Rubisco '87: Anything New ?

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is probably the most extensively studied plant enzyme. Although much is known, a workshop entitled *Rubisco—Genes, Proteins and the Regulation of Activity* held last April in Tucson, Arizona, demonstrated that work on this enzyme is far from being complete. In fact, some surprises are still in store. While the last conference on the topic, at Brookhaven in 1978, focused mainly on physiological and biochemical approaches, the advent of molecular biological techniques has broadened the field. Contributions to the conference showed that research conducted with different techniques appears to merge into a unifying molecular approach. The workshop focused on the following aspects: *Ecology* (chairperson: B. Osmond), *Activity* (N. E. Tolbert), *Biochemistry* (F. Hartman), *Subunit Functions* (G. Lorimer), *Biosynthesis and Assembly* (H. Roy), *Structure* (D. Eisenberg), *Genetics and Genes* (D. von Wettstein), and *Mutagenesis and Expression* (B. McFadden).

Ecology and Activity. Photosynthetic CO_2 fixation has been modeled from both economic and field communities of plants. Utilization of the kinetic and biosynthetic properties of the Rubisco protein has revolutionized plant modeling. It is possible to analyze CO_2-response curves of leaves in terms of biochemical responses and to account for many of the changes in photosynthesis during plant development and senescence in terms of the properties of Rubisco. (Contributors: J. Berry, G. Farquhar, R. Jensen, B. Osmond, J. Seemann, T. Sharkey, S. von Caemmerer). Incorporation of oxygenase kinetics in models accurately predicts the O_2 dependence of the slope of the CO_2-response curve leading to enhanced understanding of photorespiration. Activity measurements of Rubisco in

Abbreviations: CABP, 2-carboxy-arabinitol-1,5-bisphosphate; CA1P, 2-carboxyarabinitol-1-phosphate; LS, small subunit of ribulose-1,5-bisphosphate carboxylase; Rubisco, ribulose-1,5-bisphosphate carboxylase; SS, small subunit of ribulose-1,5-bisphosphate carboxylase.

the plant indicated that the enzyme exhibits changes in performance. Under light limitation of photosynthesis, the activation of Rubisco is reduced proportionately, indicating the importance of activity versus enzyme amount. Activation of Rubisco occurs by carbamylation of the ε-amino group of a lysine by CO_2. The CO_2 forming the carbamate is a different molecule from the CO_2 of carboxylation (G. Lorimer, H. Miziorko). The active site analog, 2-carboxy-arabinitol 1,5-bisphosphate (CABP), binds almost irreversibly to the protein and its [^{14}C] label is being used as a specific measure of the amount of active sites in leaf extracts.

How do plants cope with the "inefficiencies" of the enzyme *in vivo*? The C_4 pathway serves as a CO_2 compressor to service Rubisco confined in the bundle sheath cells. This along with other biochemical accessories, such as malate accumulation in C_4 and CAM plants and decarboxylation cycles and the CO_2-concentrating mechanisms in unicellular algae, have evolved to reduce the effects of the low affinity of Rubisco for CO_2 relative to O_2. N. E. Tolbert pointed out that evolutionary arguments disprove rather than support the hypothesis that Rubisco can be improved by engineering. Rather than generating Rubisco mutants, plants appear to have evolved CO_2-concentration mechanisms (carbonic anhydrase, C_4, CAM). M. O'Leary seconded this notion by pointing out that the access of either CO_2 or O_2 to the site of action is hardly amenable to natural selection.

In many plants an inhibitor is synthesized in low light and darkness. The inhibitor 2-carboxyarabinitol 1-phosphate (CA1P), which binds tightly to the enzyme (though not as tight as CABP), is degraded in light (J. Servaites). Although not yet fully apparent, its function may be to keep Rubisco in the active state in the dark, ready to resume carboxylation or to modulate carboxylation, depending on demands of the sink. Evidence suggests that different plant species use different combinations of activation (carbamylation) or inhibitor binding to adjust Rubisco to changing light intensities (J. Seemann, T. Sharkey). Rubisco activity is also connected to sink demand, probably as a response to the phosphate status of the chloroplast.

The activity of purified Rubisco can be greatly modified by the competitive binding of phosphorylated sugars at the active site (R. Chollet). In the leaf, however, relevant compounds for the modulation of activity include RuBP, 3-phosphoglycerate, and CA1P.

RuBP binds tighter to the deactivated enzyme than to the carbamylated form. In the light a rapid increase of RuBP is faster than the increase in enzyme activation by carbamylation. The enzyme-RuBP form may be activated by a specific enzyme, Rubisco activase (A. Portis). The precise physiological role of this enzyme remains elusive apart from the observation that it is essential for light activation. On the other hand, CA1P, whose biosynthetic pathway remains unknown, is turned over rapidly in the light. Inactive Rubisco may exist in two interconvertible forms in the leaf which are distinguished by different kinetics of activation (A. Keys).

Biochemistry and Functions of the Subunits. The active site(s) of Rubisco are located on the large subunit (LS). Although many methods have been utilized for characterization of the active site, the most conclusive is the location of the carbamate-forming residue (lys-201 of spinach or lys-191 of *R. rubrum*). Collective data provided by several different affinity labels revealed the necessity for two distinct, additional lysyl residues at the active site (lys-175 and -334 of spinach LS and lys-166 and -329 of *R. rubrum*) (F. Hartman). Site-directed mutagenesis of lys-166 (*R. rubrum*) indicates that it is not required for activation, but intimately involved in catalysis, probably as the base that enolizes RuBP. Lys-329 is needed for both activation and catalysis, but its precise function is unknown. The carboxylase/oxygenase specificity is related to the geometric accessibility of the RuBP enolate to CO_2 or O_2 (M. O'Leary).

ESR and NMR studies of the active site of Rubisco, using the ternary complex enzyme-A_{CO_2}-Mg^{2+} and the quaternary complex enzyme-A_{CO_2}-Mg^{2+}-CABP, indicate that activator and catalytic sites are in close proximity (H. Miziorko, J. Pierce). In the stable quaternary complex, the metal is not bonded to the activator CO_2 (A_{CO_2}) but to the $C^{2'}$, C^2, and C^3 of CABP, suggesting that the cation stabilizes the ene-diol of RuBP and may assist in the protonation to form D-PGA from the top half of the 6-carbon intermediate.

There have been many approaches to the study of the association of LS (large subunit) and SS (small subunit) to form the hexadecameric higher plant holoenzyme (T. Akazawa). Some of the reconstituted systems either fail to assemble or upon assembly give no activity. LS octamers without SS may be obtained from the high-

er plant-type Rubisco of *Synechococcus* either by mild acid precipitation of SS or expression of the cyanobacterial LS gene in *E. coli*. The carboxylation rate of the LS preparations is one percent of the native holoenzyme activity (T. Andrews, H. Bohnert). The three partial reactions of the carboxylation reaction sequence (RuBP enolization, keto-CABP hydrolysis and keto-CABP decarboxylation) were similiarly inhibited. Expression of LS alone in *E. coli* (H. Bohnert) results in an L_8 or larger core. This result was controversial since S. Van der Vies reported L_2 forms as the intermediate assembly unit from cyanobacteria. L_8 andL_2 are able to bind the substrate analog CABP ($K_d = 10^{-11}$) only at low ionic strength. Addition of SS or expression of LS-SS in bacteria results in stabilization of the active site conformation indicating a possible structural function of SS.

Structure. The session on structure was one of the highlights of the conference. F. Whatley reviewed the different crystal forms obtained so far and their relation to isolation methods and proteolytic digestion, and the still unresolved question about whether LS is processed in the holoenzyme of higher plants. G. Schneider presented refinements in the crystallographic structure of the L_2 enzyme from *R. rubrum*. The active site is composed of protein domains overlapping two LS subunits: an amino-terminal domain in one subunit and a carboxy-terminal domain in the second subunit. This carboxy-terminal portion of the active site forms a β-barrel at the "bottom" of which the substrate RuBP is bound. The group at UCLA (D. Eisenberg, M. Chapman) showed the structural features of a higher plant Rubisco (tobacco, L_8S_8). Their data demonstrate the basic similarity of LS conformation with the conformation of the bacterial L_2 enzyme. In the higher plant enzyme the active sites of two large subunits are assembled from the N-terminal of one and the C-terminal domains of a second LS. Thus, the unit size in the higher plant holoenzyme is 4 x L_2 with a surprisingly large amount of "empty" space between the four L_2 pairs. Part of this space is occupied by SS. It appears that, at least in higher plants, SS provides interactions important to the stability of L_8S_8. The model makes it clear, moreover, that the small subunits cover the poles of four L_2 units as S4 on either pole. This implies that L_8 might be a stable assembly intermediate via four L_2.

Genes and Genetics. Some 20 LS and SS sequences each are known and the number is increasing. Sequence homologies are very high (≈80%) amongst the various LSs. Recognizable homologies are also found between the bacterial gene for L2 and the higher plant and cyanobacterial *rbc*L gene. Within the much more divergent SS sequences from cyanobacteria and higher plants, three regions of particularly high sequence identity are observed: close to the N-terminus (amino acids 11 to 19), the middle of the sequence of the mature subunit (aa 67 to 75), and close to the C-terminus (aa 101 to 121). Several participants pointed out that there is a stretch of amino acids (in the pea sequence aa 51 to 64) which represents an insertion in all *rbc*S genes sequenced so far which are transported that is not found in cyanobacterial-type genes. Much work is now concentrated on studying the expression of LS, which appears to be controlled mainly by post-transcriptional mechanisms (D. Klessig, R. B. Hallick), and SS genes (C. Kuhlemeijer), which appear controlled mainly at the level of transcription. The control regions include sequence motifs for light induction and for recognition by other protein factors.

Differences in the structure and expression of cyanobacterial, cyanelle, and higher plant genes were summarized with emphasis on the *Anabaena-Azolla* symbiosis (S. Nierzwicki-Bauer). From the sequence comparisons alone, it appears clear that we will need not only bacterial and higher plant enzyme structures, but also a cyanobacterial model to explain biochemical differences between enzymes. R. A. Cattolico presented a passionate plea for the study of Rubisco from the red, brown, and yellow-green eukaryotic algae, which have been largely neglected. It appears that LS and SS genes are both located on the plastid DNA in many of these algae and the two genes are probably transcribed bicistronically. These observations are changing our view about the evolution of the chloroplast compartment in the various branches of the plant/algal kingdom. Expression of genes and pathways of assembly of the nuclei and plastids of Rubisco in these algal groups will likely be different from those in higher plants, which poses an intriguing question about how the nuclei and plastids of these algae "talk" to each other.

Viable mutants of Rubisco are rare events. L. Mets developed a screening system for the selection of Rubisco mutants in *Chlamydomonas* which was used to measure the site of the mutation, whether it be in the synthesis or stability of the protein or in an assembly

pathway. A similar scheme (R. Spreitzer), based on temperature sensitivity, measures the turnover of mutant (LSU or SS) subunit proteins which fail to assemble.

Expression. It is popular to express proteins in heterologous systems. Considering that few Rubisco mutants have been generated and that a heterologous expression system removes control circuits that normally act in a CO_2-fixing cell, expression in heterologous systems may be the best procedure for generating mutants of this protein. This appears to be true even when the resident genes have been replaced by a gene construction. The success of the approach depends, however, on the demonstration that heterologously expressed Rubisco is as good as the wild type enzyme. Several groups reported on that topic (S. Van der Vie, A. Gatenby, J. Andrews, F. R. Tabita, B. Bowien, J. Pierce, H. Bohnert). It seems that the time has passed where *E. coli*-made Rubisco may be viewed as the inferior product: the problems with controlled over-expression of the proteins have been solved. Protein similar to that from cyanobacteria can be recovered from transformed *E. coli* in amounts sufficient for biochemical studies. This enzyme is free from the presumed interference of regulatory circuits which normally exist in a carbon-fixing organism. The enzymatic parameters of synthesized Rubisco vary as much as the enzyme preparations obtained from natural sources.

B. McFadden presented evidence that isolated etioplasts can take up and express DNA. This technique may be a useful tool for the transient expression of Rubisco genes *in vitro*. It is still impossible to obtain active higher plant enzyme in *E. coli* because of the absolute insolubility of the higher plant LS. S. Van der Vies reported that higher plant LS from maize formed large complexes in the bacteria without gaining activity when SS was present. An interesting speculation is that this feature of LS may explain the necessity for the LS-binding protein in higher plants.

Transport and Assembly. Transport and assembly properties were covered by S. Van der Vies, T. Gatenby, S. Hemmingsen, M. Mishkind, K. Keegstra, H. Roy. Contradicting reports from several groups, S. Hemmingsen reported the absence of immunologically detectable LS-binding protein in cyanobacteria. He also described the sequence of an LS-binding protein from a higher plant

predicted from its cDNA. Homologies of 66 percent were found between the sequence of this gene and a portion of an *E. coli* gene controlling the elongation of mRNA molecules, and 50% homology with a gene for a major mycobacterial antigen. The transport of SS (K. Keegstra) appears to be mediated by a receptor. Crosslinking studies of pre-SS with the envelope resulted in the immunological detection of one predominant protein species to which the labelled SS protein was attached. Results with several proteins in which a transit peptide was fused to a (foreign) "passenger" protein led Keegstra to conclude that not all the essentials are yet known: some protein fusions are inefficiently transported, even though a functional transit peptide was provided. The heterologous transport system (M. Mishkind) using *Chlamydomonas* preSS for transport into higher plant chloroplasts was refined by an *in-vitro* processing assay. Import of barley SS into pea chloroplasts resulted in the assembly of the barley SS into holoenzyme, while little *Chlamydomonas* SS was assembled.

The Workshop paid homage to Sam Wildman, who was the discoverer of "fraction 1 protein", as Rubisco was originally known. As controversial as ever, Sam presented his movie (prepared in the early 70's) in which he shows us that chloroplasts and mitochondria (and possibly other particles) are fluctuating in a non-random way and that organelles appear to fuse, stay in contact, bud off and segregate again. The view is timely in light of increasing evidence that DNA in plants is promiscuous, such that mtDNA may represent a "junkyard" of genes or at least a melange of DNA pieces in plants.

Apart from the concise reviews summarizing the increase in knowledge since the last Rubisco meeting, the Workshop indicated that we shall see much more biochemistry of Rubisco done with proteins or mutant proteins expressed in bacterial or other systems. A high resolution structure of the ligand-free protein, nearly completed, will allow direct visualization of the functional groups and their spatial relationships for direction of the mutagenesis/expression experiments. Assembly of subunits, both in cyanobacteria and higher plants, will provide the data on essential protein contact domains. Further topics likely to bring significant new results concern the characteristics of SS transport and receptors. Answers to other questions of general importance, not only pertinent to Rubisco, will lead to a better understanding of interactions between cytosol and chloroplast. What are the connections in higher plants amongst the syn-

thesis of LS in the chloroplast, the expression of *rbcS*, the synthesis of preSS, and the transport and assembly pathways? Rubisco appears to offer the best system for studying how plant compartments "talk" to each other.

Finally, despite justifiable pessimism expressed by some of the participants, many of the others appeared nonetheless to be convinced that ultimately Rubisco will be engineered to make a more effective enzyme. One contribution (C. van Assche) claimed that O-*p*-nitrophenylhydroxylamine changes the partition coefficient expressing the preference of the enzyme for CO_2 over O_2 by reducing the $K_m(CO_2)$. Ω

Hans J. Bohnert and Richard G. Jensen
Department of Biochemistry
University of Arizona
Tucson, Arizona, 85721, U.S.A.

Hans Bohnert and Richard Jensen were the organizers of *Rubisco '87*.

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