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Review

Microbial ribulose 1,5-bisphosphate carboxylase/oxygenase: A different perspective

F. Robert Tabita
Department of Microbiology, The OSU Biochemistry and OSU Molecular, Cellular, and Developmental Biology Programs, and The Plant Biotechnology Center, The Ohio State University, 484 West 12th Avenue, Columbus, OH 43210-1292, USA (fax: (614) 292-6337; e-mail: tabita.1@osu.edu)

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Abstract

Marine and terrestrial photosynthetic and chemoautotrophic microorganisms assimilate considerable amounts of carbon dioxide. Like green plastids, the predominant means by which this process occurs is via the Calvin-Benson-Bassham reductive pentose phosphate pathway, where ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) plays a paramount role. Recent findings indicate that this enzyme is subject to diverse means of control, including specific and elaborate means to guarantee its high rate and extent of synthesis. In addition, powerful and specific means to regulate Rubisco activity is a characteristic feature of many microbial systems. In many respects, the diverse properties of microbial Rubisco enzymes suggest interesting strategies to elucidate the molecular basis of CO_2/O_2 specificity, the 'holy grail' of Rubisco biochemistry. These systems thus provide, as the title suggests, 'different perspectives' to this fundamental problem. These include vast possibilities for imaginative biological selection using metabolically versatile organisms with well-defined genetic transfer capabilities to solve important issues of Rubisco specificity and molecular control. This review considers the major issues of Rubisco biochemistry and regulation in photosynthetic microoganisms including proteobacteria, cyanobacteria, marine nongreen algae, as well as other interesting prokaryotic and eukaryotic microbial systems recently shown to possess this enzyme.

Abbreviations: CBB – Calvin-Benson-Bassham pathway; CCM – carbon concentrating mechanism; PRK – phosphoribulokinase; RuBP – ribulose 1, 5-bisphosphate; Rubisco – ribulose 1,5-bisphosphate carboxylase/oxygenase; UAS – upstream activating sequence

Introduction

Both terrestrial and marine microorganisms contribute much to the overall carbon balance and play important roles in facilitating the conversion of oxidized CO_2 to reduced organic carbon on earth. Because of the varied environments in which the CO_2 fixation catalysts have evolved, prokaryotic photosynthetic and related autotrophic prokaryotes provide many important advantages for detailed investigation of Rubisco biochemistry and function, including a capacity for genetic manipulation. There are at least four major mechanisms by which prokaryotic microorganisms plus marine and freshwater 'nongreen' algae metabolize CO_2 (Fuchs et al. 1987); however, as in terrestrial environments, the predominant route is the Calvin-Benson-Bassham (CBB) reductive pentose phosphate pathway. Aside from some interesting idiosyncrasies, the overall process is similar in all organisms that use this scheme. Depending on its source, however, the key catalyst, Rubisco, may possess significantly different properties, as can the enzyme phosphoribulokinase (PRK), which catalyzes the synthesis of ribulose 1,5-bisphosphate (RuBP), the CO_2 acceptor. This review is confined to the structure, function and regulation of RuBP carboxylase/oxygenase (Rubisco) in

prokaryotes, primarily phototrophs, as well as 'non-green' algae, a large number of which are associated with marine environments and play important roles in oceanic ecosystems. The following pages will concentrate on defining unique aspects of these microbial systems for the study of Rubisco function. Hopefully, the contrasts and parallels between these and previously studied terrestrial plant and aquatic green algal systems (discussed in the review by Spreitzer 1999) will illustrate the many ways in which these different systems might be exploited to further our knowledge of this important catalyst and the regulation of CO_2 fixation. A recent News Focus in *Science* (Mann 1999) accentuates the importance of Rubisco bioengineering.

Diversity of Rubisco molecules

Rubisco from most organisms is classically comprised of both large (catalytic) and small subunits to form a hexadecameric structure with a M_r of about 550 000, with eight copies of each protein in an L_8S_8, or more accurately, $(L_2)_4(S_4)_2$ structure (Knight et al. 1990). This is the structural form typically found in terrestrial plants and virtually all eukaryotic algae, cyanobacteria, and phototrophic and chemoautotrophic proteobacteria. Originally termed peak I or type I or form I Rubisco (Gibson and Tabita 1977; Tabita 1988), this structural form is distinguished from the type II or form II Rubisco that is sometimes encountered. A smaller Rubisco holoenzyme protein was initially indicated from the work of Anderson et al. (1968) and Akazawa et al. (1970), where molecular weight estimates of partially purified or crude preparations from the bacterium *Rhodospirillum rubrum* were variously estimated to range from 64 000 to 120 000. Eventually, homogeneous *Rs. rubrum* Rubisco was isolated as a homodimer of large subunits only, with a native molecular weight estimated by light scattering to be 110 000 (Tabita and McFadden 1974 a, b). Soon after, Rubisco from the related organism, *Rhodobacter* (once called *Rhodopseudomonas*) *sphaeroides*, was isolated as two peaks of activity from ion exchange columns; the first peak contained a form I like protein while the second peak was comprised of a *Rs. rubrum*-like form II Rubisco (Gibson and Tabita 1977). Subsequent studies showed that the two *Rb. sphaeroides* Rubisco proteins are distinct gene products with different physiological roles and distinct properties (discussed below). A number of nonsulfur photosynthetic bacteria also synthesize both form I and form II Rubisco (Tabita 1995). The occurrence of both forms of Rubisco has now been established for several chemoautotrophic bacteria as well (Yaguchi et al. 1994; Hernandez et al. 1996; Shively et al. 1998), including some interesting symbionts that provide carbon for invertebrates in hydrothermal and other marine environments (Robinson et al. 1998). In addition, we now know that several marine eukaryotic dinoflagellates contain, exclusively, a nuclear-encoded form II Rubisco gene (Morse et al. 1995; Whitney et al. 1995; Whitney and Yellowlees 1995; Rowan et al. 1996). Despite the rapid loss of Rubisco activity in *Amphidinium carterae* (dinoflagellate) extracts, this nuclear-encoded form II Rubisco might be somewhat better adapted to function in aerobic atmospheres than its form II bacterial homologs (Whitney and Andrews 1998). Further studies, both physiological and biochemical, on this interesting eukaryotic form II protein are awaited with great interest.

Form I and form II Rubisco molecules possess both similar and distinctive properties (Table 1). While form II enzymes seem to possess uniform catalytic features, including a low CO_2/O_2 substrate specificity and poor affinity for CO_2 (see Rubisco structure-function relationships), form I Rubisco exhibits great variation in these and other parameters depending on the source of the enzyme. The molecular underpinnings of these various idiosyncratic properties are quite fundamental to understanding Rubisco structure-function relationships(as discussed later). Pertinent to these considerations, the *Rs. rubrum* form II Rubisco gene was isolated (Somerville and Sommerville 1984) and its amino acid sequence, both deduced and determined (Hartman et al. 1984; Nargang et al. 1984), showed only slight homology to large subunits of plant Rubisco. This was not unexpected due to the rather specific properties of form II Rubisco (Tabita and McFadden 1974a, b; Gibson and Tabita 1977) (Table 1). These initial sequence results, however, emphatically established the universality of key residues important in the catalytic mechanism of all Rubisco enzymes, and this general pattern has been observed for the many sequences now available in the database. From the available sequence database, it is apparent that all of the form II Rubisco genes subsequently isolated show very close identity. Aside from conserved catalysis-related residues, all form II subunits differ substantially from form I large subunits (which are all clearly related), whether the form I subunits are derived from bacteria or plants (Delwiche and Palmer

Table 1. Comparisons and distinctive characteristics of form I and form II Rubisco proteins

Property	Rubisco type	
	I	II
Quarternary structure	L_8S_8	$(L_2)_x$
X-ray structures available	Yes	Yes
Chaperonin-assisted folding	Yes	Yes
Regulated synthesis	Yes	Yes
Carbamate formation	Yes	Yes
Metal specificity for carboxylase acitivity	$Mg^{2+}>Mn^{2+}>Ni^{2+}>Co^{2+}$	$Mg^{2+}>Mn^{2+}$ (Co^{2+} inhibits)
Metal specificity for oxygenase activity	$Mn^{2+}>Mg^{2+}>?$	$Mn^{2+}>Mg^{2+}>Co^{2+}$
CO_2/O_2 specificity (Ω)	25–240	10–15
K_{CO_2} (μM)	5–175	100–250
Fallover	Yes and no	No
Inhibition by RuBP	Yes and no	No
Inhibition by sugar phosphates (activated enzyme)	Yes	Slight

1996; Watson and Tabita 1996, 1997). With the isolation and sequencing of Rubisco genes from a wide diversity of microorganisms, it has become apparent that form I large and small subunits may be subdivided into at least two major subgroups, a 'green' or 'red' category, which itself contains two subclasses, IA and IB, plus IC and ID, respectively (Tabita 1995) (Figure 1). The major green and red classes refer to the predominant types of Rubisco-containing organisms; i.e., green plants, green algae, and cyanobacteria (blue-green algae) for the 'green' class and red algae and 'purple' bacteria, for the 'red' class. Finally, representatives from the 'third kingdom of life', the archaea, also contain Rubisco-like sequences (Bult et al. 1996; Klenk et al. 1997). For the archaea, Rubisco activity and antibody cross reactivity was first noted in extracts of extreme halophiles (*Haloferax* spp.) (Altekar and Rajagopalan 1990). We verified the presence of Rubisco in *Haloferax* in our laboratory and also established that this enzyme has an extremely high salt requirement for maximum activity (Daniels and Tabita, unpublished results). The genomes of other archeae have been sequenced, and a putative Rubisco gene was first uncovered from the strictly anoxic thermophilic methanogen *Methanococcus jannaschii* (Bult et al. 1996). The deduced *rbcL* sequence only slightly resembles existing form I and form II Rubisco molecules, a result which has been buttressed by two other potential Rubisco genes from *Archaeoglobis fulgidus* (Klenk et al. 1997) (Figure 1) and several species of *Pyrococcus*. Results from our laboratory indicate that the *M. jannaschii* gene encodes a functional enzyme with unusual and very interesting properties (Tabita 1998; Watson et al. 1999). Indeed, as will be discussed later, the isolation of Rubisco from an organism that never encounters molecular oxygen may provide unusual insights to various key properties of this enzyme.

Organization of Rubisco genes in proteobacteria and cyanobacteria

In proteobacteria and cyanobacteria, the form I Rubisco large and small subunit genes are always cotranscribed and part of an operon regulated by a single promoter (Tabita 1994, 1995; Gibson 1995; Gibson and Tabita, 1996; Kusian and Bowien 1997; Shively et al. 1998). The Rubisco subunit genes in proteobacteria are in fact often part of a much larger operon that contains structural genes that encode other enzymes of the CBB cycle, including phosphoribulokinase (*cbbP*), fructose 1,6-/sedoheptulose 1,7-bisphosphatase (*cbbF*), aldolase (*cbbA*), transketolase (*cbbT*), glyceraldehyde 3-phosphate dehydrogenase (*cbbG*), pentose 5-phosphate 3-epimerase (*cbbE*), and other enzymes (Figure 2). In some cases, one or more of these and other CBB enzymes are located in separate operons (Gibson and Tabita 1996; Kusian and Bowien 1997; Shively et al. 1998). In systems where regulation has been extensively studied, i.e., in nonsulfur purple photosynthetic bacteria

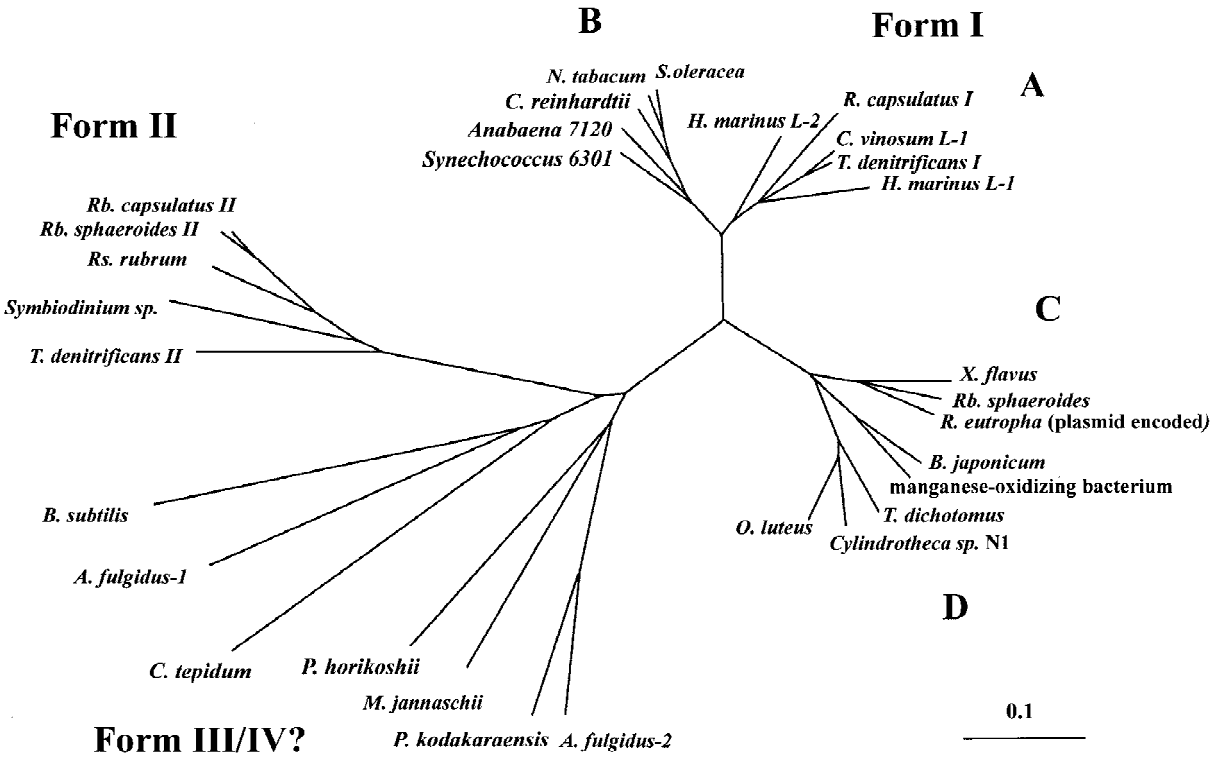

Figure 1. Molecular phylogenetic tree of selected deduced Rubisco large subunit amino acid sequences. The marker in the lower right corner refers to 0.1 substitutions per site. Multiple sequence alignments of this unrooted tree were performed using ClustalW software; tree topology and evolutionary distances estimations were performed by the neighbor-joining method using Kimura distances and Phylip 3.5 as previously described (Watson and Tabita 1996, 1997). Form I large subunits. are divided into a 'green-like' group (Delwiche and Palmer 1996; Watson and Tabita 1996, 1997), comprising subgroup A, including various proteobacterial and marine cyanobacterial large subunits, and subgroup B, including green plastid and the bulk of cyanobacterial large subunits. The 'red-like' form I large subunits comprise subgroup C, which includes various proteobacterial large subunits, and subgroup D, which includes large subunits from chromophytic and rhodophytic algae. There are two sequences that might form another subgroup between C and D, the large subunits from *B. japonicum* (Horken and Tabita, 1999) and a marine manganese-oxidizing bacterium (Caspi et al. 1996), however this classification is tentative as these are the only such sequences that have been reported thus far. Form II Rubisco sequences, from all sources, are closely related and, with the limited sequences available, have not been divided into subclasses. The archaeal Rubisco sequences currently available form two apparent major groups, with the proteobacteria *Bacillus subtilis* and *Chlorobium tepidum* each containing an archaeal-like Rubisco sequence (see www.pasteur.fr/Bio/SubtiList.html and www.tigr.org/tdb/mdb.html).

such as *Rb. sphaeroides*, *Rb. capsulatus*, and *Rs. rubrum* (Falcone and Tabita 1993; Gibson and Tabita 1993; Paoli et al. 1998a, b), as well as the purple sulfur bacterium *Chromatium vinosum* (Viale et al. 1991) and chemoautotrophic bacteria such as *Alcaligenes (Ralstonia) eutropha* (Windhovel and Bowien 1991), *Xanthobacter flavus* (van den Bergh et al. 1993), or *Thiobacillus ferrooxidans* and other thiobacilli (Kusano and Sugawara 1993; Shively et al. 1998), the major operons are regulated by the positive transcriptional regulator protein CbbR, whose gene (*cbbR*) is usually adjacent and divergently transcribed from the structural genes of the *cbb* operon (Figure 2). All of the genes of the operons controlled by CbbR carry the prefix *cbb* to denote the fact that they are Calvin-Benson-Bassham (CBB) pathway structural genes (Tabita et al. 1992). Rubisco operons of cyanobacteria, such as various *Synechococcus* and *Anabaena* species, do not contain other structural genes of the CBB cycle (Tabita 1994; Gibson and Tabita 1996; Kaplan and Reinhold 1998), and because of this, the Rubisco genes are typically termed *rbcL* and *rbcS*, much like their plant counterparts which they greatly resemble. An interesting exception is the situation in certain marine *Synechococcus* species (Figure 2). Here the *rbcLS* genes are cotranscribed along with *ccmK* (Watson and Tabita 1996), a gene that encodes a protein of the carbon concentrating system of cyanobacteria (Friedberg

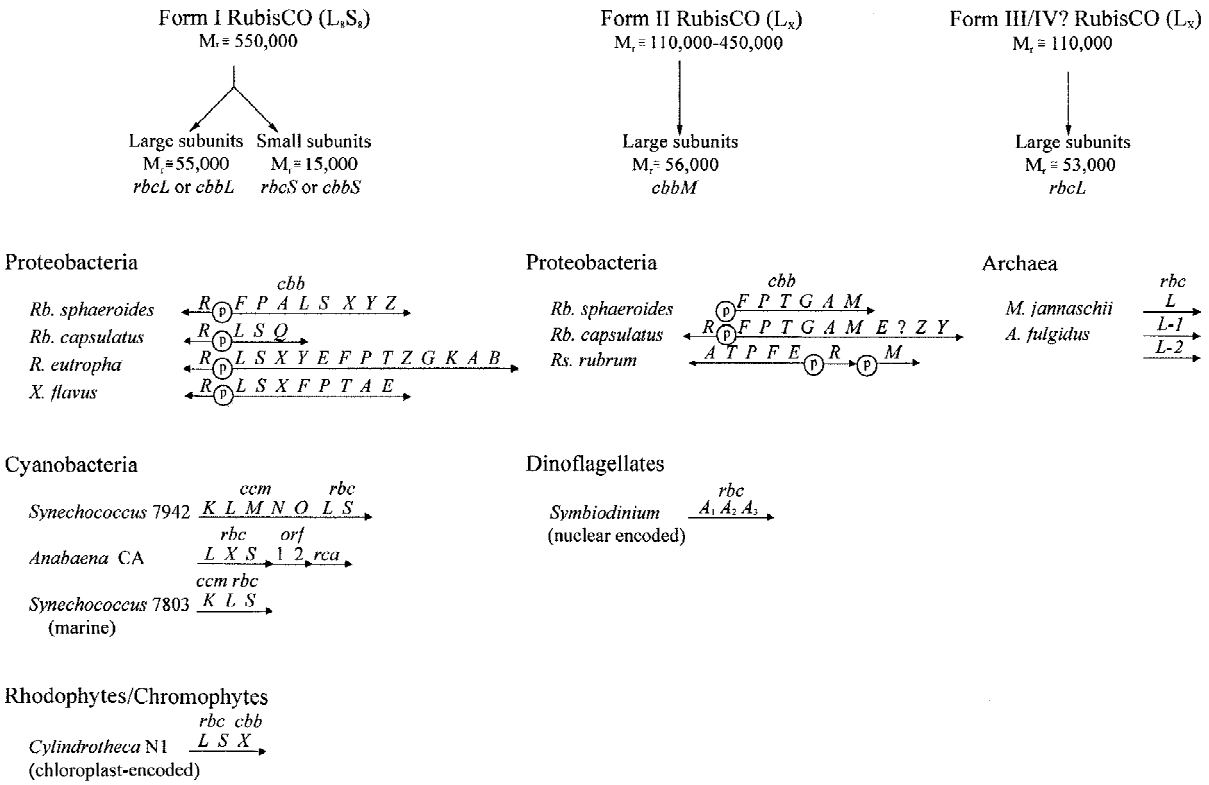

Figure 2. Organization of microbial Rubisco genes from proteobacteria, cyanobacteria, archaea, and eukaryotic rhodophytes, chromophytes, and dinoflagellates. Depicted are the different forms of Rubisco and the genes that encode them. Arrows refer to the direction of transcription, with the arrowhead delimiting the various gene clusters in proteobacteria comprising individual operons; p depicts promoter sequences controlling transcription. In the proteobacteria, the form I Rubisco genes (*cbbLS*) are located in an operon with other CBB structural genes, as is the form II Rubisco gene (*cbbM*) (Gibson 1995). These include fructose 1,6/sedoheptulose 1,7 bisphosphatase (*cbbF*), phosphoribulokinase (*cbbP*), aldolase (*cbbA*), phosphoglycolate phosphatase (*cbbZ*), transketolase (*cbbT*), glyceraldehyde phosphate dehydrogenase (*cbbG*), pentose 5-phosphate 3-epimerase (*cbbE*), phosphoglycerate kinase (*cbbK*) and genes of unknown function (*cbbX*, *cbbY*, *cbbA*, and *cbbB*). In all cases, transcription of the *cbb* operons is controlled by the product of the divergently transcribed *cbbR* gene. In *Synechococcus* 7942, the *rbc* genes are not cotranscribed with the *ccm* genes, but *ccmK* is cotranscribed with *rbcLS* in marine cyanobacterial WH strains (Watson and Tabita 1996). Only in heterocystous *Anabaena* species, among cyanobacteria, is there evidence for a Rubisco activase-like gene (*rca*) (Li et al. 1993) downstream from the *rbc* genes, but in a separate transcriptional unit (Li and Tabita 1994). A *cbbZ* sequence is invariably found downstream from the form I *rbcLS* genes of eukaryotic nongreen algae.

et al. 1989; Price et al. 1993). This protein resembles an integral protein of the Rubisco-bounded intracellular prokaryotic 'organelle' or carboxysome (English et al. 1994). There is an interesting variation in filamentous and heterocyst-forming *Anabaena* spp. In these organisms, and perhaps *Synechocystis* sp. strain PCC 6803, the *rbc* transcript contains a gene, *rbcX* (Larimer and Soper 1993; Li and Tabita 1994), which encodes a protein that seems to influence the folding of Rubisco, at least when the *Anabaena* genes are expressed in *E. coli* (Li and Tabita 1997). *Anabaena* spp., so far as known, are unique in that a monocistronic operon containing the *rca* gene, encoding Rubisco activase, is found adjacent to the *rbc* operon (Li et al. 1993). The *rbc* and *rca* operons are differentially regulated (Li and Tabita 1994). Finally, sequencing of the *Synechocystis* sp. strain PCC 6803 genome (*www.kazusa.or.jp/cyano/cyano.orig.html*), and recent studies with *Synechococcus* sp. strain PCC 7002 (Price et al. 1998), indicate that there are *cbbR*-like genes in these cyanobacteria. At this time any potential function in regulating CO_2 fixation has not been established, beyond a recently cited personal communication relative to its potential role in controlling the expression of the *cmp* operon in *Synechocystis* 6803 at low CO_2 levels (Price et al. 1998).

In many instances strong secondary structures (or hairpin/stem-loop structures) are observed 3′ to genes

encoding Rubisco in proteobacteria. Although Rubisco may be encoded by genes that are cotranscribed with other CBB cycle genes of the same operon in proteobacteria, the intracellular level of Rubisco far exceeds that of other CBB cycle enzymes (Gibson et al. 1991; Meijer et al. 1991; Schaferjohann et al. 1995). Thus, it has been postulated that the hairpin structures may serve to stabilize the Rubisco transcript following posttranscriptional processing of a large operonic message, especially in operons where the Rubisco genes are situated at the 3′ end of the operon (Tabita 1995). In the case of *R. eutropha*, the stem-loop structure acts as a transcriptional terminator (Schaferjohann et al. 1996). This makes sense because, in this organism, the *cbbLS* genes are promoter proximal in the plasmid and chromosomal *cbb* operons; premature termination following *cbbLS* transcription would then account for the differential expression of *cbb* genes observed in *R. eutropha*. These various scenarios used by proteobacteria to insure the production of large amounts of Rubisco protein are probably much appreciated by the cell since Rubisco is such a poor catalyst, with a turnover number of only 3–5 sec^{-1} (Hartman and Harpel 1993, 1994).

With respect to form II Rubisco, encoded by the *cbbM* gene in *Rhodobacter* and other nonsulfur purple photosynthetic bacteria (Gibson 1995), additional *cbb* structural genes are cotranscribed with *cbbM*; this is perhaps the situation with other autotrophic proteobacteria that contain form II Rubisco as well. The form II or cbb_{II} operon is also controlled by either the same *cbbR* gene that regulates the form I or cbb_I operon, as in *Rb. sphaeroides* (Gibson and Tabita 1993), or a *cbbR* gene separate from the one that controls cbb_I transcription, as in *Rb. capsulatus* (Paoli et al. 1998a, b) (Figure 2).

Organization of Rubisco genes in nongreen algae

Interestingly, the deduced sequences of nongreen algal Rubisco large and small subunits (class ID) closely resemble deduced sequences of bacterial enzymes in class IC (Figure 1). Form I Rubisco molecules from these organisms are encoded by chloroplast-encoded *rbcLS* genes that are cotranscribed and part of an operon in all nongreen chromophytic and rhodophytic algae examined to date (Newman et al. 1989; Newman and Cattolico 1990; Douglas and Turner 1991). These organisms also tend to contain a bacterial *cbbX*-like gene immediately downstream from the *rbcLS* genes. This scenario is obviously different from the situation in green algae and higher plants, where the small subunit is nuclear encoded (Spreitzer 1993). However, other CBB cycle structural genes appear to be nuclear encoded in nongreen algae as well. As noted above, at least some dinoflagellates contain a nuclear-encoded form II gene (Morse et al. 1995; Whitney et al. 1995; Whitney and Yellowlees 1995; Rowan et al. 1996). This is the only instance where this kind of Rubisco has been found in eukaryotes; the association of the form II Rubisco gene with other CBB genes has not been established.

What is Rubisco doing in archaea?

When Rubisco is found in microorganisms, including bacteria and eukaryotic algae, the enzyme invariably plays a key role in the ability of such organisms to employ CO_2 as a source of carbon via the CBB reductive pentose phosphate pathway. In the archaea, the enzyme was initially described in crude extracts of extreme halophiles (Altekar and Rajagopalan 1990). As described above, evidence for putative gene(s) that encode Rubisco was provided by genomic sequencing of the anoxic methanogen *Methanococcus jannaschii* (Bult et al. 1996), and by sequencing of the genome of the anoxic sulfate-reducing organism *Archaeoglobis fulgidus* (Klenk et al. 1997). More recently, additional archaeal and archaeal Rubisco-like sequences have been deposited in the database. The dilemma is that there is no apparent reason for the presence of the enzyme, or the gene that encodes it, in these organisms. In the halophiles, no capacity for CO_2-dependent growth has been demonstrated. The anoxic archaea, which do grow using CO_2 as a carbon source, apparently use a modified acetyl CoA CO_2 fixation pathway (Shieh and Whitman 1987; Sprott et al. 1993) and presumably enzymes of the reductive tricarboxylic acid pathway to obtain needed intermediates from CO_2. In the pages that follow, it will be apparent that the *M. jannaschii* and *A. fulgidus* genes have the capacity to encode bonafide Rubisco, yet the physiological basis for the enzyme's presence is not understood. In no case has a phosphoribulokinase (PRK) gene or its enzymatic activity (Bult et al. 1996; Klenk et al. 1997) been demonstrated (Selkov et al. 1997), nor have there been whole-cell CO_2 fixation studies performed that indicate metabolism through the CBB pathway (Sprott et al. 1993). In part, the absence of recognizable sequences encoding PRK in the *M. jannaschii* and *A. globus* genomes may be due to the fact that only a few microbial PRK sequences are currently available in the

database. Because proteobacterial PRK and plant PRK deduced amino acid sequences show only 13% identity (Tabita 1994, 1995; Gibson 1995), a large enough diversity of sequences may not have been examined at this time to allow one to recognize putative PRK sequences in these organisms. It is also possible that some other kinase enzyme has the capacity to catalyze the formation of RuBP. Although this is certainly a feasible scenario, the alternative is that the Rubisco genes do nothing in these organisms or the enzyme has a function completely divorced from CO_2 fixation as we know it. These intriguing questions invite further investigation, with preliminary results from our laboratory at this time showing the potential for Rubisco gene transcription in anoxic archaea.

What are archaeal-like Rubisco sequences doing in proteobacteria including Chlorobium?

The interesting situation of archaeal-like Rubisco sequences in proteobacteria should also be considered. Again, as a result of genomic sequencing, a Rubisco-like sequence was found in *Bacillus subtilis*, an organism that has no recognized capacity for CO_2-dependent growth. In this organism, the putative Rubisco-like sequence shows 36% identity to the deduced *A. fulgidus rbcL1* amino acid sequence and from 27% to 32% identity to other archaeal deduced sequences (see www.pasteur.fr/Bio/subtilist.html). The potential *B. subtilis* Rubisco is the first gene of an operon containing three other unknown open reading frames under control of a conserved leader region sequence designated the S box that is responsive to methionine availability (Grundy and Henkin 1998). Perhaps, this regulatory feature might give insights into the function of this unusual Rubisco sequence.

Especially intriguing is the situation in the green sulfur photosynthetic bacteria, which use the reductive tricarboxylic acid (RTCA) cycle for bulk CO_2 assimilation (Fuchs et al. 1980a, b). Considerable controversy over the presence of Rubisco activity in extracts of *Chlorobium* was prevalent in the 1970s and early 1980s. In one instance, Tabita et al. (1974) detected weak and labile activity in extracts of *Chlorobium limicola*. This was not reproduced by other investigators (Buchanan and Sirevag 1976) and, because of the preponderence of prevailing labeling data (Fuchs et al. 1980a, b), it has become accepted that green sulfur bacteria use only the RTCA cycle for CO_2 fixation. Interestingly, genomic sequencing of the related organism *Chlorobium tepidum* uncovered an archaeal-like Rubisco sequence (www.tigr.org/tdb/mdb.html) which appears to be cotranscribed with a glucose dehydrogenase gene and an open reading frame of unknown identity. This sequence has been isolated from genomic DNA, its sequence verified (Hanson and Tabita, unpublished results) and experiments initiated to determine if this sequence encodes bonafide Rubisco activity. Moreover, *C. tepidum* is an organism for which genetic manipulations are quite feasible (Wahlund and Madigan 1995) and specific knockout strains are being constructed to help elucidate the function of this putative Rubisco. It would appear that the presence and significance of Rubisco in these organisms is still an open question, however now there is an identifiable Rubisco sequence to consider.

Biological strategies to maximize CO_2 fixation in microorganisms

As might be expected for such a fundamental process, there are several layers of control, all of which contribute to maximize CO_2 fixation, and Rubisco function in particular. This is particularly true of prokaryotic photosynthetic and nonphotosynthetic systems, where several levels of regulation are manifest including: control of gene transcription and posttranscriptional processing of specific Rubisco messages, control by processes which maximize the folding and assembly of Rubisco, interesting means of sequestering Rubisco in prokaryotic 'organelles', and multifaceted means to control enzymatic activity, including posttranslational effects on Rubisco function.

Molecular regulation of Rubisco gene expression

Several reviews on the control of Rubisco gene expression in proteobacteria and cyanobacteria have appeared within the last few years (Tabita 1994, 1995: Gibson 1995; Gibson and Tabita 1996; Kusian and Bowien 1997; Shively et al. 1998); thus there is no need to reiterate this information here. Although little information is available about the control of Rubisco gene expression in eukaryotic 'nongreen' algae, beyond perhaps a recent study on photoperiod responses (Doran and Cattolico 1997), suffice it to say that the *cbb* operons of facultatively autotrophic phototrophic and chemotrophic proteobacteria, which use a diverse menu of carbon substrates for growth (including CO_2), are highly regulated. The finding of CbbR-dependent

cbb gene expression in these organisms was an important breakthrough as this indicated that discrete signals could be transduced to a protein always present in the cell, which subsequently binds specific sequences and then turns on the *cbb* system. Since these initial findings, however, more pertinent questions have been posed and current studies in several laboratories are devoted to elucidating the entire regulatory cascade and the precise mechanism for controlling the capacity for CO_2-dependent growth. For example, although CbbR is a positive regulator, the nature of the molecular signal that causes this protein to suddenly turn on *cbb* transcription, when organisms are placed under conditions where CO_2 is the carbon source, is not understood at this time. In addition, earlier work in *Rb. sphaeroides* indicated that up to 30 percent of CbbM (form II Rubisco) may be synthesized in a *cbbR* knockout strain (Gibson and Tabita 1993). What then are the regulatory processes that function independent of CbbR?

To answer the first of these questions, namely the nature of the molecular signal that turns on CbbR, it should first be noted that CbbR is part of a wider class of transcriptional regulator molecules found in proteobacteria, the so-called LysR Transcriptional Regulators or LTTR molecules (Schell 1993). Characteristically, these proteins are constitutively synthesized (although exceptions are found) and they usually employ a coinducer molecule, which when bound to the protein, effects a conformational state such that the LTTR is able to activate transcription. Nucleotide specificity and binding requirements were noted for purified CbbR preparations (van den Bergh et al. 1993; Kusian and Bowien 1995) and, at least for *Xanthobacter flavus* CbbR, NADPH appears to enhance binding by over three-fold in gel shift experiments (van Keulen et al. 1998). The enhancement by NADPH appears to be related to CbbR-induced bending of the DNA. However, in other bacterial systems, NADPH does not seem to be effective (Kusian and Bowien 1997; Dubbs and Tabita, unpublished results) so there may be some organism-dependent specificity in the 'coinducer' used to modulate the function of CbbR. For *Rhodobacter* and *Alcaligenes (Ralstonia)*, the two other well-studied bacterial systems, a coinducer molecule has not been identified. Moreover, for all LTTR molecules, the precise mechanism by which the small molecule modifies the structure of the protein to allow it to activate transcription is largely unknown. Usually the coinducer molecule is a product or metabolite of the pathway that is regulated (Schell 1993).

Recent studies indicate that there are regulatory processes independent of, or that function in addition to, CbbR. Studies with mutants of *Rb. sphaeroides* led to the discovery of a two-component signal transduction system that greatly influences *cbb* gene transcription in this organism (Qian and Tabita 1996). This two-component regulatory system (Sganga and Bauer 1992; Eraso and Kaplan 1994; Mosley et al. 1994) is composed of a membrane-associated sensor kinase (RegB or PrrB), which autophosphorylates itself in an ATP-dependent reaction. RegB then catalyzes the transfer of phosphate to a soluble response regulator (RegA or PrrA) before RegA$\sim$P can activate transcription (Inoue et al. 1995; Bauer and Bird 1996). The involvement of this system in CO_2 fixation was an unexpected finding as the Reg/Prr system had previously been shown to control transcription of operons involved in the biosynthesis of the photosystem of *Rb. capsulatus* and *Rb. sphaeroides* (Bauer and Bird 1996 and references therein). This same system was also shown to regulate nitrogenase biosynthesis and N_2-dependent growth (Joshi and Tabita 1996) and *nif* transcription (Qian 1997) in *Rb. sphaeroides*, making the Reg/Prr system truly a global two-component signal transduction system important for regulating a variety of processes in these organisms. The Reg/Prr system was stated to be solely involved in processes related to photosynthesis (Ogara et al. 1998), however dark aerobic *cbb* transcription is controlled by this system in chemoautotrophically-grown *Rb. sphaeroides* (Qian and Tabita 1996) and *Rb. capsulatus* (Vichivanives and Tabita, unpublished observations). Moreover, the very fact that the Reg/Prr system is involved in nitrogen fixation control in these organisms, and in the nonphotosynthetic organism *Bradyrhizobium japonicum* via an analogous regulatory system (Bauer et al. 1998), indicates that the Reg/Prr system and its homologs are global control systems not totally specific to photosynthetic processes. This is also supported by the discovery of a Reg/Prr homolog, ActSR, which is involved in acid tolerance in *Rhizobium meliloti* (Tiwari et al. 1996). The importance of sequences (306 bp) upstream from the cbb_I promoter of *Rb. sphaeroides* has become evident. These upstream activating sequences (UAS) greatly enhance cbb_I promoter activity under all growth conditions tested (Dubbs and Tabita 1998). The results of gel mobility shift assays (Dubbs and Tabita 1998) and Dnase I footprinting studies indicate that CbbR does not bind to the UAS. Obviously, one potential candidate to bind the UAS is RegA/PrrA, because this protein is involved in cbb_I transcrip-

tional control (Qian and Tabita 1996). Studies (Dubbs and Tabita, unpublished results) indicate that RegA from *Rb. capsulatus* specifically binds to the UAS of *Rb. sphaeroides* and to the cbb_I and cbb_{II} promoter-operator regions of *Rb. capsulatus* (Vichivanives and Tabita, unpublished results). These results thus show a direct involvement of the RegA and CbbR proteins in binding to specific sequences to regulate transcription. Exactly how small 'signal' metabolites and co-inducer molecules fit into this regulatory scenario and whether other genes and gene products interact with this system is under intense study. As would be suggested of a global regulatory system such as Reg/Prr, all available evidence points to the cellular redox potential influencing this regulatory cascade (Joshi and Tabita 1996; O'Gara et al. 1998; Zeilstra-Ryalls et al 1998). One might thus expect that there should be something that mediates signal transduction through the membrane-spanning RegB protein (O'Gara et al. 1998) because RegB∼P catalyzes the phosphorylation of RegA such that RegA∼P binds to UAS and other sequences important for CO_2 fixation. One might also expect that RegA and CbbR exhibit synergistic interactions, the potential for which was shown by the discovery of an additional RegA∼P binding site that overlaps that for CbbR, as well as potential direct interactions of RegA∼P with CbbR *in vitro* (Dubbs and Tabita, manuscript in preparation).

These recent results are approaching what might be termed the 'central dogma' of *cbb* and Rubisco gene regulation in photosynthetic proteobacteria and a model illustrating the interaction of the above components in the regulation of CO_2 fixation may be considered (Figure 3). Not elaborated here are potential posttranscriptional mechanisms affecting differential expression of *cbb* genes and over-synthesis of Rubisco, as alluded to earlier. In *Rb. capsulatus*, there is an inverted repeat preceded by a sequence that matches a concensus RNase E cleavage site [(G/A)AUU(A/U)] (Ehretsmann et al 1992) within the 83-nucleotide *cbbP-cbbT* intergenic region (Paoli et al. 1998b). This is reminiscent of the RNase cleavage site important in the processing of *puf* mRNA from this same organism (Fritsch et al. 1995), indicating that the above sequence is worth considering for similar transcript stability control for the cbb_{II} operon of *Rb. capsulatus* and *Rb. sphaeroides*. Presumably, this and other potential cleavage sites within the *cbb* operons allow a large primary transcript to be cleaved, followed by 3′-exonuclease digestion of newly exposed unprotected RNA (Gibson et al. 1991; Gibson 1995). By virtue of secondary structure, or lack thereof, at the 3′ end of the processed transcripts, individual messages could be protected to different degrees from exonuclease attack, allowing for the observed differential expression of *cbb* genes, especially the abundant synthesis of Rubisco protein. Further investigation of the regulation of posttranscriptional processing of operonic *cbb* transcripts would seemingly be a very fruitful and important endeavor which, to this day, has not been stressed in studies of transcriptional control of Rubisco gene expression.

The close and interactive control of the *cbb* and *nif* systems (Joshi and Tabita 1996; Qian, 1997; Qian and Tabita 1998) is also intriguing, yet it makes physiological sense, as shown in Rubisco-deficient strains of *Rhodobacter* and *Rhodospirillum* which have lost their capacity to use CO_2 as an electron acceptor (Figure 3). In the nonsulfur purple bacteria, photoheterotrophic growth is dependent on the CBB cycle to funnel reducing equivalents to CO_2, an important function of this pathway in addition to its role in allowing CO_2 to be used as the carbon source to support growth in the absence of organic carbon. If the CBB cycle is disrupted, by knocking out Rubisco function, some way to replace CO_2 as an electron acceptor must be attained, otherwise growth is impossible. The production of large quantities of H_2 gas by some Rubisco-deficient strains of *Rb. sphaeroides* and *Rs. rubrum* gave a clue as to how these organisms might accomplish this feat. Normally the nitrogenase complex of photosynthetic bacteria is involved in the evolution of hydrogen by these organisms (Hillmer and Gest 1977), however the nitrogenase system is repressed when cells are cultured with ammonia as the nitrogen source. Since the Rubisco-deficient strains were all cultured in the presence of ammonia and evolved copious quantities of hydrogen, it was suspected that these strains somehow derepress nitrogenase synthesis. This is exactly what occurs (Joshi and Tabita 1996). The nitrogenase enzyme complex catalyzes the reduction of protons to H_2 gas during nitrogen fixation according to the following relationship (Burris 1991):

$$N_2 + 16\,\mathrm{ATP} + 8e^- + 10\,H^+ \rightarrow 2NH_4^+ + 16\ \mathrm{ADP} + 16\ \mathrm{Pi} + H_2$$

At least 25% of the electron throughput is used to reduce protons to molecular hydrogen (Simpson and Burris 1984), which is normally released from photosynthetic bacteria under anoxic conditions (Hilmer and Gest 1977). In the absence of nitrogen gas, the only substrates available to the nitrogenase complex

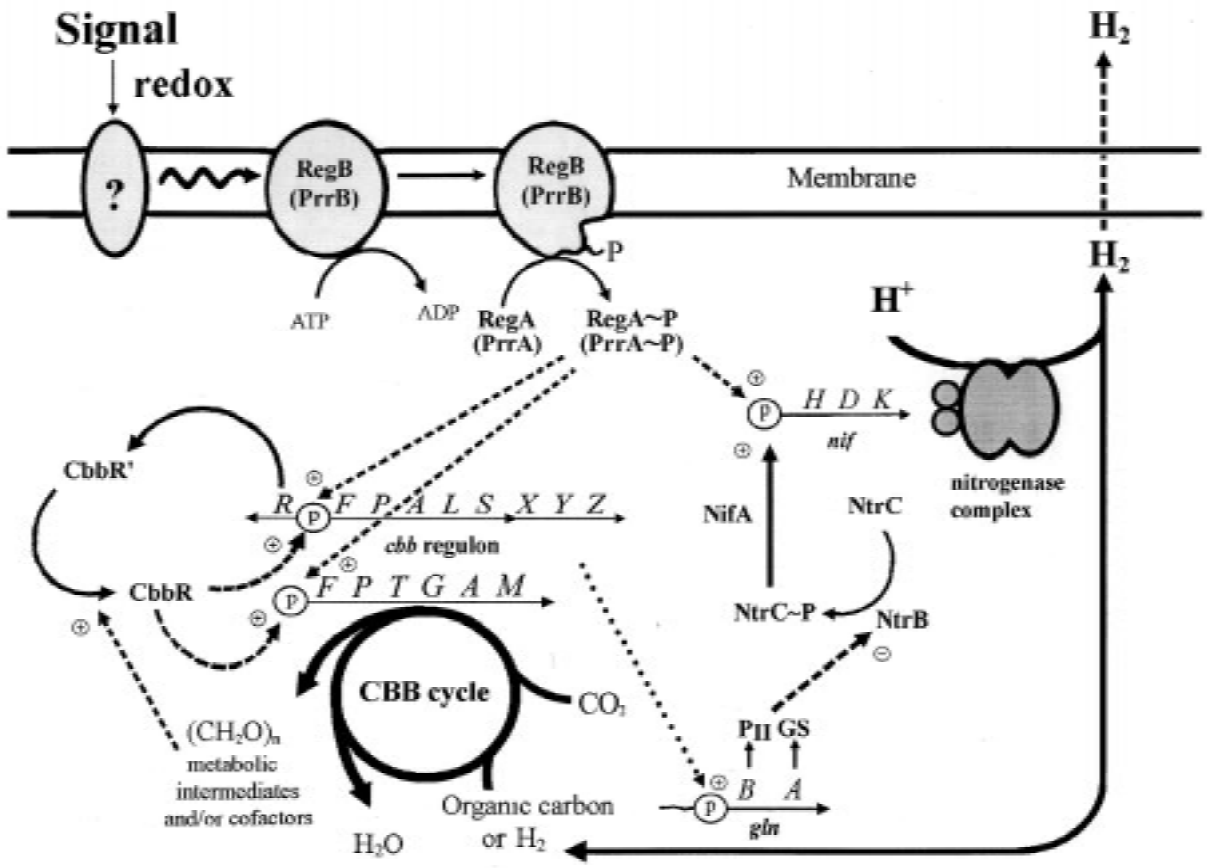

Figure 3. Conceptual model showing the interplay of various factors involved in the regulation of Rubisco gene expression in *Rb. sphaeroides*. The link between the CO_2 (*cbb*) and nitrogen regulatory system, including the nitrogen fixation (*nif*) genes is shown. Primary signals are received at the cytoplasmic membrane. This is thought to affect the redox potential of some key component (?) influencing RegB/PrrB autophosphorylation and the subsequent formation of RegA∼P (PrrA∼P). RegA∼P (PrrA∼P) interacts directly with the *cbb* and *nif* operator-promoter regions (Dubbs and Tabita, submitted for publication). Positive regulation is thus conferred both by the CbbR protein and RegA∼P(PrrA∼P), the phosphorylated response regulator of the Reg(Prr) two-component regulatory system. CbbR′ is converted to CbbR (the transcriptionally active form of this molecule), presumably by virtue of binding a coinducer molecule produced under CO_2 fixation conditions or other growth conditions that favor *cbb* transcription. The expression of *glnB* is affected by the *cbb* system (Qian and Tabita 1998) with *glnB* influencing *nif* derepression through the Ntr system and NifA. Blockage of the CBB pathway results in hydrogen evolution by virtue of the hydrogenase activity of the derepressed nitrogenase complex (Joshi and Tabita 1996). The nitrogenase complex and its inherent hydrogenase activity thus serves to remove excess reducing equivalents not dissipated in strains unable to use CO_2 as an electron acceptor. p, refers to promoter-operator regions that are activated in a positive manner (+).

are protons. Thus, H_2 gas is evolved via the reduction of protons, presumably supported by the large amounts of reducing equivalents obtained via photosynthesis and carbon oxidation. It was speculated that the reducing equivalents normally shunted to CO_2 are funneled to the nitrogenase complex in these particular Rubisco-deficient strains (Joshi and Tabita 1996). To accomplish this, the normal mechanisms of nitrogen control must be circumvented to allow for nitrogenase synthesis in the presence of ammonia (Figure 3). These results point to a link between *cbb* and *nif* control and point to the specific involvement of the Reg/Prr system in regulating these responses, since both processes are affected by mutations in the Reg/Prr system. Moreover, recent results show that there is a direct interaction of RegA∼P with the *cbb* and *nif* promoter-operator regions (Figure 3). It is envisioned that there is an activation of the nitrogen regulatory cascade in these strains due to the requirement for a functional CBB cycle for *glnB* transcription, the absence of which starts the regulatory cascade leading to nitrogenase synthesis (Qian and Tabita 1998) (Figure 3). Thus, it is very convenient for Rubisco-deficient strains to abrogate normal control mechan-

isms that prevent *nif* transcription in the presence of ammonia; i.e., so that these mutants have a way to remove reducing equivalents in the absence of a functional CBB cycle. It is quite telling that if a functional Rubisco gene is added back to these Rubisco-deficient strains, thus completing the CBB cycle, the normal controls over *nif* and *gln* transcription are restored (Joshi and Tabita 1996; Qian and Tabita 1998). That being said, there may also be other means to reduce the level of excess reducing equivalents in the absence of a functional CBB pathway, simply because Rubisco deficient strains have been isolated that do not derepress nitrogenase synthesis (Tichi and Tabita, unpublished results). These latter strains must then use some unknown process to dissipate the reducing equivalents in these cells. The interactive control of the *cbb* and *nif* systems, as well as other systems that allow these organisms to regulate redox poise, is thus extremely important relative to any consideration of how to maximize CO_2 fixation in bacteria. It would not be surprising if the CBB cycle serves an important role in regulating redox poise in plants and algae as well.

Rubisco structure-function relationships

The utilization of recombinant DNA procedures, in combination with X-ray structural models of representative form I and form II Rubisco proteins, has facilitated many interesting approaches to elucidating various aspects of catalysis and the properties and roles of the individual subunits of this enzyme. As noted previously, several rather extensive reviews have already considered many aspects of this issue (Hartman and Harpel 1993, 1994; Spreitzer 1993, 1998; Gutteridge and Gatenby 1995; Cleland et al. 1998), including reviews devoted solely to cyanobacterial and proteobacterial Rubisco (Tabita 1994, 1995). In the pages that follow, potential strategies for solving fundamental problems and reaching important objectives of particular interest to Rubisco from photosynthetic microorganisms will be stressed, with some consideration given to the use of other prokaryotic systems. In many respects, the prejudices of this reviewer will be apparent; however, it is hoped that readers will gain an appreciation of how diverse microbial systems present unusual opportunities for such investigations. Hopefully, investigators will be stimulated to either refute or further develop the ideas that follow, such that the whole field of Rubisco biochemistry will be enriched.

Folding and assembly of Rubisco

In microorganisms, factors influencing the folding and subsequent assembly of Rubisco have been investigated chiefly with bacterial systems. Indeed, virtually all studies have focused on recombinant protein produced in *Escherichia coli*. Several prior reviews (Roy and Canon 1988; Gatenby 1992; Ellis 1994; Gutteridge and Gatenby 1995), stressed the importance of the 'Rubisco-binding protein' and chaperone system of plants. In addition the involvement of the major chaperone machine of *E. coli* [i.e., the GroEL (cpn60) and GroES (cpn10) proteins] in the assemblage of a correctly folded and functional *Rs. rubrum* protein *in vitro* is well documented. However, it should be stressed that similar *in vitro* studies with the more complex bacterial L_8S_8 or form I Rubisco protein have never been reported. Although it is well established that the GroEL and GroES proteins of *Escherichia coli* are required for *in vivo* folding and construction of form I proteins (Goloubinoff et al. 1989), studies performed in Viale's laboratory (Dionisi et al. 1996; Checa and Viale 1997) indicate the additional importance of the DnaK, DnaJ, and GrpE chaperone machine for the formation of functional recombinant *Chromatium vinosum* and *Synechococcus* 6301 Rubisco *in vivo*. They employed *E. coli* mutants in *dnaK* and *dnaJ* to show the importance of the products of these genes. A similar study, with recombinant *Rb. sphaeroides* form I Rubisco and the *Synechococcus* 6301 enzyme, also indicated the importance of these gene products, however the requirement for DnaK and DnaJ could be partially overcome by overexpressing the GroEL and GroES proteins (Lee and Tabita, unpublished results). Despite these studies, successful folding of cyanobacterial large subunits *in vitro* has not been reported, even in experiments using denatured *Synechococcus* 6301 large subunits and an *in vitro* system that facilitates the folding of denatured *Rs. rubrum* subunits. Perhaps the inability to obtain productive folding of form I large subunits in vitro indicates the requirement for additional factors, as exemplified by *in vivo* recombinant protein folding studies with DnaK and DnaJ mutants. Potential involvement of the two chaperone machines must also be verified with the native organism, because folding with *E. coli* proteins *in vivo* or *in vitro* does not take into account other specific factors that might be involved. For example, the *rbcX* gene of some cyanobacterial *rbc* operons is often juxtaposed between the *rbcL* and *rbcS* genes (Figure 2) (Larimer and Soper 1993; Li and Tabita 1994) and

appears to be required for maximum activity of recombinant *Anabaena* Rubisco (Li and Tabita 1997). Likewise, the product of *cbbQ*, a gene found immediately downstream from the *cbbLS* genes of some bacteria, facilitated the folding of fully active recombinant *C. vinosum* and *Pseudomonas hydrogenothermophila* Rubisco (Hayashi et al. 1997), yet CbbQ apparently has no effect on the recombinant *Rb. capsulatus* form I enzyme (Horken and Tabita, unpublished results). Presumably, these studies may be extrapolated to Rubisco folding in the native organism, but like the *groESL* and *dnaKJ* genes, positive involvement must await studies with native chaperone genes within the environment where they are purported to function. With the finding of two sets of *groESL* operons in *Rb. sphaeroides* (Lee et al. 1997), and the isolation of the *dnaKJ* operon of this organism (Lee and Tabita, unpublished results), direct involvement of these genes in Rubisco folding in *Rb. sphaeroides* will be sought.

Presumably chaperones could be involved with the folding of small subunits as well, although this has not been demonstrated in a system free from large subunits. It should also be stressed that small subunit monomers (Paul et al. 1991) spontaneously associate with the L_8 octameric large subunit core.

Based on these recent studies, one might modify the original Rubisco assembly model (Goloubinoff et al. 1989) to include the involvement of additional chaperone proteins to assist in folding dynamics required for the construction of the $(L_2)_4$ catalytic core of large subunits, in steps before or beyond the formation of the basic dimer, which is clearly formed (at least for the *Rs. rubrum* enzyme) through the mediation of GroEL and GroES. Obviously, from the foregoing, much additional research is needed to fully elucidate all the factors and proteins required for the folding and subsequent assembly of such a complex hexadecameric protein. Prokaryotic systems seem to be the obvious systems of choice for these studies.

Prokaryotic 'organelles' of CO_2 fixation

Many chemoautotrophic proteobacteria and all cyanobacteria have been observed to contain discrete intracellular polyhedral-shaped inclusion bodies (Shively et al. 1988, 1996). The reader is also encouraged to consult a recent review of the significance of inclusion bodies and its relationship to CO_2 assimilation in eukaryotic algae (Badger et al. 1998). In bacteria, inclusion bodies were initially isolated from sulfur-oxidizing bacteria and were shown to contain massive amounts of Rubisco (Shively et al. 1973). This is true for cyanobacteria as well (Codd and Marsden 1984). Careful fractionation of the isolated inclusion bodies or 'carboxysomes' indicated that Rubisco large and small subunit polypeptides are not the only proteins present, but from 7 to 15 additional polypetides may be resolved, several of which appear to comprise the outer proteinaceous shell of the carboxysomes (Shively et al. 1998). Large amounts of carbonic anhydrase fractionated with carboxysome preparations of some cyanobacteria (Price et al. 1992), in support of the interesting model proposed by Reinhold et al. (1991), in which the carboxysomes and carbonic anhydrase are thought to play a crucial role in a CO_2 concentrating mechanism (CCM) that functions to transport HCO_3^- and provide high levels of CO_2 to the active site of Rubisco. A pump to provide high concentrations of CO_2 was hypothesized to be necessary to overcome the poor K_{CO_2} of cyanobacterial Rubisco, which, in several reports, ranges from 150–250 μM (Tabita 1994). The existence of the CCM and its association with carboxysomes is supported by many studies where mutants requiring high CO_2 concentrations for growth mapped to known carboxysome genes (for a general review see Kaplan and Reinhold 1998); such mutants contained defects in carboxysome structure (Friedberg et al. 1989; Price and Badger 1989; English et al. 1994; Marco et al. 1994; Ronen-Tarazi et al. 1995; Martinez et al. 1997). Several additional studies support the association of carbonic anhydrase with cyanobacterial (*Synechococcus* PCC 7942) carboxysomes (for example So and Espie 1998; Sultemeyer et al. 1998); however, there is no evidence for carbonic anhydrase in proteobacterial carboxysomes, and this appears to be true for other cyanobacteria as well (Ingle and Colman 1975; Firus et al. 1985; Lanaras et al. 1985; Codd 1988; Bedu et al. 1992; Suzuki et al. 1994). Thus, Shively et al. (1998) question the evidence and need for carbonic anhydrase and note that the carboxysome shell exhibits little selectivity for Rubisco substrates and products (Satoh et al. 1997). An intriguing alternate hypothesis was invoked, namely that carboxysomal Rubisco might be altered such that its substrate specificity (or Ω value) is greatly enhanced over the free enzyme by virtue of the enzyme's association with some component of the carboxysome. As nuclear-encoded gene products influence Rubisco substrate specificity in *Chlamydomonas* (Chen et al. 1990; Gotor et al. 1994), this idea of a postranslational effect that enhances Rubisco specificity in the carboxysomes deserves considera-

tion. Future developments relative to the interesting carboxysome-associated Rubisco in both cyanobacteria and proteobacteria are awaited with great interest. It will be interesting if some universal mechanism to explain the function and role of carboxysomes in CO_2 metabolism evolves for both groups of organisms or if separate mechanisms might account for the discrepant results obtained for some oxygen-evolving photosynthetic cyanobacteria and chemoautotrophic oxygen-consuming proteobacteria. Studies of the control of carboxysome synthesis as a function of the organic carbon and/or level of CO_2 provided to cultures will be most important, especially because CO_2 limitation seems to result in increased carboxysome synthesis in all systems (see review by Shively et al. 1998). An organism such as *Thiobacillus intermedius* would be particularly useful in these endeavors as carboxysome synthesis is completely repressed when organic carbon is added to cultures, while there is de novo synthesis when the organism is grown under autotrophic conditions (Purohit et al. 1976). It appears that form I Rubisco, or the regulated expression of its genes, is intimately involved in the assembly of a functional carboxysome; specific inactivation of the *cbbLS* genes in *T. neapolitanus* yielded a mutant that could grow only with high levels of CO_2, without synthesizing carboxysomes. This mutant was able to grow because it synthesized form II Rubisco, which was induced only in response to a lack of form I Rubisco (Baker et al. 1998).

Uniformity of catalytic mechanism but differences nonetheless

Despite the very different Rubisco primary structures represented by the form I and form II enzymes, and perhaps 'form III and form IV' proteins of archaea, mechanistic studies, using primarily the plant/green algal and *Rs. rubrum* enzymes, have established that the catalytic process is uniformly conserved. This whole aspect of Rubisco biochemistry has been reviewed extensively (Hartman and Harpel 1993, 1994; Cleland et al. 1998) and it is well established that this enzyme employs several partial reactions to assimilate CO_2 and/or fix O_2. In addition, all Rubisco enzymes must be activated or carbamylated before catalysis ensues. Residues important for key aspects of catalysis are conserved throughout evolution, however there are some important idiosyncratic properties that vary in even phylogenetically close proteins that exhibit over 85% sequence identity (Tabita 1995). This is perhaps best exemplified by examining the key parameters of CO_2/O_2 substrate specificity (Ω or τ), the K_{CO_2}, and the ability of the enzyme to exhibit 'fallover' by phylogenetically related enzymes (Table 2). Rubisco catalysis, either carboxylation (v_c) or oxygenation (v_o), is dependent on the inherent ability of the enzyme to discriminate between CO_2 or O_2 (the Ω or τ value) and the relative concentration of CO_2 and O_2 employed in a particular reaction:

$$v_c/v_o = \Omega[CO_2]/[O_2]$$

where

$$\Omega = v_c[O_2]/v_o[CO_2] = V_cK_o/V_oK_c$$

with V_c and V_o representing maximum velocities for carboxylation (V_c) and oxygenation (V_o) and K_c and K_o representing the values for K_{CO_2} and K_{O_2} respectively.

Jordan and Ogren (1981) initially showed that Rubisco enzymes from divergent sources possess different Ω values, providing the first evidence that this is not an immutable property. The product of the oxygenase reaction, 2-phosphoglycolate, becomes dephosphorylated via a specific phosphatase (encoded by *cbbZ*, see Figure 2) and, depending on the organism, may be further oxidatively metabolized, or excreted as glycolate. This results in a diminished capacity to retain carbon for biosynthesis and growth. The obvious importance of the Ω value, then, is that it provides some quantitative measure of Rubisco efficiency and represents a property that goes to the very core of successful CO_2-dependent growth. Thus, those enzymes that favor carboxylation have higher Ω values. While several revealing studies have identified specific residues on Rubisco that influence Ω (reviewed in Hartman and Harpel 1993, 1994), many, if not all of these residues, are conserved throughout the different evolutional forms of Rubisco. Moreover, in many instances, residues that positively or negatively affected Ω in the *Chlamydomonas* enzyme, did not result in similar effects after residue changes were created by site-directed mutagenesis in the phylogenetically related *Synechococcus* 6301 enzyme. In fact, the resultant properties (Parry et al. 1992; Gutteridge et al. 1993; Lee et al. 1993; Kane et al. 1994; Read and Tabita 1994; Ramage et al. 1998) were totally different from those of the *Chlamydomonas* enzyme (Chen and Spreitzer 1989; Chen et al. 1991; Zhu and Spreitzer 1996). From these results, and the fact that such residues are often conserved in enzymes that possess

Table 2. Summary of key kinetic constants of enzymatically characterized Rubisco enzymes. Enzymes are classifed according to their sequence relatedness as depicted in Figure 1. Ω values (rounded and averaged) for purified enzymes were obtained by the dual label specificity assay in this laboratory (Lee et al. 1991; Read and Tabita 1992a, b; 1994; Hernandez et al. 1996; Horken and Tabita 1999, and unpublished results) except the *Anabaena* (Larimer and Soper 1993), vent symbiont (Stein and Felbeck 1993), *H. marinus* (Igarashi and Kodama 1996), *C. reinhardtii* (Jordan and Ogren 1981), *G. partita* and *C. caldarium (*Uemura et al. 1997), and *C. vinosum* (Jordan and Chollet 1985) enzymes which were determined by this (Jordan and Ogren 1981; Jordan and Chollet 1985) and other methods (Larimer and Soper 1993; Stein and Felbeck 1993; Igarashi and Kodama 1996; Uemura et al. 1997) elsewhere. ND, not determined. Putative Type III/IV Rubisco sequences are from Bult et al. 1996, Klenk et al. 1997, or from existing sequence databases

Rubisco type	Organism	$V_{CO_2}K_{O_2}/V_{O_2}K_{CO_2}$ (Ω)	K_{CO_2} (μM)
Type IA	*Rhodobacter capsulatus*	25	30
	Hydrogenovibrio marinus	25	ND
	Chromatium vinosum	40	35
	Thiobacillus denitrificans I	45	140
	Vent symbiont	30	80
Type IB	Cyanobacteria		
	Synechococcus 6301	40	175
	Anabaena 7120	35	150
	Green algae		
	Chlamydomonas reinhardtii	60	30
	Plants – many species	80	10–30
Type IC	Purple bacteria class		
	Bradyrhizobium japonicum	75	65
	Xanthobacter flavus	45	100
	Rhodobacter sphaeroides	60	25
	Ralstonia eutropha	75	ND
Type ID	Marine nongreen algae		
	Cylindrotheca sp. strain N1	105	30
	Olisthodiscus luteus	100	60
	Porphyridium cruentum	130	20
	Cylindrotheca fusiformis	110	35
	Cyanidium caldarium	225	5
	Galdieria partita	240	5
Type II	*Rhodospirillum rubrum*	15	100
	Rhodobacter sphaeroides II	10	100
	Thiobacillus denitrificans II	10	250
Type III/IV?	*Methanococcus jannaschii*		
	Archaeoglobus fulgidus 1		
	Archaeoglobus fulgidus 2		
	Pyrococcus horikoschii		
	Pyrococcus kodakaraensis		
	Bacillus subtilis		
	Chlorobium tepidum		

different Ω values (Table 2), strong arguments may be made for the importance of other residues to influence Ω. Support for this idea is also strongly provided from *rbc* suppressor mutants of *Chlamydomonas* (Spreitzer 1998). It is clear that very closely related enzymes may possess vastly different Ω values (Table 2). Form I enzymes in class IC possess Ω values (Ω = 75) (Lee et al. 1991; Horken and Tabita, 1999) that approximate values obtained for higher plant Rubisco (Ω = 78–82) (Jordan and Ogren 1981; Read and Tabita 1992b) but range to a low value of Ω = 45 for the *Xanthobacter flavus* enzyme and an intermediate value of Ω = 55–60 for the *Rb. sphaeroides* form I enzyme (Jordan and Ogren 1981; Horken and Tabita 1999). The form I large subunit from *Rb. sphaeroides* (Ω = 55–60) shares 86% amino acid sequence identity with the large subunit of the *X. flavus* enzyme (Ω = 45), while the *R. eutropha* enzyme (Ω = 75) shows 83% amino acid sequence identity to the *X. flavus* large subunit. Thus it is apparent that differences in Ω (Table 2) must be attributed to either different residues, or to specific conformations that are not obvious from structural models. Large subunits, primarily, contribute to differences in Ω (reviewed in Hartman and Harpel 1993, 1994), however small subunits (Read and Tabita 1992a, b; Getzoff et al. 1998), or the products of nuclear-encoded genes in *Chlamydomonas*, may influence CO_2/O_2 substrate specificity and/or other aspects of catalysis (Spreitzer 1993). The small subunit amino acid sequences of type IC Rubisco are less similar than their corresponding large subunits; however as a first approximation, it may be fruitful to first consider differences in large subunits in any studies (discussed later) designed to elucidate the basis for Ω variance among representative enzymes of this class of form I Rubisco. The K_{CO_2} values for Rubisco enzymes of class IC vary and this important property generally differs for closely related enzymes of several classes of Rubisco. When one compares the two key kinetic constants (Ω and K_{CO_2}) over the broad expanse of enzymatically characterized Rubisco molecules (Table 2), it is apparent that these properties evolved in phylogenetically similar and diverse organisms, all of which must have been exposed to selective pressures that caused the enzyme to change according to the organisms' specific need for CO_2 assimilation in a particular environment. The fact that the closely related Type IC enzymes possess such different Ω and K_{CO_2} values is deemed a potentially significant finding, because, unlike other related Rubisco homologs that possess different kinetic properties (i.e., Type IB), the ease of genetic manipulation of Type IC organisms present a unique opportunity to glean information relative to the structural basis for such kinetic variance. This issue will be discussed in greater detail below. It should be stressed, however, that available tertiary structures of divergent form II (i.e., *Rs. rubrum*) and form I (*Synechococcus* 6301) and higher plant (spinach and tobacco) Rubisco molecules have failed thus far to point out a structural basis for the diverse specificities (Ω values) exhibited by these proteins. As it is clear (Table 2) that Nature has figured out how to put together Rubisco molecules with diverse substrate specificity (for whatever purpose), it should be feasible to elucidate the basis for such structural alterations.

Posttranslational control of Rubisco activity in vivo

Other than inherent changes in the kinetic properties of Rubisco, there are several means by which prokaryotic photosynthetic and chemoautotrophic bacteria regulate Rubisco activity *in vivo* (reviewed by Tabita 1988, 1994, 1995). These mechanisms involve some form of posttranslational modification and interaction with metabolites, stimulated by some physiological change that occurs after the organism is placed in a challenging environment. For example, Rubisco in *Rs. rubrum* is subject to oxidative modification, which appears to 'mark' the enzyme for proteolytic degradation after cells are switched from anoxic photosynthetic conditions to an aerobic environment (Cook and Tabita 1988; Cook et al. 1988). Also, *Rb. sphaeroides* form I Rubisco was subject to reversible inactivation and modification involving phosphorylated compounds that appear to bind noncovalently to the enzyme after metabolizable organic carbon compounds are added to cultures (Jouanneau and Tabita 1987; Wang and Tabita 1992a, b, and unpublished results). As form I Rubisco from *Rb.sphaeroides* is greatly inhibited by RuBP (Gibson and Tabita 1977), there may be a Rubisco activase-like enzyme that catalyzes the removal of substrate and/or other tightly-binding phosphorylated metabolites from form I Rubisco in this organism. Other examples of specific posttranslational regulation of Rubisco activity have been noted (Tabita 1994, 1995) and a particularly intriguing system is that of cyanobacteria belonging to the genus *Anabaena* (Li et al, 1993; Li 1994; Li and Tabita 1994). Here, Rubisco activase (*rca*) genes are located downstream, yet closely juxtaposed to the *rbcLS* operon, but are separately transcribed from *rbcLS*. Because cyanobacterial Rubisco does not exhibit fallover or RuBP-mediated

inhibition, the need for Rubisco activase in *Anabaena* is not obvious. Subsequent studies (Li 1994) showed that the growth rate and *in situ* Rubisco activity of a specific *rca* knockout strain of *Anabaena variabilis* was somewhat diminished. These results were interpreted to indicate that some high affinity phosphorylated compound, other than RuBP, may inhibit the *Anabaena* enzyme *in vivo*, with this metabolite removed through the action of Rubisco activase. There is some indication that this organism synthesizes 2-carboxyarabinitol monophosphate (J. Servaites, personal communication), a compound known to regulate the activity of plant and green algal Rubisco (Portis 1992).

Exploitation of microbial systems and development of novel approaches to study Rubisco function

The ease of expressing bacterial Rubisco genes using many of the extraordinarily powerful *E. coli* expression vectors is a compelling reason to consider using such microbial systems for structure-function studies. With regard to Rubisco, many fundamental issues of catalysis have been resolved by this approach (reviewed in Hartman and Harpel 1993, 1994). Available X-ray structural models may point the way to important specific residues for further study. However, a discrete molecular rationale that provides an understanding of why, for example, plant Rubisco has a much higher CO_2/O_2 substrate specificity value than the structurally similar cyanobacterial Rubisco has yet to be formulated. Likewise, there is no known basis for the high K_{CO_2} of the cyanobacterial enzyme (Table 2). Finally, there is no defined reason why this enzyme does not exhibit 'fallover'; i.e., the characteristic time-dependent decrease in enzymatic activity exhibited by the plant enzyme and most microbial form I enzymes. Indeed, comparisons of very closely related bacterial enzymes of Type IC clearly show very different enzymatic properties (Table 2), as eluded to earlier. Thus, it is apparent that additional approaches are needed to solve the specificity issue and other aspects of catalysis that are not readily approached with the procedures currently employed. In many respects, the problem comes down to the fact that site-directed mutagenesis procedures are excellent if one knows what residue(s) or sequence(s) to alter, but the whole approach is useless when one does not know upon which residue(s) to focus. One can gaze at sequences and, indeed, choose potentially important residues based on structural considerations and from comparisons of sequences of high specificity and low specificity enzymes. However, at this time, the fundamental question of CO_2/O_2 specifity remains unsolved. Moreover, when residues that contribute to specificity have been identified via affinity labeling and/or other chemical and mutagenesis approaches, invariably these results are tempered by a drastic loss in the k_{cat} of the enzyme. Obviously, some kind of controlled approach that mimics what Nature must do to select for Rubisco of desired specificity and high k_{cat} must be conceived. A variation of this approach using the highly productive *Chlamydomonas* system (Spreitzer 1998) provided much important information that would not have been realized by more standard approaches; i.e., the discovery of residues that influence the capacity of Lys-335 to affect CO_2/O_2 discrimination. In this section, the use of prokaryotic systems to approach the issue of Rubisco specificity is considered, such that advantage may be taken of the physiological diversity of organisms that support CO_2-dependent growth. In addition, the relative ease of genetic manipulation, combined with convenient growth conditions, indicates that certain prokaryotic systems might provide an important additional means to approach this most fundamental issue of Rubisco function.

Approaches to take advantage of microbial systems

There are basically three approaches that one might employ to gain a better understanding of Rubisco function. Certainly, there is the 'traditional' approach where one may focus on a particular enzyme and employ all relevant and available biochemical techniques, backed up by directed mutagenesis, to learn more about a particular aspect of catalysis. A second tack emphasizes the potential of genetically manipulable proteobacteria that contain closely related enzymes of different specificity. The third approach relates to 'avenues of opportunity' that arise as a result of serendipitous observation.

Despite a desire to employ all types of imaginable scenarios to solve the substrate specificity 'holy grail', it must be stressed that basic biochemistry provides the underpinning upon which all Rubisco knowledge is based. Thus, new concepts continue to emerge. This is exemplified by recent findings that provide a better understanding of how Rubisco may modify the stability of the transition state to accommodate the synthesis of different reaction products (Harpel et al. 1995; Kane et

al. 1998). Such studies go a long way towards enhancing knowledge of how Rubisco might discriminate between CO_2 and O_2. Indeed, discovering exactly how a particular enzyme, for example the *Rs. rubrum* Rubisco, performs most of its interesting chemisty could provide a molecular rationale for many of the interesting variations seen in Table 2. However, as discussed earlier, often a particular residue or residue(s) shown to play an important role in one Rubisco is not directly applicable to Rubisco from a different source. This is obviously because other structural aspects, perhaps not important in the first instance, play an important part of the overall catalytic scenario for the second Rubisco. This is best exemplified by the finding that residues in and around loop 6 of the *Chlamydomonas* enzyme, previously shown to influence Ω, do not have the same effect on the closely related *Synechococcus* enzyme, undoubtedly because of other structural constraints not appreciated at this time.

A convenient bacterial system to randomly select alterations in Rubisco function

So, how may one take advantage of the fact that closely related Rubisco enzymes exhibit different specificity (Table 2)? To answer this question, one is led to the second approach, namely the use of genetic selection procedures with prokaryotic systems to discern a molecular basis for Rubisco specificity. This approach might be likened to laboratory efforts to duplicate what Nature has already done, with the important difference that in the laboratory one can control all the parameters and elucidate exactly what is required for the changes that are selected. There are several scenarios that lend themselves to these procedures, any one of which might be modified and employed according to the investigator's wishes. What is required, however, is a good understanding of the physiology of the organisms that will be employed in these manipulations, as well as the ability to perform facile molecular gene transfers. Organisms containing Type IC Rubisco fit these specifications to a tee. Nonsulfur purple photosynthetic bacteria, i.e. *Rhodobacter* species, are especially germane as they are perhaps the most metabolically versatile organisms found on earth (Madigan and Gest 1979). These organisms are capable of both anoxygenic photosynthetic metabolism and aerobic respiration in the dark, and they are capable of growing at the expense of CO_2 (using the CBB cycle) as the sole carbon source, both in the presence or absence of O_2, in the dark or in the light, respectively. In addition, these organisms may grow at the expense of organic carbon such that the CO_2 fixation system is dispensible, under both photosynthetic growth conditions or under conditions where oxygen is the terminal electron acceptor in the dark. The capacity for growth in the absence of CO_2 fixation is very important for the facile verification of gene transfer and expression under conditions where Rubisco is not essential, as will be discussed below. What is first needed is an organism that will serve as the host for all subsequent manipulations and selections. Studies on the molecular control of CO_2 fixation in *Rhodobacter* (Gibson 1995; Tabita 1995; Gibson and Tabita 1996) have provided mutant strains of *Rhodobacter sphaeroides* (Falcone and Tabita 1991) (strain 16), *Rhodospirillum rubrum* (Falcone and Tabita 1993) (strain I-19), and now *Rhodobacter capsulatus* (Paoli et al. 1998b) (strain SBI-II), that are Rubisco deficient because of specific knockouts of both form I (*cbbLS*) and form II (*cbbM*) Rubisco genes in *Rb. sphaeroides* and *Rb. capsulatus* and the single *cbbM* gene of *Rs. rubrum*. In addition, a Rubisco expression vector is available that contains the extremely potent *Rs. rubrum cbb* promoter to allow for controlled expression of the desired Rubisco genes in *Rhodobacter* strains 16 and SBI-II. These strains appear more suitable and versatile than *Rhodospirillum rubrum* strain I-19 for this purpose. Introduction of the Rubisco expression plasmid into the Rubisco-deficient strains allows the genes to be expressed while complementing the organism to CO_2-dependent growth.

Selection at different CO_2/O_2 ratios

For many purposes, aerobic chemoautotrophic growth would be advantageous, that is, nonphotosynthetic growth at defined CO_2/O_2 ratios under conditions where molecular H_2 serves as the energy source. Despite common misconceptions, various photosynthetic nonsulfur purple bacteria are well known to grow in the dark under these aerobic conditions using CO_2 as sole carbon source, much like nonphotosynthetic hydrogen bacteria. *Rb. capsulatus* grows well but *Rb. sphaeroides* grows poorly, if at all, under these conditions (Madigan and Gest 1979). However, a 'gain-of-function' spontaneous mutant strain of *Rb. sphaeroides* was isolated that acquired the ability to grow chemoautotrophically in a $H_2/CO_2/O_2$ atmosphere (Paoli and Tabita 1998). As much more is known of the regulation of CO_2 fixation in *Rb. sphaeroides*, including the locus of a gene encod-

ing phosphoglycolate phosphatase (Gibson and Tabita 1997), the acquisition of the chemoautotrophic-competent *Rb. sphaeroides* strain was deemed a significant development.

Growth in a CO_2/O_2 atmosphere, and CO_2-dependent growth in the absence of O_2, presents some interesting possibilities relative to selecting Rubisco of altered specificity. As discussed earlier, Ω relates the initial velocities of carboxylation and oxygenation to the relative concentration ratio of CO_2 and O_2, such that $\Omega = v_c[O_2]/v_o[CO_2]$. Because the oxygenase reaction causes the eventual loss of carbon from the cell, v_c/v_o must exceed some critical value in order for Rubisco to catalyze net carbon assimilation. For example, in higher plants it has been estimated that 0.5 carbons are lost for each O_2 fixed (Gutteridge et al. 1989). Therefore, v_c/v_o must be greater than 0.5 in order for Rubisco to catalyze net carbon fixation and support autotrophic growth in the absence of some CO_2 concentrating mechanism (Lorimer et al. 1993). Accordingly, the $[CO_2]/[O_2]$ ratio can be adjusted such that v_c/v_o would be growth limiting. Incubation of an organism at the growth-limiting condition should provide a means for biological selection of Rubisco with increased substrate specificity. The $[CO_2]/[O_2]$ ratio at which growth limitation would occur would thus depend upon the Ω of the Rubisco synthesized. Using strains 16 and SBI/II complemented with, for example, a typical high Ω bacterial form I Rubisco gene inserted into our expression vector, should result in a $[CO_2]/[O_2]$ ratio at which the form I-containing strain will grow. However, a strain containing a low Ω form II Rubisco gene inserted in the vector will not grow. As there does not appear to be any appreciable CO_2 concentration system, such a $[CO_2]/[O_2]$ ratio would be ideal to select a form II, or low specificity form I Rubisco (Table 2), with increased substrate specificity after random mutagenesis of the Rubisco gene. One would only need to score for growth at the above CO_2/O_2 ratio. Perhaps the nuclear encoded dinoflagellate form II Rubisco, which reportedly possesses a CO_2/O_2 substrate specifity in crude extracts that is about two fold higher than the typical bacterial form II enzyme (Whitney and Andrews 1998), is just such a protein, and has naturally evolved in this way. The above discussion represents just one scenario where this system might be useful and involves a positive selection, as only those mutants that can grow would be examined further, the idea being that differences in growth should reside in the inherent substrate specificity of the particular Rubisco synthesized. Preliminary experiments indicate that concentrations at or near 0.25% CO_2 and 10.5% O_2 may be a gas ratio to effect such selection (Paoli and Tabita 1998).

Can one take advantage of kinetic anomalies?

There are several additional selection scenarios that are suggested by the idiosyncratic properties of various bacterial Rubisco proteins which may be used to obtain enzymes that complement strains 16 and SBI-II to either photoheterotrophic, photoautotrophic, or aerobic chemoautotrophic growth. We previously noted that the high K_{CO_2} of the cyanobacterial *Synechococcus* 6301 Rubisco (on plasmid pRPS75) precluded complementing *R. sphaeroides* strain 16 to photoheterotrophic growth when cultures were sparged with argon to maintain anaerobiosis (Falcone and Tabita 1991; Tabita 1994). This was attributed to the inability of the enzyme to capture CO_2 produced as a result of the oxidation of the organic carbon source, malate, in the absence of an active CO_2 pump mechanism in this organism. Only upon the addition of exogenous CO_2 or HCO_3^- could the organism grow using this Rubisco. This physiological quirk suggests a ready means to select for enzyme molecules that can better cope with lower levels of CO_2. We have also found (S.A. Smith and F.R. Tabita, unpublished results) that it may be possible to select for mutant enzymes after mutagenizing plasmid pRPS75 and plating strain SBI/II (transformed with mutagenized pRPS75) on minimal medium-containing plates under photosynthetic conditions in a CO_2-rich atmosphere. Colonies that exhibited both normal and aberrant growth were obtained after random mutagenesis of the cyanobacterial *rbcLS* genes; the colonies that grew poorly appeared to reflect potential Rubisco mutants that were negatively affected in the ability to support growth. Other studies indicated that both photoheterotrophic growth and CO_2-dependent growth was diminished when strain 16 was complemented with sequences that have site-directed changes in either large (Read and Tabita 1994; Ramage et al. 1998) or small (Read and Tabita 1992a) subunits of the *Synechococcus* 6301 Rubisco that negatively affect *in vitro* activity. The potential to isolate altered enzymes via the generation of internal suppressor mutations, to allow for better growth than the original mutant, is also very feasible, much like in *Chlamydomonas* (Spreitzer 1998).

The form I enzymes of Class IC (Table 2) are obvious candidates for further study. Indeed, the first biological selection of random mutations in any Ru-

bisco gene was reported in 1979 when Andersen mutagenized cultures of *Alcaligenes eutrophus* (now *Ralstonia eutropha*) and selected for lack of growth under chemoautotrophic conditions (Andersen 1979). Several of the mutants that were isolated had alterations in Rubisco, the basis for which was never determined. Some 20 years later, the opportunities for achieving selective changes in Rubisco are enhanced by employing procedures that mutagenize only the *rbc* or *cbb* genes. This is followed by reintroduction of the mutated genes via a plasmid or via insertion into the chromosome; the recent genetic results certainly point out the feasibility of doing such experiments with the *Rhodobacter* strain 16/strain SBI-II system.

DNA shuffling (Crameri et al. 1998) has also become a method of choice to isolate random chimera molecules of related sequences. Because the *cbbLS* genes that encode enzymes of Type IC are so closely related, it should be feasible to screen, for example, chimeric enzyme molecules that support growth in CO_2/O_2 atmospheres that favor either high or low Ω enzymes. This powerful means to obtain multiple domain shifts might be just what is needed to find out why, for example, the *X. flavus* enzyme, to the exclusion of the other related enzymes of this class, has such a low Ω value (Table 2). Manual domain switches may also be made, using convenient restriction sites. Having the organism, however, select for the type of enzyme it needs, under the growth conditions chosen, precludes unanticipated folding or other problems that may be encountered by the manual domain switch approach.

Additional prokaryotic selection scenarios

There are many additional variations of the random mutagenesis and selection procedures discussed above; i.e., the use of bacterial mutants in glycolate-oxidoreductase (Andersen et al 1986) to screen for Rubisco mutants that produce varying amounts of glycolate. In addition, the use of chemostats under growth-limiting conditions is a classical way to obtain mutants in genes that encode proteins that cause growth limitation. Combined with random mutagenesis protocols, this should be a very powerful way to select for Rubisco alterations that might not be noted otherwise. A particularly interesting modification of the *Synechocystis* sp. PCC 6803 'cyanorubrum' strain (Pierce et al. 1989) was described by Amichay et al. (1993), in which a mutant was constructed, Syn6803Δrbc, from which the entire *rbc* operon was replaced by the *Rs. rubrum cbbM* (form II) Rubisco gene. This new cyanobacterial construct is quite amenable to the introduction of foreign Rubisco genes, and with two of the genes for the carbon concentrating mechanism (CCM) also knocked out, resulting in strain Syn6803Δ*rbc::*Δ*ccmM*Δ*ccmN* (M. Gurevitz, personal communication), autotrophic growth is entirely dependent on CO_2 levels provided to the culture. The double *ccmMccmN* knockout also prevents the generation of spontaneous mutants in the CCM, thus any strain that shows altered growth characteristics must necessarily have a mutation in the Rubisco genes, which are separately mutagenized and inserted into the *rbc* region of the chromosome by homologous recombination. Mutations that confer both poor and good growth at low and high CO_2 concentrations should allow for the facile selection of Rubisco molecules altered in k_{cat}, K_{CO_2}, and/or Ω. The potential to employ DNA shuffling protocols, using, for example Rubisco genes from class IC, is currently being employed with this system under these selection conditions (Gurevitz and Tabita, unpublished results).

Potential for novel eukaryotic selection systems

The interesting high specificity form I Rubisco enzymes of nongreen algae should be emphasized in further molecular-based studies. The problem here lies in the inability thus far to express the chloroplast-encoded *rbcLS* operon in a suitable host. In our laboratory, no success in producing properly folded *Cylindrotheca* N1 Rubisco in *E. coli* was realized (Hwang and Tabita 1991) although there is a report that low activities of *Olisthodiscus* Rubisco were obtained in *E. coli* (Newman and Cattolico 1988). Attempts to express these genes in *Chlamydomonas* chloroplasts and in the cytoplasm of the yeast *Pichia* have met with limited success (Zianni and Tabita, unpublished observations), although we have produced soluble diatom (*Cylindrotheca*) recombinant small subunits in *Pichia.* If a suitable eukaryotic, or even prokaryotic, expression system could be developed, opportunities would abound for constructing site-directed mutations and for performing other experiments to examine the properties of these enzymes. Thus, approaches that require large amounts of protein, which are currently difficult to obtain from the native organism, would become available. Moreover, the interesting dinoflagellate form II Rubisco has eluded purification to high specific activity (Whitney and Andrews 1998). The similarity of the primary structure of this en-

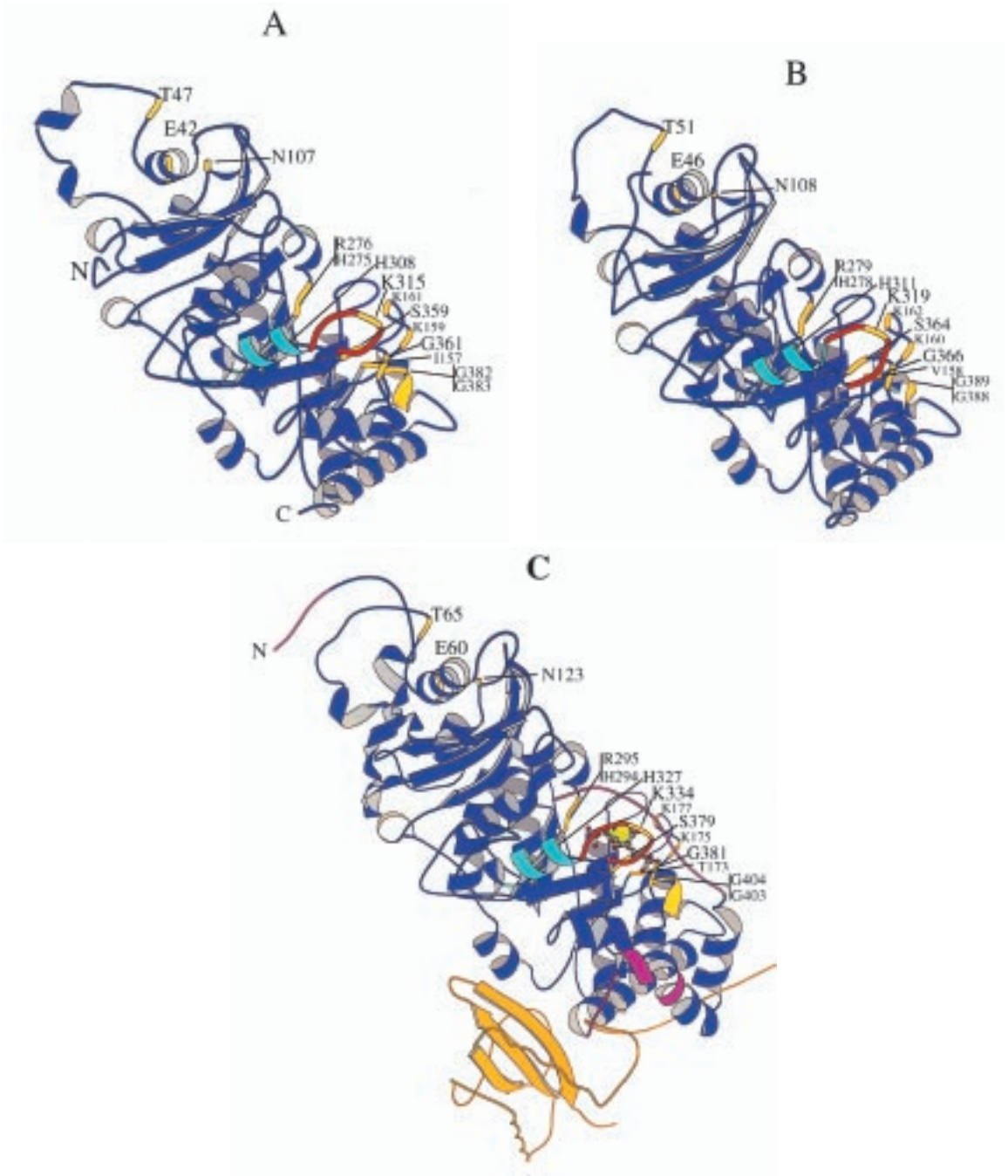

Figure 4. Tertiary structure predictions of the *M. jannaschii* (A) and the *A. fulgidus rbcL2* (B) sequences compared to the known structure (Newman and Gutteridge 1993) of the *Synechococcus* 6301 large subunit (C). A small subunit is also shown to the lower left of (C) in amber. Label sizes and shading reflect the distance from the viewer with the smaller and darker, respectively, being further from the viewer. Yellow, active-site residues within 3.3 Å of bound transition state analog (Newman and Gutteridge 1993) in the *Synechococcus* enzyme and the equivalent residues in the *M. jannaschii* and *A. fulgidus* sequences; red, loop 6 region; cyan, highly divergent α-helix-6 region; purple, residues that appear to be absent in the *M. jannaschii* and *A. fulgidus* sequences (eight residues at the N terminus of the *Synechococcus* enzyme were not resolved in the structure determination and therefore are not shown here). Mg^{2+} is represented as a green sphere and CABP is represented as a ball-and-stick model in (C). No prediction was returned for the first 6 and last 19 amino acids of the *A. fulgidus* structure and the first residue and last 18 amino acids of the *M. jannaschii* structure. From Watson et al. 1999, with permission.

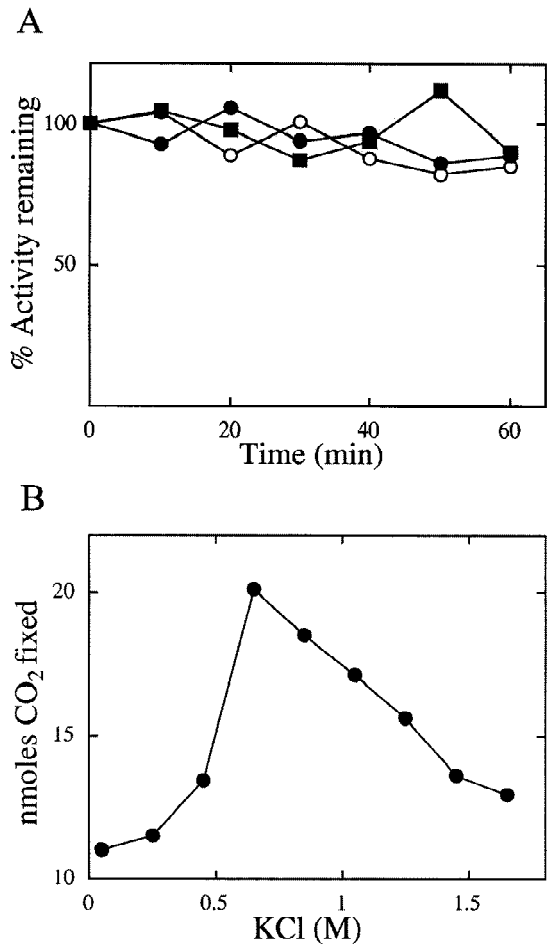

Figure 5. Stability of the *M. jannaschii* recombinant Rubisco at high temperatures (A) and stimulation of activity by high concentrations of KCl (B). In (A), the enzyme was incubated in 80 mM HEPES-NaOH, pH 7.2, under anoxic conditions at 65 °C in the presence of 0.6 mM KCl (■), at 85 °C in the presence (●) or absence (○) of 0.6 mM KCl. Assays were performed at 65 °C. In (B), the enzyme was assayed at 65 °C at the indicated concentrations of KCl . From Yu and Tabita, unpublished results.

zyme to other form II Rubisco enzymes indicates that the dinoflagellate gene might be used to complement Rubisco deficient mutants of *Rhodobacter* to autotrophic growth, using the previously constructed Rubisco expression vector specific to these organisms (Falcone and Tabita 1991). Perhaps this system could then be used to produce recombinant dinoflagellate enzyme of high specific activity to verify and study recently attributed properties, as well as obtain specific mutants.

Nature's little surprise for Rubiscologists

Two hyperthermophilic archaeons, *Methanococcus jannaschii* and *Archaeoglobus fulgidus*, contain putative Rubisco large subunit genes (Bult et al. 1996; Klenk et al. 1997), with *A. fulgidus* possessing two separate rubisco sequences that show only 41% and 45% identity to the putative *M. jannaschii* gene. Since these reports, other putative archaeal Rubisco genes have also been placed in the database. This serendipitous observation suggests a third means to employ microbial systems to advantage for studies of Rubisco specificity. The putative Rubisco molecules from *M. jannaschii* and *A. fulgidus* (and recently reported sequences from other organisms), as discussed earlier, may be placed in completely separate categories relative to the phylogenetic relationship of known form I and form II large subunits (Figure 1). Indeed, the distinctness of the *M. jannaschii* and *A. fulgidus* deduced sequences are such that one might even question whether they could encode functional proteins. Because *M. jannaschii* and *A. fulgidus* are, respectively, anoxic methanogenic and sulfate-reducing organisms that fix CO_2 by acetyl CoA- and reductive tricarboxylic acid-like pathways (Sheih and Whitman 1987; Sprott et al. 1993), it was surprising to find that both genomes contain sequences that potentially encode the large subunit of Rubisco. If these are bonafide Rubisco enzymes, the fact that these molecules evolved in organisms that never come into contact with O_2 would, presumably, provide a wonderful system to study the nature of the active site and its ability to interact with CO_2 and O_2. In no instance has Rubisco ever been obtained from an obligate anaerobe, and when one considers that archaea of this type are probably the most oxygen-sensitive organisms known, applications to the CO_2/O_2 specificity issue arise. Current dogma states that the oxygenase activity of Rubisco has evolved as a consequence of the reactivity of the carbanion of RuBP to either CO_2 and O_2 (Lorimer and Andrews 1973). However, Schloss and colleagues (Hixon et al. 1996) found that similar carbanion-like substrates of other enzymes may not necessarily interact with O_2 and, from mechanistic similarities to Rubisco chemistry, suggest that there may be sources of Rubisco that do not use O_2 as a substrate. Alternatively, if this is true, it may not be beyond the realm of possibility that some Rubisco molecule might be engineered that reacts poorly, if at all, with O_2. Because of these considerations, it would be most interesting to examine the putative archaeal Rubisco molecules.

There is near absolute conservation of known critical residues in the *M. jannaschii* and *A. fulgidus rbcL2* deduced sequences, strongly suggesting that selective pressures have maintained at least partial functionality of these proteins. Further *'in silico'* analyses of the potential Rubisco proteins indicated that virtually all the known key active site residues were in the correct loci relative to structural models that were generated from known X-ray structures (Figure 4). Further work has shown that the genes from both organisms unequivocally encode proteins with bonafide Rubisco activity, with the *M. jannaschii* recombinant protein fairly well characterized (Tabita 1998; Watson et al. 1999). Interestingly, the native *M. jannaschii* protein is a dimer of large subunits, like the *Rs. rubrum* enzyme, however its properties are completely unrelated to any Rubisco previously studied. Stoichiometric amounts of 3-phosphoglyceric acid (3-PGA) are produced under anoxic conditions and the enzyme has a reasonable k_{cat}. The enzyme is stable at 85 °C for 60 min and it requires high levels of KCl for maximum activity (Figure 5), in keeping with the known physiology and intracellular milieu of the native organism. Most interesting is the reversible inhibition of this enzyme by air levels of oxygen, yet the enzyme is weakly able to use oxygen as substrate under simultaneous carboxylase/oxygenase assay conditions employing [l-^{3}H]-RuBP (Watson et al. 1999). It appears that this enzyme has a very low K_i for O_2, with O_2 inhibiting to such an extent that the low residual activity that remains barely is able to discriminate between the two gaseous substrates. Aside from these initial studies, it is apparent that this unusual enzyme will provide a most interesting system to ascertain how the active site of Rubisco has evolved to accommodate oxygen. Moreover, conceptual details of how Rubisco is engineered by the archaea to withstand high temperatures and salt concentrations (Figure 5) may also provide fundamental insights into Rubisco chemistry.

As this review went to press, an interesting report appeared of a high activity, high CO_2/O_2 substrate specificity Rubisco from the hyperthermophilic archaeon *Pyrococcus kodakaraensis* (Ezaki et al. 1999). This enzyme, and its gene, were expressed in *P. kodakaraensis*, while the recombinant protein exhibited properties that were considerably different from the *M. jannaschii* enzyme (Tabita 1988; Watson et al. 1999), perhaps consistent with the low identities (44%) of these proteins. Noteworthy was the octameric structure and high Ω value of 310 at 90 °C, however no mention of the oxygen sensitivity of the enzyme from this obligate anaerobe was made.

Conclusions

Exploitation of the natural biodiversity of Rubisco molecules may be an important part of future strategies to solve the molecular basis of CO_2/O_2 specificity. If existing approaches are not giving us all the answers, why not attempt to determine how Nature has solved the specifity issue for different sources of enzyme? Many different CO_2 fixing microorganisms, which employ the CBB cycle, have adapted to a plethora of different environments, some of which are of the extreme variety. Indeed, the very existence of Rubisco in organisms of this type often, by definition, indicates obvious and important modifications of Rubisco function. The major take-home lesson here is that the wide biodiversity of microbial systems known to contain Rubisco, in combination with the usual protein structure-function and molecular approaches, should provide answers to the mysteries of Rubisco specificity. For example, one might logically ask what might be the consequences of Rubisco evolving in the absence of oxygen, at temperatures that approach boiling water, in an intracellular milieu of high ionic strength? Also significant is that closely related form I Rubisco molecules have now been described that possess CO_2/O_2 substrate specificities that range from low values to specificities which approximate the higher values obtained for plant Rubisco. These results indicate that procedures of DNA shuffling might be employed to produce *in vivo* chimeras to provide clues to the molecular basis for Rubisco specificity. This approach is now feasible due to the ability to perform facile genetic manipulations in appropriate bacterial hosts, the recent construction of specific expression vectors, and the ongoing development of selection strategies to facilitate such studies. Knowledge of the molecular regulation of CO_2 fixation has been an important part of these advances and these approaches may greatly complement more traditional biochemical investigations devoted to this challenging enzyme.

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