Regulation of carbon dioxide fixation in the chemoautotroph Xanthobacter flavus

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2000

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):
Keulen, G. V. (2000). Regulation of carbon dioxide fixation in the chemoautotroph Xanthobacter flavus. s.n.

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CHAPTER 1

General introduction

SOMETHING FROM ALMOST NOTHING: Carbon Dioxide Fixation in Chemoautotrophs

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ABSTRACT

The last decade has seen significant advances in our understanding of the physiology, ecology, and molecular biology of chemoautotrophic bacteria. Many ecosystems are dependent on CO₂ fixation by either free-living or symbiotic chemoautotrophs. CO₂ fixation in the chemoautotroph occurs via the Calvin-Benson-Bassham cycle. The cycle is characterized by three unique enzymatic activities: ribulose bisphosphate carboxylase/oxygenase, phosphoribulokinase, and sedoheptulose bisphosphatase. Ribulose bisphosphate carboxylase/oxygenase is commonly found in the cytoplasm, but a number of bacteria package much of the enzyme into polyhedral organelles, the carboxysomes. The carboxysome genes are located adjacent to cbb genes, which are often, but not always, clustered in large operons. The availability of carbon and reduced substrates control the expression of cbb genes in concert with the LysR-type transcriptional regulator, CbbR. Additional regulatory proteins may also be involved. All of these, as well as related topics, are discussed in detail in this review.

INTRODUCTION

Since the previous comprehensive reviews (79, 159) were published, there has been an explosion of information regarding bacterial CO₂ fixation. The vast majority of the articles referenced in these two reviews were published prior to 1990. Thus, the preparation of a new review seemed timely. In view of the mass of information, space limitations, and our own particular interest, the scope of this review has been limited to CO₂ fixation in chemoautotrophs. Other autotrophs are compared/contrasted only where deemed appropriate.

Chemoautotrophic bacteria were some of the first bacteria studied. Now, over a century later, they still retain a great deal of scientific interest. In recent years, major advances have been made in understanding CO₂ fixation in these organisms—including the physiology, biochemistry, and molecular biology of the fixation process; the regulation of, and interaction between, the Calvin cycle and central metabolic pathways; and the involvement of the specialized organelle unique among prokaryotes, the carboxysome—and a clearer understanding of the various ecological niches occupied by these organisms has been gained.

THE CALVIN CYCLE

Occurrence in Bacteria

FACULTATIVE AND OBLIGATE AUTOTROPHS Chemoautotrophic bacteria are subdivided into two major groups: obligate chemoautotrophic bacteria, which are completely dependent on CO₂ fixation, and facultative chemoautotrophs, which assimilate CO₂ via the Calvin cycle and in addition have the ability to use a wide range of other growth substrates.

Obligate chemoautotrophs are specialists. Their metabolism is optimized for the utilization of a small number of reduced substrates (6, 75). In general these bacteria have a high affinity for reduced growth substrates and display high growth rates compared with nonspecialists. In addition, obligate chemoautotrophs growing under nutrient limiting conditions are able to oxidize 300–400% more reduced substrate than is available in the medium. The high growth rate and respiratory overcapacity allow the specialist to rapidly respond to sudden changes in the availability of reduced substrates because the metabolic pathways required for their utilization are already present and need not be induced.

Facultatively chemoautotrophic bacteria display an enormous metabolic versatility, which allows them to grow on a wide range of substrates. In contrast to obligate autotrophs, catabolic and anabolic pathways of facultatively autotrophic bacteria are inducible. A
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A characteristic of these bacteria is the ability to grow mixotrophically, i.e. substrates supporting autotrophic and heterotrophic growth are used simultaneously (e.g. 25). Both specialist and facultative strategies can be advantageous, depending on the growth conditions (6). As a consequence of their metabolic characteristics, obligate and facultative chemoautotrophs have different environmental niches. The former are encountered where there is a continuous or fluctuating supply of reduced inorganic compounds with a low turnover of organic compounds, whereas the latter thrives when both inorganic reduced substrates and organic compounds are present.

**AUTOTROPHIC GROWTH SUBSTRATES** Molecular hydrogen and reduced nitrogen (e.g. NH$_4^+$, NO$_2^-$), sulfur (e.g. S$_2$O$_3^{2-}$, H$_2$S), metals (e.g. Fe$^{2+}$, Mn$^{2+}$), and carbon (e.g. CO, CH$_4$, CH$_3$OH) compounds serve as electron donors for chemoautotrophic bacteria. A variety of anthropogenic, biological, and geological processes are responsible for the introduction of reduced compounds in the biosphere. These include industry and agriculture, anaerobic metabolism in sediments and animal guts, and volcanic activity (75).

Phylogenetic studies based on 16S rRNA sequences of autotrophic bacteria showed that nitrifying bacteria and sulfur and iron oxidizers are not limited to one phylogenetic group. Ammonia oxidizers belong to the β- and γ-subdivision of the proteobacteria; nitrite oxidizers are found in the α-, β-, γ-, and δ-subdivisions (163). Representatives of iron and sulfur oxidizers are present in all subdivisions of the proteobacteria and in the gram-positive division (53, 84, 108).

**FREE-LIVING AUTOTROPHIC BACTERIA** Free-living autotrophic bacteria require a sufficient supply of reduced organic and inorganic compounds to serve as electron donors as well as a supply of substrates, which may serve as electron acceptors. Because many reduced substrates are produced in vastly different, sometimes harsh environments, chemoautotrophic bacteria display a staggering physiological diversity that includes acidophiles, thermophiles, and psychrophiles. For example, the optimum growth temperature and pH of sulfur reducing bacteria varies between 25°C–72°C and pH 2–8. Many reduced substrates are produced anaerobically, in sediments for example, which subsequently accumulate and diffuse to aerobic layers. Substrates such as sulfide and ferrous iron are rapidly and spontaneously oxidized under aerobic conditions (16, 152). Chemoautotrophic bacteria generally require oxygen as an electron acceptor and are therefore limited to the oxic layer, where they have to compete with the chemical oxidation of reduced inorganic compounds. Thus, it is not surprising that chemoautotrophic bacteria depending on oxygen-labile compounds as electron donors position themselves at the oxic/anoxic interface of sediments or stratified lakes (33, 107). Because this interface is usually highly dynamic, autotrophic bacteria are generally chemotactic toward the chemocline. A number of H$_2$S-oxidizing bacteria, such as *Beggiatoa* and *Thiovulum* spp., form microbial mats or veils, which are surrounded by a thin, unstirred layer. This provides the bacteria with a stable microenvironment, which offers protection against sudden changes in H$_2$S and O$_2$ concentrations and therefore represents an adaptation to life at the interface between H$_2$S and O$_2$ (66). Some chemoautotrophic bacteria are able to replace oxygen as terminal electron acceptors with, for example, nitrate or ferric iron. *Thiobacillus ferrooxidans* is able to use ferric iron as an electron acceptor during growth on elemental sulfur, although the growth yield is about twofold lower than during aerobic growth (122). Interestingly, these alternative electron acceptors are products of aerobic chemolithoautotrophic oxidation.

A remarkable adaptation required for the use of reduced iron is the ability to grow at a pH between 0.5 and 4. Iron in the biosphere is usually present as ferric iron in the form of insoluble complexes. The concentration of ferrous iron at neutral pH is generally low because at this pH ferric iron is poorly soluble and ferrous iron is rapidly oxidized. The solubility of ferric iron increases and the rate of ferrous iron oxidation strongly decreases below pH 4 (152), which explains why many iron-oxidizing bacteria are acidophiles. Iron oxidizers growing at neutral pH, such as *Gallionella ferruginea*, are usually encountered at
the oxic/anoxic interface because of the rapid oxidation of ferrous iron in the presence of oxygen.

SYMBIOTIC AUTOTROPHIC BACTERIA In 1977, hydrothermal vents with an abundance of associated fauna were discovered 2500 m below the surface of the sea (22). Because these communities thrive in complete darkness, it was obvious that the primary production was not dependent on photosynthesis. This led to the discovery of symbiosis between sulfide-oxidizing bacteria and invertebrates, in which CO₂ fixation by the Calvin cycle provides the invertebrate host with a source of organic carbon. Since then, it has been shown that this type of symbiosis is commonplace in habitats where oxygen-labile sulfide and oxygen are both present. To date, symbiotic associations involving over 200 bacterial species and hosts belonging to six different phyla have been discovered (12).

The fact that symbiosis is widespread indicates that there are distinct advantages to a symbiotic relationship. As discussed above, sulfide-oxidizing chemoautotrophic bacteria grow at the oxic/anoxic interface because of the extreme oxygen-labile nature of sulfide. The association with an invertebrate host helps sulfide-oxidizing bacteria overcome these limitations. The host will—by means of its size, movement, or burrowing—bridge the aerobic-anaerobic interface that gives the sulfide oxidizer access to both oxygen and sulfide (12). The host benefits from the presence of chemoautotrophic bacteria because they provide a source of organic substrates. In a number of cases, the symbiotic host lacks a digestive system and is completely dependent on the autotrophic activity of its symbiotic partner.

It is unclear how these symbiotic relationships originated. Phylogenetic studies showed that the symbiotic autotrophs within the bivalve families Vesicomyidae and Lucinacea are monophyletic, i.e. have a common ancestor. In addition, the available fossil record of the host and the 16S rRNA sequence data of the bacterial symbiont indicated that cospeciation of bacterial symbiont and host had occurred (27). This supports the hypothesis that symbiosis was established relatively early, with a single symbiotic ancestor from which the present symbiotic associations evolved. However, this may not be true for all symbiotic associations. Closely related chemoautotrophs were discovered in host species belonging to distantly related phyla, indicating that these bacterial symbionts have the ability to associate with more than one host (29). Conversely, symbionts of clams belonging to the bivalve genus Solemya are polyphyletic, indicating that symbiosis in Solemya originated from independent events (74). These data and the observation that symbiotic chemoautotrophic bacteria have different phylogenetic origins in the γ- or ε-subdivision of the proteobacteria indicate that many chemoautotrophic bacteria had the ability to enter a symbiotic relationship (29, 32, 74, 116, 117). Methanotrophic bacteria are also able to form a symbiotic association with marine invertebrates and, in some cases, coexist in the same cell with symbiotic chemoautotrophic bacteria (13, 28, 39). These observations indicate that current diversity in symbiotic associations results from numerous independent symbiotic events that occurred throughout evolution.

COMMUNITIES DEPENDING ON CHEMOAUTOTROPHY Primary production in the terrestrial environment is largely dependent on photosynthesis cyanobacteria, plants, and algae. Marine organisms at depths greater than 300 m live in constant darkness and are therefore dependent on input of organic material from surface waters. The deep sea is therefore similar to a desert environment. The discovery of hydrothermal vents and associated invertebrate communities 2500 m below the surface showed that oases exist in the ocean-desert (12, 52). Because these communities thrive in total darkness, it is unlikely that they are dependent on the influx of organic matter from surface waters. The fluid excreted by hydrothermal vents contains H₂S and smaller amounts of H₂, NH₄⁺, and CH₄; the fluid is anoxic, highly reduced, with temperatures ranging from 5°–25°C for “warm vents” to 350°C for “black smokers” (63). The chemical and physical characteristics of hydrothermal fluid immediately suggested that chemoautotrophic metabolism may be the basis of the food chain in these deep-sea communities (184). Large numbers of bacteria are
present in the milky-bluish waters flowing from warm vents, in microbial mats that form on virtually all areas exposed to the warm-vent plumes, and in symbiotic association with invertebrates (discussed above).

Given the variety in growth conditions of hydrothermal vents, it is hardly surprising that microbial communities associated with hydrothermal vents display a large physiological and phylogenetic diversity (110, 111, 136). However, the majority of chemoautotrophs are sulfur oxidizers because reduced-sulfur compounds are the predominant constituents of hydrothermal fluid usable as electron donors in chemosynthesis. Heterotrophic, chemoheterotrophic, and obligately chemoautotrophic sulfur-oxidizing bacteria were isolated from deep-sea hydrothermal vents of the Galapagos Rift. The latter were characterized as *Thiomicrospira* spp. and the former two as *Pseudomonas*-like and *Thiobacillus*-like bacteria, respectively. In addition, microbial mats resembling those formed by sulfur-oxidizing *Thiothrix* and *Beggiatoa* spp. were observed (136). A phylogenetic analysis, based on 16S rRNA sequences isolated from microbial mats at a hydrothermal vent system in Hawaii, showed that this community was dominated (60% of isolates) by bacteria affiliated with *Thiovulum* spp. that are in the ε-subdivision of the proteobacteria. These bacteria produce veils, which is an adaptation to the oxic/anoxic interface, as discussed earlier. Bacteria belonging to the γ- and δ-subdivisions of the proteobacteria formed the second and third largest groups (110). In contrast to the bacterial diversity of these hydrothermal vents, a mid-Atlantic Ridge hydrothermal vent community is dominated by a single bacterial species belonging to the ε-subdivision (116).

The only terrestrial ecosystem completely dependent on chemoautotrophy was recently discovered in a Romanian cave. As in the hydrothermal vents, the chemoautotrophic bacteria in this system oxidize H₂S, which is present in a stream feeding the cave system (138). The bacteria in microbial mats that form on the surface of the H₂S-rich water are predominately *Thiobacillus thioparus* (176).

**Overview of the Calvin Cycle** (97, 159)

Carbon dioxide fixation by the Calvin cycle is dependent on 13 enzymatic reactions (Figure 1). The enzyme responsible for the actual fixation of CO₂, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), catalyzes the carboxylation of ribulose-1,5-bisphosphate (RuBP) to form two molecules of 3-phosphoglycerate. The other enzymes of the Calvin cycle are dedicated to the regeneration of RuBP. Three glycolytic enzymes—phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, and triosephosphate isomerase—convert two molecules of 3-phosphoglycerate to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate at the expense of two molecules of ATP and two of NADH. This is followed by a series of rearrangement reactions that result in the production of ribulose-5-phosphate.

The rearrangement reactions are divided into two similar metabolic units, made up of an aldolase, a phosphatase, and a transketolase (APT). The first APT unit converts glyceraldehyde-3-phosphate and dihydroxyacetone phosphate into xylulose-5-phosphate and erythrose-4-phosphate, which is followed by the formation of xylulose-5-phosphate and ribulose-5-phosphate from dihydroxyacetone phosphate and erythrose-4-phosphate by the second unit. The phosphatase used in the second APT unit [sedoheptulose bisphosphatase (SBPase)] catalyzes the dephosphorylation of SBP, which is, in contrast to the gluconeogenic fructose bisphosphatase (FBPase), an activity unique to the Calvin cycle. In principle, the pentose phosphate cycle would provide an alternative for these rearrangement reactions. In this scenario, the aldolase and phosphatase of the second APT unit are replaced by transaldolase. The main difference is that the use of a phosphatase in the aldolase/SBPase variant renders the second APT unit irreversible, whereas it is reversible in the transaldolase variant. All autotrophic organisms studied to date employ the aldolase/SBPase variant of the Calvin cycle.

The sequential reactions of the two APT units result in the formation of xylulose-5-phosphate and ribose-5-phosphate. These are converted to ribulose-5-phosphate by pentose
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Figure 1 The Calvin cycle. Metabolites: DHAP, dihydroxyacetonephosphate; E-4-P, erythrose-4-phosphate; F-6-P, fructose-6-phosphate; F-1,6-P, fructose-1,6-bisphosphate; GAP, glyceraldehyde-3-phosphate; 3-PGA, 3-phosphoglycerate; 1,3-PGA, 1,3-diphosphoglycerate; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; Ru-1,5-P, ribulose-1,5-bisphosphate; S-7-P, sedoheptulose-7-phosphate; S-1,7-P, sedoheptulose-1,7-bisphosphate; Xu-5-P, xylulose-5-phosphate; Pi, inorganic phosphate. Enzymes: ALD, aldolase; FBA, fructose-1,6-bisphosphate aldolase; FBPase, fructose-1,6-bisphosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PPE, pentose-5-phosphate epimerase; PPI, pentose-5-phosphate isomerase; PRK, phosphoribulokinase; RuBisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; SBPase, sedoheptulose bisphosphatase; TKT, transketolase; TPI, triosephosphate isomerase.

phosphate epimerase and pentose phosphate isomerase, respectively. The final step in the regeneration of RuBP is catalyzed by another unique Calvin cycle enzyme, phosphoribulokinase (PRK), which phosphorylates ribulose-5-phosphate at the expense of an ATP. The net result of the activity of the Calvin cycle is the formation of one molecule of triose phosphate from three molecules of CO₂ at the expense of nine molecules of ATP and six of NADH.

In addition to the carboxylation of RuBP, RuBisCO catalyzes the oxygenation of this substrate, resulting in the formation of phosphoglycolate and 3-phosphoglycerate. The incorporation of O₂ instead of CO₂ is an intrinsic characteristic of RuBisCO, the extent of which depends on the properties of the particular enzyme studied (65). The production of phosphoglycolate during aerobic autotrophic growth is therefore unavoidable. Phosphoglycolate itself is of little use to the organism and may even be harmful, as it is a known inhibitor of triosephosphate isomerase, one of the enzymes of the Calvin cycle (89). Chemoautotrophic bacteria dispose of phosphoglycolate by using phosphoglycolate
phosphatase, which catalyzes the formation of glycolate, which is subsequently either metabolized or excreted, depending on the organism (see e.g. 21).

Enzymes (55, 153)

UNIQUE CALVIN CYCLE ENZYMES Three enzymatic activities are unique to the Calvin cycle: RuBisCO, PRK, and SBPase. The latter are discussed in the section on Isoenzymes. We only briefly discuss RuBisCO because recent reviews provide excellent overviews of its biochemistry, engineering, and structure-function relationships. Two types of RuBisCO are encountered in chemoautotrophic bacteria. The dominant form I enzyme, found in most chemoautotrophs, is similar to the enzyme present in plants, algae, and cyanobacteria. It is composed of large (L) (50–55 kDa) and small (S) (12–18 kDa) subunits in a hexadecameric (L8S8) structure. The form II enzyme, which consists of large subunits only (L2, L4, or L8), was initially discovered in phototrophic purple non-sulfur bacteria, but it has since been found in certain symbiotic and free-living chemoautotrophic bacteria and, recently, in eukaryotic dinoflagellates (14, 19, 36, 60, 109, 124, 135, 155; JM Shively, unpublished data). *Thiobacillus denitrificans*, *Thiobacillus intermedius*, *Thiobacillus neapolitanus*, and *Hydrogenovibrio marinus* possess both a form I and form II RuBisCO, which, with the exception of *Rhodospirillum rubrum*, is also the case for purple non-sulfur bacteria. The bacterial form I enzymes are similar to enzymes encountered in higher plants with respect to their structure and biochemical properties. In contrast, even though the residues involved in catalysis and activation are conserved, the amino acid sequence similarity between the form I and form II large subunits is only 35%.

The efficiency of RuBisCO is commonly evaluated by determining its specificity factor, tau (τ), i.e. the enzyme's ability to discriminate between carboxylation and oxygenation (65). The form II enzymes have a characteristically low τ value between 10 and 20 (128, 160). The form I enzymes of higher plants generally have τ values above 80 (128, 168). Interestingly, very high τ values have been recently reported in several eukaryotic algae (128, 167, 168). The form I enzymes of bacteria, including chemo- and photoautotrophic bacteria and cyanobacteria, as well as green algae, are intermediate, commonly falling between 30 and 60 (56, 128, 160). A number of factors have been shown to alter specificity, including mutations resulting in alterations of both the large and small subunits, the creation of hybrid RuBisCO, and the interaction of the enzyme with other proteins (18, 126, 127, 153). The τ values for the form I and form II enzymes makes it understandable why *H. marinus* predominantly synthesizes form II RuBisCO when grown in an atmosphere containing 10% CO2, whereas form I RuBisCO is the dominant enzyme during growth in the presence of 2% CO2 (60). A similar differential regulation of form I and form II RuBisCO was observed earlier in the phototroph *Rhodobacter sphaeroides* (68).

The second unique enzyme of the Calvin cycle, PRK, has received considerably less attention. PRK from *Ralstonia eutropha* and the photosynthetic bacteria, *Rhodopseudomonas acidophila* and *Rb. sphaeroides*, are octameric proteins with subunits of 32–36 kDa (47, 133, 150, 151, 158). In contrast to RuBisCO, bacterial PRK is not related to the dimeric enzyme (45 kDa subunits) from higher plants (73). The activity of PRK in bacteria is tightly regulated by the concentration of intracellular metabolites. With the exception of the enzyme from *T. neapolitanus* (93), activity of phosphoribulokinases from chemoautotrophic bacteria is dependent on activation by NADH (1, 70, 92, 151), which, with few exceptions, is also true for the PRK from phototrophic purple bacteria (132, 133, 158). Phosphoenolpyruvate and AMP are common inhibitors of PRK (1, 4, 46, 91, 93). The regulation of PRK activity by NADH and AMP can be rationalized as a response of the enzyme to the redox and energy state of the cell. The concentration of phosphoenolpyruvate may reflect the amount of carbon available. In addition to allosteric regulation, the activity of PRK of *R. eutropha* is apparently regulated by an additional mechanism that may involve covalent modification of the enzyme. Addition of pyruvate to a culture growing autotrophically resulted in the rapid inactivation of PRK, which was paralleled by a decrease
ISOENZYMES The most obvious change in the metabolism of facultatively autotrophic bacteria following the transition from heterotrophic to autotrophic growth is the induction of RuBisCO and PRK, which are unique to the Calvin cycle. In addition, the activity of Calvin cycle enzymes other than RuBisCO and PRK increase dramatically following the transition to autotrophic growth (102). Mutants with strongly reduced activities in phosphoglycerate kinase and triosephosphate isomerase have been isolated that only had marginally reduced growth rates while growing on heterotrophic growth substrates such as succinate and gluconate. In contrast, neither mutant was able to grow autotrophically, despite the fact that the other Calvin cycle enzymes were fully induced (99, 103). The sharp increase in activity of enzymes other than RuBisCO and PRK has therefore been interpreted as a reflection of the increased carbon flux required to assimilate sufficient CO₂ to allow autotrophic growth to proceed.

In addition to the significant increase in activity of Calvin cycle enzymes other than RuBisCO and PRK, biochemical and genetic analysis have identified isoenzyme forms of some of these enzymes. The first to be discovered was FBPase. Johnson & MacElroy (64) noted that in contrast to the gluconeogenic enzyme from heterotrophic organisms, the FBPase of T. neapolitanus was not stimulated by acetyl coenzyme A but was inhibited by phosphoenolpyruvate. It was later shown that Thiobacillus versutus, Nocardia opaca, and Xanthobacter flavus contained different FBPase activities following heterotrophic and autotrophic growth that were catalyzed by two different proteins (2, 105, 172, 185). The subsequent characterization of the FBPase proteins from N. opaca and X. flavus showed that the enzyme induced during autotrophic growth was twice as active with sedoheptulose bisphosphate as the substrate in comparison to fructose bisphosphate. The constitutive FBPase of N. opaca and X. flavus dephosphorylated both substrates in a 1:4.5 and 1:1 ratio, respectively. In addition to the differences in substrate specificity, the two forms of FBPase also displayed differences in the regulation of their activity. ATP caused a twofold increase in activity of the inducible enzyme of X. flavus; the constitutive enzyme was unaffected. The autotrophic enzyme of N. opaca was inhibited by ATP and RuBP, whereas AMP and phosphoenolpyruvate had no effect. In contrast, the constitutive form was inhibited by the latter two metabolites.

Two unrelated and mechanistically distinct types of aldolase are encountered in Bacteria, Archaea, and Eukarya. Class I aldolase forms a Schiff base between the substrate and the ε-amino group of a lysine residue during catalysis, whereas class II enzymes depend on a divalent cation as electrophile in the catalytic cycle (94). Following heterotrophic growth of X. flavus, aldolase activity was independent of the addition of Fe²⁺, whereas aldolase activity was stimulated 14-fold by Fe²⁺ following autotrophic growth (170). These data strongly suggest that X. flavus employs a constitutive class I aldolase and induces a class II aldolase during autotrophic growth.

The function of most of the isoenzymes is unknown because specific gene disruptions to study their role in metabolism have not been constructed. It is possible that these isoenzymes are merely needed to increase activity of the enzymes of the Calvin cycle, which is necessary to allow for the high rate of CO₂ fixation required to sustain autotrophic growth. Alternatively, their activity and regulation may be tailored for a role in the Calvin cycle. This certainly seems to be true for the inducible FBPase of N. opaca and X. flavus. The high SBPase activity of this enzyme, and the regulatory differences between the autotrophic and heterotrophic enzymes, indicate that the in vivo role of this enzyme is that of an SBPase, a characteristic enzyme of the Calvin cycle. The biochemical characterization of the two FBPase forms of X. flavus and N. opaca indicates that the constitutive enzyme characterized by a high FBPase-to-SBPase ratio plays a role in the first APT unit of the Calvin cycle, whereas the inducible FBPase/SBPase catalyzes the dephosphorylation of sedoheptulose bisphosphate in the second APT unit (Figure 1). In addition to the inducible FBPase/SBPase, the cbb operon also frequently
encodes transketolase and class II aldolase (see THE GENES AND THEIR LOCATION/ORGANIZATION, below). It is therefore likely that the latter enzymes also operate in the second APT unit.

CARBOXYSONES

The enzymes of the Calvin cycle are located in the cytoplasm in most autotrophic bacteria. A notable exception is RuBisCO, which in a number of chemooautotrophic bacteria and all cyanobacteria, is located in polyhedral inclusion bodies known as carboxysomes.

Occurrence

Inclusion bodies with polygonal profiles have been reported in all cyanobacteria examined, in a very limited number of other photosynthetic prokaryotes, and in many, but not all, chemooautotrophic bacteria (20, 81, 82, 147–149). Presumably, all polyhedral bodies in organisms utilizing CO₂ as a carbon source are carboxysomes. Polyhedral bodies were first isolated over 25 years ago from *T. neapolitanus*, shown to contain the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase, and named carboxysomes (144). Since then, carboxysomes have been isolated from a number of organisms, including *Chlorogloeopsis fritschii*, *Nitrobacter agilis*, *Nitrobacter hamburgensis*, *Nitrooccus nitroreducens*, *Synechococcus PCC7942*, and *Thiobacillus thyasiris* (10, 20, 30, 57, 58, 81, 82, 120, 147–149). The presence of carboxysomes in chemooautotrophs appears to be limited either to the obligate autotrophs or to facultative types that grow equally well both autotrophically and heterotrophically. Those chemooautotrophs that exhibit high heterotrophic growth yields have little need for the selective advantage gained by possessing carboxysomes.

Structure

In thin section, the carboxysomes of chemooautotrophs are most commonly regular hexagons about 120 nm in diameter, exhibiting granular substructure, and surrounded by a shell 3–4 nm across (57, 115, 145, 146). This size, 120 nm, would also be acceptable for the majority of carboxysomes, but much larger inclusions with polygonal profiles have been reported in some cyanobacteria (20, 147). In rare instances the bodies appear elongated in one dimension (145).

To date, the question of whether RuBisCO fills the carboxysome completely or just lines the inner surface of the carboxysome shell has not been resolved. The appearance of a uniform, granular substructure in a multitude of thin sections of carboxysomes within cells suggests to most researchers that the inclusions are filled with RuBisCO. Immunogold labeling of numerous thin sections of cyanobacterial carboxysomes is even more supportive (98, 157). However, assuming a shell thickness of 3.5 nm, a RuBisCO diameter of 11 nm, and a 60- to 80-nm average thickness for the thin sections, one might argue that obtaining an exact center section would be an unlikely event. Furthermore, the appearance of this center section within the cell might be missed unless one is specifically searching for that particular image. The arrangement of RuBisCO in rows inside of isolated carboxysomes of *T. neapolitanus* as observed in negative stained preparations prompted Holthuijzen and coworkers (57) to hypothesize that RuBisCO lines the inner surface of the shell as a monolayer. In their opinion, this hypothesis was strongly supported by the discovery of a RuBisCO-to-shell peptides ratio of 1:1 and by the extremely tight association of the small subunit of RuBisCO with the shell in broken carboxysomes (57, 58). These authors did not speculate on the possible contents or function, if any, of the resulting center cavity.

Examination of thin sections of purified carboxysomes of *T. neapolitanus* revealed three distinct profiles: hexagons with completely empty centers, hexagons with centers of decreased staining intensity, and hexagons of even staining intensity (145; JM Shively,
unpublished observations). These observations seem to suggest that RuBisCO might line the inner surface of the shell. However, these structures might represent different stages of carboxysome breakage or may simply be an artifact created by the isolation and/or electron microscopy preparation procedures. The RuBisCO-lining-the-shell model is attractive because it eliminates the necessity for the extensive diffusion of the enzyme substrates/products in and out of a filled and crowded structure, i.e. the fixation of CO2 becomes a “surface”—albeit inner surface of the shell—phenomenon. Recent evidence does suggest that the diffusion of the substrate RuBP and the product PGA play a role in the activity of carboxysomal RuBisCO, but this observation neither favors nor discounts either of the structural possibilities (139). An attempt to differentiate between the two structural possibilities using the stoichiometry of the *T. neapolitanus* carboxysome polypeptides elucidated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) in conjunction with a variety of other known carboxysome parameters was unsuccessful (148).

Finally, it should be noted that the much larger polyhedral bodies found in some cyanobacteria possess an even, granular substructure throughout, thereby providing evidence for a filled structure. One should keep in mind, however, that a polyhedral body is not necessarily a carboxysome; the polyhedral bodies of enteric bacteria (see below) and the carboxysomes of chemoautotrophs provide a perfect example. One might also consider that two polyhedral bodies with RuBisCO, i.e. carboxysomes from chemoautotrophs and cyanobacteria, may not necessarily possess identical structure/function. However, this seems unlikely because carboxysome-possessing chemoautotrophs and cyanobacteria for all practical purposes face the same dilemma, the overriding presence of O2.

RuBisCO is the only enzyme whose presence in the carboxysome has been unequivocally established. Carbonic anhydrase (CA) was reported to be present in the carboxysomes of *Synechococcus* PCC7942, but the preparations were grossly contaminated with cytoplasmic/outer membrane, a common cellular location of carbonic anhydrase (120). Attempts to show CA activity in carboxysome fractions of *Chlorella fritschii* were unsuccessful even though the activity was easily demonstrated in whole cells and broken cell fractions (83).

Purified carboxysomes of *T. neapolitanus*, three *Nitrobacter* spp., and *C. fritschii* were shown several years ago to possess from 7 to 15 polypeptides (7, 10, 30, 58, 81). The molecular masses of these polypeptides were from 10 to 120 kDa. No additional information has been reported for the carboxysomes of either *C. fritschii* or *Nitrobacter*. Recently the presence of eight polypeptides in the carboxysomes of *T. neapolitanus* has been shown (149). The existence of three additional polypeptides is deemed likely, i.e. a total of 11 (149; JM Shively, unpublished data). Two of the identified polypeptides with molecular masses of 51 and 9 kDa are the L and S subunits of RuBisCO, respectively. Because the six identified polypeptides have not been shown to possess any enzymatic activity, they have been designated with Cso for carboxysome, S for shell, and numbers and letters for further identification (35, 149). These six polypeptides, with molecular masses—as determined by SDS-PAGE—of 130 kDa (CsoS2B), 80 kDa (CsoS2A), 44 kDa (CsoS3), 15 kDa (CsoS1B), and 5 kDa (CsoS1A and CsoS1C), are believed to be shell components and are thought to be glycosylated (10, 35, 58, 149; JM Shively, unpublished data). The established presence of these peptides was based on the determination of the polypeptide composition of purified carboxysomes using SDS-PAGE and on genetic analyses (see below). CsoS1A, B, and C are structural homologs resulting from the transcription/translation of a three-gene repeat (35). Sequencing of the N terminus of CsoS2A and B yielded the same 15 amino acids, indicating they are products of the same gene; at least one and possibly both are the result of posttranslational processing (149; JM Shively, unpublished data). One of the additional polypeptides (38 kDa) appears to be a component of the mature carboxysome, but its corresponding gene is yet to be identified (see below). The other two polypeptides, structural homologs resulting from the transcription/translation of a duplicated gene (ORFA and B), have not been observed by SDS-PAGE of purified carboxysomes. Their presence is inferred from their being part of a putative carboxysome operon (149; see below). One needs to
consider that some polypeptides may be required solely for the assembly of the carboxysome. The existence of other quantitatively minor but functionally essential components is always possible. Although a number of putative carboxysome genes have been elucidated in *Synechococcus* PCC7942, the direct identification of polypeptides as components of the purified carboxysome has not been forthcoming.

**Hypothesized Function(s)**

The regulation of RuBisCO synthesis, as well as its packaging into the carboxysome, via the availability of CO₂ to growing cultures of *T. neapolitanus* strongly suggested an active role for the carboxysome in carbon dioxide fixation (5). Under CO₂ limitation, the cells respond by synthesizing elevated levels of RuBisCO and sequestering a greater percentage of the enzyme into the carboxysome. Under conditions of excess CO₂ the reverse is true: Less RuBisCO is synthesized and a lower percentage is packaged. Furthermore, in the facultative autotroph, *T. intermedium*, both the enzyme and the carboxysomes are, for all practical purposes, totally repressed when the culture is supplied with suitable organic nutrients (148). More importantly, alleviating the repression by transferring the cells to an autotrophic growth environment results in the immediate formation of carboxysomes, i.e. a large pool of enzyme is not accumulated before packaging begins (JM Shively, unpublished data). The availability of carbon is also a controlling factor in the sequestering of RuBisCO into the carboxysomes of cyanobacteria (98, 165). Compelling evidence for the carboxysome’s active role in carbon fixation has been garnered via genetic manipulation and analysis. Insertion mutation of carboxysome genes in both *T. neapolitanus* and *Synechococcus* results in a reduction in number, a structural modification, or a total loss of carboxysomes, resulting in a requirement of elevated CO₂ for growth (34, 42, 95, 96, 118, 134; JM Shively, unpublished data). Thus, it is now widely accepted that the carboxysome is not only actively involved in the fixation of CO₂, it somehow enhances the CO₂-fixing ability of its RuBisCO. The possible presence of CA inside the carboxysomes of the cyanobacterium *Synechococcus* PCC7942 provides the basis for an interesting and attractive hypothesis regarding carboxysome function (69, 119, 129). Essentially, the mechanism for CO₂ fixation enhancement calls for the diffusion of HCO₃⁻ into the interior of the carboxysome, where it is rapidly converted to CO₂ by the CA. The CO₂ is fixed by RuBisCO before outward diffusion can take place. The uptake of HCO₃⁻ into the carboxysome would have to be driven by a concentration gradient of some type. This gradient would be created, at least in part, by the rapid conversion of HCO₃⁻ to CO₂ by CA and the concomitant fixation of the formed CO₂ by RuBisCO. It should be noted that the production of protons by the RuBisCO reaction itself could promote the formation of CO₂ without the necessity of CA. Furthermore, as reported above, the presence of CA has not been unequivocally established. Also, it is somewhat bothersome that the carboxysome shell appears to exhibit little if any selective permeability for the substrates and/or products of RuBisCO (139, 148). O₂ can readily diffuse into the carboxysome. An alternate, and as yet untested, hypothesis to account for the fixation enhancement is the creation of an improved τ value for RuBisCO. This might result from the association of the enzyme with a certain polypeptide(s) in the assembled carboxysome. The very tight association of the small subunit of RuBisCO with components of the carboxysome shell has been reported (57, 58). Chen and coworkers (18) suggest that a nuclear-encoded protein in *Chlamydomonas reinhardtii* might be able to influence the structure (conformation) of RuBisCO, which in turn alters its τ factor.

**Relationship of Other Polyhedral Bodies to Carboxysomes**

Inclusions with polygonal profiles have recently been described in *Salmonella enterica* (*Salmonella typhimurium*), in *Klebsiella oxytoca*, and in *Escherichia coli* (146), and their presence in other enteric bacteria seems likely. The polyhedral bodies of enteric organisms, also surrounded by a shell or envelope, are commonly more irregular, slightly smaller, and somewhat less dense in appearance than those of their chemoautotrophic counterparts.
(146). Extensive electron microscopy studies suggest that the bodies of enteric bacteria are filled with a proteinaceous material (HC Aldrich, personal communication). Although these bodies are obviously not carboxysomes, they are definitely related structurally (see below). The inclusions are formed only when the organisms are grown anaerobically with either ethanolamine or propanediol as the energy source (17, 154). S. typhimurium with mutations in either the ethanolamine (eut) or propanediol (pdu) operons are unable to form the inclusions (17, 154). The degradation of both ethanolamine and propanediol proceeds via a coenzyme vitamin B12-dependent pathway, thereby providing a common thread for their occurrence/structure. No information is available on the peptide composition of these polyhedral bodies. However, genetic studies demonstrate that the probable shell polypeptides PduA and CchA, coded for by genes in the propanediol and ethanolamine operons, respectively, have significant sequence similarity with the CsoS1 carboxysome polypeptides of T. neapolitanus and with putative carboxysome polypeptides of Synechococcus PCC7942 (146). Interestingly, it was proposed that the polyhedral bodies of S. typhimurium might serve to protect the Pdu (propanediol) enzymes from O2 (17). Alternatively, the microcompartments might serve to protect the enzymes of ethanolamine and propanediol metabolism from toxic aldehydes (154).

THE GENES AND THEIR LOCATION/ORGANIZATION

Calvin:Benson:Bassham (cbb) Genes

The organization of the Calvin cycle genes of both photo- and chemoautotrophic bacteria has been recently reviewed in detail (80). Thus, our coverage is limited to Figure 2, which shows gene location/organization, some summary statements, and a few comments that provide essential information for other parts of the review. The genes for the bacterial enzymes that function exclusively in the Calvin-Benson-Bassham cycle are given the designation cbb (161). (The gene/enzyme designations are explained in Figures 1 and 2.) The cbb genes may be plasmid encoded (Oligotropha carboxidovorans), chromosome encoded (common), or both (N. hamburgensis, R. eutropha). A number of organisms exhibit cbb gene duplications. The presence of the cbbM gene for RuBisCO is more common than originally believed, with a number of bacteria possessing both cbbLS and cbbM. The cbbR gene, encoding the transcriptional regulator of the cbb genes, is present in all but three of the bacteria thus far examined, and in the latter bacteria cbbR may be located elsewhere on the genome. The cbbR does not have to be directly adjacent to the genes it regulates. With the exception of cbbR in Rs. rubrum, cbbR, when adjacent to cbb genes, is always transcribed in the opposite direction.

Carboxysome Genes

The carboxysome (cso) genes of T. neapolitanus reside immediately downstream of, and form an operon with, the genes for the large (cbbL) and small (cbbS) subunits of RuBisCO in the following order: csoS2, csoS3, ORFA, ORFB, csoS1C, csoS1A, and csoS1B (35, 149; JM Shively, unpublished data; see Figure 2). These genes code for their corresponding proteins described in the section on carboxysome structure. The location and function of the products of the gene repeat ORFA and B have not been determined. Probing genomic DNA of several thiobacilli and nitrifying and ammonia oxidizing bacteria with csoS1A yielded positive results, indicating that these carboxysome formers all have the well-conserved csoS1. The same gene organization observed in T. neapolitanus has been elucidated in T. intermedia K12 (JM Shively, unpublished data). In T. denitrificans, however, the csoS1 three-gene repeat is located in closer proximity to cbbM, the gene for the form II RuBisCO (JM Shively, unpublished data). Obviously, the carboxysome genes will also be located differently in Nitrobacter vulgaris (see Figure 2). The putative genes (ccmK, ccmL, ccmM,
Figure 2 Calvin cycle genes and their location/organization. Each letter identifies a \textit{cbb} gene. Gene and product abbreviations: \textit{cbb}LS, the large and small subunits of form I RuBisCO, respectively; \textit{cbb}M, form II RuBisCO; \textit{cbb}R, LysR-like transcriptional activator; \textit{cbb}F, FBPase/SBPase; \textit{cbb}P, PRK; \textit{cbb}T, TKT; \textit{cbb}A, FBA/SBA; \textit{cbb}E, PPE; \textit{cbb}K and \textit{pgk}, PGK; \textit{cbb}Z, PGP (phosphoglycolate phosphatase); \textit{cbb}B, X, Y, and Q, unknown. See Figure 1 for enzymes. Gene size is not depicted here. (arrow) Operons and transcriptional direction. \textit{R}. \textit{eutroph}-\textit{C} and -\textit{P} are chromosome and plasmid, respectively. Carboxysome genes are located downstream of \textit{cbb}LS in \textit{Thiobacillus intermedium} and \textit{Thiobacillus neapolitanus}. (From References 11, 49, 50, 77, 80, 113, 137, 174, 175, 187, 188.)

c\textit{ccm}N\text{, and c}\textit{ccm}O\text{) for the carboxysomes of \textit{Synechococcus} PCC7942 lie upstream (in descending order) from \textit{cbb}LS but constitute a separate operon (42, 121, 134). \textit{Synechocystis} PCC6803 has four copies of \textit{ccm}K and a \textit{ccm}O; none are located close to \textit{cbb}LS (146). As indicated above, the CcmK and CcmO polypeptides have a considerable amount of similarity to the CsoS1 polypeptides of \textit{T. neapolitanus} as well as to the CchA and PduA polypeptides of \textit{S. typhimurium} (146). Watson & Tabita (180) recently reported the presence of a \textit{ccm}K directly upstream and in the same operon with \textit{cbb}LS in a marine cyanobacterium. The product of this \textit{ccm}K has greater homology to the CsoS1A of \textit{T. neapolitanus} than to the CcmK of both \textit{Synechococcus} and \textit{Synechocystis} (146). All of the carboxysome-containing chemoautotrophs thus far examined possess at least one \textit{cbb}R. The location varies (Figure 2) and the regulation of the expression of carboxysome genes by CbbR is yet to be determined.
Phylogeny—Origin of the Calvin Cycle

The phylogeny of RuBisCO and the implications for the origins of eukaryotic organelles was recently extensively reviewed (24, 181), and therefore we only present the major conclusions regarding RuBisCO. A phylogenetic analysis of the large subunit of RuBisCO reveals two groups of form I RuBisCO: green-like and red-like (Figure 3). The green-like RuBisCO proteins are subdivided into a cluster containing proteins of cyanobacteria and of plastids of plants and green algae and into a cluster containing representatives of the α-, β-, and γ-subdivisions of the proteobacteria and cyanobacteria. The red-like RuBisCO proteins are also subdivided into two clusters: One contains proteins of non-green algae and one contains sequences of the α- and β-subdivisions of proteobacteria. Clearly, the RuBisCO phylogeny is at odds with 16S rRNA–based phylogenies, which led Delwiche & Palmer (24) to propose at least four lateral gene transfers to explain the dichotomy between red- and green-like RuBisCO sequences. One of the best examples of lateral gene transfer of form I RuBisCO is the presence of a green-like RuBisCO in *Rhodobacter capsulatus*, whereas *Rb. sphaeroides* has a red-like protein. In addition, both species contain a closely related form II RuBisCO. This indicates that the *Rhodobacter* ancestral species contained form II RuBisCO and subsequently acquired form I RuBisCO via lateral gene transfer (114). The phylogenetic tree of CbbR has the same topology as the RuBisCO tree, which includes grouping of *Rb. capsulatus* CbbR, which is encoded upstream from cbbLS, with proteins encoded upstream from green-like RuBisCO. This strongly suggests that cbbR-cbbLSQ of *Rb. capsulatus* was obtained by lateral gene transfer from a bacterium containing green-like RuBisCO (114).

Chapter 1
Figure 3 Phylogenetic relationships of form II and the large subunit of form I ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO). The unrooted phylogenetic tree is based on a distance matrix calculated using the Kimura model and constructed with the neighbor joining method as implemented in the PHYLIP 3.5c software package. Insertions and deletions were not taken into account. For clarity, only sequences of proteobacteria are indicated. 1, Ralstonia eutropha (chromosomal); 2, R. eutropha (plasmid); 3, Rhodobacter sphaeroides; 4, Xanthobacter flavus; 5, Hydrogenovibrio marinus; 6, Thiobacillus neapolitanus; 7, Nitrobacter vulgaris; 8, Thiobacillus denitrificans; 9, Chromatium vinosum; 10, Thiobacillus ferroxidans; 11, Pseudomonas hydrogenothermophila; 12, Rhodobacter capsulatus; 13, C. vinosum; 14, H. marinus; 15, T. denitrificans; 16, H. marinus; 17, Rh. sphaeroides; 18, Rh. capsulatus; 19, Rhodospirillum rubrum.

glyceraldehydephosphate dehydrogenase and phosphoglycerate kinase genes (cbbKG) into the cbb operon, whereas the closely related X. flavus employs the gap-pgk operon for both heterotrophic and autotrophic growth. The cbb operon is frequently found on mobile genetic elements of both proteobacteria and gram-positive bacteria, which provides a means for the lateral transfer of autotrophy (31, 43, 179).

REGULATION OF GENE EXPRESSION

Growth Conditions and Expression of Calvin Cycle Genes

The fixation of three molecules of CO_2 by the Calvin cycle requires the investment of nine molecules of ATP and six of NADH to obtain one molecule of glyceraldehyde-3-phosphate. It is obvious that this is an expensive process that has to be regulated carefully. The Calvin cycle is usually not induced during growth of facultatively autotrophic bacteria on substrates...
supporting fast heterotrophic growth, for example succinate or acetate (26, 45, 51). Growth of *R. eutropha* on fructose, glycerol, or gluconate, substrates that allow only intermediate or low growth rates leads to intermediate or high Calvin cycle enzyme levels (45). However, heterotrophic derepression of the Calvin cycle is only observed in strains harboring a megaplasmid containing one of the two *cbb* operons of *R. eutropha* (9). The facultatively autotrophic bacterium *Pseudomonas oxalaticus* simultaneously uses acetate and formate in batch cultures. Under these growth conditions formate is only used as an ancillary energy source and the Calvin cycle is not induced (26). The repression of the Calvin cycle is less severe during growth under carbon-limiting conditions (25, 44). Addition of formate to the feed of an acetate-limited continuous culture of *P. oxalaticus* resulted in simultaneous and complete utilization of the two substrates. Interestingly, the enzymes of the Calvin cycle remained absent at formate concentrations below 40 mM, whereas at higher concentrations they were induced. Because the bacterial dry mass of the culture increased by 40% when the formate concentration was increased from 0 to 40 mM, it was concluded that the use of formate as an additional energy source allowed a decreased dissimilation (via the citric acid cycle) and an increased assimilation (via the glyoxylate pathway) of acetate. Interestingly, growth of obligately autotrophic bacteria under CO₂ limitation also leads to increased activities of the Calvin cycle (5).

Although growth under carbon limitation conditions alleviates repression of the Calvin cycle, carbon starvation does not lead to the induction of the Calvin cycle (45). Under these conditions, the presence of reduced compounds supporting autotrophic growth are required, which generally stimulates the expression of the Calvin cycle. For example, the Calvin cycle is rapidly induced to autotrophic levels following the addition of formate, methanol, or H₂ to *X. flavus* growing on gluconate (102).

The results of these experiments show that two physiological parameters, the availability of carbon sources (including CO₂) and reduced (in)organic substrates supporting autotrophic growth, control the expression of the Calvin cycle in chemoautotrophic bacteria. This is most likely mediated by metabolites originating from carbon and energy metabolism. A reduced metabolite originating from the oxidation of reduced compounds (e.g. H₂S) is most likely responsible for the induction of the Calvin cycle. Chemostat experiments showed that methanol is a more potent inducer than formate, even though these compounds are metabolized via the same linear pathway (23). However, the oxidation of methanol generates three reducing equivalents whereas formate oxidation only produces one. The importance of reducing equivalents in the regulation of the Calvin cycle is also evident from an interesting series of experiments involving the phototrophs *Rb. capsulatus* and *Rb. sphaeroide*. Anaerobic photoheterotrophic growth of these bacteria depends on the Calvin cycle to dispose of excess reducing equivalents. Consequently, the activity of the Calvin cycle is higher during growth on butyrate than on the less-reduced substrate malate. Conversely, the expression of Calvin cycle enzymes is dramatically reduced in the presence of alternative electron acceptors, e.g. dimethyl sulfoxide (54, 131, 178).

The search for the identity of the repressor intermediate derived from carbon metabolism has focused on the end products of the Calvin cycle or closely related compounds. During growth of *R. eutropha* on fructose, the levels of RuBisCO increased fivefold upon addition of sodium fluoride. This compound inhibits, among other things, the activity of enolase, which catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate (61). An increase in RuBisCO activity was also observed in a phosphoglycerate mutase mutant of *R. eutropha* grown on fructose (130). The reduced ability to convert fructose to 2-phosphoglycerate and phosphoenolpyruvate apparently alleviates repression of the Calvin cycle enzymes. Mutants of *X. flavus* that are devoid of either phosphoglycerate kinase or triosephosphate isomerase activity display enhanced repression of the Calvin cycle by gluconeogenic substrates (99, 103 (Chapter 2)). The metabolic block introduced by the *pgk* and *tpi* mutations are likely to cause a buildup of intermediary metabolites during gluconeogenic growth. These data strongly suggest that the concentration of an intermediary metabolite, most likely phosphoenolpyruvate, signals the carbon status of the cell. However, the results of experiments involving an isocitrate lyase mutant of *P. oxalaticus* indicate that a metabolite
related to acetyl coenzyme A, rather than phosphoenolpyruvate, fulfills this signaling role (104).

Transcripts

THE CBB PROMOTERS The cbb operons of the chemoautotrophs *T. ferrooxidans*, *X. flavus*, and *R. eutropha* are transcribed from a single promoter (77, 78, 101). In contrast to the -10 region, which is poorly conserved, the -35 regions of the cbb promoters of these bacteria are virtually identical and resemble the *E. coli* $\sigma^{70}$ consensus sequence (Figure 4). A characteristic of the cbb operon is the close proximity of the divergently transcribed cbbR gene (Figure 2). The intergenic region between cbbR and the cbb operon can be as small as 89 bp, which indicates that the promoters of cbbR and the cbb operon may have regulatory elements in common. This was recently established for *R. eutropha* (78). Analysis of cbbR transcripts and regulation studies involving transcriptional fusions between lacZ and cbbR showed that cbbR of *R. eutropha* is constitutively transcribed during heterotrophic and autotrophic growth, giving rise to a monocistronic transcript of 1.4 kb. Surprisingly, cbbR is transcribed from two different $\sigma^{70}$ promoters, depending on the growth conditions. Promoter P$_{Rp}$ is located 120 bp upstream from the cbbR gene, which is within the transcribed region of the cbb operon. As a consequence, P$_{Rp}$ is not active during autotrophic growth because of the high activity of the cbb operon promoter. Under these conditions, therefore, cbbR is transcribed from the alternative promoter P$_{Ra}$, 75 bp upstream from the cbbR gene, which partially overlaps the promoter of the cbb operon. The activity of the cbbR promoters is only 4% of the promoter of the cbb operon, which could be the result of the low similarity to the *E. coli* $\sigma^{70}$ consensus sequence.

<table>
<thead>
<tr>
<th>R-site</th>
<th>A-site</th>
</tr>
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<tbody>
<tr>
<td>TF</td>
<td>$\text{ATA}\text{GATAACCA-}T\text{GTAAATCATATA}T\text{GTAAATATA}TT\text{TTAACTTTTAATA}$</td>
</tr>
<tr>
<td>XF</td>
<td>$\text{CACTTTCAATT-}T\text{CTGAAATGCC-}T\text{ACCATTATAAATTACCTGAAAT}$</td>
</tr>
<tr>
<td>RE</td>
<td>$\text{CACTTTAGGATTCTTTACAG-GGGCTTTAAGAATTCTGAAATTTACCTTTATG}$</td>
</tr>
</tbody>
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+1, Transcription initiation site.

Figure 4 Alignment of the promoter of the cbb operon of *Thiobacillus ferrooxidans* (TF), *Xanthobacter flavus* (XF), and *Ralstonia eutropha* (RE). (brackets) Position of a LysR-motif (T-N$_{11}$-A). (bold and underlined) Putative CbbR binding sites. (bars) Position of the -35 and -10 regions of the $\sigma^{70}$ promoter.

MRNA PROCESSING AND TRANSCRIPTION TERMINATION The insertion of antibiotic resistance markers or transposons in cbb genes of *R. eutropha*, *X. flavus*, and *Rb. sphaeroides* prevents the expression of cbb genes downstream from the insertion. These experiments revealed that the cbb genes are organized in large operons, which in *R. eutropha* may encompass as much as 15 kb (8, 49, 100, 141, 182). However, although cbbLS mRNA is relatively abundant in cells following autotrophic growth, mRNA containing all cbb genes has not been detected (3, 36, 59, 77, 101). Analysis of cbb transcripts of *X. flavus* showed that cbbLS mRNA encompassing the 5' end of the cbb operon is six times as abundant as the 3' end (101). Similar observations were made in *R. eutropha* and
interpreted as a premature transcription termination at a sequence resembling a terminator structure downstream from the \textit{cbb\textsubscript{LS}} genes (140). Sequences that may form a hairpin structure are also present downstream of the \textit{cbb\textsubscript{LS}} genes of \textit{T. ferrooxidans}, \textit{T. denitrificans}, and \textit{X. flavus} (56, 123). RuBisCO is usually synthesized to high levels during autotrophic growth because the enzyme is a poor catalyst. Since all \textit{cbb} genes are transcribed from a single promoter, differential \textit{cbb} gene expression in \textit{X. flavus} and \textit{R. eutropha} seems to be achieved by a relative abundance of \textit{cbb\textsubscript{LS}} mRNA.

\textbf{The LysR-Type Regulator CbbR}

\textbf{PRIMARY STRUCTURE} To date, \textit{cbbR} genes have been cloned and sequenced from the chemoautotrophs \textit{R. eutropha}, \textit{X. flavus}, \textit{T. denitrificans}, \textit{T. neapolitanus}, \textit{T. intermedius}, \textit{T. ferrooxidans}, and \textit{N. vulgaris} (76, 90, 101, 156, 171; JM Shively, unpublished results) and from the phototrophic bacteria \textit{Rs. rubrum}, \textit{Rb. capsulatus}, \textit{Rb. sphaeroides}, and \textit{Chromatium vinosum} (38, 48, 114, 175). The predicted molecular mass of the CBBR proteins ranges from 31.7 to 35.9 kDa. CbbR is a LysR-type transcriptional regulator, a protein family that includes over 70 proteins from gram-positive and gram-negative bacteria (142). LysR-type regulators are dimeric or tetrameric proteins, which contain a conserved amino-terminal DNA binding domain. The central and carboxy terminal domains are involved in ligand binding and multimerization, respectively. Because LysR-type regulators control a wide range of cellular processes, they bind a variety of chemically unrelated compounds. It is therefore not surprising that the central ligand binding domain is not conserved. Interestingly, the CbbR proteins are similar (35\% identity) throughout the sequence, indicating that these proteins bind the same or related ligands. The structure of the ligand binding domain of CysB (amino acids 88–324), which is closely related to CbbR, was recently solved by Tyrrell et al (166). The CysB monomer contains two α/β domains enclosing a coinducer binding cavity. One sulfate anion was found in the coinducer binding cavity between the two domains in each monomer. All four threonine residues involved in ligand binding by CysB are located in three regions that are conserved in all CbbR proteins (Figure 5). This strongly suggests that these conserved residues also play a role in ligand binding by CbbR. In addition, a fourth region of CysB involved in ligand response and/or multimerization is also conserved in all CbbR proteins. Because the level of expression of CbbR is too low for biochemical studies, the proteins of \textit{R. eutropha}, \textit{T. ferrooxidans}, \textit{C. vinosum}, and \textit{X. flavus} have been overexpressed in \textit{E. coli}. Only the proteins from \textit{R. eutropha} and \textit{X. flavus} have been purified to homogeneity (79, 173). The CbbR proteins from \textit{R. eutropha} and \textit{X. flavus} are dimers in solution, although the protein from \textit{R. eutropha} forms a tetramer at 4°C (79, 173).

\begin{figure}
\centering
\includegraphics[width=\textwidth]{alignment.png}
\caption{Alignment of CysB of \textit{Klebsiella aerogenes} with CbbR of \textit{Ralstonia eutropha} (RE), \textit{Xanthobacter flavus} (XF), \textit{Rhodospirillum rubrum} (RR), \textit{Rhodobacter sphaeroides} (RS), \textit{Rhodobacter capsulatus} 1 (RC-1), \textit{Rb. capsulatus} 2 (RC-2), \textit{Chromatium vinosum} (CV), and \textit{Thiobacillus ferrooxidans} (TF). Threonine residues forming a complex with sulfate in CysB(88-324) are shown (boldfaced). \textbullet\textcdot\textbullet, Hydrophobic residue (GALIVFPYWTMS); \textasteriskcentered, identical residue; \textdagger, charged/hydrophilic residue (HNDKRE); X, any residue. (See References 112, 164 for alignment methods.)}
\end{figure}
GENES UNDER CBBR CONTROL

**Autoregulation of cbbR gene expression** The cbbR gene of *R. eutropha* is constitutively transcribed from two promoters 75 and 120 bp upstream from the cbbR gene. DNA footprinting studies (see below) showed that CbbR binds to a DNA fragment 52–104 bp upstream from the cbbR gene; this indicates that CbbR binding may repress transcription from the cbbR promoters. Removal of the CbbR binding site nearest to the cbbR gene resulted in a 4- and 35-fold increase in cbbR expression during autotrophic and heterotrophic growth, respectively (78, 79). This shows that the overlap between CbbR binding sites and promoters creates an autoregulatory circuit in which cbbR expression is repressed by CbbR. The CbbR binding sites of *T. ferrooxidans* and *X. flavus* are also adjacent to cbbR, indicating that transcription of cbbR in these organisms is regulated in a similar manner.

**Induction and super induction of Calvin cycle genes** Disruption of cbbR by insertion of Tn5 or an antibiotic resistance gene completely abolished the expression of the cbb operons of *R. eutropha* and *X. flavus* (171, 183). The fact that both the chromosomal and plasmid operons of *R. eutropha* were affected showed that the activity of both cbb promoters is dependent on chromosomally encoded CbbR protein. The similarity in genetic organization and regulation of the cbb operons indicates that the dependence on CbbR for transcription of the cbb operon is a common characteristic of chemoautotrophic bacteria. In addition to regulating transcription of the cbb operon and cbbR, CbbR has been shown to control the expression of at least one other transcriptional unit. The gap-pgk operon of *X. flavus* is constitutively expressed and superinduced following a transition to autotrophic growth conditions (106). The cbbR mutant strain failed to superinduce this operon, indicating that CbbR is required for autotrophic regulation of gap-pgk.

**MECHANISM OF ACTION**

**Interactions between CbbR and the cbb promoter** Bandshift assays and footprinting experiments were used to analyze binding of CbbR to the promoter region of the cbb operon of *R. eutropha*, *X. flavus*, *T. ferrooxidans*, *T. neapolitanus*, *T. denitrificans*, and *T. intermedius* (76, 79, 171, 173, 183; JM Shively, unpublished results). Footprinting experiments showed that CbbR of *T. ferrooxidans* protected nucleotides from position -76 to -14 relative to the cbbL1 transcriptional start site (76). CbbR from *R. eutropha* and *X. flavus* protected similar regions that are located between -74 and -29 and between -75 and -23 relative to the transcriptional start site of the cbb operon, respectively (79, 173). Increasing concentrations of CbbR of *R. eutropha* protected additional nucleotides up to position +13. These experiments show that the binding site of CbbR overlaps the -35 region of the cbb promoter. This facilitates contact with the α subunit of RNA polymerase, which is essential for transcriptional activation by LysR-type regulators (162).

Bandshift assays using DNA fragments containing segments of the binding sites of *R. eutropha* or *X. flavus* revealed the presence of two subsites (R- and A-site; Figure 4) (79, 169, 171). These experiments showed that CbbR from *R. eutropha* and *X. flavus* have different DNA binding characteristics. CbbR from *R. eutropha* is able to bind to both subsites, regardless of the protein concentration or the presence of a ligand, which is similar to NodD (40). The protein from *X. flavus* behaves like TrpI, which has a high affinity for the promoter distal site (R-site) and only binds to the promoter proximal site (A-site) at higher protein concentrations or in the presence of a ligand (15).

Comparison of the DNA sequences that are protected in footprinting experiments using CbbR of *T. ferrooxidans*, *X. flavus*, and *R. eutropha* reveal a number of interesting similarities (Figure 4). The R-site of the cbb operon promoter of *X. flavus* and *T. ferrooxidans* contains a LysR-motif (T-N11-A), which forms the core of an inverted and a direct repeat, respectively. A related motif (T-N12-A) is present in the imperfect inverted repeat of the R-site of *R. eutropha*. Comparison of these repeats reveals the CbbR consensus sequence TnA-N7/8-TnA. Interestingly, the consensus sequence is both a direct and an inverted repeat. CbbR of *X. flavus* and *R. eutropha* is able to bind to a DNA fragment containing only the R-
site. Furthermore, mutagenesis of the T in the LysR-motif of *X. flavus* abolishes binding of CbbR, which strongly suggests that the TnA-N7-TnA sequence represents the CbbR binding site (Chapter 6).

The A-site of *R. eutropha* and *X. flavus* contains two partially overlapping TnA-N7-TnA sequences (Figure 4). The A-site of *T. ferrooxidans* has overlapping TnA-N7-TnA and TnA-N7-ANa sequences. The sequences containing the CbbR-consensus are related to those of the R-site. Most striking is the conservation of the right half-site of the putative CbbR binding site of the R-site (GTAAA, *T. ferrooxidans*; CTGAA, *X. flavus*; CTTAT, *R. eutropha*). Interestingly, this sequence is repeated in the -10 region of *R. eutropha*, which may account for CbbR binding in this region at high protein concentrations.

The CbbR consensus sequences in the R- and A-sites are separated by two and three turns of the DNA-helix and are located on the same side. Insertion of two additional nucleotides between the R- and A-sites of *R. eutropha* did not abolish DNA binding of CbbR, although the *cbb* promoter was no longer active (79). This indicates that protein-protein contacts, which are dependent on the proper positioning of the two CbbR molecules, are essential for transcriptional activation. DNase I hypersensitive sites were observed between the R- and A-sites at positions -47 and -48 in the *cbb* promoter of *R. eutropha* and *X. flavus*, which could be the result of DNA bending induced by binding of CbbR (79, 173 (Chapter 4)). Bandshift assays using circular permutated DNA fragments showed that CbbR of *X. flavus* induces a 64° DNA bend upon binding (173 (Chapter 4)). Protein-induced DNA bending is frequently observed in LysR-type proteins (142).

**Ligands of CbbR** As discussed above, the expression of the Calvin cycle genes in autotrophic bacteria depends on the availability of suitable carbon and energy sources. The discovery that CbbR is a transcriptional regulator of the *cbb* operons in autotrophic bacteria strongly suggests that CbbR is required for the transduction of these signals to the transcription apparatus. Bandshift assays using purified CbbR of *X. flavus* showed that DNA binding of this protein is increased threefold following the addition of 200 µM NADPH to the binding assay. In addition, CbbR-induced DNA bending is decreased by 9° in the presence of NADPH (173 (Chapter 4)). Similar changes in DNA binding characteristics following binding of ligands was previously observed for other LysR-type regulators (e.g. 177).

Although these in vitro experiments do not prove that the in vivo expression of the *cbb* operon is controlled by the intracellular concentration of NADPH, a number of experiments indicate that autotrophic growth is associated with elevated concentrations of NADPH. The transition from heterotrophic to autotrophic growth of *P. oxalaticus* is accompanied by an increase in the NADPH-to-NADP ratio (71). Furthermore, NADP is completely reduced when *Rs. rubrum* is incubated under anaerobic conditions in the light, when cells normally induce the *cbb* operon. NADPH was subsequently oxidized within 1 min when the cells were exposed to oxygen or incubated in the dark, growth conditions under which *Rs. rubrum* does not have an active Calvin cycle (62).

**IN RELATION TO PHOTOAUTOTROPHIC BACTERIA** The same principles that apply to transcriptional regulation of Calvin cycle genes by CbbR in chemoeutrophs also apply to photoautotrophic bacteria. The expression of the *cbbM* of *Rs. rubrum* and of the form I *cbb* operon of *Rb. sphaeroides* depends on the presence of a functional CbbR protein (38, 48). However, these phototrophs are metabolically extremely versatile. It is therefore not surprising that there are some interesting differences with chemoeutrophic bacteria regarding transcriptional regulation of the Calvin cycle genes.

Like *R. eutropha*, *Rb. sphaeroides* also has two *cbb* operons and only one *cbbR* gene (48). However, in contrast to *R. eutropha*, the transcription of only one *cbb* operon is completely dependent on CbbR. The form II operon was still expressed at 30% of the level found in the wild type in a *cbbR* mutant strain. As a result, photoautotrophic growth was completely abolished. However, photoheterotrophic growth on malate or butyrate was still possible, albeit at a reduced growth rate. Exposure to oxygen completely repressed the synthesis of form II RuBisCO, indicating that in addition to CbbR, other control mechanisms exist in
purple-nonsulfur bacteria. Two genes of unknown function, orfU and orfV, are located upstream from the form II operon of \textit{Rb. sphaeroides} (186). Using transcriptional fusions with \textit{xylE} it was shown that the expression of these genes was increased in a \textit{cbbR} mutant, indicating that the expression of these two genes is repressed by CbbR. No biochemical studies of CbbR of phototrophic bacteria have been reported to date. However, the extensive similarities in primary structure between CbbR from chemosynthetic and photoautotrophic bacteria indicate that the molecular mechanism underlying transcriptional regulation by CbbR are similar in both types of autotrophic bacteria.

\textit{Additional Transcriptional Regulators?}

\textit{REPRESSORS?} There is only circumstantial evidence for the presence of a repressor of the \textit{cbb} operon. The presence of an additional \textit{cbb} operon located on an indigenous megaplasmid or a broad host range plasmid results in heterotrophic derepression of the Calvin cycle in \textit{R. eutropha} and \textit{X. flavus}, respectively (9, 88). Fusions between the \textit{cbb} promoter of \textit{R. eutropha} and \textit{X. flavus} and \textit{lacZ} are also active during heterotrophic growth when present in multiple copies (78, 101). The anomalous activity of the \textit{cbb} promoter under these conditions is likely to be due to titration of a repressor molecule by multiple repressor binding sites.

\textit{Tn5} mutagenesis of \textit{R. eutropha} identified a gene that is required for autotrophic growth. The mutant (a) failed to induce the Calvin cycle during heterotrophic growth on gluconeate or formate, (b) displayed reduced glycolytic activity, and (c) altered colony morphology. Interestingly, the \textit{cbb} operon was still inducible by formate. Southern hybridization showed that the \textit{aut} gene is also present in \textit{Pseudomonas} sp. and \textit{T. intermedius}, and possibly also in \textit{Rb. capsulatus} and \textit{Paracoccus denitrificans}. The Aut protein displays similarities with cytidyltransferases; however, its function remains unknown (41).

\textit{PHOSPHO-RELAY SYSTEM IN PHOTOAUTOTROPHS} A global signal transduction system that includes the two-component phospho-relay system, RegA-RegB, has been identified in purple nonsulfur bacteria that integrates CO$_2$ fixation with other important processes such as photosynthesis and nitrogen fixation (67, 125, 143). The RegA-RegB system is involved in the positive regulation of the \textit{cbb} operons of \textit{Rs. rubrum} and \textit{Rb. sphaeroides} as well as in the expression of genes important for the alternative CO$_2$ fixation pathway (67, 125). RegB, the sensor kinase, is autophosphorylated in response to an unidentified (external) signal (37) and is required for the phosphorylation of the response regulator RegA, which in turn controls photosynthetic gene expression. RegA does not have a DNA-binding domain, which indicates that an additional factor must be involved in transducing the signals to the transcription machinery.

\textit{Model of Regulation of \textit{cbb} Gene Expression}

The data from physiological experiments strongly suggest that a reduced metabolite derived from the oxidation of autotrophic substrates is responsible for induction of the Calvin cycle. Genetic and biochemical data show that the transcriptional regulator CbbR binds to \textit{cbb} promoters and regulates the expression of Calvin cycle genes. Furthermore it has been shown that CbbR interacts with NADPH, which enhances DNA binding and reduces DNA bending by CbbR. Based on this we propose the model shown in Figure 6 for the regulation of the expression of the Calvin cycle in chemosynthetic bacteria. During heterotrophic growth of facultatively autotrophic bacteria, reducing power and ATP is generated via catabolic pathways and oxidative phosphorylation, which is subsequently used for the assimilation of organic substrates into biomass via anabolic pathways. Catabolic and anabolic reactions in the cell are thus balanced. The balance between catabolism and anabolism is disturbed following the transition from heterotrophic to autotrophic growth conditions.
The oxidation of reduced (in)organic compounds supporting autotrophic growth leads to the production of reducing equivalents. However, since organic carbon sources are not available, anabolic reactions do not proceed, which leads to a buildup of reducing equivalents such as NADPH, which may be formed from NADH by transhydrogenase. The increased intracellular concentration of NADPH in the cell favors the formation of an NADPH-CbbR complex, which activates transcription of the cbb operon and in X. flavus also of the gap-pgk operon. The subsequent fixation of CO$_2$ via the Calvin cycle provides the cell with a source of carbon, which allows anabolism to proceed and dissipates excess reducing equivalents.

The extent by which reducing power is dissipated depends on the carbon flux through the Calvin cycle. CO$_2$ limitation reduces the flow of carbon through the Calvin cycle and hence reduces the rate by which reducing equivalents are oxidized. As a result, the NADPH concentration increases, which shifts the equilibrium between CbbR and NADPH-CbbR to the latter. This results in an enhanced transcription of the Calvin cycle.

The available data indicate the presence of a repressor protein that may respond to the intracellular concentration of an intermediary metabolite such as phosphoenolpyruvate. Because actively growing cells contain NADPH, part of the CbbR population will be activated following binding of NADPH, which may lead to a low level expression of the Calvin cycle during heterotrophic growth. The role of the repressor protein may therefore be the prevention of cbb transcription during heterotrophic growth.

**Figure 6** Model for the regulation of expression of the Calvin cycle genes. C$_3$, Triosephosphate, a metabolite intermediary to the Calvin cycle and intermediary metabolism; PEP, phosphoenolpyruvate; CoA, coenzyme A. The helices represent the cbb operon, cbbR, and the gap-pgk operon. (1) The gap-pgk operon has only been identified in Xanthobacter flavus.

**CONCLUDING REMARKS**

A wealth of new information contributing to our understanding of carbon dioxide fixation in chemoautotrophs has been gathered since the previous comprehensive reviews on the subject. However, for every question answered, many others have been unmasked. For example, how does the carboxysome enhance CO$_2$ fixation? How is the expression of the
carboxysome genes regulated? How is the carboxysome assembled? Where are the Calvin cycle genes organized in chemoautotrophic bacteria in which the \( cbb \) genes are not clustered with other \( cbb \) genes? Is \( CO_2 \) fixation controlled by a phospho-relay system as is the case in \( RB. \) \textit{sphaerooides}? Does NADPH activate CbbR in vivo? What is the mechanism by which CbbR activates transcription of the Calvin cycle genes? Is CbbR involved in the regulation of carboxysome gene expression? How did the Calvin cycle evolve? The answer to these, as well as to many other questions, will likely be the subject of a future review.

ACKNOWLEDGMENTS

Some of the material in this article is based on work supported by the Cooperative State Research Service, US Department of Agriculture, under agreement No. 92-37306-7663 (JMS); a National Science Foundation Grant, MCB-9513481 (JMS); a grant from the Royal Society (WGM); and a European Union HumanCapital and Mobility Institutional Grant (ERBCHBGCT930293) (WGM). We wish to thank K. Verschueren and T. Wilkinson of the Department of Chemistry, University of York, for helpful discussions on CysB structural data.

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Chapter 1


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