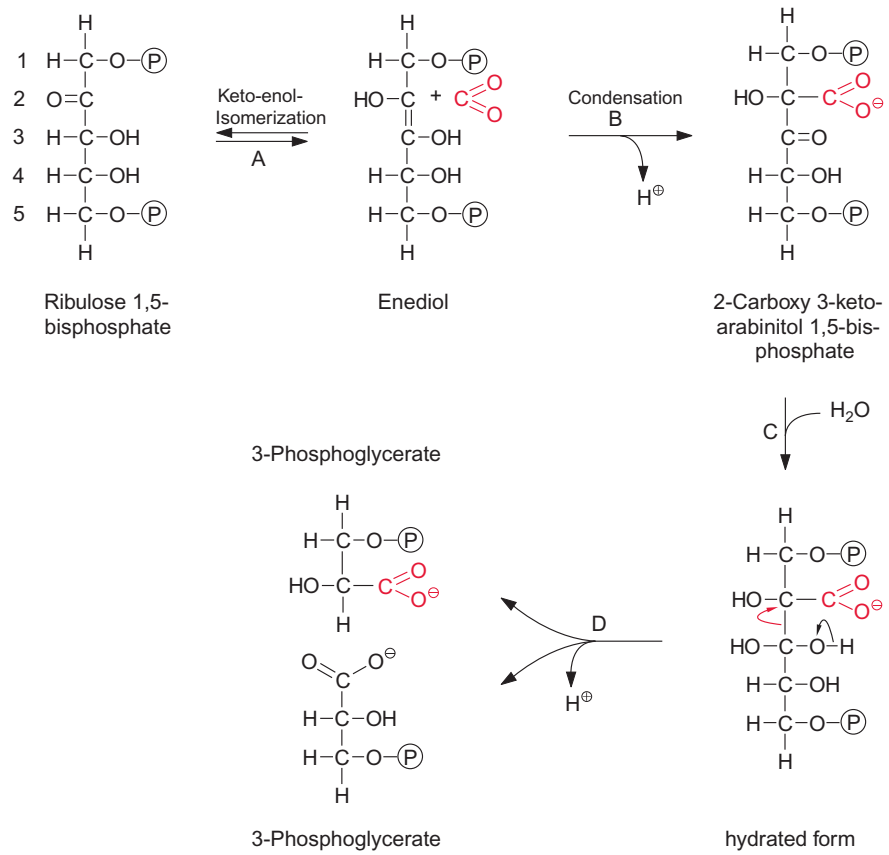


Figure 6.5 Reaction sequence of the carboxylation of RuBP by RubisCO. For the sake of simplicity, $-PO_3^{2-}$ is symbolized as -P. An enediol, formed by keto-enol-isomerization of the carbonyl group of the RuBP (A), allows the nucleophilic reaction of CO_2 with the C-2 atom of RuBP by which 2-carboxy 3-ketoarabinitol 1,5-bisphosphate (B) is synthesized. After hydration (C), the bond between C-2 and C-3 is cleaved and two molecules of 3-phosphoglycerate are released (D).

enzyme is therefore a prerequisite for the existence of the present life on earth. In plants and cyanobacteria it consists of eight identical **large subunits** (depending on the species of a molecular mass of 51–58 kDa) and eight identical **small subunits** (molecular mass 12–18 kDa). **With its 16 subunits, RubisCO is one of the largest enzymes in nature**. In plants the genetic information for the large subunit is encoded in the plastid genome and for the small subunit in the nucleus. Each large subunit contains one catalytic center. The function of the small subunits is not yet fully understood. It has been suggested that the eight small subunits stabilize the complex of the eight large subunits. Apparently the small subunit is not essential for the process of CO_2 fixation *per se*. RubisCO occurs in some phototrophic

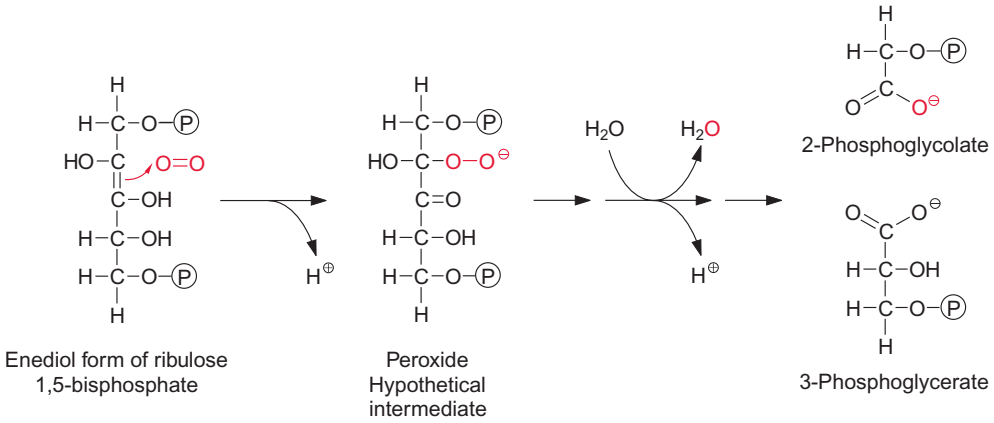


Figure 6.6 Oxygenation reaction of RubisCO with the substrate RuBP. P symbolizes $-PO_3^{2-}$.

purple bacteria as a dimer of only large subunits, but the catalytic properties of the corresponding bacterial enzymes are not basically different from those in plants. The bacterial enzymes consisting of only two large subunits, however, exhibit a higher ratio of oxygenase versus carboxylase activity than the plant enzymes, which consist of eight large and eight small subunits.

The oxygenation of ribulose bisphosphate: a costly side-reaction

Although the CO_2 concentration required for half saturation of the enzyme (K_M [CO_2]) is much lower than that of O_2 (K_M [O_2]) (Table 6.1), the velocity of the oxygenase reaction is very high. This high velocity is a consequence of the different atmospheric concentrations; the concentration of O_2 in air amounts to 21% and that of CO_2 to only 0.035%. Moreover, the CO_2 concentration in the gaseous space of the leaves can be considerably lower than the CO_2 concentration in the atmosphere. For these reasons, the ratio of oxygenation to carboxylation during photosynthesis of a leaf at 25°C is in the range of **1:4** to **1:2**, which implies *that every third to fifth ribulose 1,5-bisphosphate molecule is consumed in the side-reaction*. When the temperature rises, the CO_2/O_2 specificity of the RubisCO (Table 6.1) decreases, and as a consequence, the ratio of oxygenation to carboxylation increases. On the other hand, a rise in the CO_2 concentration in the atmosphere lowers oxygenation, which in many cases leads to higher plant growth. Moreover, the concentration of CO_2 in water (thus also in cellular water) which is in equilibrium with the atmospheric concentration decreases with increasing temperature more strongly than that of O_2. Both effects result in an increase

Table 6.1: Kinetic properties of ribulose bisphosphate carboxylase/oxygenase (RubisCO) at 25°

Substrate concentrations at half saturation of the enzyme

K_M [CO_2] : 9 μmol/L*

K_M [O_2] : 535 μmol/L*

K_M (RuBP) : 28 μmol/L

Maximal turnover (related to one subunit)

Kcat [CO_2] : 3.3 s^{-1}

Kcat [O_2] : 2.4 s^{-1}

$$CO_2/O_2 \text{ specificity} = \left(\frac{K\text{cat}[CO_2]}{K_M[CO_2]} \Big/ \frac{K\text{cat}[O_2]}{K_M[O_2]} \right) = 82$$

* For comparison:

In equilibrium with air (0.035% = 350 ppm CO_2, 21% O_2) the concentrations in water at 25°C amounts to

CO_2 : 11 μmol/L

O_2 : 253 μmol/L

(Data from Woodrow and Berry, 1988)

of the oxygenation/carboxylation ratio due to the increasing temperature. In greenhouses the oxygenation can be decreased by an artificial increase of the atmospheric CO_2 concentration to obtain higher plant growth.

It will be shown in Chapter 7 that recycling of the by-product 2-phosphoglycolate, produced in very large amounts, is a very costly process for plants. This recycling process requires a metabolic chain with more than 10 enzymatic reactions distributed over three different organelles (chloroplasts, peroxisomes, and mitochondria), as well as very high energy consumption. Section 7.5 describes in detail that about a **third of the photons absorbed** during the photosynthesis of a leaf are consumed to reverse the consequences of oxygenation.

Apparently evolution has not been successful in eliminating this costly side-reaction of ribulose bisphosphate carboxylase. The ratio of the carboxylase and oxygenase activities of RubisCO is only increased by a factor of less than two when enzymes of cyanobacteria and of higher plants are compared. It seems as if the evolutionary refinement of a key process of life has reached its limitation due to the chemistry of the reaction. It is speculated that the early evolution of the RubisCO occurred at a time when there was no oxygen in the atmosphere. A comparison of the RubisCO proteins from different organisms leads to the conclusion that this enzyme was already present about three and a half billion years ago, when the first chemolithotrophic bacteria evolved. When more than one and a half billion years later, due to photosynthesis, oxygen appeared in the atmosphere in higher concentrations,

the RubisCO protein probably had reached such a complexity that it was no longer possible to change the catalytic center to eliminate the oxygenase activity. Experimental results support this conception. A large number of experiments, in which genetic engineering was employed to obtain site-specific amino acid exchanges in the region of the active center of RubisCO, were unable to improve the ratio between the activities of carboxylation and oxygenation. The only chance of lowering oxygenation by molecular engineering may lie in simultaneously exchanging several amino acids in the catalytic binding site of RubisCO, which would be an extremely unlikely event in the process of evolution. Section 7.7 will show how plants make a virtue of necessity, and use the energy-consuming oxygenation to eliminate surplus NADPH and ATP produced by the light reaction.

Ribulose bisphosphate carboxylase/oxygenase: special features

The catalysis of the carboxylation of RuBP by RubisCO is very slow (Table 6.1): the turnover number for each subunit amounts to $3.3\,s^{-1}$. This implies that at substrate saturation only about three molecules of CO_2 and RuBP are converted per second at one catalytic site of RubisCO. In comparison, the turnover numbers of dehydrogenases and carbonic anhydrase are in the order of $10^3\,s^{-1}$ and $10^5\,s^{-1}$, respectively. Because of the extremely low turnover number of RubisCO, very large amounts of enzyme protein are required to catalyze the fluxes necessary for photosynthesis. RubisCO can account for **50% of the total soluble proteins** in leaves. The wide distribution of plants makes RubisCO by far the **most abundant protein on earth**. The concentration of the catalytic large subunits in the chloroplast stroma is as high as $4–10 \times 10^{-3}$ mol/L. A comparison of this value with the aqueous concentration of CO_2 in equilibrium with air (at 25°C about 11×10^{-6} mol/L) shows the abnormal situation in which the concentration of an enzyme is up to 1,000 times higher than the concentration of its substrate CO_2 and at a similar concentration as its substrate RuBP.

Activation of ribulose bisphosphate carboxylase/oxygenase

All the large subunits of RubisCO contain a lysine in position 201 of their 470 amino acid long sequence. RubisCO is active only when the ε-amino group of this lysine reacts with CO_2 to form a **carbamate** (carbonic acid amide), to which an Mg^{++} ion is bound (Fig. 6.7). The activation is due to a change in the conformation of the protein of the large subunit. The active conformation is stabilized by the complex formation with Mg^{++}. This carbamylation is a prerequisite for the activity of all known RubisCO proteins.

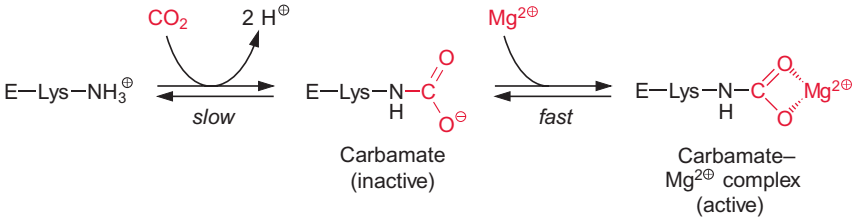


Figure 6.7 RubisCO is activated by the carbamylation of a lysine residue.

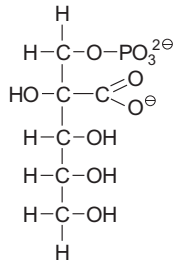


Figure 6.8 2-Carboxyarabinitol 1-phosphate is an inhibitor of RubisCO.

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.

The activation of RubisCO requires ATP and is catalyzed by the enzyme **RubisCO activase**. The noncarbamylated, inactive form of RubisCO binds RuBP very tightly, resulting in the inhibition of the enzyme. Upon the consumption of ATP, the activase releases the tightly bound RuBP and thus enables the carbamylation of the free enzyme. The regulation of RubisCO activase is discussed in section 6.6.

RubisCO is inhibited by several hexose phosphates and by 3-phosphoglycerate, which all bind to the active site instead of RuBP. A very strong inhibitor is **2-carboxyarabinitol 1-phosphate** (CA1P) (Fig. 6.8). This compound has a structure very similar to that of 2-carboxy 3-ketoarabinitol 1,5-bisphosphate (Fig. 6.5), which is an intermediate of the carboxylation reaction. CA1P has a 1,000-fold higher affinity to the RuBP binding site of RubisCO than RuBP. In a number of species, CA1P accumulates in the leaves during the night, blocking a large number of the binding sites of RubisCO and thus inactivating the enzyme. During the day, CA1P is released by RubisCO activase and is degraded by a specific phosphatase, which hydrolyzes the phosphate residue from CA1P and thus eliminates the effect of the RubisCO inhibitor. CA1P is synthesized from fructose 1.6-bisphosphate with the intermediates hexosephosphates hamamelose bisphosphate and hamamelose monophosphate. Since CA1P is not formed in all plants, its role in the regulation of RubisCO is still a matter of debate.

6.3 The reduction of 3-phosphoglycerate yields triose phosphate

For the synthesis of dihydroxyacetone phosphate the carboxylation product 3-phosphoglycerate is phosphorylated to 1,3-bisphosphoglycerate by the enzyme **phosphoglycerate kinase**. In this reaction, with the consumption of ATP, a mixed anhydride is formed between the new phosphate residue and the carboxyl group (Fig. 6.9). As the free energy for the hydrolysis of this anhydride is similarly high to that of the phosphate anhydride in ATP, the phosphoglycerate kinase reaction is reversible. An isoenzyme of the chloroplast phosphoglycerate kinase is also involved in the glycolytic pathway proceeding in the cytosol, where it catalyzes the formation of ATP from ADP and 1,3-bisphosphoglycerate (section 13.3).

The reduction of 1,3-bisphosphoglycerate to D-glyceraldehyde 3-phosphate is catalyzed by the enzyme **glyceraldehyde phosphate dehydrogenase** (Fig. 6.9). The carboxylic acid phosphoanhydride reacts with an SH-group of a cysteine residue in the active center of the enzyme to form a thioester intermediate with the release of the phosphate group (Fig. 6.10). The free energy for the hydrolysis of the thioester so formed is similarly high to that of the anhydride ("energy-rich bond"). When a thioester is reduced, a thiosemiacetal is formed which has low free energy.

Through the catalysis of phosphoglycerate kinase and glyceraldehyde phosphate dehydrogenase, the large difference in redox potentials between the carboxylate and the aldehyde in the course of the reduction of 3-phosphoglycerate to glyceraldehyde phosphate is overcome by the consumption

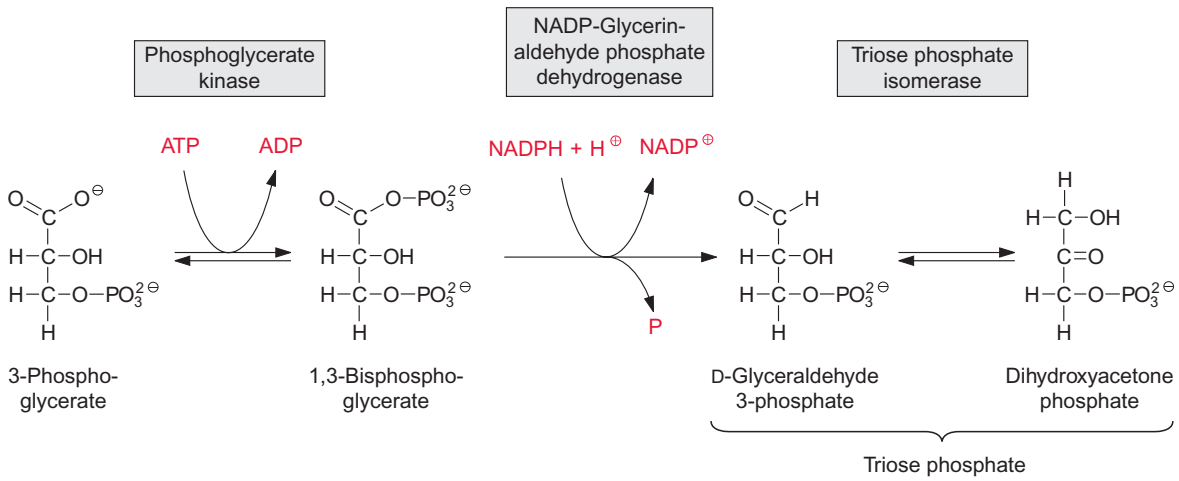


Figure 6.9 Conversion of 3-phosphoglycerate into triose phosphate.

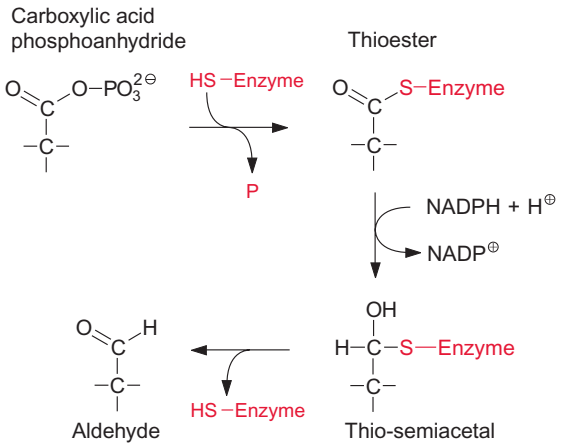


Figure 6.10 Reaction sequence catalyzed by glyceraldehyde phosphate dehydrogenase. HS-enzyme symbolizes the sulfhydryl group of a cysteine residue in the active center of the enzyme.

of ATP. It is therefore a reversible reaction. A glyceraldehyde phosphate dehydrogenase in the cytosol catalyzes the conversion of glyceraldehyde phosphate to 1,3-bisphosphoglycerate as part of the glycolytic pathway (section 13.3). In contrast to the cytosolic enzyme, which catalyzes mainly the oxidation of glyceraldehyde phosphate using NAD^+ as hydrogen acceptor, the chloroplast enzyme uses NADPH as a hydrogen donor.

This is an example of the different roles that the NADH/NAD^+ and NADPH/$NADP^+$ systems play in the metabolism of eukaryotic cells. Whereas the NADH system is specialized in collecting reducing equivalents to be oxidized for the synthesis of ATP, the NADPH system mainly gathers reducing equivalents to be donated to synthetic processes. Figuratively speaking, the NADH system has been compared with a **hydrogen low pressure line** through which reducing equivalents are pumped off for oxidation to generate energy, while the NADPH system is a **hydrogen high pressure line** through which reducing equivalents are pressed into synthesis processes. Usually the reduced/oxidized ratio is about 100 times higher for the NADPH system than for the NADH system. The relatively high degree of reduction of the NADPH system in chloroplasts (about 50–60% reduced) allows the very efficient reduction of 1,3-bisphosphoglycerate to glyceraldehyde-3-phosphate.

Triose phosphate isomerase catalyzes the isomerization of glyceraldehyde phosphate to dihydroxyacetone phosphate. This conversion of an aldose into a ketose proceeds via a 1,2-enediol as intermediate and is basically similar to the reaction catalyzed by ribose phosphate isomerase. The equilibrium of the reaction lies towards the ketone. Triose phosphates, as a collective term, comprise about 96% dihydroxyacetone phosphate and only 4% glyceraldehyde phosphate.

6.4 Ribulose bisphosphate is regenerated from triose phosphate

The fixation of three molecules of CO_2 in the Calvin cycle results in the synthesis of six molecules of phosphoglycerate which are converted to six molecules of triose phosphate (Fig. 6.11). Of these, only one molecule of triose phosphate is the actual gain, which is provided to the cell for various biosynthetic processes. The remaining five triose phosphates are needed to regenerate three molecules of ribulose bisphosphate so that the Calvin cycle can continue. Figure 6.12 shows the metabolic pathway of the conversion of the five triose phosphates (white boxes) to three pentose phosphates (red boxes).

The two trioses dihydroxyacetone phosphate and glyceraldehyde phosphate are condensed in a reversible reaction to fructose 1,6-bisphosphate, as catalyzed by the enzyme **aldolase** (Fig. 6.13). Figure 6.14 shows the reaction mechanism. As an intermediate of this reaction, a protonated Schiff base is formed between a lysine residue of the active center of the enzyme and the keto group of dihydroxyacetone phosphate. This Schiff base enhances the release of a proton from the C-3 position and enables a nucleophilic reaction with the C atom of the aldehyde group of glyceraldehyde phosphate. Fructose 1,6-bisphosphate is hydrolyzed by **fructose 1,6-bisphosphatase** in an irreversible reaction to fructose 6-phosphate (Fig. 6.15).

The enzyme **transketolase** transfers a carbohydrate residue with two carbon atoms from fructose 6-phosphate to glyceraldehyde 3-phosphate yielding xylulose 5-phosphate, and erythrose 4-phosphate in a reversible

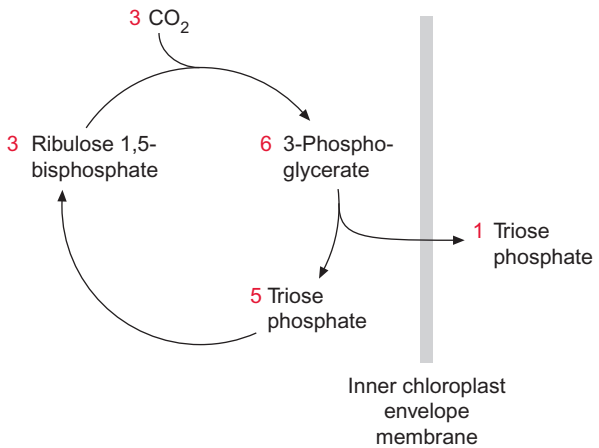


Figure 6.11 Five of the six triose phosphates formed by photosynthesis are required for the regeneration of ribulose 1,5-bisphosphate. One molecule of triose phosphate represents the net product and can be utilized by the chloroplast for biosynthesis or be exported.

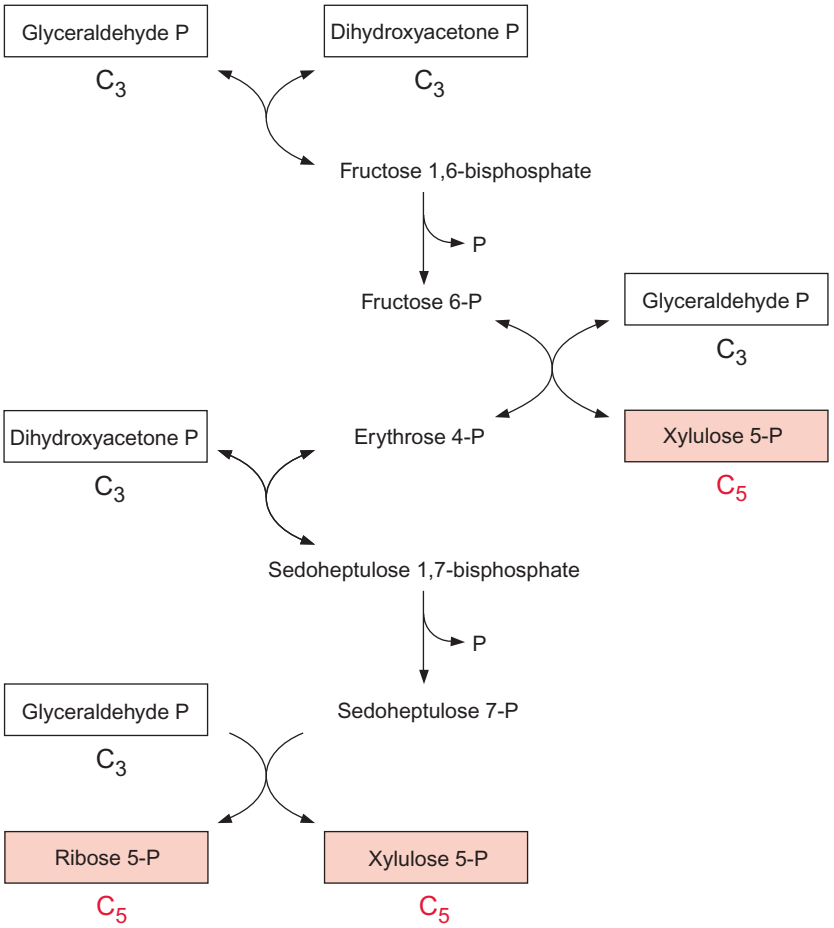


Figure 6.12 Reaction chain for the conversion of five molecules of triose phosphate into three molecules of pentose phosphate. P symbolizes $-PO_3^{2-}$.

reaction (Fig. 6.16). Thiamine pyrophosphate (Fig. 5.5), already discussed as a reaction partner of pyruvate oxidation (section 5.3), is involved as the prosthetic group in this reaction (Fig. 6.17).

Once more an **aldolase** (Fig. 6.13) catalyzes a condensation reaction; erythrose 4-phosphate is combined with dihydroxyacetone phosphate to form sedoheptulose 1,7-bisphosphate. Subsequently, the enzyme **sedoheptulose 1,7-bisphosphatase** catalyzes the irreversible hydrolysis of sedoheptulose 1,7-bisphosphate. This reaction is similar to the hydrolysis of fructose 1,6-bisphosphate, despite the fact that both reactions are catalyzed by different enzymes. Again, a carbohydrate residue of two C atoms is transferred by **transketolase** from sedoheptulose 7-phosphate to dihydroxyacetone phosphate to form ribose 5-phosphate and xylulose 5-phosphate (Fig. 6.16).

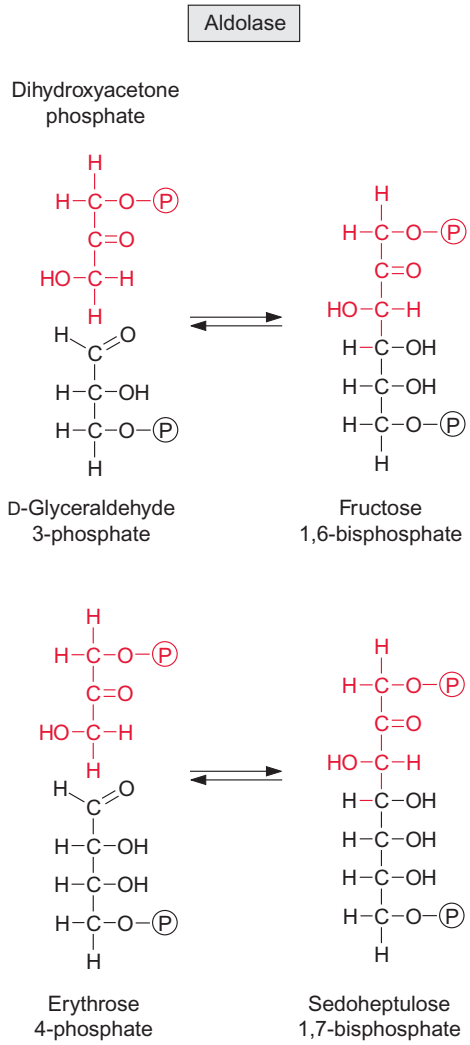


Figure 6.13 Aldolase catalyzes the condensation of dihydroxyacetone phosphate with the aldoses glyceraldehyde 3-phosphate or erythrose 4-phosphate. Ⓟ symbolizes $-PO_3^{2-}$.

The three pentose phosphates synthesized are then converted to ribulose 5-phosphate (Fig. 6.18). The conversion of xylulose 5-phosphate is catalyzed by **ribulose phosphate epimerase**; this reaction proceeds via a keto-enol isomerization and a 2,3-enediol intermediate. The conversion of the aldose ribose 5-phosphate to the ketose ribulose 5-phosphate is catalyzed by **ribose phosphate isomerase**, again via an enediol as intermediate, although in the 1,2-position. The three molecules of ribulose 5-phosphate formed in this way are converted to the CO_2 acceptor ribulose 1,5-bisphosphate upon consumption of ATP by **ribulose phosphate kinase** (Fig. 6.19).

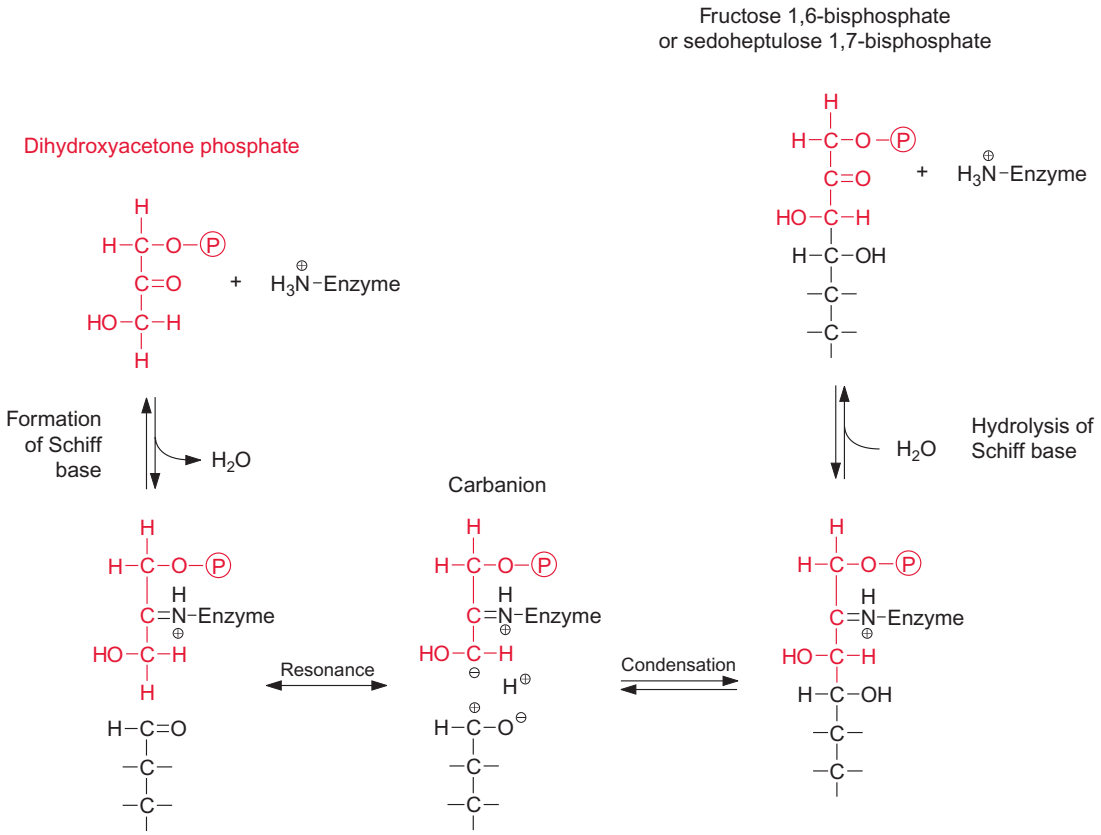


Figure 6.14 Pathway of the aldolase reaction. Dihydroxyacetone phosphate forms a Schiff base with the terminal amino group of a lysine residue of the enzyme protein. The positive charge at the nitrogen atom favors the release of a proton at C-3, and thus a carbanion is formed. In one mesomeric form of the glyceraldehyde phosphate, the C atom of the aldehyde group is positively charged. This enables condensation between this C atom and the negatively charged C-3 of the dihydroxyacetone phosphate. After condensation, the Schiff base is cleaved again and fructose 1,6-bisphosphate is released. Sedoheptulose 1,7-bisphosphate is synthesized by the same enzyme which catalyzes the reaction with erythrose 4-phosphate. The aldolase reaction is reversible. P symbolizes $-PO_3^{2-}$.

This kinase reaction is irreversible, since a phosphate of the "energy-rich" anhydride in the ATP is converted to a phosphate ester with a low free energy of hydrolysis.

The scheme in Figure 6.20 presents a summary of the various reactions of the Calvin cycle. Four irreversible steps are indicated in the cycle (bold arrows): carboxylation, hydrolysis of fructose- and sedoheptulose bisphosphate,

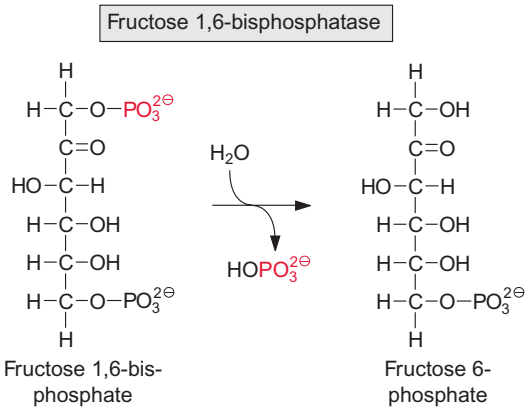


Figure 6.15 Hydrolysis of fructose 1,6-bisphosphate by fructose 1,6-bisphosphatase.

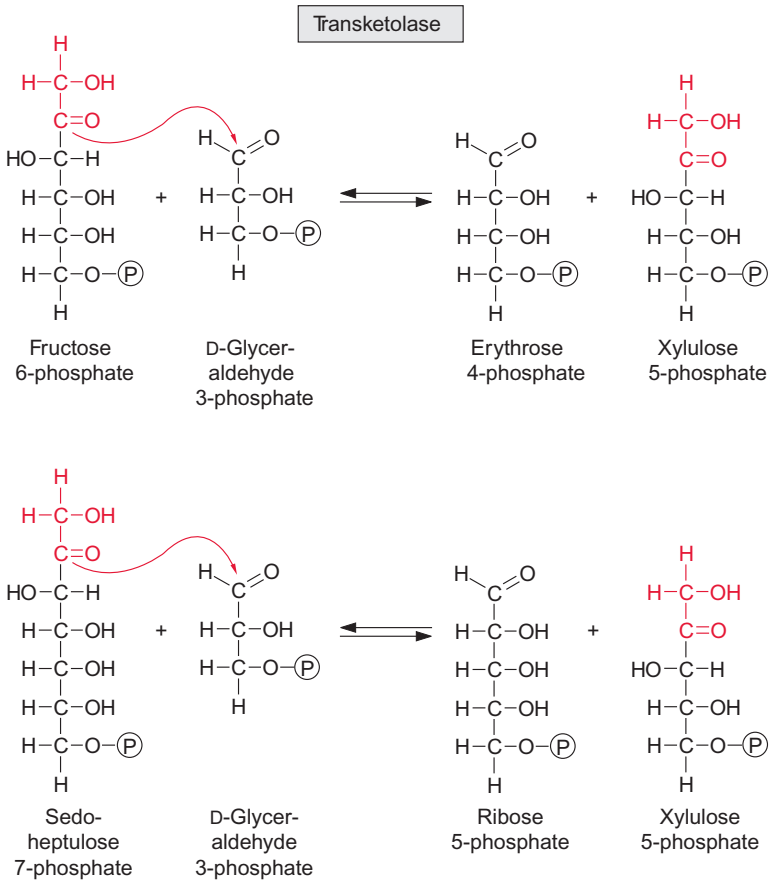


Figure 6.16 Transketolase catalyzes the transfer of a C_2 moiety from ketoses to aldoses. P symbolizes $-PO_3^{2-}$.

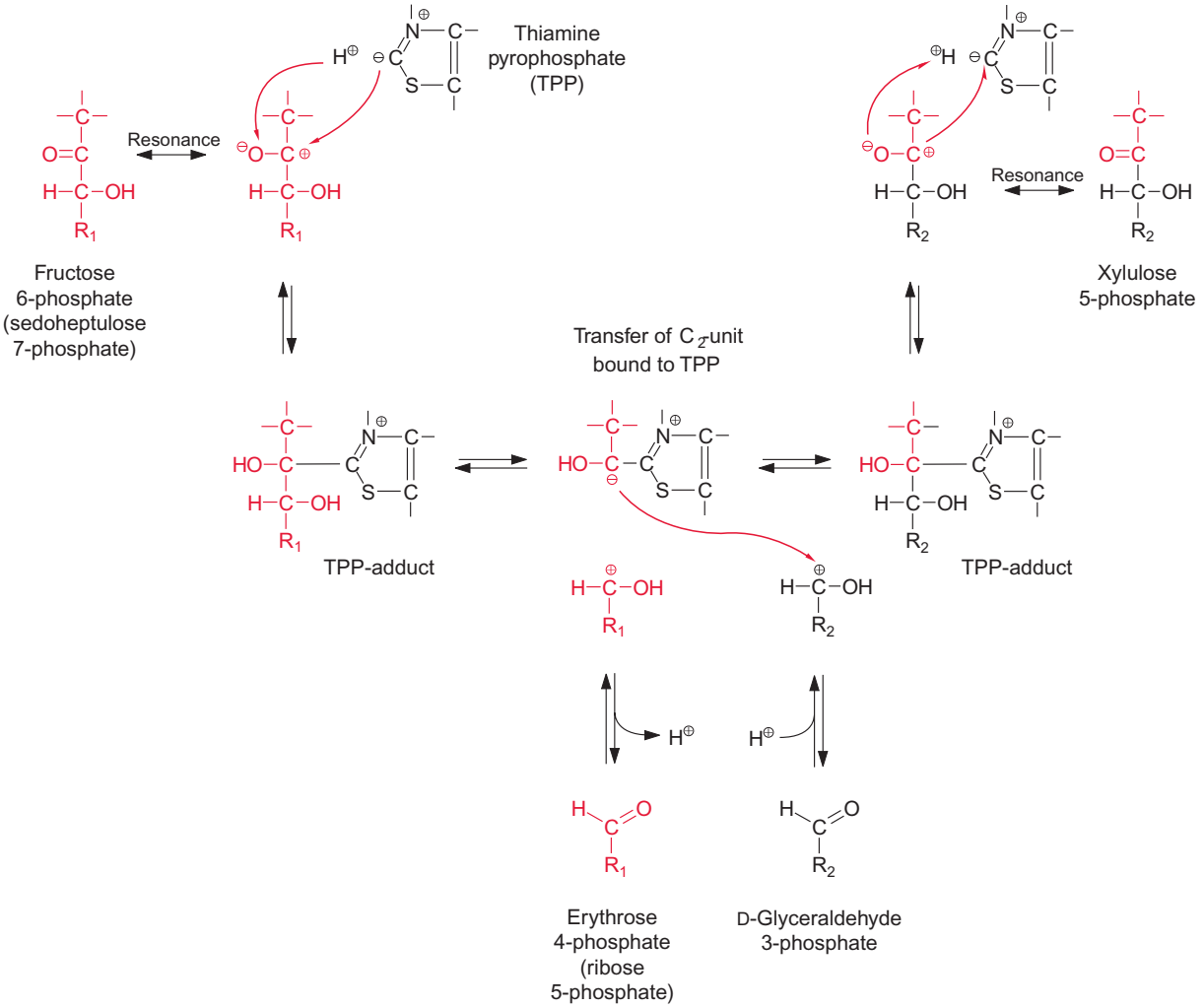
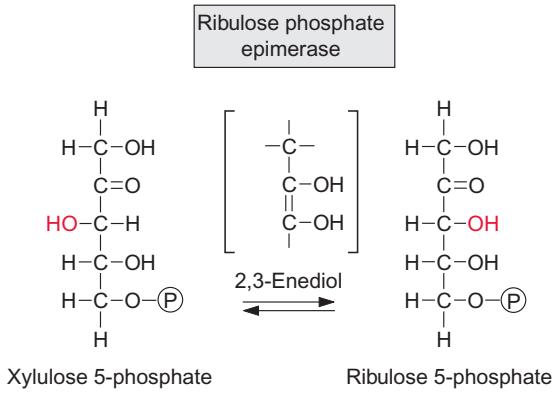


Figure 6.17 Mechanism of the transketolase reaction. The enzyme contains thiamine pyrophosphate as a prosthetic group, the thiazole ring is the reactive component. The positive charge of the N atom in this ring enhances the release of a proton at the neighboring C atom, resulting in a negatively charged C atom (carbanion). The partially positively charged C atom of the keto group binds the substrate. The positively charged N atom of the thiazole favors the cleavage of the carbon chain, resulting in an carbanion at the carbon atom in position 2. The reaction mechanism is basically the same as that of the aldolase reaction in Figure 6.14. The C_2 carbohydrate moiety bound to the thiazole is transferred to the C-1 position of the glyceraldehyde 3-phosphate.



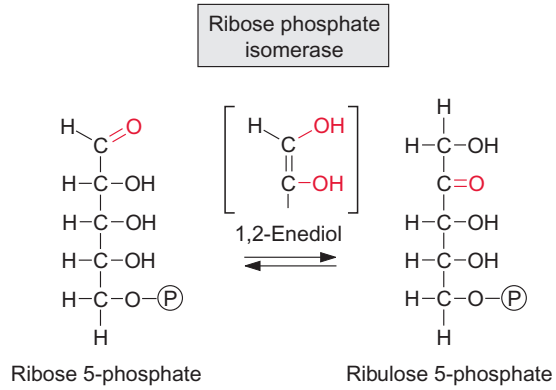


Figure 6.18 The conversion of xylulose 5-phosphate and ribose 5-phosphate to ribulose 5-phosphate. In both reactions a *cis*-enediol is formed as intermediate. Ⓟ symbolizes $-PO_3^{2-}$.

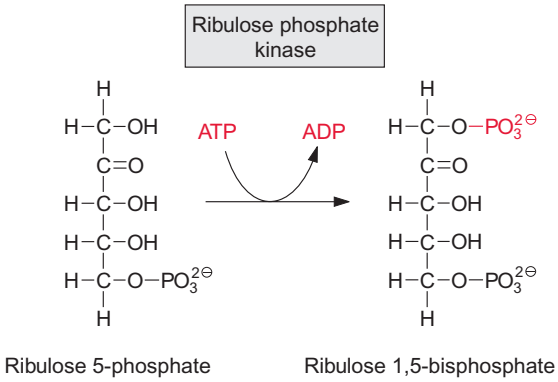


Figure 6.19 Ribulose phosphate kinase catalyzes the irreversible synthesis of ribulose 1,5-bisphosphate.

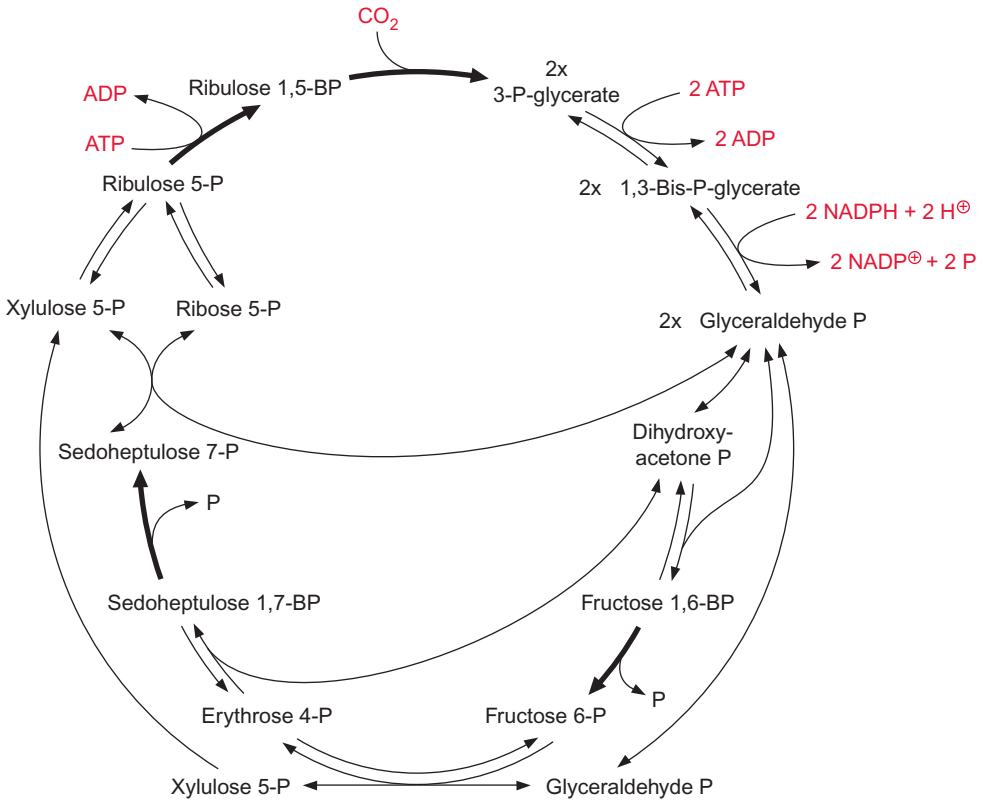


Figure 6.20 The Calvin cycle (reductive pentose phosphate pathway). P, phosphate; BP, bisphosphate. Bold arrows indicate irreversible reactions.

and phosphorylation of ribulose 5-phosphate. The fixation of one molecule of CO_2 requires in total two molecules of NADPH and three molecules of ATP.

6.5 Besides the reductive pentose phosphate pathway there is also an oxidative pentose phosphate pathway

Besides the reductive pentose phosphate pathway discussed in the preceding section, the chloroplasts also contain the enzymes of an oxidative pentose phosphate pathway. This pathway, which occurs both in the plant and animal kingdoms, oxidizes an **hexose phosphate** to a **pentose phosphate**

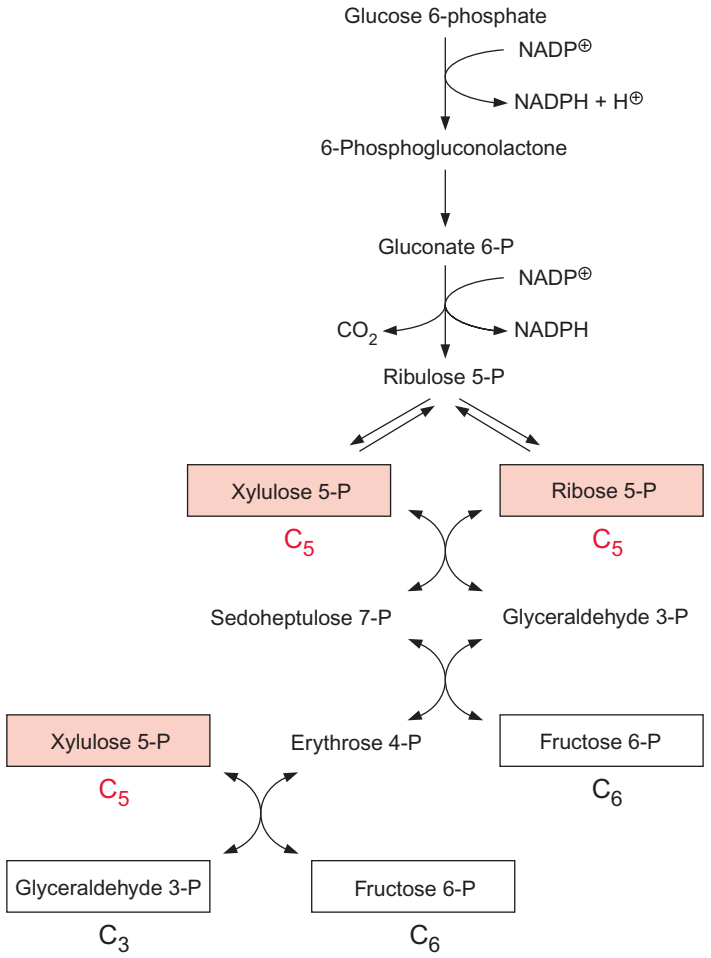


Figure 6.21 Oxidative pentose phosphate pathway.

with the release of one molecule of CO_2. This pathway provides **NADPH** as "high pressure hydrogen" for biosynthetic processes (Fig. 6.21). Glucose 6-phosphate is first oxidized by **glucose 6-phosphate dehydrogenase** to 6-phosphogluconolactone (Fig. 6.22). This reaction is highly exergonic and therefore not reversible. 6-Phosphogluconolactone, an intramolecular ester, is hydrolyzed by **lactonase**. The gluconate 6-phosphate thus synthesized is oxidized to ribulose 5-phosphate by the enzyme **gluconate 6-phosphate dehydrogenase**. In this reaction, CO_2 is released and NADPH is produced.

In the oxidative pathway, xylulose 5-phosphate and ribose 5-phosphate are isomerized from ribulose 5-phosphate by **ribulose phosphate epimerase** and **ribose phosphate isomerase**, respectively. These two products are then converted by **transketolase** to sedoheptulose 7-phosphate and glyceraldehyde

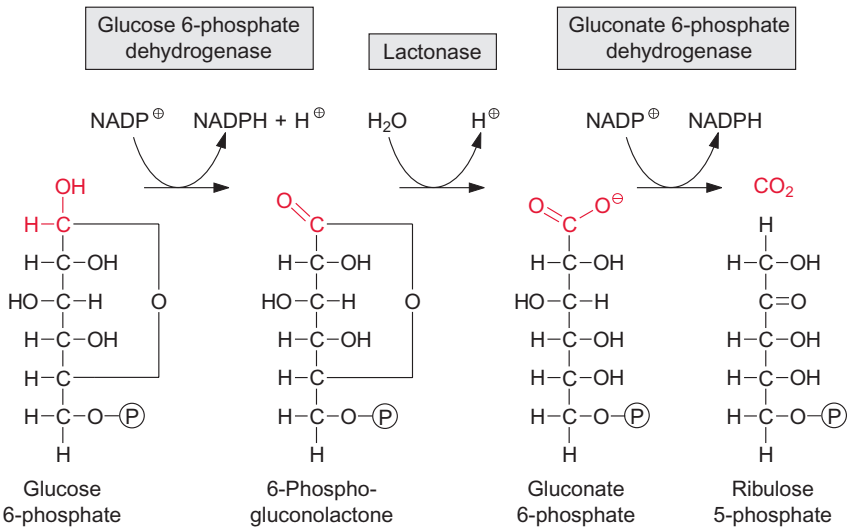


Figure 6.22 The two oxidation reactions of the pentose phosphate pathway. Ⓟ symbolizes $-PO_3^{2-}$.

3-phosphate. This transketolase is TPP-dependent and transfers a C_2 moiety (see Figs. 6.17, 5.4 and 5.5A). This reaction sequence is a reversal of the reductive pentose phosphate pathway. The next reaction is a special feature of the oxidative pathway: **transaldolase** transfers a nonphosphorylated C_3 moiety from sedoheptulose 7-phosphate to glyceraldehyde 3-phosphate, synthesizing fructose 6-phosphate and erythrose 4-phosphate (Fig. 6.23). The reaction mechanism is basically the same as in the aldolase reaction (Fig. 6.13), the only difference is that after the cleavage of the C-C bond, the remaining C_3 moiety continues to be bound to the enzyme via a Schiff base, until it is transferred. Erythrose 4-phosphate reacts with another xylulose 5-phosphate via a **transketolase reaction** to synthesize glyceraldehyde 3-phosphate and fructose 6-phosphate. In this way two hexose phosphates and one triose phosphate are formed from three pentose phosphates:

$$3\,C_5\text{-P} \rightleftarrows 2\,C_6\text{-P} + 1\,C_3\text{-P}$$

This chain of reactions is reversible. It allows the cell to provide ribose 5-phosphate for nucleotide biosynthesis even when no NADPH is required.

In the oxidative pathway, two molecules of NADPH are gained from the oxidation of glucose 6-phosphate and the release of one molecule of CO_2, whereas in the reductive pathway the fixation of one molecule of CO_2 requires not only two molecules of NADPH but also three molecules of ATP (Fig. 6.24). With the expenditure of energy it is possible for the reductive

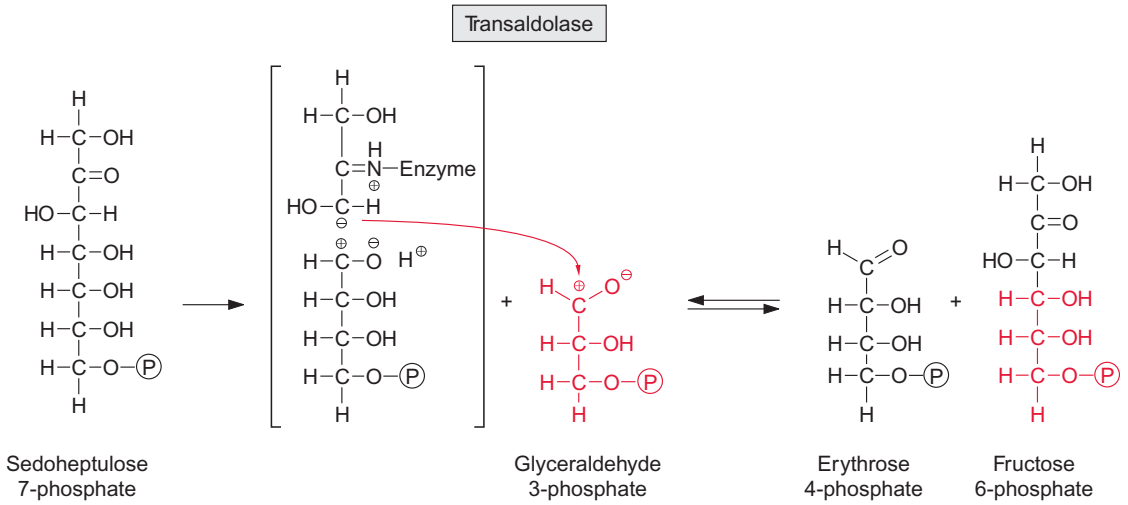


Figure 6.23 Transaldolase catalyzes the transfer of the C_3 moiety from a ketone to an aldehyde. The reaction is reversible. The reaction mechanism is the same as with aldolase, except that after the cleavage of the C-C bond the C_3 moiety remains bound to the enzyme and is released after the transfer to glyceraldehyde phosphate as fructose 6-phosphate.

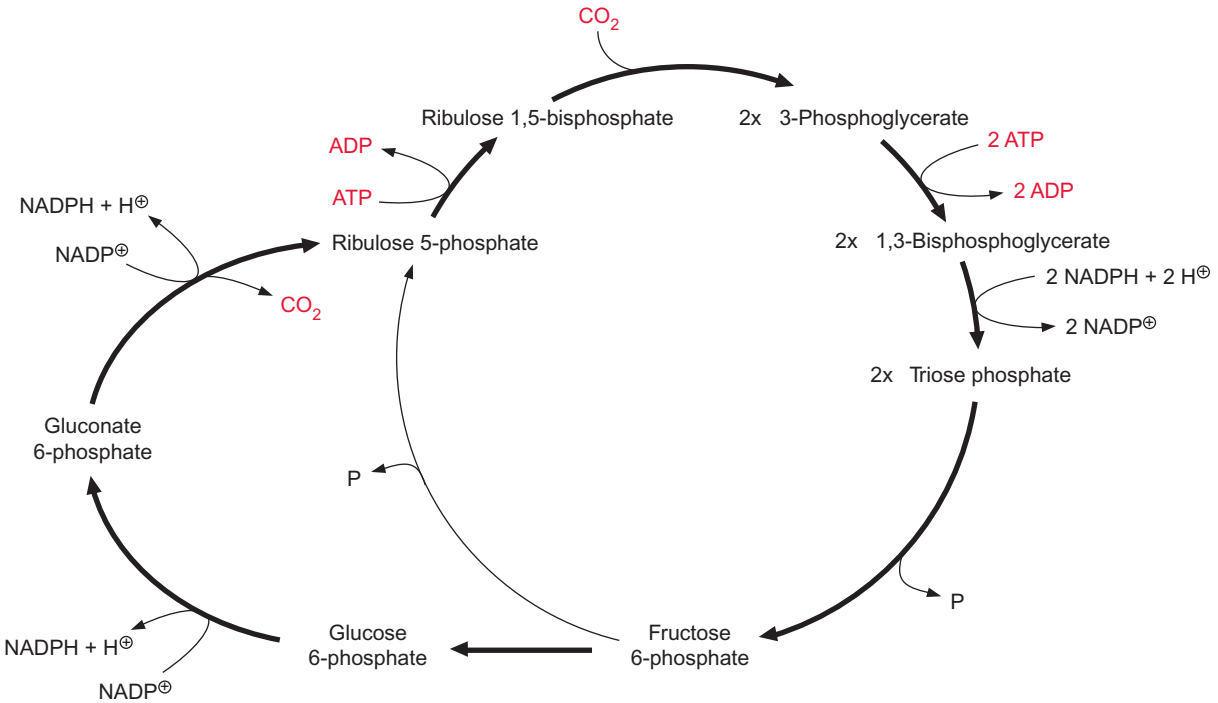


Figure 6.24 A simultaneous operation of the reductive and the oxidative pentose phosphate pathway would result in a futile cycle with the waste of ATP.

pentose phosphate pathway to proceed with a very high flux rate in the opposite direction to the oxidative pathway.

6.6 Reductive and oxidative pentose phosphate pathways are regulated

The enzymes of the reductive as well as the oxidative pentose phosphate pathways are located in the chloroplast stroma (Fig. 6.24). A simultaneous operation of both metabolic pathways, in which one molecule of CO_2 is reduced to a carbohydrate at the expense of three ATP and two NADPH (reductive pentose phosphate pathway), and then reoxidized by the oxidative pathway to CO_2, yielding two molecules of NADPH, would represent a futile cycle. This futile cycle would waste three molecules of ATP in each turn. This is prevented by metabolic regulation, which ensures that key enzymes of the reductive pentose phosphate pathway are active only during illumination and are switched off in darkness, whereas the key enzymes of the oxidative pentose phosphate pathway are only active in the dark.

Reduced thioredoxins transmit the signal "illumination" to the enzymes

An important signal for the state "illumination" is provided by photosynthetic electron transport as reducing equivalents such as reduced thioredoxin (Fig. 6.25). Electrons of reducing equivalents are transferred from ferredoxin to thioredoxin by the enzyme **ferredoxin-thioredoxin reductase**, an iron-sulfur protein of the 4Fe-4S type.

Thioredoxins form a family of small proteins, consisting of about 100 amino acids, which contain as a reactive group the amino acids **Cys-Gly-Pro-Cys**, located at the periphery of the protein. Due to the neighboring cysteine side chains, the thioredoxin can occur in two redox states: the reduced thioredoxin with **two SH-groups** and the oxidized thioredoxin in which the two cysteines are linked by a **disulfide (S-S) bond**.

Thioredoxins are found in all living organisms from archaebacteria to plants and animals. They function as **protein disulfide oxido-reductases**, in reducing disulfide bonds in target proteins to the -SH form and reoxidizing them again to the S-S form. Despite their small size, they possess a relatively high substrate specificity. Thioredoxins participate as redox carriers in the reduction of high as well as low molecular compounds (e.g., the reduction of

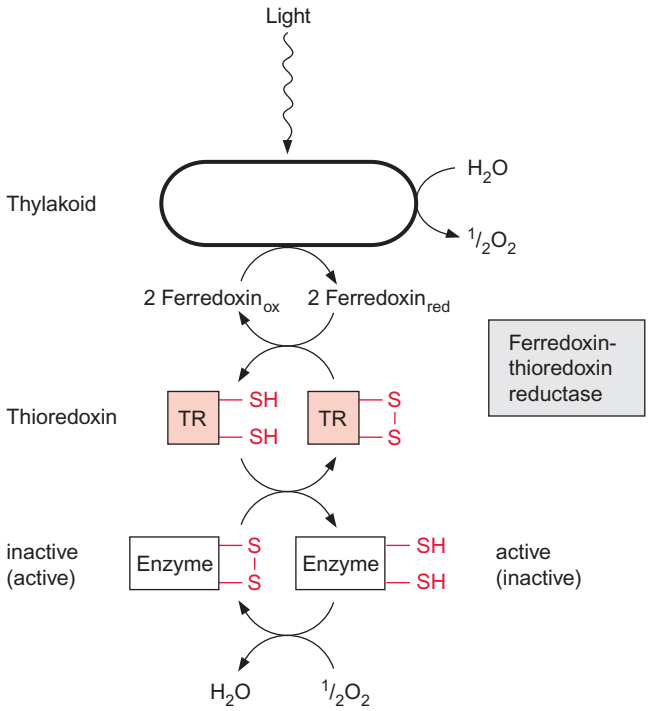


Figure 6.25 The light regulation of chloroplast enzymes is mediated by reduced thioredoxin.

ribonucleotides to deoxyribonucleotides; the reduction of sulfate, a process occurring in plants and microorganisms (section 12.1); and the reductive activation of seed proteins during germination). Furthermore, other processes are known in which thioredoxins play an essential role, for instance the assembly of bacteriophages, and hormone action or the blood-clotting process in animals.

The involvement of thioredoxins in the light regulation of chloroplast enzymes is a very special function which might have occurred during evolution in addition to their general metabolic functions. The chloroplast enzymes **ribulose phosphate kinase**, **sedoheptulose 1,7-bisphosphatase**, **NADP-glyceraldehyde phosphate dehydrogenase** and the chloroplast isoform of **fructose 1,6-bisphosphatase** are converted from an inactive state to an active state via reduction with thioredoxin and are thus switched on by light. This also applies to other chloroplast enzymes such as **NADP-malate dehydrogenase** (section 7.3) and **F-ATP synthase** (section 4.4). Reduced thioredoxin also converts **RubisCO activase** (section 6.2) from a less active state to a more active state. Reduced thioredoxin can also inactivate enzymes, e.g., **glucose**

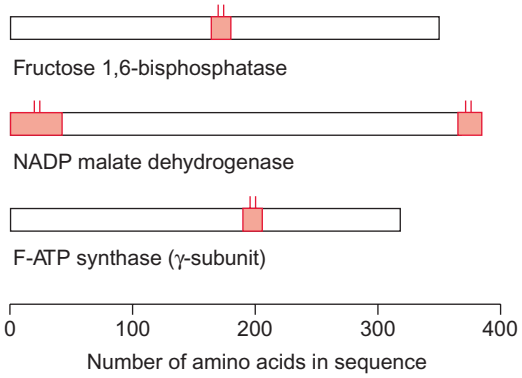


Figure 6.26 In contrast to the nonplastid isoenzymes, several thioredoxin-modulated chloroplast enzymes comprise additional amino acid sequences (shown in red) in which two cysteine residues are located. (After Scheibe, 1990.)

6-phosphate dehydrogenase, the first enzyme of the oxidative pentose phosphate pathway.

The thioredoxin modulated activation of chloroplast enzymes releases a built-in blockage

Important knowledge of the mechanism of thioredoxin action on the chloroplast enzymes has been obtained from comparison with the corresponding isoenzymes from other cellular compartments. Isoenzymes of chloroplast fructose 1,6-bisphosphatase, glyceraldehyde phosphate dehydrogenase and malate dehydrogenase exist in the cytosol and are not regulated by thioredoxin. This also applies to F-ATP synthase in the mitochondria. Comparison of the amino acid sequences shows that at least in some cases the chloroplast isoenzymes possess **additional sequences** at the N- or C-terminus, or in an internal region of their sequence, which provide **two cysteine residues** (Fig. 6.26). The SH-groups of these cysteine residues can be oxidized and form a disulfide bond, which is the substrate for the disulfide oxidoreductase activity of thioredoxin.

Upon exchange of the cysteine residues involved in the regulation by genetic engineering (Chapter 22) enzymes were obtained that are fully active in the absence of reduced thioredoxin. Under oxidizing conditions, the enzymes regulated by thioredoxin are forced by the formation of a disulfide bridge into a conformation in which the catalytic center is inactivated. The reduction of this disulfide bridge by thioredoxin releases this blockage and the enzyme is converted into a relaxed conformation in which the catalytic center is accessible.

The light activation discussed so far is not an all or nothing effect. It is due to a continuous change between the thioredoxin-mediated reduction of the enzyme protein and its simultaneous oxidation by oxygen. The

degree of activation of the enzyme depends on the rate of reduction. This is not only due to the degree of the reduction through thioredoxin (and thus to the degree of reduction of ferredoxin), but also to the presence of other metabolites, e.g., the reductive activation of fructose and sedoheptulose bisphosphatase is enhanced by the corresponding bisphosphates. These effectors cause a decrease of the redox potential of the SH-groups in the corresponding enzymes, which enhances the reduction of the disulfide group by thioredoxin. In this way the activity of these enzymes is increased when the concentration of their substrates rises. Thus the reductive activation of NADP malate dehydrogenase is decreased by the presence of $NADP^+$. This has the effect that the enzyme is only active at a high NADPH/$NADP^+$ ratio. On the other hand, the reductive inactivation of glucose-6-phosphate dehydrogenase is increased by NADPH. Thus with a sufficient supply of NADPH the activity of the oxidative pentose phosphate pathway is turned down. In contrast, the oxidative activation of glucose 6-phosphate dehydrogenase is enhanced by $NADP^+$ which increases the activity of the oxidative pentose phosphate pathway when there is a demand for NADPH.

Multiple regulatory processes tune the reactions of the reductive pentose phosphate pathway

An additional light regulation of the Calvin cycle is based on the effect of light-dependent changes of chloroplast enzyme activities due to the stromal proton and Mg^{++} concentrations. When isolated chloroplasts are illuminated, the acidification of the thylakoid space (Chapter 3) is accompanied by an alkalization and an increase in the Mg^{++} concentration in the stroma. During the dark/light transition, the pH in the stroma may change from about pH 7.2 to 8.0. This correlates with the pH optimum of the CO_2 fixation of isolated chloroplasts of about pH 8.0 with a sharp decline towards the acidic range. An almost identical pH dependence is found with the light-activated enzymes **fructose 1,6-bisphosphatase** and **sedoheptulose 1,7-bisphosphatase**. Moreover, the catalytic activity of both these enzymes is increased by the light-dependent increase of the stromal Mg^{++} concentration. The light activation of these enzymes due to the thioredoxin system and light-induced changes of the stromal pH and Mg^{++} concentration is a very efficient system for switching these enzymes on and off, according to demand. During darkness this system results in an extensive inactivation of the corresponding enzymes.

The activities of several stromal enzymes are also regulated by metabolite levels. The chloroplast **fructose 1,6-bisphosphatase** and **sedoheptulose**

1,7-bisphosphatase are inhibited by their corresponding products, fructose 6-phosphate and sedoheptulose 7-phosphate, respectively. Thus the accumulation of the products has negative effects on the activity of these enzymes. **Ribulose phosphate kinase** is inhibited by 3-phosphoglycerate and also by ADP. Inhibition by ADP is important for coordinating the two kinase reactions of the reductive pentose phosphate pathway. Whereas ribulose phosphate kinase catalyzes an irreversible reaction, the phosphoglycerate kinase reaction is reversible. If both reactions were to compete for ATP in an unrestricted manner, in the case of a shortage of ATP the irreversible phosphorylation of ribulose 5-phosphate would be at an advantage, resulting in an imbalance of the Calvin cycle. A decrease in the activity of ribulose phosphate kinase at an elevated level of ADP can prevent this.

Finally, fructose 1,6-bisphosphatase and sedoheptulose 1,7-bisphosphatase are strongly inhibited by glycerate. As shown in section 7.1, glycerate is an intermediate in the recycling of phosphoglycolate formed by the oxygenase activity of RubisCO. The accumulation of glycerate slows down the regeneration of RuBP and its carboxylation. In this way also the accompanying oxygenation is lowered, decreasing the synthesis of glycolate which is the precursor of glycerate.

Also, **RubisCO** is subject to regulation. Experiments with whole leaves demonstrated that the degree of the activation of RubisCO correlates with the intensity of illumination and the rate of photosynthesis. The activation state of RubisCO is adjusted via a regulation of the **RubisCO activase** (section 6.2). RubisCO-activase is activated by **reduced thioredoxin** and is also dependent on the **ATP/ADP** ratio. When there is a rise in the ATP/ADP ratio in the stroma, the activity of the activase also rises. This scenario explains how the activity of RubisCO can be adjusted to the supply of ATP delivered by the light reaction of photosynthesis. However, many observations suggest that this cannot be the only mechanism for a light regulation of RubisCO, for example the RubisCO activase is regulated by the light-dependent proton gradient across the thylakoid membrane, and the activity of RubisCO is inhibited by its product **3-phosphoglycerate**. In this way the activity of RubisCO could be adjusted according to the product accumulation

Figure 6.27 presents a scheme summarizing the various factors that influence the regulation of the enzymes of the reductive and oxidative pentose pathways. A multitude of regulatory processes ensures that the various steps of both reaction chains are adjusted to each other and to the demand of the cell.

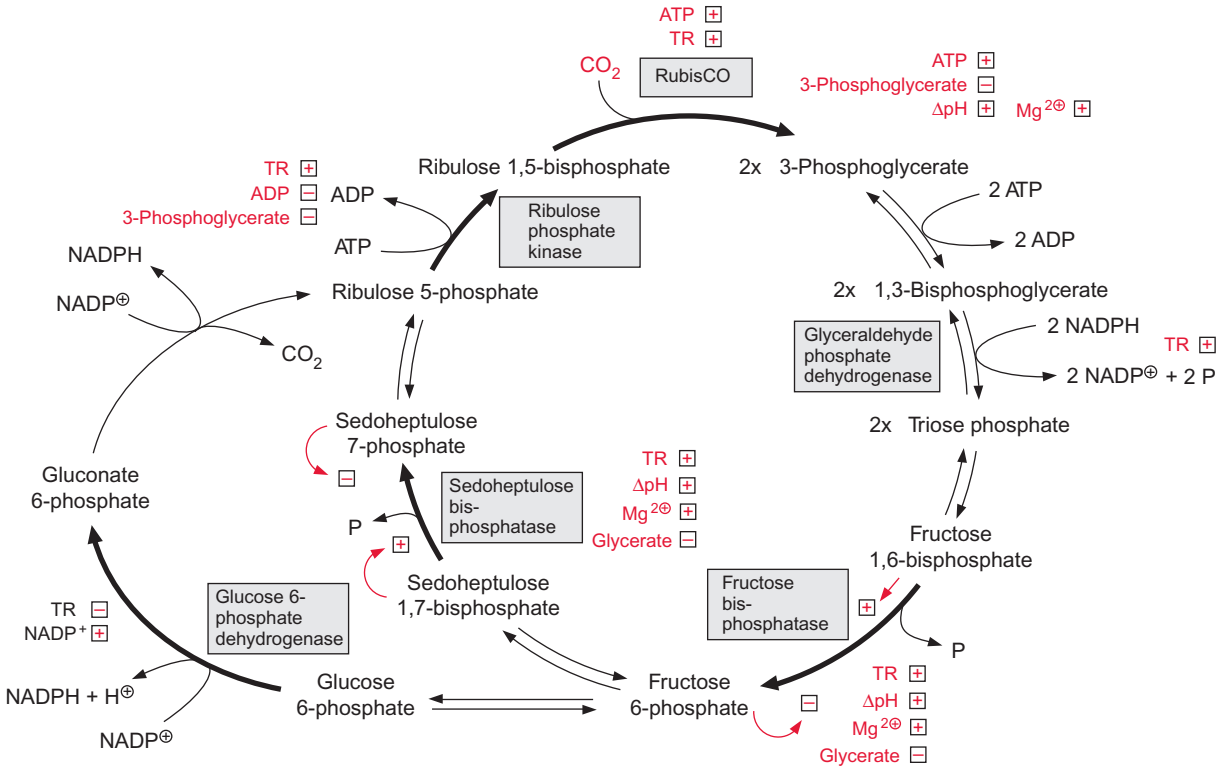


Figure 6.27 Regulation of the reductive and oxidative pentose phosphate pathways. Both pathways are represented in a simplified scheme. Only those enzymes for which regulation has been discussed in the text are highlighted. [+] increase and [−] decrease of activity caused by the factors written in red, such as reduced thioredoxin (TR), light-dependent alkalization (ΔpH), the increase in Mg^{++} concentration in the stroma and the presence of metabolites. The regulation of RubisCO proceeds via a regulation of the RubisCO activase.

Further reading

Allen, D. J., Ort, D. Impact of chilling temperature on photosynthesis in warm-climate plants. Trends in Plant Science 6, 36–42 (2002).

Andersson, I., Backlund, A. Structure and function of RubisCO. Plant Physiology Biochemistry 46, 275–291 (2008).

Andralojc, P. J., Keys, A. J., Kossmann, J., Parry, M. A. J. Elucidating the biosynthesis of 2-carboxyarabinitol-1-phosphate through reduced expression of chloroplastic fructose-1.6-bisphosphate phosphatase and radiotracer studies with $^{14}CO_2$. Proceedings National Academy of Science USA 99, 4742–4747 (2002).

Buchanan, B. B., Balmer, Y. Redox regulation: A broadening horizon. Annual Reviews Plant Biology 56, 187–220 (2005).

Gulliaume, G., Tcherkez, B., Farquhar, G. D., Andrews, T. J. Despite low catalysis and confused substrate specificity, all ribulose bisphosphate carboxylases may be nearly

perfectly optimized. Proceedings National Academy of Science USA 103, 7252–7257 (2006).
Hisabori, T., Motohashi, K., Hosoya-Matsuda, N., Ueoka-Nakanishi, H., Romano, P. G. Towards a functional dissection of thioredoxin networks in plant cells. Photochemistry Photobiology 83, 145–151 (2007).
Kruger, N. J., von Schaewen, A. The oxidative pentose phosphate pathway: Structure and organisation. Current Opinion Plant Biology 6, 236–246 (2003).
Lemaire, S. D., Michelet, L., Zaffagnini, M., Massot, V., Issakidis-Bourguet, E. Thioredoxins in chloroplasts. Current Genetics 51, 343–365 (2007).
Raines, C. A. Transgenic approaches to manipulate the environmental responses of the C3 carbon fixation cycle. Plant Cell Environment 29, 331–339 (2006).
Spreitzer, R. J., Salvucci, M. E. RubisCO: Structure, regulatory interactions and possibilities for a better enzyme. Annual Reviews Plant Biology 53, 449–475 (2002).
Trost, P., Fermani, S., Marri, L., Zaffagnini, M., Falini, G., Scagliarini, S., Pupillo, P., Sparla, F. Thioredoxin-dependent regulation of photosynthetic glyceraldehyde-3-phosphate dehydrogenase: Autonomous vs. CP12-dependent mechanisms. Photosynthesis Research 89, 263–275 (2006).
Von Caemmerer, S., Quick, W. P. RubisCO: Physiology in vivo. In Photosynthesis: Physiology and Metabolism, R. C. Leegood, T. D. Sharkey, S. von Caemmerer (eds.), pp. 85–113. Dordrecht, The Netherlands: Kluwer Academic Publishers. (2000)
Zhang, N., Kallis, R. P., Ewy, R. G., Portis, A. R. Light modulation of Rubisco in Arabidopsis requires a capacity for redox regulation of the larger Rubisco activase isoform. Proceedings National Academy of Science USA 99, 3330–3334 (2002).