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Personal perspective

Research on carbon dioxide fixation in photosynthetic microorganisms (1971–present)

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

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Abstract

This paper presents my personal account of research on CO_2 fixation from when I began these studies as a postdoctoral student in the early 1970s. It traces interests in microbial ribulose bisphosphate carboxylase/oxygenase (Rubisco) and considers early breakthroughs on the isolation, characterization, and significance of this enzyme from nonsulfur purple photosynthetic bacteria and other phototrophic organisms. This article also develops a historical perspective as to how recent efforts may lead to an understanding of molecular mechanisms by which the synthesis of this enzyme and other proteins of the pathway are regulated at the molecular level. In addition, how these studies impinge on the interactive control of CO_2 fixation, along with nitrogen fixation and hydrogen metabolism, is also considered. Finally, CO_2-fixation studies in green sulfur photosynthetic bacteria and the discovery of the rather surprising Rubisco-like protein are described.

Abbreviations: bp – base pair(s); DMSO – dimethyl sulfoxide; LTTR – LysR-type transcriptional regulator; PRK – phosphoribulokinase; RLP – Rubisco-like protein; RTCA – reverse tricarboxylic acid; Rubisco – ribulose bisphosphate carboxylase/oxygenase; RuBP – ribulose bisphosphate; TPP – thiamine pyrophosphate

Early interests

Howard Gest and Govindjee asked me to write an article concerning my experiences in photosynthesis and to consider our laboratory's contributions to the field. While I am indebted and grateful for being asked to write such a retrospective, I must admit I really know little about the topic. Rather, our work and interests have always been confined to the 'dark side' of what transpires when a photosynthetic organism performs its magic to convert light energy to chemical energy. Thus, this article will consider our research on CO_2 fixation in photosynthetic microorganisms.

As an undergraduate student, I was fascinated by organic chemistry, especially how complex compounds could be synthesized from simple building blocks. However, any inklings of following a career in synthetic organic chemistry ended after I took my first undergraduate microbiology course, as it did not take long for me to realize that the best synthetic chemists (by far) are microbes. So, it was apparent that to really appreciate how to best synthesize diverse compounds, one would be well advised to understand microbial metabolism. While undergoing this scientific epiphany, I became aware of the incredible contributions of Melvin Calvin, Andrew A. Benson (see Benson 2002) and James A. Bassham (see Bassham 2003) and learned that certain bacteria, like plants, could convert the most oxidized and ubiquitous source of carbon in the biosphere, CO_2, to reduced organic matter. This I found astounding; and I still do because the energetic cost is enormous. As the most fundamental of

synthetic processes, learning more about CO_2 reduction seemed a good way to indulge one's interests in biosynthesis, especially if one could develop systems where the process could be controlled. Clearly, prokaryotic organisms are the most versatile in this regard and as my interests matured, I became particularly enamored by the physiology and biochemistry of organisms, for example, facultative autotrophs, and especially photosynthetic bacteria, that exhibit the capacity to turn on or turn off the ability to use CO_2 as sole carbon source. These fascinating organisms and their metabolic versatility have never ceased to amaze me. The following retrospective stresses how we have studied these organisms over the years and is a very personal account that makes no attempt to serve as a comprehensive review. Admittedly, I was reluctant to take time away from other projects to write this account, but I have found this endeavor to be quite enjoyable; yet while it is fun to look back, I am by no means ready to hang up the 'pipette person' and look forward to many new adventures with our active research group.

Postdoctoral work and serendipitous introduction to the metabolic versatility of photosynthetic bacteria (1971–1973)

My doctoral work in the late Don Lundgren's laboratory at Syracuse University introduced me to the metabolism and biochemistry of autotrophic bacteria. While considering potential postdoctoral opportunities, I realized that my interests would be best served by improving my expertise in enzymology and protein chemistry. Bruce McFadden's group in the Chemistry Department at Washington State University (WSU) had at that time published a series of very interesting articles on ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) from aerobic hydrogen bacteria (Kuehn and McFadden 1969a, b). Bruce (Figure 1) was clearly one of the leading investigators in the autotrophy field; he was also one of the few individuals who took an enzymological approach. Thus, a postdoctoral position with Bruce seemed like the perfect match, as I would be able to learn more protein chemistry/enzymology, while also pursuing my interests on the biochemistry of autotrophic bacteria. After meeting Bruce at a national American Society of Microbiology (ASM) meeting in Boston, a postdoctoral position was arranged and this turned out to be a wonderful and profitable experience, as I learned much from my colleagues and friends at WSU.

Figure 1. Bruce McFadden (right) with the author (center) and Mike Lord (left) in a typical pose at the 2nd International Photosynthetic Prokaryotes Symposium in Dundee, Scotland in August 1976.

"When you reach a fork in the road, take it!" *Rhodospirillum rubrum* and butyrate

Some will recognize the author of the fork in the road statement as coming from that great American philosopher, Yogi Berra, one of the heroes of my youth in New York City (Berra 1998). I began postdoctoral work trying to synthesize compounds that might specifically and irreversibly modify the active site of Rubisco. These studies first involved the enzyme from *Hydrogenomonas eutropha* and *H. facilis* (the taxonomists have since had a wonderful time with these organisms, first changing *H. eutropha* to *Alcaligenes eutrophus* and now *Ralstonia eutropha* and *H. facilis* to *Pseudomonas facilis* and probably something else by now). For these studies, we needed lots of enzyme. Like plant Rubisco, the bacterial large molecular weight protein has a molecular weight of about 550,000 and is comprised of large (catalytic) and small subunits in an L_8S_8 stoichiometry. Taking advantage of this large molecular size, we modified a procedure from Lawrence Bogorad's laboratory (Goldthwaite and Bogorad 1971) and used sucrose density centrifugation to purify fairly large amounts of highly purified protein in a single day (Tabita and McFadden 1974a). This procedure turned out to be a simple way to purify most bacterial Rubisco enzymes and it enabled subsequent investigations in several laboratories on the biochemistry of large molecular weight prokaryotic (form I) Rubisco.

We noted earlier reports from T. Akazawa's (Akazawa et al. 1970) and R. Clint Fuller's (Anderson

et al. 1968) laboratories in which Rubisco from crude extracts or partially purified preparations from nonsulfur purple bacteria such as *Rhodospirillum rubrum* and *Rhodopseudomonas* (now *Rhodobacter*) *sphaeroides* appeared to be considerably smaller in molecular weight than the plant or bacterial enzymes with which we were working. When we reported on the utility of the sucrose density method to purify large molecular weight forms of bacterial Rubisco, we verified that the *R. rubrum* and *R. sphaeroides* proteins migrated differently in these gradients, as if they were definitely smaller in size, with the *R. rubrum* protein seemingly the smallest of all. I thus became intrigued by the enzyme from photosynthetic bacteria and wondered about the structure of such seemingly different enzymes. In addition, at about the same time, we noted differences in the sensitivity of lower molecular weight photosynthetic bacterial Rubisco towards phosphorylated effectors such as 6-phosphogluconate; for example, these enzymes were relatively insensitive to such effectors after the enzyme was activated (carbamylated) while the large molecular weight plant and bacterial Rubisco proteins were markedly inhibited by these effectors (Tabita and McFadden 1972). Realizing that these studies might suggest potentially significant differences in the geometry at the active site, but also recognizing that these earlier studies with photosynthetic bacterial proteins were performed with crude material, we set out to purify these proteins. Because of the small size of the *R. rubrum* Rubisco, we focused on this enzyme.

While these studies were going on in earnest, Bruce left for a year-long sabbatical in England, so I had to grow up in a hurry. Obviously, the simple sucrose gradient procedure would not work because the enzyme from *R. rubrum* fractionated just like the bulk (lower molecular weight) soluble proteins. In addition, when I learned how to grow nonsulfur purple photosynthetic bacteria, I followed protocols set forth by people who worked extensively with these organisms and used electron donors like malate to obtain large amounts of photosynthetically grown cells. After several trials, I was uniformly disappointed by the very low levels of Rubisco that were obtained; thus purifications were always unsuccessful since by the time I ran columns, the amount of enzyme obtained was vanishingly small and not very active. Louise Anderson and Clint Fuller found that growing *R. rubrum* with H_2 as electron donor yielded much higher levels of enzyme (Anderson and Fuller 1969), yet despite working in a laboratory that historically and routinely grew organisms with explosive mixtures of hydrogen and oxygen, there was no one around at that time that had that experience. Thus, I was not about to mess with hydrogen, especially after hearing embellished stories from labmates about former students who at one time had hair, two intact eyes, unmarked faces, etc. Anyway, I did what any self-respecting microbiologist would do; I grew the organisms with different organic carbon sources, hoping that I would somehow hit on a particular condition that would result in enhanced enzyme synthesis. I made different media and used substrates like acetate, succinate, lactate, and of course malate, the usual carbon source. I also added a bit of bicarbonate. One of my colleagues in the lab, John Rogers, was working on isocitrate lyase from *Pseudomonas indigofera* (the other enzyme Bruce studied) and he routinely used butyrate to induce the glyoxylate cycle and isocitrate lyase in this organism prior to purification. Anyway, since there was plenty of it around I figured I would try butyrate as well. Then I read that many years before Hans Gaffron (Gaffron 1935) and Cornelis B. Van Niel (Van Niel 1936) noted that growth of photosynthetic bacteria on reduced electron donors such as butyrate, or even longer chain fatty acids, resulted in significantly enhanced CO_2 uptake. So I became very curious as to how this might relate to Rubisco activity levels. While *R. rubrum* did not grow as well on butyrate, I was excited to see that radiometric Rubisco assays of extracts from these cells pegged the scintillation counter to the right side of the meter, such that the Rubisco activity levels were basically off scale. At first I did not believe these results and thought I might have forgotten to quench the reaction and acidify the Rubisco reaction mixture (acid is used to stop the assay and drive off any unreacted radioactive bicarbonate so that only acid stable phosphoglyceric acid is counted). Several repeat assays, however, and several additional growth experiments verified the initial results. Needless to say, I was excited because now I could obtain large amounts of cell material, with high levels of enzymatic activity, without having to work with hydrogen gas. From such induced cells, I quickly purified the enzyme and determined that the *R. rubrum* protein had a unique quaternary structure and lacked small subunits; it turned out to be a homodimer of large catalytic subunits with a native molecular weight of about 110,000. Most important, I was able to verify its unique catalytic properties (Tabita and McFadden 1974b, c). This simple experiment, where I discovered that growth with a reduced electron donor up-regulates Rubisco synthesis, has really been the

foundation for my entire career with photosynthetic bacteria as it made it possible to carry out experiments on the enzymology of Rubisco and other enzymes of the pathway; these initial findings also spurred subsequent studies on the regulation of CO_2 fixation and the control of Rubisco synthesis. This was one fork I was glad I took!

The Texas years (1973–1988)

The Department of Microbiology at the University of Texas at Austin was looking for someone who did research with autotrophic bacteria, to replace and carry on the tradition of Derek Hoare who was a well-known microbial biochemist who had suddenly passed away. Fortunately for me, there were not many people who fit these criteria and I was hired after a candidate who was supposed to come the year before suddenly changed his mind and took another position. I was fortunate to have lined up this Assistant Professor position about a year prior to my actual start date in the fall of 1973. This allowed me to complete a lot of work in Pullman without worrying about where I was going next. By this time, I was hooked on the photosynthetic bacteria and decided I would continue working on Rubisco as an independent investigator. I thought that the *R. rubrum* protein would serve as an excellent model system as the protein was infinitely simpler than the widely studied plant enzyme, yet it catalyzed the same reaction. I was also intrigued that *R. sphaeroides* and *R. capsulatus* appeared to possess enzymes that were intermediate in size from the small and structurally simple *R. rubrum* enzyme and the large hexadecameric plant-type enzyme found in most bacteria. In addition, the ability to turn up or tamp down Rubisco synthesis would become the rationale for studying the molecular basis for regulation in photosynthetic bacteria. In the pages that follow, I would like to stress that whatever contributions we have made in these areas, and others, were clearly brought about by an excellent and wonderful group of graduate students and postdoctoral associates that have worked in our lab over the years, both at the University of Texas (UT) and Ohio State University (OSU). Indeed, my first three PhD students at UT, William (Barny) Whitman, Gary Stacey, and Janet Gibson were gems and got things going on the right track.

Different forms of Rubisco

When I got set up in Austin, I first performed experiments with *R. capsulatus* and *R. sphaeroides* and noted that these organisms, like *R. rubrum*, produced high levels of Rubisco when cells were grown with butyrate as electron donor. Thus, it appeared that it would be feasible to purify the enzyme from these organisms and find out about what appeared to be still another unique structural form of Rubisco. While *R. capsulatus* grew faster, the enzyme appeared to be more stable in extracts of *R. sphaeroides*, so we concentrated on that organism. Jan Gibson was given the problem of purifying and characterizing the enzyme from *R. sphaeroides*. When extracts from induced cells were passed over a DEAE-cellulose column, we were amazed that two well-separated peaks of enzymatic activity eluted from the column when the usual NaCl gradient was used during purification. We decided to purify the enzyme from each peak separately (peaks I and II). This worked nicely and when the purified peak I and peak II enzymes were analyzed, it was shown that the peak I enzyme resembled a typical plant-type Rubisco in that it had a high molecular weight of about 500,000 and was comprised of both large and small subunits. By contrast, the peak II enzyme was comprised only of large subunits (somewhat larger than the peak I large subunits), much like the *R. rubrum* enzyme, and the native molecular weight was estimated to be about 240,000 (Gibson and Tabita 1977a). However, the peak II enzyme has the interesting propensity to aggregate into larger active forms, in multiples of 120,000, at high pH. Thus this organism appeared to synthesize two distinct forms of the enzyme, which have since been termed form I and form II. In fact, this nomenclature has survived over the years for these two different structural forms of Rubisco. The form I and form II enzymes had different catalytic properties; in addition our generalization about sensitivity to effectors held. Interestingly, antibodies directed against the native form I and form II proteins from *R. sphaeroides* do not cross react. While this caused us to suspect that these enzymes, or more specifically, the large subunits of form I and form II, might be distinct proteins and different gene products, there was always the possibility that the different conformation and assemblage of the form I and II proteins might explain the antibody results. In the absence of genetics at that time, Jan Gibson, and our technician Florence Waddill, carefully separated the large and small subunits of the form I enzyme and compared its peptide maps to that of the form II protein. These results were published some time later but clearly indicated that the form I and form II large subunits were different gene products (Gibson and Tabita 1985). In this age of molecular biology, I would hesitate to ask

if students could determine, in the absence of gene knockouts, whether two similar proteins are encoded by the same or different genes.

Meeting the phototrophic prokaryote scientific community

In 1975, soon after our work on the *R. rubrum* enzyme appeared, I got a call from Howard Gest, telling me that he was running a Photosynthetic Bacteria meeting at Bloomington later that year. As a beginning Assistant Professor, I was thrilled that Howard knew who I was and that he had invited me to attend and present our work. It was also great to meet all the US scientists, as well as some international visitors in the field that spring. After the Indiana meeting, I received an invitation to attend the Second International Symposium on Photosynthetic Prokaryotes the next year in Dundee, Scotland. This was my first international trip and by the time I reached London I was pretty frazzled. But armed with exacting instructions from Howard as to how to reach a comfortable and reasonable London hotel, that I only partially messed up, I finally arrived. At the hotel I was met by Howard and Jan, who proceeded to show me a wonderful and relaxing time in London prior to the Dundee meeting. This was most appreciated and greatly helped me get settled prior to my first international presentation. The meeting was notable by the excellent science and free-flowing Scotch whisky, not to mention our host, Bill Stewart, playing the bagpipes at the banquet. This meeting, followed by a short satellite workshop on cyanobacteria at Oxford University, had a lasting impression as I learned early on that not all the leading scientists in the international phototrophic prokaryote field were as forthcoming or as personable as Howard.

Continuing with the R. rubrum *enzyme*

Will Whitman's PhD studies clearly verified the basic premise that the simple *R. rubrum* enzyme would prove useful for detailed structure–function studies of Rubisco. He also provided one of the first thorough active site modification studies performed with this enzyme and showed that one could stoichiometrically and specifically tag the active site (Whitman and Tabita 1978a, b), demonstrating the importance of a specific lysine at the active site. Of course, further detailed mechanistic studies and additional active site modification and residue alteration experiments were subsequently performed with this enzyme by other investigators, especially Fred Hartman, George Lorimer, and John Andrews (reviewed in Cleland et al. 1998), resulting in a fairly extensive understanding of the mechanism of Rubisco catalysis. Indeed, it has always been quite satisfying to realize that much of this work would not have been possible without the availability of the *R. rubrum* enzyme, the subsequent cloning and expression of its gene to form active recombinant enzyme in *Escherichia coli* (Somerville and Somerville 1994), and the eventual solving of the protein's three-dimensional structure by C-I. Branden's group (Schneider et al. 1986). Most plant biochemists were slow to recognize the utility of the *R. rubrum* enzyme and I recall Sam Wildman suggesting after my talks at meetings in Hawaii in 1975 and in Tuscon in 1987 that structurally distinct bacterial enzymes (that differed from the typical plant fraction I protein) were really not Rubisco, but enzymes with other catalytic potentials. (For his discovery of the fraction 1 protein, see Wildman 2002.) To this day, simple isolation of highly active recombinant eukaryotic Rubisco is still a dream and, although prejudiced, it seems apparent that *R. rubrum* contributed more than any other source of enzyme to the ability of Rubiscologists to better understand structure–function relationships, not to mention the role this protein played as a substrate for George Lorimer's elegant studies on chaperonin-assisted protein folding (Goloubinoff et al. 1989).

In the early to mid-1970s, William (Bill) Ogren and his colleagues discovered that plant Rubisco, in addition to the carboxylation reaction, also catalyzed an internal monooxygenase reaction, a fundamental discovery that explained much about the physiological significance of Rubisco to aerobic organisms (Ogren 2003). Aside from a couple of short notes relative to the fact that the *R. rubrum* enzyme could catalyze RuBP-dependent O_2 uptake, including work by McFadden using enzyme I had sent to him while on sabbatical (McFadden 1974), much was not understood relative to the oxygenase activity of this enzyme. Melinda Martin came to the laboratory and she thoroughly examined the oxygenase activity of the *R. rubrum* enzyme, and together with Whitman, was able to show that under the appropriate conditions, both activities were subject to the influence of effectors (Whitman et al. 1979), debunking an idea that had been offered at that time that small subunits were somehow involved with the response to effectors. Pete Robison then joined the group as our first Rubisco postdoc and he and Martin provided the first solid evidence for differential effects on the carboxylase and oxygenase activities, from metal interaction studies with *R. rubrum* Rubisco (Robison et al. 1979).

Regulation of Rubisco synthesis and modulation of activity

Antibodies to the form I and form II enzymes of *R. sphaeroides* and the form II enzyme from *R. rubrum* provided highly specific reagents that allowed us to follow enzyme synthesis in these organisms. For *R. sphaeroides*, this was particularly interesting as we could track whether synthesis of the two enzymes was differentially controlled, hopefully providing some indication why there might be two Rubiscos in this organism and in *R. capsulatus* (Gibson and Tabita 1977b). Yves Joanneau joined us as a postdoc and he provided his immunochemical expertise to carefully show that both the rate and extent of form I and form II accumulation differed when cultures were switched from repressive to induction growth conditions (Joanneau and Tabita 1986). Clearly, the form I and form II genes were differentially expressed. Similar induction studies were performed by Linda Sarles Cook with *R. rubrum*, where she made the very interesting observation that the level of CO_2 supplied greatly influences the amount of Rubisco synthesized by photoautotrophically grown cultures (Sarles and Tabita 1983) (note: I was no longer afraid of hydrogen because my students and postdocs were doing the actual experiments!). Cook was also able to show that the *R. rubrum* enzyme became oxidatively modified when cultures were oxygenated (Cook and Tabita 1988; Cook et al. 1988), and Joanneau found that the addition of certain sources of organic carbon yielded modified and inactivated form I enzyme of *R. sphaeroides* (Jouanneau and Tabita 1987). Thus, posttranslational modification appeared to be another means to influence the activity of Rubisco.

The beginning of molecular and genetic studies

Never having had a real molecular genetics course, I was slow to realize the potential that this approach might play in facilitating both our enzymological and regulation studies. Fortunately, a precocious student, Rob Quivey, joined us in 1979, after earning an MS in Genetics with Ed Stevens at Penn State. After struggling for about a year with physiological regulation, Quivey emphatically stated that we needed to clone the Rubisco genes from our organisms. After assuring him he would have to seek real professional advice, Rob was able to enlist the help of Richard Meyer, a plasmid biologist in the Microbiology Department, and eventually Quivey cloned the *R. sphaeroides* form II gene and expressed it in an active form in *E. coli* (Quivey and Tabita 1984), a bit after Chris and Shauna Somerville cloned the *R. rubrum* gene (Somerville and Somerville 1984). When I saw how easy it was to obtain purified recombinant enzyme and the interesting questions we could address, I soon became an advocate for molecular biology. About a year after Quivey joined us, Keith Weaver, also from Stevens' laboratory, became a graduate student with us. Weaver was able to obtain the first regulatory mutants involved with CO_2 fixation and also prepared a most useful cosmid library from *R. sphaeroides* that we still use (Weaver and Tabita 1983, 1985). Certainly, at about this time in the late 1970s and early 1980s, genetics and molecular biology were beginning to be exploited by Barry Marrs (Marrs 2002) and Sam Kaplan (Kaplan 2002) with *R. capsulatus* and *R. sphaeroides*, respectively; we were able to use this to advantage for our work, particularly when Jan Gibson returned to Texas in 1984 after 4 years of postdoctoral work with Mary Jane Osborne (at the University of Connecticut Health Sciences Center).

The form I Rubisco genes were cloned (Gibson and Tabita 1986) and this was followed by the finding that the two Rubisco genes were part of separate operons (cbb_{I} and cbb_{II}), along with other structural genes of the CBB pathway (Gibson and Tabita 1987, 1988). [Note in honor of Calvin, Benson, and Bassham, and because the structural genes of the pathway are clustered in operons, the bacterial CO_2 fixation community has taken the convention of calling these the *cbb* genes (Tabita et al. 1992).] At about this time, our very close colleagues, Botho Bowien in Germany, and Wim Meijer in Holland, were also showing interesting and similar gene organizations in facultative chemolithoautotrophs, *Alcaligenes* (*Ralstonia*) *eutropha* and *Xanthobacter flavus*, respectively (Kusian and Bowien 1997; Shively et al. 1998), indicating that most autotrophs organize key *cbb* genes in operons, and as discussed later, are controlled by similar regulator molecules. Dean Falcone then joined the laboratory; his transposon insertions into the Rubisco genes indicated that the absence of one gene caused the other to be over-expressed to compensate (Falcone et al. 1988), again showing that there was something responsible for regulating these operons. The operons and genes were sequenced (by hand of course), beginning at UT, and then completed at OSU [the form II Rubisco gene was done mainly at Penn State (Wagner et al. 1988)], and it became clear that transcriptional control was most important (Zhu and Kaplan 1985; Gibson et al. 1990, 1991; Chen et al. 1991).

Fun with cyanobacteria and algae

The University of Texas housed virtually the entire world's expertise on the physiology and optimized growth of cyanobacteria, then called blue-green algae. Jack Myers (Myers 2002) was in the Zoology department and of course remains one of the giants of photosynthesis; his postdoc, Ed Stevens, became a very close friend (note: he was the source of the aforementioned Penn State student pipeline to UT). Stevens did his PhD with Chase Van Baalen, who was himself a former student of Jack's and resided at the UT Marine Sciences Institute on the Texas Gulf Coast in Port Aransas. Richard Starr was in the Botany department and was the curator of the world famous UT algal/cyanobacterial culture collection and he, and Harold Bold, also in the Botany department, were acknowledged international leaders on algal taxonomy. Myers, Starr and Bold were members of the National Academy of Sciences. Van Baalen (with his students Lonnie Ingram and Ed Stevens), among other firsts with cyanobacteria, was the first to show that one could isolate auxotrophic mutants in cyanobacteria and Stevens, after he arrived at Penn State, went on to show that high efficiency transformation was feasible with an organism that Van Baalen had originally isolated that grew with a 2-h generation time. With that kind of talent and expertise around, and watching Stevens, and his wife Kathryn, culture cyanobacteria as easy as *E. coli* in Myers' chambers across the street, it seemed obvious that we should begin to consider studying cyanobacterial Rubisco, which we did (Tabita et al. 1974, 1976). We still use the cyanobacterial Rubisco as a model system, as discussed below, but when we and others found that one could express both the large and small subunit genes from *Synechococcus* in *E. coli* and obtain highly active recombinant protein (Gatenby et al. 1985; Tabita and Small 1985), a very nice and closely related homolog of the plant enzyme became available for molecular manipulation, which was exploited by several groups, including our own.

Perhaps the most meaningful part of this sojourn into the cyanobacteria became my relationship with Chase Van Baalen (Figure 2). He was, and always will be, the person that most influenced me as a scientist, and he became my closest colleague and best friend at UT, albeit some 200 miles away. Chase never took any garbage from anyone and he was one of the most careful and critical experimenters I've ever known; we also both liked a good time, while talking science over beers and poker and fishing in the Texas Gulf and in Lake Buchanan. We began interacting in 1974; then Chase and Pat Parker (Director at the UT Marine Institute) invited me to teach my microbial ecology course during the summer of 1975. Parker, an isotope chemist, and Van Baalen enlisted me to help jointly supervise a student, Marilyn Estep, who began to examine carbon isotope fractionation by purified Rubisco. This resulted in the initial indication that form I and form II Rubisco do this differently (Estep et al. 1978). After that summer of 1975, a long and cherished collaborative arrangement with Van Baalen flourished and we went on to jointly supervise students and postdocs, and share common grants on cyanobacterial metabolism. This whole scenario, however, had a major catalyst, Gary Stacey. Stacey was a student from Ohio, of all places, who wanted to learn some marine microbiology in the summer program at Port Aransas prior to entering the graduate program in microbiology at Austin, Texas. In Van Baalen's summer course, Stacey took the beginning stages of an enrichment culture from Van Baalen's technician, obtained from microbial mat material at the seashore, and began to isolate nitrogen-fixing cyanobacteria. Stacey continued to work on this during the summer and eventually obtained a heterocystous organism that appeared to grow much faster than anything available at that time. After the summer, Stacey obtained an axenic

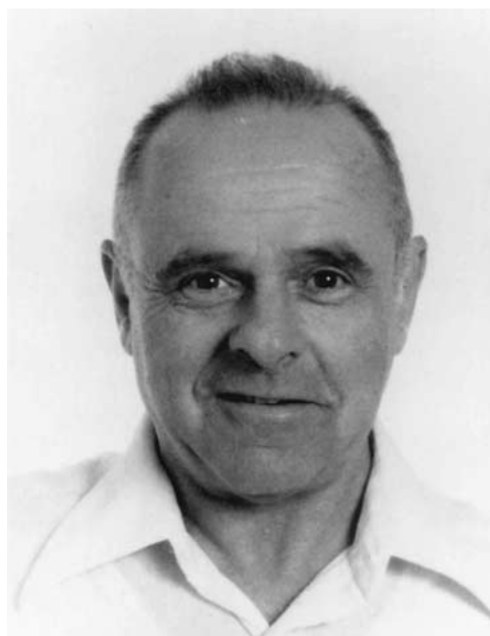

Figure 2. Chase Van Baalen (1926–1986).

culture in my laboratory (Stacey et al. 1977) and decided to characterize this organism (which we called *Anabaena* strain CA) and work on nitrogen fixation in Austin for his PhD in microbiology, jointly supervised by Van Baalen and myself. Stacey, and his work, begat a whole series of joint publications (some 25 or so) by the Austin-Port Aransas axis on cyanobacterial nitrogen fixation, hydrogen metabolism and heterocyst formation, and a wonderful group of students and postdocs followed Stacey on this project, including John Gotto, Gail Donaldson, Pete Bottomley, John Grillo, Dhruv Kumar, Ashok Kumar, Phil Pienkos, Stephan Bodmer, David Alexander, Z. Xiankong, Ben Haskell, Russell Smith, Dave Ramsden, Jerry Chen and Ann Chow. Space considerations force me to focus this review on CO_2 fixation, however several interesting findings developed from these studies with *Anabaena* CA. Peter Wolk even took my picture for his scrap book, or rogue's gallery of cyanobacteriologists, after we began to work in this field. Finally, Rob Beudeker joined the laboratory from Holland and proceeded to do a beautiful job studying the photorespiratory nitrogen cycle in a 100% oxygen-resistant mutant of *Chlorella* (Beudeker and Tabita 1983), an organism obtained from Warren Pulich at Port Aransas, which we originally thought might have an altered Rubisco to account for its phenotype.

A minisabbatical

By 1986, the laboratory was well infused with molecular biology procedures; the problem was that the major professor had never so much as run an agarose gel! A former undergraduate student researcher in the laboratory, Brian Lawlis, who went on to do his PhD with Bruce McFadden, was a group leader at Genencor, then part of Genentech, in south San Francisco, California. I was well familiar with the tremendous strides that their enzymologists, like Jim Wells and Dave Estelle, had made relative to the use of site-directed and random mutagenesis to examine enzyme mechanisms. Assisted by Lawlis, a short sabbatical was arranged for the fall of 1987, where I worked with Genencor's scientists and learned how to do site-directed mutagenesis, plus I was able to get back into the laboratory. I believe this was the first time I had a semester free from teaching while at Texas. I worked with Randy Berka, who kindly took me under his wing and proceeded to show me the ropes, with the hope that I could bring this knowledge back and commence to perform site-directed mutagenesis with Rubisco genes in our laboratory. A very bright and talented student, Bonggeun Lee, joined our group soon after I returned and he took up the project of using site-directed mutagenesis to examine the function of the small subunit of Rubisco. I had also at this time checked out and become highly impressed with an endowed Chair position at The Ohio State University, and after performing the prescribed post-sabbatical year's service to UT in 1988, moved the laboratory to Columbus, Ohio, in December of that year.

The Ohio State years (1989–present)

Six of us moved to The Ohio State University at Columbus: Jan Gibson, and graduate students Deane Falcone, Shin-Rong Hwang, Bonggeun Lee, Lih-Ann Li and I. We all met at 4 A.M. on 8 December 1988 at the laboratory in Austin; each person had their own vehicle that carried a large ice chest filled with dry ice and our valuable strains, plasmids, enzymes, and antibodies that we were not about to trust to any professional carrier. We then drove to Columbus as a caravan; I carried an extra large chest filled with additional dry ice to replenish that which dissipated during the journey.

Continuing with Rubisco

While I did not have any tangible results from my short sabbatical, I had learned valuable techniques and Genencor was nice enough to bundle me off with several mutagenic primers, which we could use to alter residues of the small subunit. Andrews (1988) expressed large and small subunit genes separately and demonstrated that recombinant large subunits, albeit weakly, were able to catalyze the reaction in the absence of small subunits. Bonggeun Lee took this somewhat further as he was able to prepare a purified catalytic core (L_8) of large subunits that showed interesting properties in the absence of small subunits (Lee and Tabita 1990; Lee et al. 1991b). Of course, this L_8 core could be functionally complemented with separate preparations of small subunits. Most importantly, Lee made the first mutations in small subunits that affected activity but yielded normally folded and properly assembled holoenzyme (Lee et al. 1991a). This was followed up by the work of a postdoc, Betsy Read, who showed that residues in the small subunit actually could influence key kinetic properties such as the affinity for CO_2 (Read and Tabita 1992a). Read also found that a cyanobacterial large subunit/diatom

small subunit hybrid chimera greatly influenced the specificity towards CO_2 and O_2, a property thought to be exclusively attributed to large subunits (Read and Tabita 1992b). At about this time, the structure of L_8S_8 (plant) Rubisco became available and it was clear that the small subunits contributed nothing to the active site, yet the structural model did indicate that small subunits could potentially influence the active site (Knight et al. 1990). Thus, our results agreed well with this idea of small subunits influencing the active site from afar. Subsequent studies from many other laboratories support this concept.

We had interesting experiences with Rubisco from nongreen algae. Van Baalen had isolated several fast growing diatoms from the Texas Gulf Coast. At about this time, Rose Ann Cattolico had found that chloroplasts from several species of nongreen algae differed from plants and green algae in that the plastid DNA encoded both large and small subunit genes (Reith and Cattolico 1985). Shin-Rong Hwang joined our group to work with Van Baalen and myself, but after Chase (1926–1986) passed away, Hwang came to Austin and showed that the chloroplasts of one of these diatom strains, *Cylindrotheca* strain N1, also encoded both *rbcL* and *rbcS* genes, and these genes were organized in an operon much like in bacteria (Hwang and Tabita 1989). When the genes were sequenced from this organism in Columbus, Ohio (Hwang and Tabita 1991), and elsewhere with other nongreen algal Rubisco genes, it became apparent that deduced amino acid sequences of nongreen algal Rubisco proteins greatly resembled form I enzymes of *Rhodobacter*, *Ralstonia*, and *Xanthobacter*. Most importantly, our work showed that Rubisco from several nongreen algae possessed a higher specificity (CO_2/O_2 discrimination factor or Ω value) than any of the many other sources of Rubisco previously encountered (Read and Tabita 1994). This work gave credence to the idea that specificity might be engineered beyond what is found in higher plants; it also became clear that we could classify the form I enzymes into four different subclasses (forms IA, IB, IC, and ID) along with the separate group of highly conserved form II proteins (Tabita 1995, 1999). Another student, Kemp Horken, showed that closely identical prokaryotic Rubisco proteins from *Rhodobacter*, *Ralstonia*, *Xanthobacter* and *Bradyrhizobium* have vastly different Ω values, suggesting that molecular techniques and chimera construction protocols might be devised to probe the specificity issue (Horken and Tabita 1999; Tabita 1999).

During this time period, other aspects of the Rubisco saga were being addressed, including the assembly and posttranslational inactivation of the form I enzyme from *R. sphaeroides*. Xing Wang was able to show that this was a physiologically significant, reversible phenomenon that appeared to be due to noncovalent interaction with a phosphorylated molecule (Wang and Tabita 1992a). Kathy Terlesky purified the chaperonin 60 and chaperonin 10 proteins from *R. sphaeroides* (Terlesky and Tabita 1991) and Ted Lee completed work that Kathy started to clone the *groESL* genes from this organism (Lee et al. 1997). Meanwhile, Xing Wang found that the reactivation of inactivated form I enzyme was ATP-dependent and that active form I Rubisco and chaperonin 60 (GroEL) interacted in the presence of ATP (Wang and Tabita 1992b). The form I enzyme is also subject to fallover, a phenomenon originally described for plant Rubisco, which is basically the time-dependent loss of activity with time (Portis and Salvucci 2002). This inactivation is due to the accumulation of phosphorylated misfire products that are formed during catalysis and bind at the active site of the enzyme (Edmondson et al. 1990). In plant chloroplasts, Rubisco activase catalyzes the removal of inhibitory phosphorylated intermediates, thus reactivating the enzyme. While we never systematically examined whether this enzyme or an analogous activity is present in *R. sphaeroides*, clearly something is going on relative to the reactivation of inactivated form I enzyme. On the other hand, the molecular basis for the curious lack of fallover for all types of cyanobacterial Rubisco has never been explained (Li and Tabita 1997), despite the fact that the plant and cyanobacterial proteins are close homologs. Interestingly, an analog of Rubisco activase is present in heterocystous cyanobacteria, but not unicellular cyanobacteria (Li et al. 1993) and while knocking out the activase gene seemed to have a physiological effect *in vivo* in *Anabaena variabilis* (Li et al. 1999), no role or actual need for this enzyme has been shown *in vitro*. Clearly, there are several issues that remain to be addressed relative to the means by which Rubisco activity is modulated in prokaryotic cells.

Towards the holy grail of Rubiscology

Despite the fact that so much is known of the structure, function and mechanism of Rubisco catalysis (Cleland et al. 1998), structural biology approaches have basically failed to provide an answer for the most important question, namely the molecular basis

for CO_2/O_2 specificity. Why do enzymes that are so identical in primary sequence, such as the cyanobacterial and plant enzymes, or some of the bacterial enzymes, and whose structures are virtually superimposable, have such different catalytic properties? I have always been impressed with Bob Spreitzer's work, in which the isolation of intragenic suppressors of a Rubisco-dependent, temperature-sensitive phenotype in the green alga *Chlamydomonas reinhardtii* is used as a basis to select for mutant forms of Rubisco (Spreitzer 1999). In the early 1990s, when Deane Falcone was preparing Rubisco knockout strains of *R. sphaeroides*, while showing that various Rubisco genes could complement the defect using a vector containing a CO_2-responsive promoter (Falcone and Tabita 1991), we realized that it might be feasible to develop a prokaryotic bioselection system to probe some of the mysteries of Rubisco function. Such a system could offer additional and perhaps greater flexibility to select for enzymes that have residue alterations not predicted from the structural model that influence key parameters such as k_{cat} (turnover number), Ω (specificity; omega to honor Ogren), K_C (K_m for CO_2), K_{RuBP} (K_m for RuBP), or other properties important for catalysis. These efforts were spurred by a recent PhD student, Stephanie Smith; her work has shown that interesting mutant forms of bacterial Rubisco can be selected using an *R. capsulatus* Rubisco-deficient host (Smith and Tabita 2003). These studies have greatly contributed to current efforts by Stephanie Scott, a postdoc in the lab, as well as other students (Todd Smith, Nathan Kreel and Brian Witte) and another postdoc (Zhandong Zhao) currently beginning to use this system of directed enzyme evolution.

A complex means to regulate a simple process

The move to OSU at Columbus, Ohio, saw our program on the molecular regulation of CO_2 fixation in nonsulfur purple bacteria become a major part of our overall research effort. Deane Falcone and Jan Gibson became the lynchpins for these efforts, assisted by a new graduate student, George Paoli, who had made arrangements to join us after we arrived. In addition, Wim Meijer, who worked on the regulation of CO_2 fixation in *Xanthobacter* at Groningen (The Netherlands), joined us as a postdoc a short while later.

Transcriptional regulators

A divergently transcribed open reading frame (*cbbR*) was discovered upstream of the first gene of the cbb_I operon of *R. sphaeroides* that encodes a homolog of a typical LysR-type transcriptional regulator (LTTR). When *cbbR* was inactivated, autotrophic growth became impossible while photoheterotrophic growth was greatly diminished due to a complete loss of cbb_I transcription and about a 70% reduction in cbb_{II} transcription (Gibson and Tabita 1993). Furthermore, the compensatory effect of knocking out one Rubisco gene by over-expressing the other was abolished in the *cbbR* strain. These results indicated that CbbR is a positive regulator for *cbb* transcription, as it is in other facultative autotrophs (Shively et al. 1998). Paoli and Falcone showed that *cbb* gene organization differed somewhat in *R. capsulatus* and *R. rubrum*, however regulation in both organisms involved the mediation of *cbbR*, with *R. capsulatus* possessing separate divergently transcribed *cbbR* genes to activate each cognate operon (Falcone and Tabita 1993; Paoli et al. 1998). The *cbb* operons of *R. sphaeroides*, localized on separate genetic elements (Suwanto and Kaplan 1989), are obviously coordinately controlled through CbbR, yet each operon is independently regulated as well. Howard Xu and Jim Dubbs joined the group and found interesting upstream promoter elements that play important roles in transcriptional control of both the cbb_I (Dubbs and Tabita 1998) and cbb_{II} (Xu and Tabita 1994) operons. The cbb_I promoter was especially interesting and was found to be comprised of both a promoter proximal region (between +1 and −100 bp) that binds CbbR, sufficient to confer low level and CbbR-dependent regulated expression of cbb_I, and a promoter distal region (between −280 and −636 bp) that does not bind CbbR. The distal region enhanced cbb_I expression some 42-fold, with a 50-bp sequence most responsible for this effect.

Related to the above studies, graduate student Yilei Qian, while working on a totally unrelated problem, uncovered the importance of the RegA/B (PrrA/B) two-component system for regulating *cbb* transcription (Qian and Tabita 1996). Of course the Reg or Prr system had been previously shown by Bauer and Kaplan to be important for controlling the transcription of genes involved in photosystem biosynthesis in *R. capsulatus* and *R. sphaeroides*, respectively (Eraso and Kaplan 1994; Mosley et al. 1994). Because of the involvement of the upstream activation sequence of the cbb_I operon, and due to the requirement for the

Reg/Prr system for maximal regulation, we initiated experiments to determine if these two observations were related. Both Jim Dubbs and graduate student Pui Vichivanives spent 2 weeks in Carl Bauer's laboratory at Bloomington, Indiana, learning how to purify and prepare constitutively active and phosphorylated RegA from *R. capsulatus* for use in DNase I footprint experiments. Once back in Columbus, Dubbs was able to show that the cbb_I upstream activation sequence contained multiple RegA binding sites, of which an extremely high affinity site was found to be localized to the 50-bp sequence that most enhanced cbb_I transcription (Dubbs et al. 2000). While *R. capsulatus* did not show evidence for upstream regulatory sequences in its promoter regions, RegA did control *cbb* transcription and was shown to bind to these promoters *in vitro* (Vichivanives et al. 2000). Interestingly, upstream activation sequences associated with the cbb_{II} operon of *R. sphaeroides* also bind RegA (Dubbs and Tabita 2003).

Carbon and redox mediated control of the cbb *operons of* R. sphaeroides

Work from Samuel Kaplan's laboratory had indicated that mutations in the cytochrome oxidase operon, especially in *ccoP*, allowed aerobic derepression as well as enhanced anaerobic expression of photosystem biosynthesis genes that are regulated by *prrA* (*regA*). Since photosystem gene expression is normally off under aerobic conditions, these results led to the prevailing view that under normal aerobic conditions electron flow through the cytochrome oxidase transmits a signal to sensor kinase PrrB, resulting in a lack of phosphorylation of PrrB and a subsequent interruption in the PrrB/PrrA regulatory cascade (O'Gara et al. 1998). We were interested in testing this hypothesis with respect to *cbb* gene expression since the Prr system is intimately involved with controlling these genes as well. The upshot of some very careful experiments by Gibson and Dubbs, using *ccoP* and *prrA/ccoP* mutant strains, was that this theory appeared to hold for the cbb_{II} operon but was not relevant with respect to the cbb_I operon, suggesting that additional transcriptional regulator(s) are important under aerobic chemoautotrophic growth conditions (Gibson et al. 2002). Indeed, an alternative model was developed whereby a signal derived from cytochrome oxidase may be funneled to some additional regulator (not PrrB), which could then activate the cbb_I promoter (Figure 3).

The model of Figure 3 suggests that both the redox potential of the cell and the source of carbon influence *cbb* expression. CbbR is synthesized constitutively in *R. sphaeroides* and presumably responds to the carbon status of the cells; and both Mary Tichi and Stephanie Smith, with *R. capsulatus* and *R. sphaeroides*, respectively, were able to implicate the product of the *cbbP* gene (namely RuBP) as a potential effector for CbbR (Smith and Tabita 2002; Tichi and Tabita 2002). This is in keeping with the fact that LTTR proteins are often influenced by effector molecules that are intermediates of the pathway that is regulated (Schell 1993). Indeed, *in vitro* experiments with purified CbbR proteins from both organisms support the potential of RuBP to act as a positive effector (P. Vichivanives, J. Dubbs and D. Dangel, manuscripts in preparation). Drew Dangel has also obtained a number of constitutively active CbbR proteins that will facilitate an understanding of the structural basis for effector-mediated CbbR transcription activation. From these results, we believe that clarification of this entire complex regulatory mechanism will be attained and this will occupy much of our future efforts.

Interactive control of important metabolic processes

As noted above, Deane Falcone constructed the first Rubisco knockout strains in *R. sphaeroides* and *R. rubrum*. Under photoheterotrophic growth conditions, the CBB (to repeat, Calvin–Benson–Bassham) pathway functions to effectively balance the redox potential of the cell by allowing CO_2 to be utilized as an electron acceptor for the removal of excess reducing equivalents. These reducing equivalents are generated from the oxidation of the organic electron donor/carbon source supplied to the cells. The *R. sphaeroides* Rubisco knockout (strain 16) does not grow photoheterotrophically on malate unless an exogenous electron acceptor, such as dimethylsulfoxide (DMSO) is supplied to the cells. However, Deane was always a persistent and deliberate researcher and he was determined to find out whether strain 16 really was impaired in its ability to grow in the absence of an added electron acceptor. Thus, he grew the organism aerobically on complex medium and inoculated a large number of cells into a defined malate medium in the light. Nothing grew for several weeks but repeatedly after about 6 weeks, growth did commence and after streak purification, a strain was isolated that maintained its Rubisco negative phenotype but grew

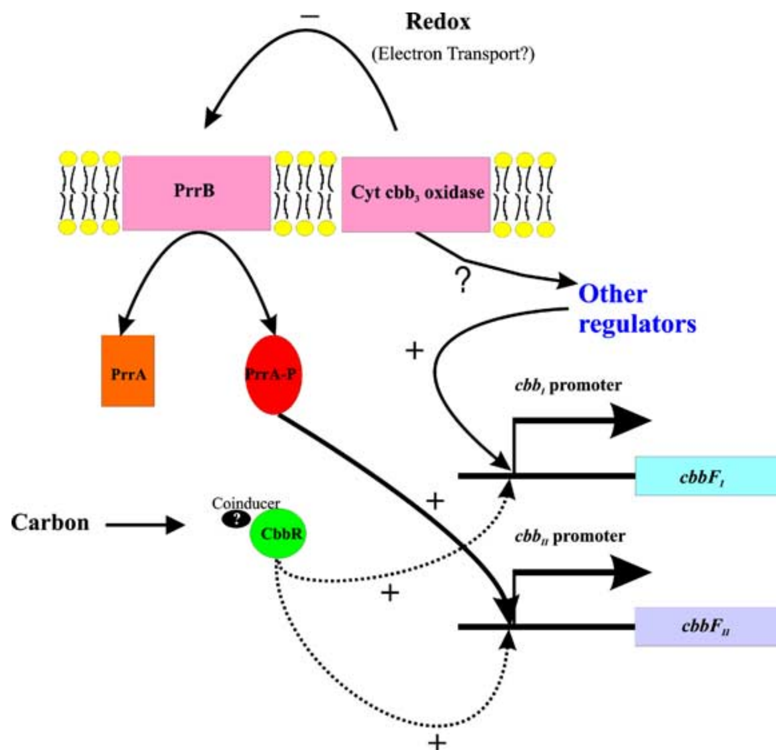

Figure 3. Proposed model of *cbb* gene regulation in *R. sphaeroides* during chemoautotrophic growth (from Gibson et al. 2002). The cbb3 cytochrome oxidase transmits an inhibitory signal to PrrB resulting in a shift in the equilibrium towards unphosphorylated PrrA and subsequent lack of activation of the cbb_{II} promoter. Also shown is a signal derived from cbb3 cytochrome oxidase activity going to an alternate transcriptional regulator that in turn activates the cbb_{I} promoter. The model depicts transcriptional activation of both *cbb* operons by CbbR in response to a signal that presumably reflects the carbon status of the cell. For a color version of this figure, see http://www.kluweronline.com/issn/0166.8595.

well under photoheterotrophic conditions on malate. This was strain 16PHC (Wang et al. 1993), which appeared to be a true genetic variant. Similar strains have since been isolated from *R. capsulatus* and *R. palustris*. Hema (Modak) Joshi joined the laboratory as a postdoc at about the time Deane was finishing his graduate work and she noticed that cultures of strain 16PHC invariably broke or cracked tightly capped screw-cap vessels. These cultures were quite evidently producing copious amounts of gas and this turned out to be hydrogen. The only thing wrong with this observation was that the organism was cultured with high levels of ammonia as the nitrogen source. Any student of photosynthetic bacteria is cognizant of the fact that Howard Gest, in his PhD research with Martin Kamen, had found a relationship between hydrogen evolution and nitrogen fixation (Gest et al. 1950) and most microbiologists appreciate that ammonia classically represses nitrogenase synthesis. So, the question we asked is whether *nif* transcription and nitrogenase synthesis might be derepressed in strain 16. This turned out to be the case (Joshi and Tabita 1996) and the same phenomenon was discovered for other Rubisco knockout strains of *R. capsulatus*, *R. rubrum*, and *R. palustris*. Thus, we feel that the absence of the CBB pathway causes the organism to seek or select other means to remove excess reducing equivalents under photoheterotrophic growth conditions; one means to do this is to abrogate the normal means to control *nif* transcription in the presence of ammonia. Thus, it is apparent that nitrogenase acts as a hydrogenase *in vivo*, basically to reduce protons to hydrogen gas in the absence of molecular nitrogen, in support of Howard Gest's early ideas. If one repairs the defect in the CBB pathway by complementing the organism with an active Rubisco gene, ammonia control reverts back to the usual wild type scenario. Furthermore, Joshi showed that the Reg/Prr system was required for derepression (Joshi and Tabita 1996), which led to the initial finding that the Reg/Prr system is truly a global regulatory system, well reiterated by many subsequent studies by Bauer's group (Bauer

2001; also see Bauer this volume). These studies point out that there is an exquisite control of redox balancing systems in these bacteria and the regulation of CO_2 reduction, N_2 fixation, and H_2 evolution are all intertwined.

Other enzymes of the CBB pathway

It has always been attractive to consider studying the other enzymes that are encoded by genes in the *cbb* operons, especially those that catalyze key conversions such as phosphoribulokinase (PRK) and transketolase, products of the *cbbP* and *cbbT* genes, respectively. I have been proud that this work resulted in several firsts. Thus, despite being an enzyme that is found in virtually all organisms, our CbbT sequence was the first transketolase sequence reported from any source (Chen et al. 1991). For PRK, three successive Christmas breaks free from teaching resulted in the initial purification and description of the quaternary structure of PRK from any source (Tabita 1980). More recently, John Novak (the younger brother of one of my best friends from graduate school) followed up on an observation that Jan Gibson made relative to the PRK isozymes of *R. sphaeroides*. PRKI, encoded by the cbb_{I} operon of *R. sphaeroides* has nearly an absolute requirement for NADH for activity, while PRKII, encoded by the *cbbP* gene of the cbb_{II} operon, is active in the absence of NADH (Gibson and Tabita 1997). For his postdoctoral work, Novak showed that he could prepare recombinant chimeras of the *cbbP* genes and elucidate residues important for the differential ability of NADH to regulate activity of the isozymes (Novak and Tabita 1999). As for CbbT, Cedric Bobst, a current student in the laboratory whose work is being readied for publication, has shown the importance of an interesting cysteine residue in the active site that is responsible for cofactor (TPP) binding. He has also shown that CbbT and the normal heterotrophic transketolase have different catalytic properties.

Rhodopseudomonas palustris

Perhaps the most metabolically versatile of all the nonsulfur purple bacteria is *R. palustris*, an organism that shows the same characteristics of the other well-studied organisms, but also has the ability to oxidize lignin monomers, both aerobically and anaerobically. *R. palustris* also has a well-developed autotrophic potential as it can grow with reduced sulfur compounds such as thiosulfate as well as on hydrogen as electron donor. Its genome suggests that it is really a photosynthetic *Bradyrhizobium*. Research with this fascinating organism has accelerated rapidly as we have a very active consortium comprised of our group along with those of Carrie Harwood, Tom Beatty, Frank Larimer, Jim Liao and Joe Zhou cooperating on these studies (see http://genome.ornl.gov/microbial/rpal/1/rpal_1.html).

Other ways to fix CO_2 and an interesting surprise

Green sulfur photosynthetic bacteria

At the International Symposium on Phototrophic Prokaryotes in Amherst in 1992, I was impressed by a poster by Mike Madigan and his student Tom Wahlund on the natural transformation system exhibited by the fast growing green sulfur photosynthetic bacterium *Chlorobium tepidum*. I realized this would be an excellent organism to use as a model system to study the enzymology and regulation of the reductive tricarboxylic acid (RTCA) pathway of CO_2 fixation. Tom came to my lab as a postdoc and established this system (Wahlund and Tabita 1997); he was followed by another postdoc, Ki-Seok Yoon, who went on to purify and characterize virtually every protein involved with this process (Yoon et al. 1999, 2001). The enzymes that catalyze reductive carboxylation in this pathway, originally discovered by Bob Buchanan and Daniel I. Arnon (Buchanan et al. 1972), have turned out to be rich sources to study the enzymology of low potential iron sulfur proteins. Things really got interesting, however, when the first data on the genomic sequence of this organism began to become available from The Institute of Genomic Research (TIGR). Tom Hanson joined our lab in 1998 and when I asked him to check out the TIGR database for Rubisco sequences, low and behold the computer gave us a positive indication that such a sequence was present. Interestingly, several of the important residues known to be important for Rubisco activity were altered in this protein. In addition, when Hanson expressed this gene in *E. coli* and purified the recombinant protein, it failed to catalyze any activity. Thus, we had a protein that looked like Rubisco but is not Rubisco; hence we call it the Rubisco-like protein (RLP). It does, however, appear to be a potential evolutional precursor to Rubisco, so maybe Samuel G. Wildman was on the

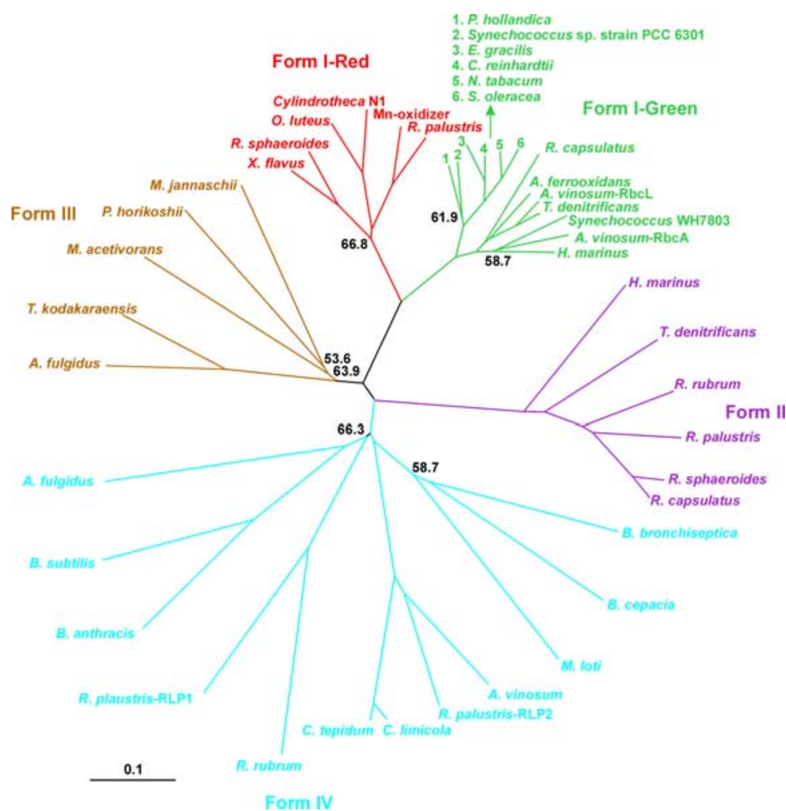

Figure 4. Unrooted neighbor joining phylogenetic tree (from Tabita and Hanson 2003) showing the relationship of *bona fide* Rubisco sequences (form I – red and green, form II – purple, form III – bronze) and the emerging Rubisco-like protein sequences (form IV – cyan). For a color version of this figure, see http://www.kluweronline.com/issn/0166.8595.

right track after all, just the wrong bacterial system. Indeed, knocking out the RLP gene in *C. tepidum* resulted in a mutant strain that was defective in normal sulfur metabolism, while exhibiting a profound stress response, presumably as a result of its problems with the ability to properly oxidize reduced sulfur compounds (Hanson and Tabita 2001). RLP genes have since been found in the genomes of several interesting prokaryotes, including archaea, and may be classified as a form IV Rubisco to distinguish this group of proteins from bona fide form I, II, and III Rubiscos (Figure 4).

Those other things that look like bacteria

As archaeal genomes became available in the mid-to-late 1990s, several putative Rubisco sequences were observed. This was unexpected because these organisms apparently do not use the CBB pathway to fix CO_2. Greg Watson and J-P Yu [my 'scientific grandson,' who received his PhD from my first student Will (Barny) Whitman] were able to show that the *Methanococcus jannaschii* gene encoded a bona fide Rubisco, which exhibited excellent activity when expressed in *E. coli* (Watson et al. 1999); similar results were obtained for other archaeal sequences (Ezaki et al. 1999). Indeed, Mike Finn was able to show that the archaeal Rubisco genes were active in the host organisms and the gene could complement *R. sphaeroides* and *R. capsulatus* Rubisco knockout strains, suggesting that the archaeal genes are fully able to function in a physiologically relevant way (Finn and Tabita 2003). At this point, the relevant question relates to the role of Rubisco in archaea since there is no identifiable sequence for phosphoribulokinase, the enzyme that catalyzes the formation of Rubisco's substrate. Finn will provide the answer for his PhD dissertation. (For a perspective on the discovery of 'Archea,' see Woese this issue.)

Forays into molecular ecology

To illustrate how things somehow fall into place, at a national ASM meeting some years ago in Atlanta, a group of us, including my good friend and former teacher at Syracuse, Ralph Slepecky, went to an Atlanta Hawks basketball game one evening. There I met Ralph's nephew John Paul, a marine microbiologist at the University of South Florida, who had recently devised methods to extract and amplify nucleic acid sequences from the open ocean. Although the basketball game was awful, John and I had a fascinating discussion as we formulated a collaborative plan to examine the presence of Rubisco transcripts in the marine environment. This joint project has been going on for some years now, with several joint publications, and has resulted in molecular tools to show how and when different organisms contribute to CO_2 fixation and has approached the question as to how photosynthetic marine organisms, including cyanobacteria, regulate Rubisco gene expression in the natural environment (Wawrick et al. 2002).

Quo vadis?

So what do we know and what will we do in the future? Or to get back to the Yogi Berra analogy, what forks will we take now that we have become seasoned veterans? Our future research efforts will certainly continue the strong directions we currently follow. However, as I hope was conveyed throughout this piece, we have always tried to keep an open mind and will not predict which lines of research will be the most interesting until we actually do the experiments. One thing is certain, whatever we do will always relate to the general topic of CO_2 fixation. Clearly, I have really been fortunate over the years to have interacted with so many imaginative and hard working students and postdocs and I expect our current group and future colleagues will take us down similar interesting paths. As requested by the editors, I include a current picture of myself (Figure 5).

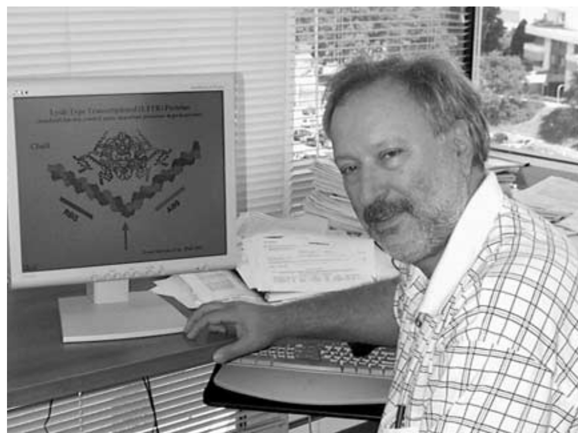

Figure 5. The author (Bob Tabita), 26 June 2003.

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