ISOTOPIC COMPOSITION OF PHOTOSYNTHETIC O\(_2\) FLASH YIELDS IN THE PRESENCE OF H\(_2\)\(^{18}\)O AND HC\(^{18}\)O\(_3\)

Richard RADMER and Otto OLLINGER
Martin Marietta Laboratories, 1450 South Rolling Road, Baltimore, MD 21227, USA

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1. Introduction

We described a mass spectrometer-inlet system for measuring the amount and isotopic composition of gases evolved and consumed by photosynthetic tissue in response to single short saturating light flashes [1]. Here we use this technique to study photosynthetic O\(_2\) evolution with \(^{18}\)O-labeled substrates. We observed the following:

1. When CO\(_2\)-depleted* chloroplasts were reactivated with \(^{18}\)O bicarbonate, all the O\(_2\) evolved had the isotopic composition of the H\(_2\)O rather than the CO\(_2\). These results support the idea that neither bicarbonate nor CO\(_2\) is the immediate source of photosynthetically evolved O\(_2\).

2. When these chloroplasts or normal non-depleted chloroplasts were flashed in the presence of H\(_2\)\(^{18}\)O added in total darkness, all of the O\(_2\), including that evolved in the first few flashes, had the isotopic composition of the added H\(_2\)O. This indicates that the S\(_2\) state does not contain a bound intermediate oxidation product of H\(_2\)O.

3. Although CO\(_2\) depletion did not affect the O\(_2\) evolution system directly, it drastically decreased the magnitude of the ferricyanide-induced 'anomalous double hit' probably due to an inhibition of reactions on the acceptor side of system II. This inhibition may provide a mechanism for regulating cyclic electron flow in vivo.

2. Materials and methods

The mass spectrometric apparatus and measuring technique were described in [1]. Special precautions were taken to assure that no light other than the actinic flashes reached the sample after the addition of bicarbonate or labeled H\(_2\)O. With hydroxylamine as the system II donor, N\(_2\) is evolved only on the first flash in the presence of DCMU [1]. This system is essentially irreversible [1,2]: tens of minutes of dark time are required before a significant N\(_2\) flash yield can again be observed. Thus, these chloroplasts can serve as a very sensitive actinometer for our system: a light flux of \(~1\) quantum trap\(^{-1}\) min\(^{-1}\) after the addition of NH\(_2\)OH and DCMU will severely depress the subsequent N\(_2\) flash yield.

Manipulation and monitoring [1] of these chloroplasts under the same conditions as those used here showed no decrease in the N\(_2\) flash yields compared to steady-state flash yields or a 'dark control'. Thus, there was apparently no discharge of any \(^{18}\)O-label incorporated into the O\(_2\)-evolving system before the evolved O\(_2\) was monitored, and the O\(_2\) observed was the first O\(_2\) evolved after the addition of the bicarbonate or labeled H\(_2\)O. Accordingly, chloroplasts were layered on the mass spectrometer 'flash yield inlet' in total darkness using an Eppendorf pipettor guided by a special bracket. The inlet and associated flash lamp were contained in a light-tight box.

CO\(_2\)-depleted chloroplasts were prepared by a procedure derived from that of Stemler and co-workers (see, e.g. [3]). Chloroplasts (0.5 mm) \(~5\) mg chl/ml, were prepared from greenhouse or market spinach [4], pelleted, and taken up in 10 ml depletion medium (100 mM potassium phosphate, 100 mM NaCl, 100 mM sodium formate, pH 5.0). After the suspen-
sion was agitated for 10 min under a stream of \( \mathrm{N}_2 \), the chloroplasts were pelleted and suspended in 0.5 ml chloroplast medium (100 mM potassium phosphate, 100 mM NaCl, 100 mM formate, 400 mM sucrose, pH 6.8). The reaction medium for the flash experiments contained 100 mM potassium phosphate, 100 mM NaCl, 100 mM sodium formate (pH 6.8). For the polarographic assays to determine the effect of \( \text{CO}_2 \) depletion, 30 mM methylamine and 1 mM \( \text{K}_3\text{Fe(CN)}_6 \) were added to the reaction medium.

During the isolation and assay procedure, great care was taken to exclude contamination by atmospheric \( \text{CO}_2 \). All procedures were done in closed vessels under a stream of \( \mathrm{N}_2 \), and transfers were effected via modified syringes. All reagents were extensively bubbled with \( \mathrm{CO}_2 \)-free \( \mathrm{N}_2 \) before use. \( \text{CO}_2 \) depletion resulted in a 7–8-fold decrease in the rate of the Hill reaction in high light. Reactivated chloroplasts typically had a rate of 60–70 \( \mu \text{mol} \ \text{O}_2 \cdot \text{mg chl}^{-1} \cdot \text{h}^{-1} \).

Depleted chloroplasts were reactivated in total darkness by adding solid \( \text{NaHCO}_3 \) to both the chloroplast preparation and the medium to 10 mM final conc. The mass spectrometer reaction vessel was filled with the \( \text{HCO}_3^- \)-spiked buffer, and the reactivated chloroplasts were layered on the inlet. The isotopic composition of the \( \text{CO}_2 \) (m/e = 44, 46, 48) was then continuously monitored during the 3–5 min settling time of the chloroplasts. After the signals stabilized, the flash yields of the various \( \text{O}_2 \) isotopic species were determined.

\([^{18}\text{O}]\) Bicarbonate was prepared by dissolving unlabeled \( \text{NaHCO}_3 \) in 95 atom\% \( \text{H}_2^{18}\text{O} \) (from Bio Rad...
Labs.), and allowing the solution to equilibrate for several days. Known quantities of solid HC\textsuperscript{18}O\textsubscript{3} (and unlabeled HCO\textsubscript{3}) free of labeled H\textsubscript{2}O were obtained by drying aliquots of the solution under a stream of N\textsubscript{2}.

3. Results and discussion

3.1. Isotopic composition of O\textsubscript{2} evolved by HCO\textsubscript{3}\textsuperscript{-}depleted chloroplasts reactivated in the dark

The hypothesis that CO\textsubscript{2} or bicarbonate is the immediate source of photosynthetically evolved O\textsubscript{2} has been long proposed (see, e.g. [5]). Using CO\textsubscript{2}-depleted chloroplasts [3], it was found that the O\textsubscript{2} evolved after reactivation with HCO\textsubscript{3} reflected the isotopic composition of the H\textsubscript{2}O and not the bicarbonate. However, because their apparatus did not have sufficient sensitivity to monitor single turnovers of the O\textsubscript{2}-evolving system, they could not eliminate the possibility that the very first molecules of evolved O\textsubscript{2} were derived from the reactivating HCO\textsubscript{3}.

Figure 1 shows the flash yield pattern of \textsuperscript{18}O\textsubscript{2} (m/e = 34) and unlabeled O\textsubscript{2} (m/e = 32) for CO\textsubscript{2}-depleted chloroplasts and depleted chloroplasts reactivated with labeled or unlabeled bicarbonate. Each pair of measurements was done on the same sample; a 5 min dark period preceded each flash sequence.

As shown on the left in fig.1, no \textsuperscript{18}O\textsubscript{2} (beyond that attributable to the natural abundance of H\textsubscript{2}O) was evolved when the CO\textsubscript{2}-depleted chloroplasts were reactivated with \textsuperscript{18}O bicarbonate. Significant labeled O\textsubscript{2} was evolved only in the presence of added H\textsubscript{2}O\textsubscript{18} (see below). A similar lack of response of the evolved O\textsubscript{2} to reactivation with labeled bicarbonate was also observed at m/e = 36, which corresponds to doubly-labeled O\textsubscript{2} (data not shown).

That the added bicarbonate did indeed reactivate the O\textsubscript{2}-evolving system is shown in fig.1 (right column), which illustrates the flash yield patterns of unlabeled O\textsubscript{2} (m/e = 32) for CO\textsubscript{2}-depleted chloroplasts and depleted chloroplasts reactivated with labeled or unlabeled bicarbonate. Each pair of measurements was done on the same sample; a 5 min dark period preceded each flash sequence.

The data of fig.1 set rather stringent limitations on the amount of O\textsubscript{2} that can be derived from CO\textsubscript{2}. At the onset of the flash sequence, the isotopic composition of the CO\textsubscript{2} in the \textsuperscript{18}O experiment was: C\textsuperscript{18}O\textsubscript{2} = 0.031, C\textsuperscript{16,18}O\textsubscript{2} = 0.320, C\textsuperscript{16}O\textsubscript{2} = 0.649. Thus, 35% of the CO\textsubscript{2} was at least singly labeled. Since we could easily discern a 2-fold increase in the output of singly labeled O\textsubscript{2}, a point we verified using H\textsubscript{3}\textsuperscript{18}O, we concluded that CO\textsubscript{2} or HCO\textsubscript{3} contributed \textless 1% to the O\textsubscript{2} evolved by single turnovers of the O\textsubscript{2} evolving system. Therefore, these data, along with earlier findings, suggest that CO\textsubscript{2} or HCO\textsubscript{3} is not a significant source of photosynthetic oxygen.

3.2. Isotopic composition of O\textsubscript{2} evolved after dark equilibrium with labeled water

At present, the nature of the intermediate oxidation states leading to photosynthetic O\textsubscript{2} evolution is unknown. There is some evidence that bound intermediate oxidation products of water participate [7]. Indeed, there has been a report (not yet confirmed, to our knowledge) that the O\textsubscript{2}-evolving complex in whole algae cannot isotopically exchange H\textsubscript{2}O with the intracellular H\textsubscript{2}O in the absence of light [8].

If the H\textsubscript{2}O were strongly bound by the O\textsubscript{2}-evolving complex, the initial O\textsubscript{2} evolved would be expected to reflect the isotopic composition of the H\textsubscript{2}O in which the chloroplasts were last illuminated. Thus, the evolved O\textsubscript{2} would not reflect the isotopic composition of H\textsubscript{2}O added in total darkness until the bound H\textsubscript{2}O had been oxidized.

Figure 2 shows the results obtained when normal chloroplasts were illuminated in the presence and absence of H\textsubscript{2}O\textsuperscript{18} added in total darkness prior to the flash sequence. (Similar results were obtained using depleted chloroplasts reactivated with unlabeled HCO\textsubscript{3}; data not shown.) In both cases the O\textsubscript{2} evolved always reflected the isotopic composition of the H\textsubscript{2}O in the medium at the onset of illumination. Note that there was no appreciable difference between the two experiments in the ratio of the first few flash yields (Y\textsubscript{2}, Y\textsubscript{3}, Y\textsubscript{4}) to the later yields, an observation which suggests that the substrate H\textsubscript{2}O is freely exchangeable with the medium. [Because of the problems in handling these small quantities of isotope, the observed 34/32 ratio did not always accurately reflect the amount of isotope previously added. This discrepancy did not compromise the validity of the conclusions, but was somewhat disturbing. We therefore monitored the H\textsubscript{2}O\textsuperscript{18}/H\textsubscript{2}O\textsuperscript{16} ratio in situ by determining the C\textsuperscript{18}O\textsubscript{2} : C\textsuperscript{16,18}O\textsubscript{2} : C\textsuperscript{16}O\textsubscript{2} ratios before illumination in preparations containing 10 mM HCO\textsubscript{3} and 100 units of carbonic anhydrase. The isotope ratio of the evolved O\textsubscript{2} in these experiments was found to agree with the measured H\textsubscript{2}O\textsuperscript{18}/H\textsubscript{2}O\textsuperscript{16} ratio.]
Fig. 2. O₂ flash yields observed when H₂¹⁴O (top) or unlabeled (bottom) H₂O was added in total darkness to dark-adapted chloroplasts. Buffer: 0.9 ml Tricine (50 mM, pH 7.4) plus 0.1 ml H₂¹⁴O (71 atom%) or H₂¹⁶O. H₂O (3 μl) was also added to 25 μl chloroplast suspension. After mixing, 10 μl of these chloroplasts were layered on the mass spectrometer inlet. The unlabeled O₂ signal was attenuated 5-fold compared to the labeled (m/e = 34) signal.

These results show that, in the dark, the O₂-evolving complex does not contain bound substrate H₂O. This in turn, suggests that the S₁ state, the predominant oxidation state in dark-adapted chloroplasts, does not contain a bound intermediate oxidation product of H₂O, and that any association between the S₁ and S₀ states and substrate H₂O is relatively short-lived.

3.3. An effect of bicarbonate depletion on the reducing side of system II

Although CO₂ apparently is not the immediate substrate for O₂ evolution, bicarbonate depletion can considerably affect the turnover of system II (reviewed [9]). During the course of the experiments in sections 3.1 and 3.2, we observed that bicarbonate depletion drastically altered the flash yield pattern in the presence of ferricyanide, which, in normal deactivated chloroplasts, causes a significant increase in the oxygen yield of the second flash [10]. This high second flash yield does not reflect the generation of S₂ in the dark, but rather, the ability of these chloroplasts to undergo a 'double hit' [11], i.e., a double turnover of the system II trap within the few μs of a single xenon flash. This phenomenon correlates well with the degree of oxidation of an endogenous chloroplast component with a midpoint potential of ~400 mV [11], which may be identical with 'C', an alternate secondary system II electron acceptor [10]. Presum-
ably, when C is in the oxidized state, it can rapidly (within a few μs) accept an electron from Q\(^{-}\) and thus allow two turnovers of each system II trap within a single (~5 ps) xenon flash.

Figure 3 shows that the availability of this electron acceptor to Q\(^{-}\) is strongly suppressed by CO\(_{2}\) depletion. Only a minimal second flash yield is evident in CO\(_{2}\)-depleted chloroplasts in the presence of ferricyanide (fig.3, top). When the chloroplasts are reactivated, a large Y\(_{2}\), comparable to Y\(_{3}\), is observed (fig.2, middle). This effect is specific for HCO\(_{3}\); with NaCl, no increase in Y\(_{2}\) is observed (bottom trace).

A major effect of bicarbonate depletion is inhibition of electron flow from Q\(^{-}\) to the intersystem electron transport pool \([12]\). The results of our experiments suggest that this inhibition is more general. Electron flow from Q–C, which normally takes place within the few μs of the actinic flash, is also apparently slowed sufficiently so that little Q–C transfer within the flash is observed.

The mechanism by which bicarbonate inhibits the suite of reactions on the donor side of system II, and indeed the occurrence and function of this phenomenon in vivo, is unknown. One could postulate that the inhibition of system II by bicarbonate depletion facilitates cyclic electron flow under CO\(_{2}\)-limited conditions (fig.4). By partially inhibiting the electron flow from H\(_{2}\)O, bicarbonate depletion could effect, in nature, the same 'poising' of the electron-transport system as that obtained in the laboratory using DCMU or low light intensities (see, e.g. \([13]\)). Since (cyclic) electrons apparently enter the transport chain at the level of plastoquinone \([13]\), the bicarbonate-sensitive reactions are properly situated to effectively regulate the flow of electrons into this cycle.

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