Dark Reactions

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The light reactions of photosynthesis transform the energy of light into high energy ATP and NADPH. The dark reactions reduced carbon dioxide to form carbohydrates. These reactions are called dark reactions because these reactions do not directly depend on photons of light. NADPH is the anabolic reducing currency of the cell.

The Calvin Cycle

The Calvin Cycle is the process by which carbon dioxide is fixed into a form that it is useful for many processes. The carbon dioxide that is fixed by this process will become the nucleic acids, proteins, carbohydrates and fats of the plant. The capacity to accumulate carbon atoms from carbon dioxide for the net synthesis of carbohydrate distinguishes the photoautotrophic from the heterotrophic. The Calvin Cycle takes place in the stroma of the chloroplast.

The Calvin cycle is named after Melvin Calvin and his colleagues at UC Berkeley who discovered it. They were investigating CO_2 fixation by tracing $^{14}CO_2$ into organic products. They discovered that the first radiolabeled compound formed was 3-phosphoglycerate.

Shown to the left is the classical experiment showing where the $^{14}CO_2$ appears within 5 seconds after adding the radiolabel. All of the radiolabel appears as 3-PG.

After 60 seconds, the radioactivity appears in the many metabolites of the Calvin Cycle.

The Calvin cycle is composed of three parts.
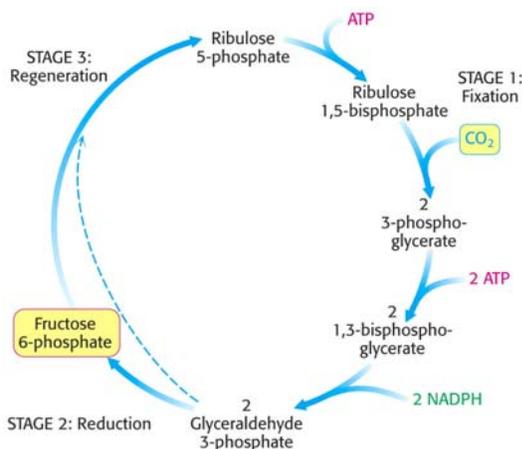
1. The fixation of CO_2 to 1,5-bisphosphoribulose to form 2 molecules of 3-phosphoglycerate.

2. The conversion of 3-phosphoglycerate to glyceraldehyde 3-phosphate which then goes on to form hexoses.

3. The regeneration of ribulose, 1,5-bisphosphoglycerate.

I. CO$_2$ Fixation

The CO$_2$ acceptor is ribulose-1-5-bisphosphate (RuBP). The enzyme catalyzing the fixation is ribulose bisphosphate carboxylase/oxygenase or **Rubisco** for short. Rubisco is found on the stromal surface of the thylakoid membrane. The carboxylase/oxygenase ending reflects the two activities of this enzyme. Rubisco catalyzes the addition of CO$_2$ or O$_2$ to RuBP. Rubisco constitutes more than 15% of the total chloroplast protein. Due to prevalence of plants in the biosphere, rubisco is world's most abundant protein. It is a large (550 kD) heteromultimeric enzyme ($\alpha_8\beta_8$) with eight identical large subunits (55 kD) and 8 small subunits (15 kD). The large subunits contain the catalytic sites and a regulatory site. The small subunits modulate the activity of the large subunit.
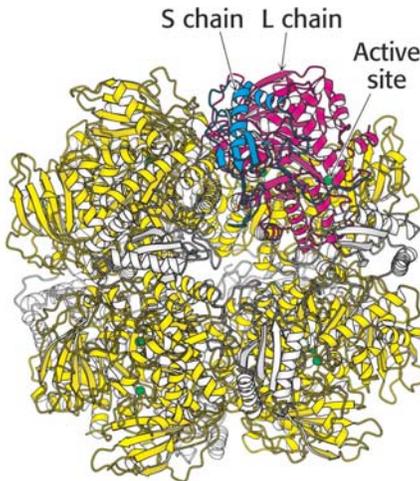

The large subunit is depicted in red.

The small subunit is depicted in blue.

The catalytic active site lies in the large subunit.

Rubisco is a relatively slow enzyme with a k$_{cat}$ of only 3s^{-1}.

Rubisco requires a bound divalent metal (Mg^{2+}) for catalytic activity. The metal ion activates the bound substrate molecules and stabilizes the negative charge developed in the transition state.
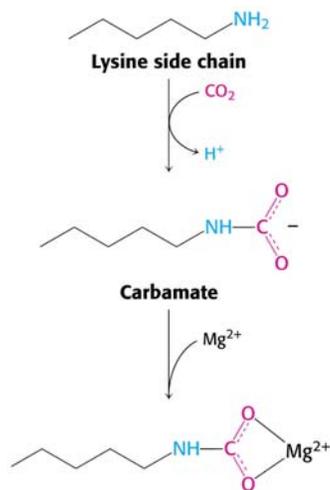
The coordination of the Mg^{2+} ion requires a CO$_2$ molecule to add to the ε-amino group of Lys201 to form a carbamylated lysine. The negatively charged carbamate coordinates to the Mg^{2+} activating the enzyme.


Rubisco exists in 3 forms.
1. Uncarbamylated, No Mg^{2+} bound, inactive, designated **E**.
2. Carbamylated, No Mg^{2+} bound, inactive, designated **EC.**
3. Carbamylated, Mg^{2+} bound, active, designated **ECM**.

The carbamylation is favored by slightly alkaline pH, 8.0.

Once carbamylated, rubisco competes to bind Mg^{2+}.

In the E form, rubisco binds ribulose-1,5-bisphosphate very tightly (K$_D$ = 20 nM), making the substrate a potent inhibitor of rubisco activity. In order to become carbamylated and active, the bound RuBP must dissociate. The release of RuBP is mediated by **rubisco activase**, a regulatory protein that binds to the E form of the enzyme and uses the energy of ATP hydrolysis to release RuBP from the active site. The enzyme can now be carbamylated and coordinate a magnesium ion to become fully active.

The metal center plays a crucial role in catalysis. Ribulose 1.5-bisphosphate coordinates to the metal through its keto group and adjacent hydroxyl group. The complex is then deprotonated to form an enediolate intermediate. The enediolate reacts with a coordinated CO_2 group forming a new carbon-carbon bond. A molecule of H_2O is then added to form an intermediate that cleaves into two molecules of 3-phosphoglycerate.
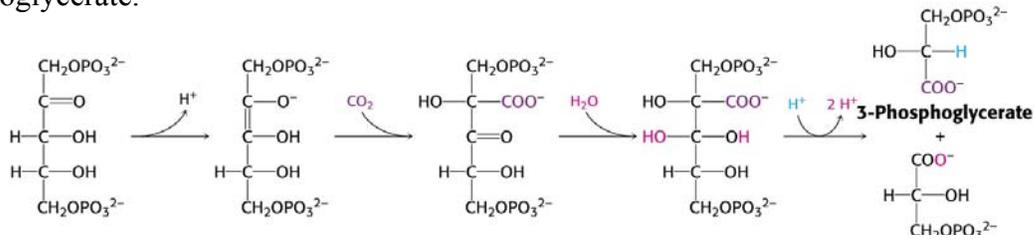

The Oxygenase Reaction of Rubisco

Sometimes the magnesium metal center of Rubisco coordinates to RuBP and oxygen instead of carbon dioxide. Rubisco then catalyzes an oxygenase reaction.
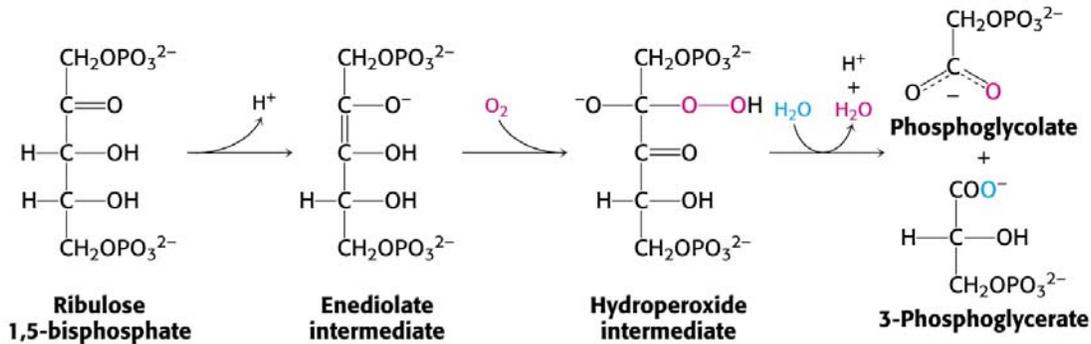
Under normal conditions the rate of the carboxylase reaction is 4 times faster than the oxygenase reaction. Normal conditions being P = 1 atm, T = 25 °C, $[CO_2]$ = 10 μM and $[O_2]$ = 250 μM. The oxygenase reaction like the carboxylase reaction requires Lys201 to be carbamylated. The carbamylated lysine is only formed in the presence of CO_2 which prevents rubisco from catalyzing the oxygenase reaction when CO_2 is absent.
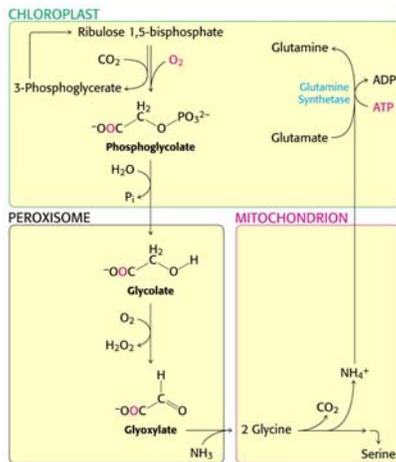

There is a salvage pathway to recover part of the carbon skeleton of phosphoglycolate. A specific phosphatase converts phosphoglycolate into glycolate. Glycolate enters the peroxisome microbodies where it is oxidized into glycolate by glycolate oxidase. Glycolate oxidase contains a FMN prosthetic group. Hydrogen peroxide is generated in this reaction which is cleaved in the peroxisome by catalase into water and oxygen. Glycolate is then transaminated to form glycine. Two molecules of glycine can be used to form serine, CO_2 and NH_4^+. Serine can then be converted in glucose.

This salvage pathway saved 3 of the four carbons of phosphoglycolate.

II. The reduction of 3-phosphoglycerate and the Synthesis of Hexose Phosphates
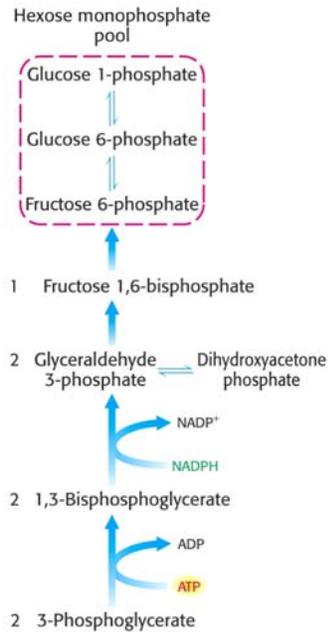
After the formation of 3-phosphoglycerate by rubisco, the next step in the Calvin cycle is the conversion of 3-phosphoglycerate into 3-phosphoglyceraldehyde.

The conversion is parallel with gluconeogenesis.

First 3-phosphoglycerate is phosphorylated by ATP to form 1,3-bisphosphoglycerate by phosphoglycerokinase.

1,3-Bisphosphoglycerate is a substrate for the chloroplast's glyceraldehyde 3-phosphate dehydrogenase.

Glyceraldehyde 3-phosphate dehydrogenase in the chloroplast is different than the cystolic glyceraldehyde 3-phosphate dehydrogenase.

The chloroplast GAPDH is specific for NADPH rather than NADH.

III. Regeneration of Ribulose 1,5-bisphosphate.

The third phase of the Calvin cycle is very similar to the second half of the pentose phosphate pathway. In fact two of the enzymes involved are none other than aldolase and transketolase. Aldolase is the enzyme we encountered in glycolysis. Transketolase is a PPP enzyme that as you all know contains the cofactor thiamine pyrophosphate and transfers two carbon groups.
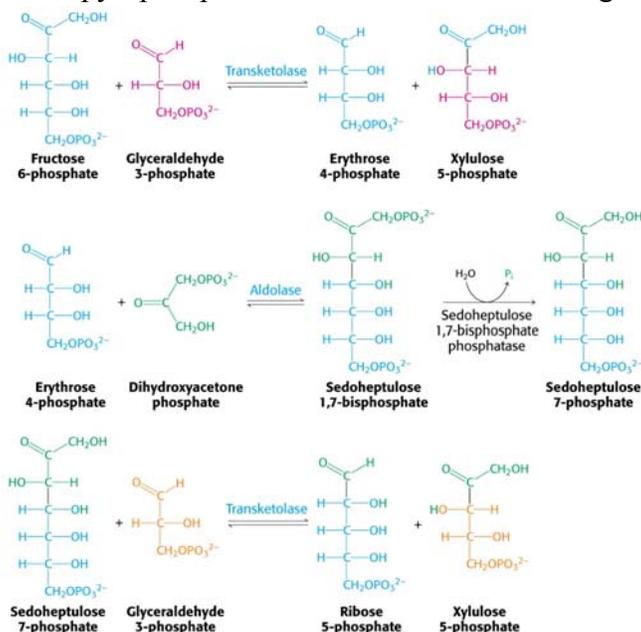
First transketolase converts fructose 6-phosphate and glyceraldehydes 3-phosphate into erythrose 4-phosphate and xylulose 5-phosphate.

Then Aldolase condenses erythrose 4-phosphate with dihydroxyacetone phosphate to form sedoheptulose 1,7-bisphosphate. Sedoheptulose 1,7-bisphosphatase removes the phosphate group from the 1 position to form Sedoheptulose 7-phosphate.

Sedoheptulose 7-phosphate then reacts with transketolase with a second molecule of glyceraldehydes 3-phosphate to form ribose-5-phosphate and xylulose 5-phosphate.

Finally, ribose 5-phosphate is converted into ribulose 5-phosphate by phosphopentose isomerase, and xylulose 5-phosphate is converted into ribulose 5-phosphate by phosphopentose epimerase. Please review the pentose phosphate pathway and the mechanisms involved in all of these reactions. The last step is the phosphorylation of ribulose 5-phosphate to form Ribulose 1,5-bisphosphate.
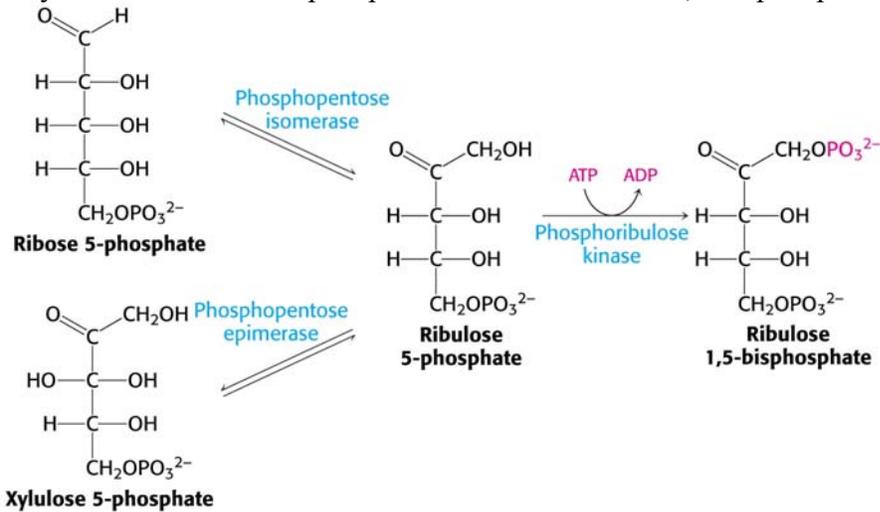
If we balance the Calvin cycle such that 6 molecules of CO_2 are fixed for the net synthesis of one hexose, then we come up with the stoichiometries shown below.

Garrett & Grisham: Biochemistry, 2/e
Table 22.1

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The Calvin Cycle Series of Reactions

Reactions 1 through 15 constitute the cycle that leads to the formation of one equivalent of glucose. The enzyme catalyzing each step, a concise reaction, and the overall carbon balance is given. Numbers in parentheses show the numbers of carbon atoms in the substrate and product molecules. Prefix numbers indicate in a stoichiometric fashion how many times each step is carried out in order to provide a balanced net reaction.

1. Ribulose bisphosphate carboxylase: $6 CO_2 + 6 H_2O + 6 RuBP \longrightarrow 12 \ 3\text{-PG}$ $6(1) + 6(5) \longrightarrow 12(3)$
2. 3-Phosphoglycerate kinase: $12 \ 3\text{-PG} + 12 \ ATP \longrightarrow 12 \ 1,3\text{-BPG} + 12 \ ADP$ $12(3) \longrightarrow 12(3)$
3. $NADP^+$-glyceraldehyde-3-P dehydrogenase:
        $12 \ 1,3\text{-BPG} + 12 \ NADPH \longrightarrow 12 \ NADP^+ + 12 \ G3P + 12 \ P_i$ $12(3) \longrightarrow 12(3)$
4. Triose-P isomerase: $5 \ G3P \longrightarrow 5 \ DHAP$ $5(3) \longrightarrow 5(3)$
5. Aldolase: $3 \ G3P + 3 \ DHAP \longrightarrow 3 \ FBP$ $3(3) + 3(3) \longrightarrow 3(6)$
6. Fructose bisphosphatase: $3 \ FBP + 3 \ H_2O \longrightarrow 3 \ F6P + 3 \ P_i$ $3(6) \longrightarrow 3(6)$
7. Phosphoglucoisomerase: $1 \ F6P \longrightarrow 1 \ G6P$ $1(6) \longrightarrow 1(6)$
8. Glucose phosphatase: $1 \ G6P + 1 \ H_2O \longrightarrow 1 \ GLUCOSE + 1 \ P_i$ $1(6) \longrightarrow 1(6)$
   The remainder of the pathway involves regenerating six RuBP acceptors (= 30 C) from the leftover two F6P (12 C), four G3P (12 C), and two DHAP (6 C).
9. Transketolase: $2 \ F6P + 2 \ G3P \longrightarrow 2 \ Xu5P + 2 \ E4P$ $2(6) + 2(3) \longrightarrow 2(5) + 2(4)$
10. Aldolase: $2 \ E4P + 2 \ DHAP \longrightarrow 2 \ \text{sedoheptulose-1,7-bisphosphate (SBP)}$ $2(4) + 2(3) \longrightarrow 2(7)$
11. Sedoheptulose bisphosphatase: $2 \ SBP + 2 \ H_2O \longrightarrow 2 \ S7P + 2 \ P_i$ $2(7) \longrightarrow 2(7)$
12. Transketolase: $2 \ S7P + 2 \ G3P \longrightarrow 2 \ Xu5P + 2 \ R5P$ $2(7) + 2(3) \longrightarrow 4(5)$
13. Phosphopentose epimerase: $4 \ Xu5P \longrightarrow 4 \ Ru5P$ $4(5) \longrightarrow 4(5)$
14. Phosphopentose isomerase: $2 \ R5P \longrightarrow 2 \ Ru5P$ $2(5) \longrightarrow 2(5)$
15. Phosphoribulose kinase: $6 \ Ru5P + 6 \ ATP \longrightarrow 6 \ RuBP + 6 \ ADP$ $6(5) \longrightarrow 6(5)$

Net: $6 \ CO_2 + 18 \ ATP + 12 \ NADPH + 12 \ H^+ + 12 \ H_2O \longrightarrow$
     $glucose + 18 \ ADP + 18 \ P_i + 12 \ NADP^+$ $6(1) \longrightarrow 1(6)$

The net reaction:

$$6CO_2 + 18\ ATP + 12\ NADPH + 12H^+ + 12\ H_2O \rightarrow C_6H_{12}O_6 + 18\ ADP + 18\ Pi + 12\ NADP^+$$
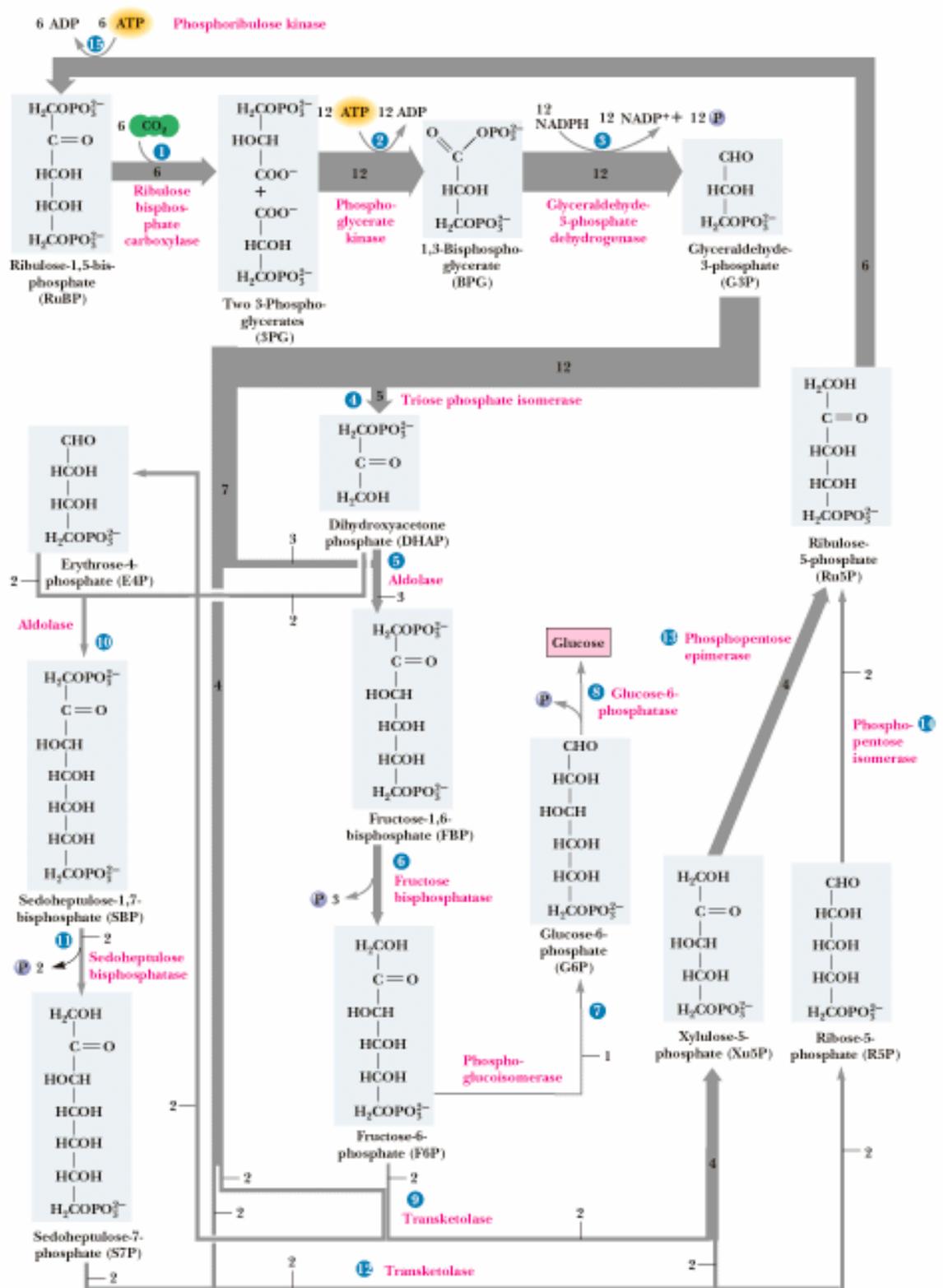
IV. Regulation of the Calvin Cycle

Plant cells are able to do glycolysis in the cytosol. Plants have mitochondria and can carry our cellular respiration. It is wasteful to have glycolysis and the citric acid cycle going at the same time as CO_2 fixation. This situation is avoided by regulation of the Calvin cycle. The enzymes of the Calvin cycle are indirectly regulated by light. When light is available to generate ATP and NADPH, the Calvin cycle enzymes are activated for carbon dioxide fixation. In the dark, when ATP and NADPH generation ceases, CO_2 fixation also ceases. Light induced changes in the chloroplast regulate key enzymes in the Calvin cycle. These light induced changes include:
- Change in the stromal pH.
- Generation of NADPH and reduced ferredoxin.
- Mg^{+2} efflux from the thylakoid lumen.
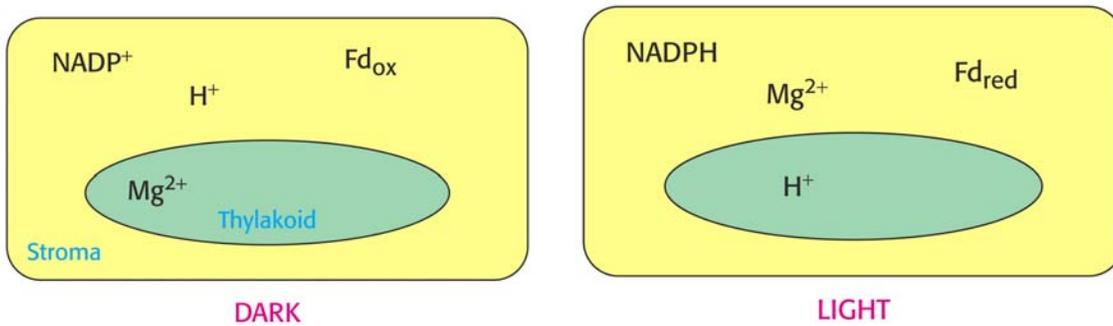
The illumination of the chloroplast leads to light driven pumping of protons from the stroma into the thylakoid lumen. The stromal pH rises, the lumen pH decreases. The stromal pH rises to around 8 pH units. At this pH, Lys201 reacts with CO_2 to form the carbamylated lysine that coordinates to the magnesium ion. In addition as the pH rises in the stroma , the activities of Fructose 1,6-bisphosphatase, ribulose-5-phosphate kinase and glyceraldehyde 3-phosphate dehydrogenase reach their maxima.

The light driven pumping of protons from the stroma to the thylakoid lumen occurs with the concomitant efflux of Mg^{2+} ions from the lumen to the stroma. The efflux of Mg^{2+} maintains electrical neutrality. Both rubisco and fructose 1,6-bisphosphatase are Mg^{2+} activated, stimulating CO_2 fixation. The rate determining step of the Calvin cycle is fructose 1,6-bisphosphatase which make this enzyme a key enzyme in Calvin cycle regulation.

Illumination of the chloroplasts activates photosynthetic electron transport, which generates reducing power in the form of NADPH and reduced ferredoxin. Several of the enzymes involved in CO_2 fixation are activated upon reduction of disulfide bonds. The most notable enzymes are fructose 1,6-bisphosphatase, sedoheptulose 1,7-bisphosphatase, and ribulose 5-phosphate kinase.

The reducing agent that activates these enzymes is thioredoxin. Thioredoxin is a small protein possessing a pair of reduced sulfhydryls which upon oxidation form disulfide bonds. Thioredoxin is the electron carrier between NADPH or Fd_{red} and the enzymes regulated by light.
Shown to the left is thioredoxin in the oxidized, disulfide form.
Reduced thioredoxin reduces disulfide bonds in certain enzymes, activating them.
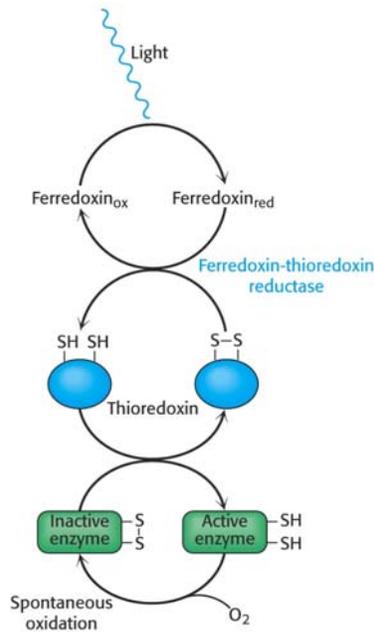
In chloroplasts thioredoxin is reduced by ferredoxin by ferredoxin-thioredoxin reductase. This enzyme has a 4Fe-4S cluster that couples the one electron transfers from reduced ferredoxin towards the two electron reduction of thioredoxin.

Phosphoribulose kinase and glyceraldehydes 3-phosphate dehydrogenase are also regulated by NADPH directly. In the dark these two enzymes aggregate with a small protein called CP12 to form a large inactive complex. NADPH binds to this complex, leading to the release of the enzymes from the complex and thereby activating them.

V. The C-4 Pathway of CO_2 Fixation

Recall the oxygenase activity of rubisco. Under normal conditions the rate of the carboxylase reaction is 4 times faster than the oxygenase reaction. Normal conditions being $P = 1$ atm, $T = 25\ ^\circ C$, $[CO_2] = 10$ μM and $[O_2] = 250\ \mu M$. When the temperature increases the rate of the oxygenase activity increases more rapidly than the carboxylase activity. Plants that grow in hot climates need a mechanism to minimize the wastefull oxygenase activity. The plants adapted to hot climates overcome this problem by creating a high local concentration of CO_2 in the stroma of the chloroplasts. These plants use four carbon compounds C_4 such as arpartate and malate to carry CO_2 from mesophyll cells, which are the cells in contact with the air, to the bundle sheath cells, which are the major sites of photosynthesis. The decarboxylation of the C_4 compound in the bundle sheath creates a high local concentration of CO_2 at the site of the Calvin cycle. The decarboxylation creates a 3 carbon compound such as pyruvate which can return to the mesophyll cell and become recarboxylated.

The C-4 pathway functions as a CO_2 delivery system, carrying carbon dioxide from the oxygen rich surface of the leaf to the bundle sheath where the oxygen concentration is lower and hence less competing with CO_2 for the binding site in rubisco.

The essential features are shown below. In the mesophyll cells, CO_2 is used to carboxylate PEP to form oxaloacetate in a reaction catalyzed by PEP carboxylase. Oxaloacetate is then reduced to malate by a NADPH specific malate dehydrogenase. Malate then diffuses from the mesophyll cell to the bundle sheath cells where it is decarboxylated by the malic enzyme to yield CO_2 and pyruvate. The resulting CO_2 is fixed via the calvin cycle. Pyruvate returns to the mesophyll cell where it is reconverted into PEP and carboxylated to form oxaloacetate.

The conversion of pyruvate to PEP is catalyzed by the plant enzyme pyruvate-Pi dikinase which takes pyruvate, ATP and Pi to form PEP, AMP and PPi..

Plants that use this pathway are called C4 plants.
Plants that use the conventional CO_2 uptake are called C3 plants.