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# Oxygen-17 Appears Only in Protein in Water-Stressed Soybean Leaves Labeled by $^{17}O_2$

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***Abstract:*** We have used a rotational-echo adiabatic-passage double-resonance $^{13}C\{^{17}O\}$ solid-state NMR experiment to prove that the glycine produced in the oxygenase reaction of ribulose bisphosphate carboxylase-oxygenase is incorporated exclusively into protein (or protein precursors) of intact, water-stressed soybean leaves exposed to $^{13}CO_2$ and $^{17}O_2$. The water stress increased stomatal resistance and decreased gas exchange so that the Calvin cycle in the leaf chloroplasts was no more than 35% $^{13}C$ isotopically enriched. Labeled $O_2$ levels were sufficient, however, to increase the $^{17}O$ isotopic concentration of oxygenase products 20-fold over the natural-abundance level of 0.04%. The observed direct incorporation of glycine into protein shows that water stress suppresses photorespiration in soybean leaves.

## Introduction

Soybean (*Glycine max*) is a $C_3$ plant which means that the first step in carbon assimilation is the production of triose phosphates by the carboxylase reaction of ribulose bisphospate carboxylase-oxygenase (Rubisco) in the Calvin cycle.[1] Rubisco is also responsible for catalyzing an oxygenase reaction which produces 2-phosphoglycolate in the chloroplasts (Figure 1). By conventional accounting,[1,2] the phosphoglycolate is transferred to peroxisomes and converted to glycine, which then moves to mitochondria. Two glycines are used to produce serine by the action of glycine decarboxylase with the release of photorespiratory $CO_2$. The serine is deaminated to glycerate in the peroxisomes and phosphorylated in the chloroplasts where it becomes part of the Calvin cycle. This entire $C_2$ cycle is usually considered an adaptation of $C_3$ plants to rescue assimilated carbon from a wasteful but unavoidable Rubisco oxygenase reaction.[2]

However, not all the glycine produced by the oxygenase pathway is recycled to the chloroplasts. We have shown that $^{13}C$-labeled glycine (free amino acid) and glycyl residues (leaf protein or protein precursors) are detected by $^{13}C\{^{15}N\}$ rotational-echo double-resonance solid-state NMR as soon as 2 min after the start of $^{13}CO_2$ labeling.[3] (The soybean plants were grown using $^{15}N$ fertilizer and so were uniformly $^{15}N$ labeled.) We have surmised that these observed glycyl residues in leaf protein are the result of a fast direct insertion of glycine from the Rubisco oxygenase reaction,[4] rather than a result of the conversion of photosynthetic sugar to pyruvate with the $C_2$ cycle run in reverse to produce glycine.

The fraction of oxygenase-pathway glycine that is used directly relative to that returned to the Calvin cycle as glycerate apparently depends on the $CO_2$ concentration within the leaf,[3,4] which suggests some sort of fast-acting control mechanism.[5,6] Under external labeling, $CO_2$ concentrations of 200−300 ppm and reduced internal $CO_2$ concentrations (which would occur naturally inside a mildly water-stressed leaf with decreased stomatal conductance), about half of the glycine is inserted into protein.[3,4]

While a significant reduction of internal $CO_2$ occurs for water-stressed leaves, internal $O_2$ levels remain high even with reduced gas exchange because of the abundance of oxygen in the atmosphere. Thus, characterization of the oxygenase activity of Rubisco is possible using $^{17}O_2$ as an NMR probe. In this report, we confirm a direct-insertion interpretation of glycine incorporation in protein by detection of $^{13}C-^{17}O$ labeled glycyl residues in intact water-stressed leaves exposed to 400-ppm $^{13}CO_2$ and 21% $^{17}O_2$, and the absence of $^{13}C-^{17}O$ labels in sugars from glycine via the $C_2$ cycle.

## Materials and Experimental Methods

**Growth of Plants.** Soybeans were grown outdoors (June−August, 2008) on the roof of the Washington University McMillen Laboratories building. The plants were grown in 30-cm-diameter pots filled with a mixture of one-third perlite and two-thirds top soil. Approximately 3 weeks after planting, the pots were fertilized each day with 100 mL of a 1 g/L $^{15}NH_4{}^{15}NO_3$ solution. The $^{15}N$ label (99 atom % $^{15}N$, Isotec, Miamisburg) was used to distinguish $^{13}C$ label in amino acids and proteins from that in organic acids. This high level of fertilizer suppressed symbiotic nitrogen fixation, as evidenced later by the scarcity of soybean root nodules. The $^{15}N$ enrichment of the leaves was approximately 50%, as determined by solid-state $^{15}N$ NMR,[4] less than that of the ammoniun nitrate

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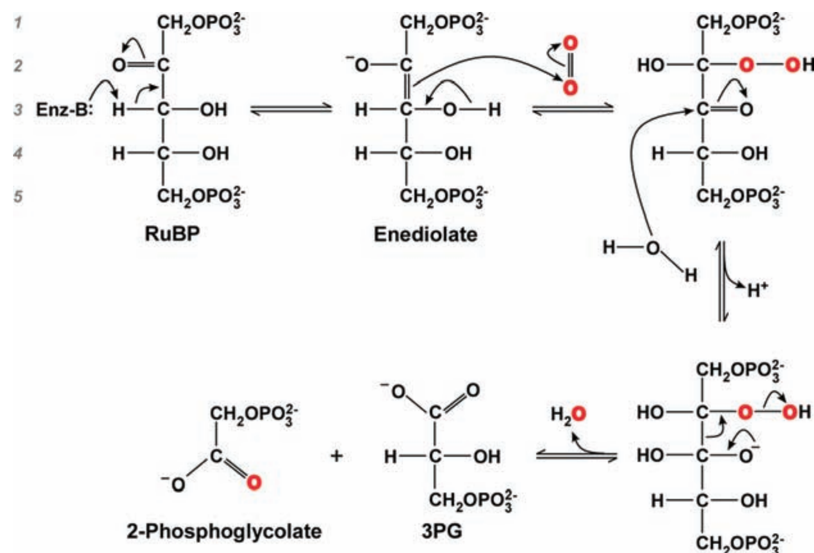


***Figure 1.*** Oxygen addition to ribulose bisphosphate catalyzed by Rubisco (after Voet and Voet). The carbon directly bonded to the red oxygen in 2-phosphoglycolate eventually becomes the C-1 carbon of $^{17}$O-labeled glycine.

because of unlabeled nitrogen sources in the top soil. The plants were watered by hand, typically every 2−4 h during the day.

**Labeling with $^{13}CO_2$ and $^{17}O_2$.** Labeling was performed approximately 8 weeks after planting. The labeling was performed between 1:00 p.m. and 3:30 p.m. on a sunny, cloud-free day (August 11, 2008) with a temperature of 84 °F at noon in St. Louis. The windy conditions on the roof of the McMillen Laboratories building resulted in soybean plants that were short (60 cm after 8 weeks) with thick stems. Water stress was induced in the plant by witholding water from the pots for 48 h. Leaves were curled but not wilted. The target soybean trifoliolate (fifth node, uniform green coloration, total surface area of about 100 cm$^2$) was held down by fishing line and enclosed within a specially constructed labeling chamber with a volume of approximately 200 mL.

The labeling was done in two parts. In the first part, labeling with 400-ppm $^{13}CO_2$, natural-abundance oxygen, and the remainder $N_2$ was begun after confirmation of some gas exchange by an immediate increase from 37% to 50% relative humidity (at 84 °F) within the chamber (Vaisala HMT330 RHT sensor, Helsinki). However, there was no accumulation of moisture on the inner surface of the chamber, as we observe for well-watered leaves. The gas mixture was in a pressurized 6-L cylinder (Praxair, Inc., Cahokia) and flowed through the labeling chamber for 1 h with no recycling. The gas flow was constant and was the equivalent of a turnover of 50 vol of the labeling chamber per minute. This allowed a rapid complete exchange of $^{13}CO_2$ for $^{12}CO_2$. An electric fan blew exiting gas away from the plants. Humidification of the labeling gas was achieved by bubbling through 50 mL of water so that the relative humidity inside the labeling chamber was maintained at 80% (at 82 °F). Exposure of the water-stressed leaves to increased humidity could increase stomatal conductance and improve carbon assimilation.[7]

Because of the water stress, we anticipated that labeled carbon assimilation would be reduced to perhaps 5−10% of that for unstressed leaves (*vide infra*). The motivation of the first part of the labeling was therefore to produce some $^{13}$C-labeled sugars within the leaf that might be used in the second part when $^{17}O_2$ was present.

The second part of the labeling was done with 400-ppm $^{13}CO_2$ (99 atom % $^{13}$C), 21% $^{17}O_2$ (90 atom % $^{17}$O), and the balance $N_2$. Because of the cost of the $^{17}O_2$ ($7000 for 0.2 L, STP, 90% $^{17}$O isotopic enrichment, Isotec, Miamisburg), use of closed-loop recirculating gas-handling was mandatory. The cost of the $^{17}O_2$ also meant that only one labeling experiment was practical. We chose water-stress conditions based on our surmise that glycine metabolism in leaves changes from that commonly characterized for well-watered leaves and full stomatal conductance by gas-exchange experiments.

The chamber was sealed by an O-ring held in place by 4 thumb screws. The chamber had a copper bottom which was in contact with a Peltier thermoelectric cooler to control the temperature.[8] The seal around the leaf stem was by an elastic gum and was leaky. A positive pressure (0.5-in. water) was maintained inside the chamber by admitting makeup gas using pressure-controlled relays (see Figure 1 in ref 8). For the first 20 min, the makeup gas was the 1 L of $^{13}CO_2$ and $^{17}O_2$ labeled gas which represented approximately 5 chamber volumes. (A video record of the beginning of this labeling is available at http:www.chemistry.wustl.edu/~schaefer/Research.htm.) For the next 60 min, the makeup gas was dry $N_2$ to extend the period that the leaf was exposed to $^{17}O_2$. There was no humidification of the labeling chamber during closed-loop operation. The relative humidity dropped from 80% (at 82 °F) at the start of closed-loop operation to 60% after 80 min.

At the end of the combined two labeling periods, the petiole was cut and the leaves were immersed in liquid nitrogen, a procedure that required less than 10 s. The frozen leaves were lyophilized, and then chopped into approximately 1-mm fragments by hand with a razor blade, packed into a magic-angle spinning rotor, and examined by solid-state NMR.

**Solid-State NMR at 500 MHz.** Spectra were obtained using a 6-frequency transmission-line probe,[9] having a 12-mm long, 6-mm inner-diameter analytical coil and a Chemagnetics/Varian magic-angle spinning ceramic stator. Lyophilized samples were contained in thin-wall Chemagnetics/Varian 5-mm outer-diameter zirconia rotors. The rotors were spun at 7143 Hz with the speed under active control to within ±2 Hz. The spectrometer was controlled by a Tecmag Libra pulse programmer. Radiofrequency pulses for $^{13}$C (125 MHz) and $^{15}$N (50.3 MHz) were produced by 2-kW American Microwave Technology power amplifiers. Proton (500 MHz) radiofrequency pulses were generated by a 2-kW Amplifier Systems tube amplifier driven by a 50-W American Microwave Technology power amplifier. Final-stage power amplifiers were under active control.[10] The $\pi$-pulse lengths were 8 $\mu$s for $^{13}$C and 9 $\mu$s for $^{15}$N.

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A 12-T static magnetic field was provided by an 89-mm bore Magnex superconducting solenoid. Proton-carbon cross-polarization magic-angle spinning transfers were made with radiofrequency fields of 62.5 kHz. Proton dipolar decoupling was 100 kHz during data acquisition.

**Rotational-Echo Double Resonance (REDOR).** REDOR was used to restore the dipolar couplings between heteronuclear pairs of spins that are removed by magic-angle spinning.[11,12] REDOR experiments are always done in two parts, once with rotor-synchronized dephasing pulses ($S$) and once without (full echo, $S_0$). The dephasing pulses change the sign of the heteronuclear dipolar coupling, and this interferes with the spatial averaging resulting from the motion of the rotor. The difference in signal intensity (REDOR difference, $\Delta S = S_0 - S$) for the observed spin in the two parts of the REDOR experiment is directly related to the corresponding distance to the dephasing spin.[12] All REDOR spectra were collected with standard xy-8 phase cycling[13] on both observed and dephasing channels. In the $^{13}C\{^{15}N\}$ REDOR experiments reported here, short dipolar evolution periods were used (16 rotor periods) so that only directly bonded $^{13}C-^{15}N$ pairs were detected.

**Solid-State NMR at 151 MHz.** Spectra were obtained using a triple-channel custom-built spectrometer with a Tecmag Libra pulse programmer and a 3.55-T magnet (proton frequency of 151.395 MHz). The triple-channel probe is a transmission-line design,[9] and incorporates a Chemagnetics 7.5-mm pencil rotor spinning assembly with a 14-mm long, 8.65-mm inner diameter, 6-turn coil made of 14-gauge wire. Radio-frequency (rf) field strengths were 114 kHz for $^1H$ decoupling, 49 kHz for $^1H-^{13}C$ cross-polarization, 49 kHz for $^{13}C$ pulses, and 48.6 kHz for the $^{17}O$ pulse. The $^{13}C$ and $^{17}O$ power amplifiers were under active control. All spectra were obtained with 1-s recycle delays and 1-ms matched Hartmann−Hahn cross-polarization transfers.

**Rotational-Echo Adiabatic-Passage Double Resonance (REAPDOR).** In contrast to the situation for $^{13}C-^{15}N$ REDOR, dephasing in $^{13}C\{^{17}O\}$ REAPDOR depends on a large number of parameters,[14−17] including the quadrupolar coupling constant and asymmetry parameter, the spinning speed, the radiofrequency irradiation strength, and the relative orientation of the $^{13}C-^{17}O$ internuclear vector and the $^{17}O$ quadrupolar tensor. Nevertheless, observed and calculated dephasing can be used to extract internuclear distances as long as the radiofrequency amplitude and the sample rotation speed satisfy an adiabaticity condition.[17] In the experiments on labeled plants, we chose a short evolution time (2.56 ms) to reveal by sizeable dephasing $^{13}C-^{17}O$ proximities of the order of 1.5−3.0 Å.

The spinning rate for the REAPDOR experiments was 3.125 kHz and was stabilized with a custom-built controller to within 0.2 Hz. With $^{13}C$ $\pi$ pulses at $T_r/2$ in the REAPDOR sequence (Figure 2), the sample-spinning rate must be controlled accurately to avoid dephasing during shift-tensor evolution.[17] Precise control of the spinning speed was accomplished by marking the sample rotor with black ink to provide an alternating pattern of five dark and five light marks. The spinning speed was monitored optically by counting the number of times the black marks appear in 1 s. This number was compared to the expected number of passing black marks for the desired spinning rate. If the optically detected number of passing marks differs from the set value, then an electronically controlled air valve adjusts the drive gas pressure accordingly to compensate for the error in the spinning rate. With 5 marks painted on the rotor and a counting time of 1 s, the spinning rate can be corrected for deviations of 0.2 Hz from the set value.

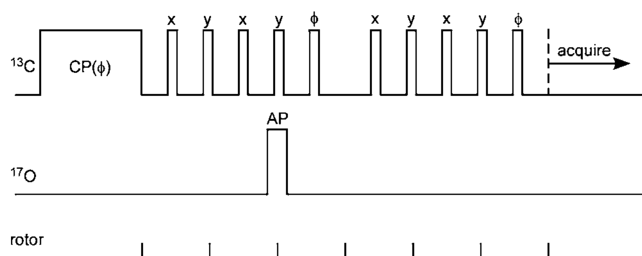


***Figure 2.*** Pulse sequence for rotational-echo adiabatic-passage double resonance (REAPDOR). The $^{17}O$ pulse was one-third of a rotor period (106.6 $\mu$s) and was centered about a $^{13}C$ $\pi$ pulse to avoid a $^{13}C\{^{17}O\}$ Bloch-Seigert phase shift (see ref 15). The matched Hartmann−Hahn cross-polarization transfer from the $^1H$ channel (not shown) used spin-temperature alternation. The xy-4 phase alternation for the $^{13}C$ $\pi$ pulses was itself cycled through x and y phases. The phase of the AP pulse was always x. The phase of the $^{13}C$ $\pi$ pulses following the xy-4 phased pulses was the same as that of the $^{13}C$ cross-polarization pulse.

The source of high-pressure air was a CompAir Hydrovane rotary-vane compressor set to maintain a pressure of 92 psi in an attached 100-gallon ballast tank. This air was delivered to a 60-gallon tank regulated at 85 psi. The gas was then delivered to another 60-gallon tank with the gas pressure regulated at 78 psi. Two 30-gallon tanks fed off the latter tank; one of these was the source for the bearing air and was regulated at 50 psi, and the other was the source for the drive air and was also regulated at 50 psi. The function of all of these ballast tanks is to smooth any pressure waves that might be generated by compressor cycling.

## Results and Discussion

**Line Assignments.** The 125-MHz $^{13}C\{^{15}N\}$ REDOR spectra of intact water-stressed soybean leaves labeled for 1 h by 400-ppm $^{13}CO_2$ (and natural-abundance $O_2$) are shown in Figure 3. The level of incorporated label was modest, and represents no more than 10% of the carbonyl and carboxy-carbon peak, centered at 175 ppm, and less than 10% of the carbohydrate peak at 72 ppm. (Natural-abundance spectra not shown.) Accurate determinations of low levels of labeling are difficult because of minor compositional variabilities from leaf to leaf. The carboxyl-carbon peak arises from glutamic and aspartic-acid residue side chains, free citric-acid-cycle organic acids, free amino acids, and possibly cell-wall and cell-wall-precursor acidic pectins. These carboxyl carbons do not contribute to the 2.24-ms $^{13}C\{^{15}N\}$ REDOR difference signal (Figure 3, top right). Methylene carbons from free glycine and glycyl residues in protein have a strong REDOR difference signal at 42 ppm (Figure 3, top left).

**REAPDOR.** The 38.1-MHz $^{13}C\{^{17}O\}$ REAPDOR spectra of water-stressed soybean leaves labeled by both $^{13}CO_2$ and $^{17}O_2$ are shown in Figure 4. The full-echo spectrum is essentially the same as the REDOR full-echo spectrum of Figure 3. There is only one REAPDOR difference peak which is centered at 171 ppm (Figure 4, top right), and shifted upfield from the center of the full-echo peak at 175 ppm (dotted line). We measured the carboxyl-carbon shift for free glycine in the solid state as 175 ppm, consistent with literature values.[18] Most free carboxyl-carbon shifts are between 176 and 178 ppm.[3] Ester carbonyl-carbon shifts are typically at 170 ppm or less.[19] The 171-ppm

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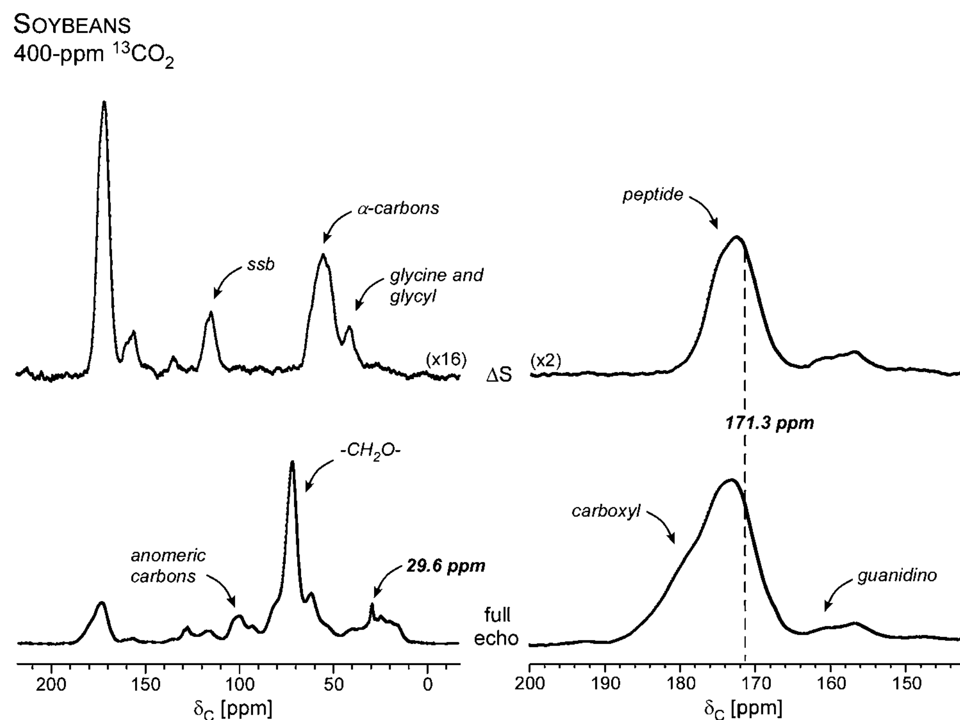


***Figure 3.*** The 125-MHz $^{13}C\{^{15}N\}$ REDOR spectra of water-stressed soybean leaves (150 mg) labeled for 1 h with 400-ppm $^{13}CO_2$. The REDOR difference ($\Delta S$) after 16 rotor periods (2.24 ms) of dipolar evolution is shown at the top of the figure, and the full echo at the bottom. The lipid peak at 29.6 ppm is an internal reference. An expansion of the low-field region is shown on the right. The difference signal arises primarily from directly bonded $^{13}C-^{15}N$ pairs. The spectra are the result of the accumulation of 51K scans. Magic-angle spinning was at 7143 Hz.

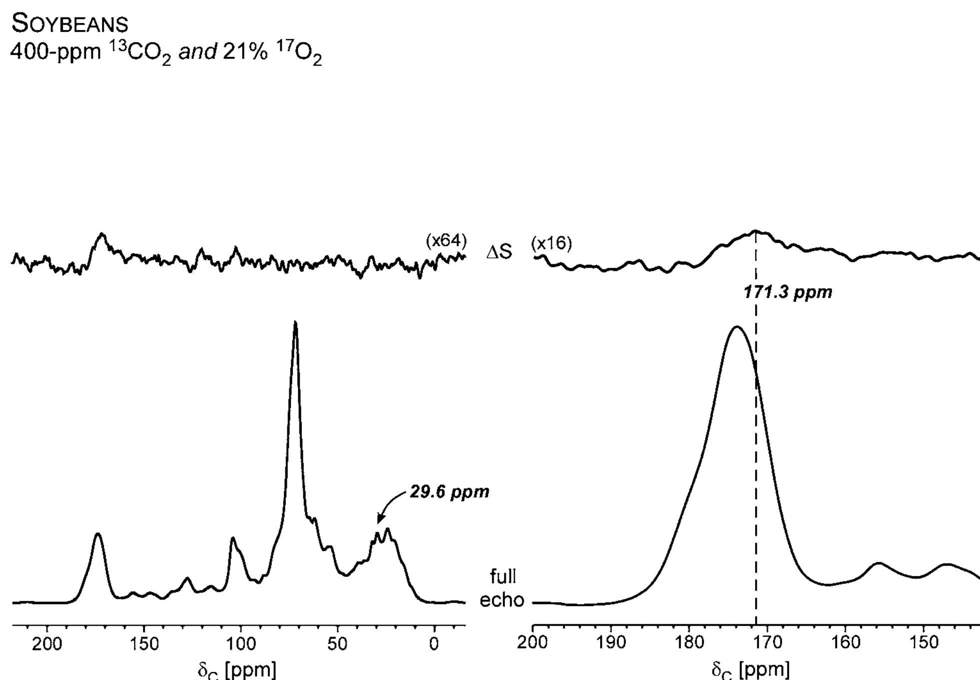


***Figure 4.*** The 38.1-MHz $^{13}C\{^{17}O\}$ REAPDOR spectra of water-stressed soybean leaves (300 mg) labeled for 1 h with 400-ppm $^{13}CO_2$ and natural-abundance $O_2$, and then for 20 min with 400-ppm $^{13}CO_2$ and 21% $^{17}O_2$. The REAPDOR difference ($\Delta S$) after eight rotor periods (2.56 ms) of dipolar evolution is shown at the top of the figure, and the full echo at the bottom. An expansion of the low-field region is shown on the right. The difference peak is shifted to high field by about 4 ppm relative to the maximum of the full-echo peak. The spectra are the result of the accumulation of 2045K scans. Magic-angle spinning was at 3125 Hz.

value is a signature solid-state chemical shift for glycyl residues.[20] We conclude that the 171-ppm shift in Figure 4 (top right) is that of glycyl residues in protein or protein precursors.[21,22]

We estimate the $\Delta S/S_0$ ratio for the protein component of the 175-ppm peak (that is, the carboxyl-carbon shoulder is excluded) as 1/150, or about 0.7% (Figure 4). This is far too large a difference to be related to natural-abundance $^{17}O$ which is only 0.04%. Thus, $^{17}O_2$ entered the leaf and labeled the oxygenase pathway (Figure 1, red highlight). This is not surprising considering that the $^{17}O_2$ concentration outside the

leaf was almost 20% for about 20 min, and that Rubisco was fully active despite the water stress and reduced stomatal conductance.[23]

The offset of the difference peak from the center of the full-echo peak means that the difference cannot be due to natural-abundance $^{13}C$ but rather is due to a labeled minor component. (A REAPDOR experiment on an unlabeled leaf revealed no peaks above the noise after 800K scans.) Gas exchange measurements prior to labeling would have been ambiguous because $CO_2$ uptake was limited and could have changed during the 80-min labeling. To determine how much $^{13}C$ label was assimilated by the leaf, we estimated the area of the full-echo carbonyl-carbon peak of Figure 3 (bottom right panel) to the right of the dotted line as 1/5 of the total, and the fraction due to label as 1/10 of the total (cf. above), or 2%. If we assume that *all* of the peak intensity to the right of the dotted line is due to glycyl residues,[24] then a $^{13}C$ isotopic enrichment of the labeled fraction of about 35% would account for the REAPDOR $\Delta S/S_0$ of Figure 4 (35% of 2% is 0.7%). This is an upper bound only. The true isotopic enrichment could be much lower.

A low isotopic $^{13}C$ enrichment is consistent with relatively little $^{13}CO_2$ entering the water-stressed leaf against high stomatal resistance. As a result, the Calvin cycle would not have been isotopically saturated,[3,25] and product hexose sugars would primarily be singly labeled at carbons 1, 3, 4, and 6. Because of slow turnover of the carboxylase pathway under these conditions, the RuBP labeled by $^{17}O_2$ would have been derived primarily from hexoses (possibly formed in the initial 60-min labeling period) via the pentose phosphate pathway,[25] resulting in $^{13}C$ labels in carbons 2, 3, and 5 (see Figure 1 for numbering). The RuBP label at C-2 would then become the C-1 carbon of phosphoglycolate (Figure 1, red highlight) and ultimately the C-1 carbon of glycine. This pattern explains the observation that there is no REAPDOR difference signal at 42 ppm: $^{17}O$-labeled glycine was not $^{13}C_2$ labeled and the probability of a single $^{13}C$ label at C-2 was low.

Figure 5 shows the $^{13}C\{^{17}O\}$ REAPDOR spectra of a mixture of $^{17}O$-labeled and $^{13}C$-labeled Ala-Ala-Ala tripeptides.[26] The pulse sequence was identical to that used for the leaf-spectra of Figure 4. The difference peaks at 48 ppm ($C_\alpha$) and 21 ppm ($CH_3$) arise from natural-abundance $^{13}C$ in the $^{17}O$-labeled peptides. Thirty-six percent of the tripeptides have an $^{17}O$ (80% of the tripeptides are labeled by $^{17}O$ with an isotopic enrichment of 45%), each of which has two nearby intramolecular methine carbons (2.4 and 2.8 Å) and three methyls (3.1, 3.3, and 3.5 Å).[27] These proximities account for the 12% dephasing[17] of the 48- and 21-ppm peaks (Figure 5), and this dephasing shows that REAPDOR difference signals from protonated carbons are readily detected.[28] All the triose, RuBP, hexose, sucrose, and starch

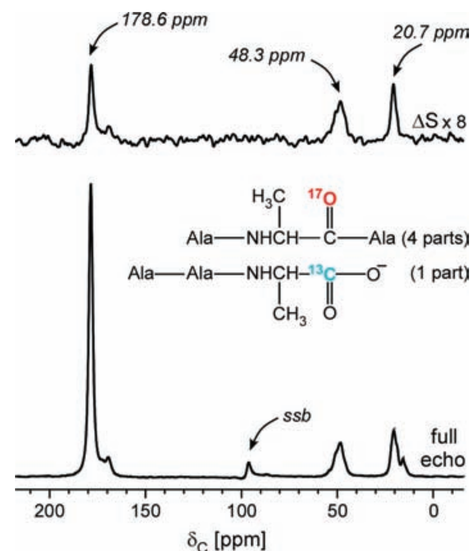


***Figure 5.*** The 38.1-MHz $^{13}C\{^{17}O\}$ REAPDOR spectra of a recrystallized mixture of 1 part of Ala-Ala-[1-$^{13}C$]Ala tripeptide and 4 parts of Ala-[1-$^{17}O$]Ala-Ala tripeptide. The REAPDOR difference ($\Delta S$) after eight rotor periods (2.56 ms) of dipolar evolution is shown at the top of the figure, and the full echo at the bottom. The methine-carbon and methyl-carbon difference peaks (48 and 21 ppm, respectively) are due to natural-abundance $^{13}C$ in the $^{17}O$-labeled tripeptide. Isotopic enrichment of the $^{17}O$ was 45%. The spectra are the result of the accumulation of 110K scans. Magic-angle spinning was at 3125 Hz.

products of the Rubisco carboxylase reaction (which was not completely shut down by water stress) have −CH(OH)− carbons that would be $^{13}C-^{17}O$ labeled if glycerate from the $C_2$ pathway were returned to the chloroplasts.[25] Most of these carbons would contribute to the 72-ppm peak in Figure 4. In addition, half of the $^{17}O$ label would survive the decarboxylation of glycine in making phosphoglycerate via serine.[25] The absence of a REAPDOR difference peak at 72 ppm in Figure 4 therefore means that the glycolate → glycine → serine → glycerate → sugar pathway (see Figure 1 in ref 4) was not active.

With all the glycine produced by the oxygenase pathway incorporated into protein (or protein precursors), there is no decarboxylation of glycine in water-stressed soybean leaves and, therefore, no photorespiration and no loss of photosynthetically assimilated carbon. Under water stress, the leaf produces glycine-rich proteins (GRPs) which include cell-wall proteins[24] and the so-called hydrophilins and dehydrins[29] associated with adaptation to hyperosmotic conditions.[30] This appears to be a short-term response to water stress by soybeans to optimize productivity, a strategy that requires stored energy reserves and ample fixed nitrogen.

## Conclusions

In water-stressed soybean leaves with hexose reserves, glycine from the Rubisco oxygenase pathway is not converted to serine with the release of photorespiratory $CO_2$ but is used exclusively in protein synthesis instead. The $C_2$ pathway appears to be under the control of the internal $CO_2$ concentration,[3,4] and glycine from the oxygenase reaction is primarily routed through glycerate

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back to the chloroplasts only in well-watered soybean leaves under external $CO_2$ concentrations greater than 300 ppm.[24] Under water stress when $CO_2$ uptake is limited, there is no photorespiration and no loss of scarce photosynthetically assimilated carbon.

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