ISOTOPIC COMPOSITION OF PHOTOSYNTHETIC O_2 FLASH YIELDS IN THE PRESENCE OF $H_2{}^{18}O$ AND $HC^{18}O_3^-$

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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_2O 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_2O. This indicates that the S_1 state does not contain a bound intermediate oxidation product of H_2O.

(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.

* We will use the terms CO_2 and bicarbonate interchangeably; no reference to the true active species involved is intended

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_2O. [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 $\sim$1 quantum . trap^{-1} . 10 min^{-1} after the addition of NH_2OH 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_2O.] 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) $\sim$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 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 CO_2 depletion, 30 mM methylamine and 1 mM $K_3Fe(CN)_6$ were added to the reaction medium.

During the isolation and assay procedure, great care was taken to exclude contamination by atmospheric CO_2. All procedures were done in closed vessels under a stream of N_2, and transfers were effected via modified syringes. All reagents were extensively bubbled with CO_2-free N_2 before use. 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 μmol O_2 . mg chl^{-1} . h^{-1}.

Depleted chloroplasts were reactivated in total darkness by adding solid $NaHCO_3$ to both the chloroplast preparation and the medium to 10 mM final conc. The mass spectrometer reaction vessel was filled with the HCO_3^--spiked buffer, and the reactivated chloroplasts were layered on the inlet. The isotopic composition of the 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 O_2 isotopic species were determined.

[^{18}O]Bicarbonate was prepared by dissolving unlabeled $NaHCO_3$ in 95 atom% $H_2{}^{18}O$ (from Bio Rad

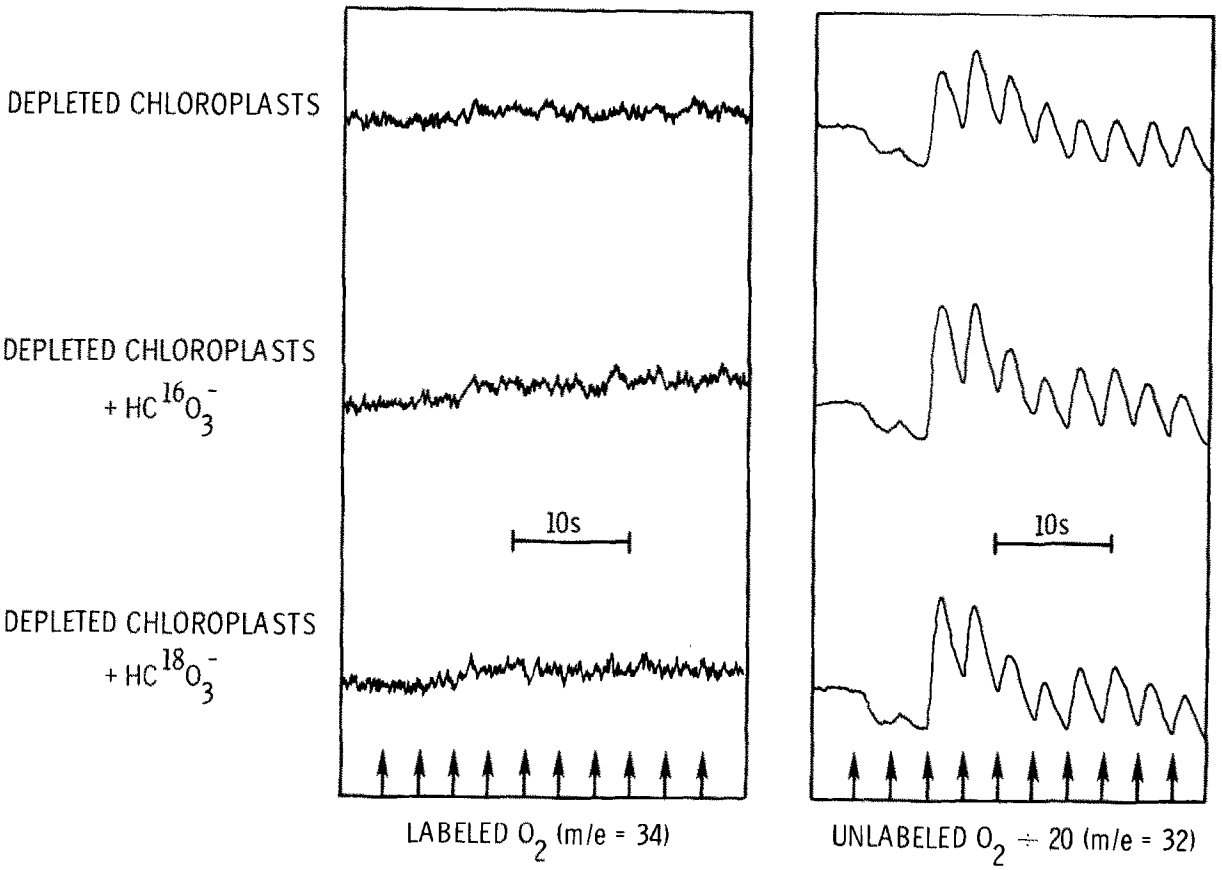

Fig.1. Evolution of $^{18}O_2$ and unlabeled O_2 elicited by short saturating light flashes in HCO_3^--depleted chloroplasts, depleted chloroplasts reactivated with unlabeled HCO_3^-, and depleted chloroplasts reactivated with $HC^{18}O_3^-$. The unlabeled O_2 signal (m/e = 32) was attenuated 20-fold compared to $^{18}O_2$ (m/e = 34) signal. The solid arrows in this and the following two figures mark the firing of the xenon flash (3 s spacing). For the $HC^{18}O_3^-$ experiment, the isotopic composition of the CO_2 at the beginning of the flash sequence was: $C^{18}O_2$ (m/e = 48), 0.031; $C^{16,18}O_2$ (m/e = 46), 0.320; $C^{16}O_2$ (m/e = 44), 0.649.

Labs.), and allowing the solution to equilibrate for several days. Known quantities of solid $HC^{18}O_3^-$ (and unlabeled HCO_3^-) free of labeled H_2O were obtained by drying aliquots of the solution under a stream of N_2.

3. Results and discussion

3.1. *Isotopic composition of O_2 evolved by HCO_3^--depleted chloroplasts reactivated in the dark*

The hypothesis that CO_2 or bicarbonate is the immediate source of photosynthetically evolved O_2 has been long proposed (see, e.g. [5]). Using CO_2-depleted chloroplasts [3], it was found that the O_2 evolved after reactivation with HCO_3^- reflected the isotopic composition of the H_2O and not the bicarbonate. However, because their apparatus did not have sufficient sensitivity to monitor single turnovers of the O_2-evolving system, they could not eliminate the possibility that the very first molecules of evolved O_2 were derived from the reactivating HCO_3^-.

Figure 1 shows the flash yield pattern of $^{18}O_2$ ($m/e = 34$) and unlabeled O_2 ($m/e = 32$) for CO_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 $^{18}O_2$ (beyond that attributable to the natural abundance of $H_2{}^{18}O$) was evolved when the CO_2-depleted chloroplasts were reactivated with [^{18}O]bicarbonate. Significant labeled O_2 was evolved only in the presence of added $H_2{}^{18}O$ (see below). A similar lack of response of the evolved O_2 to reactivation with labeled bicarbonate was also observed at $m/e = 36$, which corresponds to doubly-labeled O_2 (data not shown).

That the added bicarbonate did indeed reactivate the O_2-evolving system is shown in fig.1 (right column), which illustrates the flash yield patterns of unlabeled O_2 ($m/e = 32$) for the same samples as before after 5 min dark time. Note that the reactivation procedure resulted in a modest increase in the flash yield amplitudes and a restoration of the oscillation (compare line 1 with lines 2, 3). Both of these observations agree with earlier reports [6]. (See also fig.3 below.)

The data of fig.1 set rather stringent limitations on the amount of O_2 that can be derived from CO_2. At the onset of the flash sequence, the isotopic composition of the CO_2 in the ^{18}O experiment was: $C^{18}O_2 = 0.031$, $C^{16,18}O_2 = 0.320$, $C^{16}O_2 = 0.649$. Thus, 35% of the CO_2 was at least singly labeled. Since we could easily discern a 2-fold increase in the output of singly labeled O_2, a point we verified using $H_2{}^{18}O$, we concluded that CO_2 or HCO_3^- contributed $\lesssim 1\%$ to the O_2 evolved by single turnovers of the O_2 evolving system. Therefore, these data, along with earlier findings, suggest that CO_2 or HCO_3^- is not a significant source of photosynthetic oxygen.

3.2. *Isotopic composition of O_2 evolved after dark equilibrium with labeled water*

At present, the nature of the intermediate oxidation states leading to photosynthetic O_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_2-evolving complex in whole algae cannot isotopically exchange H_2O with the intracellular H_2O in the absence of light [8].

If the H_2O were strongly bound by the O_2-evolving complex, the initial O_2 evolved would be expected to reflect the isotopic composition of the H_2O in which the chloroplasts were last illuminated. Thus, the evolved O_2 would not reflect the isotopic composition of H_2O added in total darkness until the bound H_2O had been oxidized.

Figure 2 shows the results obtained when normal chloroplasts were illuminated in the presence and absence of $H_2{}^{18}O$ added in total darkness prior to the flash sequence. (Similar results were obtained using depleted chloroplasts reactivated with unlabeled HCO_3^-; data not shown.) In both cases the O_2 evolved always reflected the isotopic composition of the H_2O 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_2, Y_3, Y_4) to the later yields, an observation which suggests that the substrate H_2O 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 $H_2{}^{18}O/H_2{}^{16}O$ ratio in situ by determining the $C^{18}O_2$: $C^{16,18}O_2$: $C^{16}O_2$ ratios before illumination in preparations containing 10 mM HCO_3^- and 100 units of carbonic anhydrase. The isotope ratio of the evolved O_2 in these experiments was found to agree with the measured $H_2{}^{18}O/H_2{}^{16}O$ ratio.]

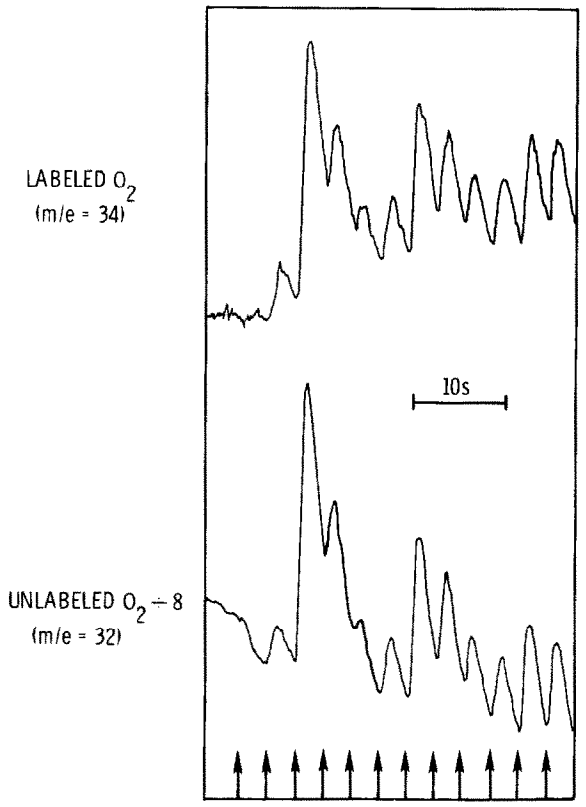

Fig. 2. O_2 flash yields observed when $H_2{}^{18}O$ (top) or unlabeled (bottom) H_2O was added in total darkness to dark-adapted chloroplasts. Buffer: 0.9 ml Tricine (50 mM, pH 7.4) plus 0.1 ml $H_2{}^{18}O$ (71 atom%) or $H_2{}^{16}O$. H_2O (3 μl) was also added to 25 μl chloroplast suspension. After mixing, 10 μl of these chloroplasts were layered on the mass spectrometer inlet. The unlabeled O_2 signal was attenuated 8-fold compared to the labeled (m/e = 34) signal.

These results show that, in the dark, the O_2-evolving complex does not contain bound substrate H_2O. This in turn, suggests that the S_1 state, the predominant oxidation state in dark-adapted chloroplasts, does not contain a bound intermediate oxidation product of H_2O, and that any association between the S_1 and S_0 states and substrate H_2O is relatively short-lived.

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

Although CO_2 apparently is not the immediate substrate for O_2 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 S_2 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-

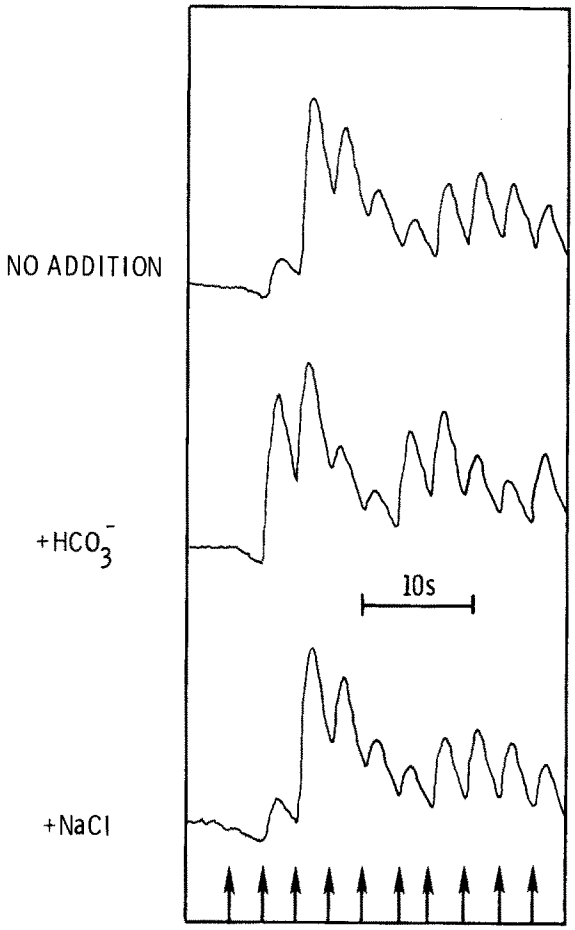

Fig. 3. Effect of bicarbonate on the second flash yield in the presence of ferricyanide. The buffer was reaction medium (see section 2) amended with 1 mM $K_3Fe(CN)_6$. Top trace, depleted chloroplasts; middle trace, depleted chloroplasts plus 10 mM $NaHCO_3$; bottom trace, depleted chloroplasts plus 10 mM NaCl.

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 μs) 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_2O, 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.

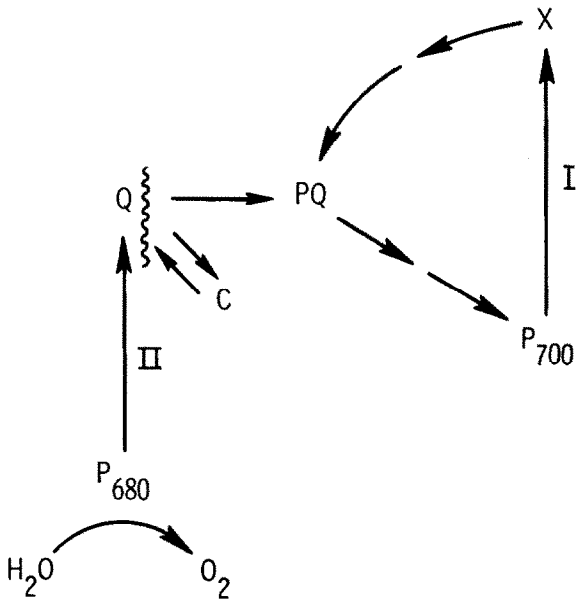

Fig.4. Electron transport scheme showing the relationship of bicarbonate-sensitive reactions (wavy line) and cyclic electron flow. P680 and P700, primary donors of photosystems II and I, respectively; Q and X, the primary acceptors; PQ (plastoquinone) and C, secondary system II acceptors.

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